This report presents the recommendations of a WHO Expert Committee commissioned to coordinate activities leading to the adoption of international recommendations for the production and control of vaccines and other biological substances, and the establishment of international biological reference materials.

Following a brief introduction, the report summarizes a number of general issues brought to the attention of the Committee. The next part of the report, of particular relevance to manufacturers and national regulatory authorities, outlines the discussions held on the development of revised WHO Guidelines. Specific discussions took place on the development of WHO guidance on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines; the quality, safety and efficacy of typhoid conjugate vaccines; and the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology.

Subsequent sections of the report provide information on the current status and proposed development of international reference materials in the areas of vaccines and related substances; blood products and related substances; in vitro diagnostic device reagents; and biotherapeutics other than blood products.

A series of annexes are then presented which include an updated list of WHO Recommendations, Guidelines and other documents on biological substances used in medicine (Annex 1), followed by a series of WHO Guidelines adopted on the advice of the Committee (Annexes 2–4). All additions and discontinuations made during the 2013 meeting to the list of International Standards and Reference Panels for biological substances maintained by WHO are summarized in Annex 5. The updated full catalogue of WHO International Reference Preparations is available at: http://www.who.int/bloodproducts/catalogue/en/.

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Sixty-fourth report

World Health Organization

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WHO Technical Report Series
987

WHO Expert Committee on Biological Standardization

Sixty-fourth report

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization

World Health Organization

WHO Library Cataloguing-in-Publication Data

WHO Expert Committee on Biological Standardization, sixty-fourth report.

(WHO technical report series ; no. 987)

1. Biological products - standards. 2. Vaccines - standards. 3. Reference standards.
4. Guideline. I. World Health Organization. II. WHO Expert Committee on Biological Standardization (2013: Geneva, Switzerland). III. Series.

ISBN 978 92 4 120987 8 (NLM classification: QW 800)
ISSN 0512-3054

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Printed in Italy

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WHO Expert Committee on Biological Standardization
21 to 25 October 2013

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Abbreviations

β-TG	β-thromboglobulin
ADC	antibody-drug/toxin conjugate
ADH	adipic acid dihydrazide
ADR	adverse drug reaction
anti-HBc	antibodies against hepatitis B core antigen
anti-HBe	antibodies against hepatitis B envelope antigen
anti-HBs	antibodies against hepatitis B surface antigen
APEC	Asia-Pacific Economic Cooperation
ASEAN	Association of Southeast Asian Nations
AVAREF	African Vaccine Regulatory Forum
B19V	parvovirus B19
BGTD	Biologics and Genetic Therapies Directorate
BRN	WHO Blood Regulators Network
BRR	biological reference reagent
BSP	Biological Standardisation Programme
CAT	Coalition Against Typhoid
CBER	Center for Biologics Evaluation and Research
CDC	United States Centers for Disease Control and Prevention
C. freundii s.l.	*Citrobacter freundii sensu lato*
CI	confidence interval
CRF	circulating recombinant form
CRM197	cross-reactive material 197
CRS	cytokine release syndrome
CT	cholera toxin
CTAB	hexadecyltrimethylammonium bromide
CTC	controlled temperature chain
CV	coefficient of variation
CYP	cytochrome P450 enzyme

D-Ag	D-Antigen
DBSQC	Division of Biological Standards and Quality Control
DCVMN	Developing Countries Vaccine Manufacturers Network
DNA	deoxyribonucleic acid
DT	diphtheria toxoid
DU	D-Antigen unit (of inactivated poliomyelitis vaccine)
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (also abbreviated EDAC)
EDQM	European Directorate for the Quality of Medicines & HealthCare
EEA	European Economic Area
EFD	embryo-fetal development
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot assay
EMA	European Medicines Agency
EMP	WHO Department of Essential Medicines and Health Products
ePPND	enhanced pre/postnatal development
FISH	fluorescence in situ hybridization
FIX	factor IX
FT-IR	Fourier transform infrared spectroscopy
FXIa	activated factor XI
G-CSF	granulocyte colony-stimulating factor
GACVS	Global Advisory Committee on Vaccine Safety
GAP	Global Action Plan
GCP	good clinical practice
GCV	geometric coefficient of variation
GLP	good laboratory practice
GMC	geometric mean concentration
GMP	good manufacturing practice
GRevP	Good Regulatory Review Practice

HAV	hepatitis A virus
HBeAg	hepatitis B envelope antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HDI	human development index
HDV	hepatitis D virus
Hib	*Haemophilus influenzae* type b
HIV	human immunodeficiency virus
HMRV	high mutant reference virus
HMWS	high molecular weight species
HPAEC–PAD	high-performance anion exchange chromatography with pulsed amperometric detection
HPLC	high-performance liquid chromatography
HPSEC	high-performance size-exclusion chromatography
HPV	human papillomavirus
IBPC	Institute for Biological Product Control
ICDRA	International Conference of Drug Regulatory Authorities
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IFAT	indirect fluorescent antibody test
IFPMA	International Federation of Pharmaceutical Manufacturers & Associations
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IHC	immunohistochemical
INN	International Nonproprietary Name
IPAC	WHO Immunization Practices Advisory Committee

IPV	inactivated poliomyelitis vaccine
IU	International Unit
KD	(distribution constant)
KFDA	Korea Food and Drug Administration (now the Ministry of Food and Drug Safety)
LAL	*Limulus* amoebocyte lysate
Lf	limit for flocculation
LMRV	low mutant reference virus
LPS	lipopolysaccharide
mAb	monoclonal antibody
MABEL	minimum anticipated biological effect level
MACE	major adverse cardiac event(s)
MALLS	multiple angle laser light scattering
MAPREC	mutant analysis by polymerase chain reaction and restriction enzyme cleavage
MCB	master cell bank
MenA pS	meningococcal serogroup A polysaccharide
MFDS	Ministry of Food and Drug Safety
MHRA	Medicines and Healthcare Products Regulatory Agency
MPL	monophosphoryl lipid A (also abbreviated MPLA)
MS	mass spectrometry
MW	molecular weight
NADFC	National Agency for Drug and Food Control
NAT	nucleic acid amplification
NCL	national control laboratory
NHP	non-human primate
NIBSC	National Institute for Biological Standards and Control
NIFDC	National Institutes for Food and Drug Control
NIFDS	National Institute of Food and Drug Safety Evaluation
NIH	National Institutes of Health

NIID	National Institute of Infectious Diseases
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
NRA	national regulatory authority
OLSS	Office of Laboratories & Scientific Services
OPA	opsonophagocytic antibody
OPV	oral poliomyelitis vaccine
PANDRH	Pan American Network for Drug Regulatory Harmonization
PATH	Program for Appropriate Technology in Health
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCR-SSCP	polymerase chain reaction single-strand conformation polymorphism
PD	pharmacodynamic(s)
PEI	Paul-Ehrlich-Institut
PF4	Platelet factor 4
PK	pharmacokinetic(s)
PPND	pre/postnatal development
qPCR	quantitative polymerase chain reaction
QTc	corrected QT interval
RCD	reverse cumulative distribution
rcDNA	residual cellular DNA
rDNA	recombinant deoxyribonucleic acid
RDT	rapid diagnostic test
rEPA	recombinant *Pseudomonas aeruginosa* exoprotein A
RFLP	restriction fragment length polymorphism
RHSC	Regulatory Harmonization Steering Committee
RNA	ribonucleic acid
RQ-PCR	real-time quantitative polymerase chain reaction
RSV	respiratory syncytial virus

SAP	Statistical Analysis Plan
SBA	serum bactericidal antibody
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
S-IPV	Sabin inactivated poliomyelitis vaccine
SPI-7	Salmonella pathogenicity island 7
SPR	surface plasmon resonance
SRID	single radial immunodiffusion
SSC	Scientific and Standardization Committee (of the International Society on Thrombosis and Haemostasis)
TCA	trichloracetic acid
TCR	tissue cross-reactivity
TIG	tetanus immunoglobulin
TK	toxicokinetics
TLR	toll-like receptor
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor alpha
TQT study	thorough QT/QTc study
TRS	Technical Report Series
TSE	transmissible spongiform encephalopathies
TT	tetanus toxoid
UHC	universal health coverage
Vi PS	Vi polysaccharide
Vi-rEPA	Vi polysaccharide conjugated to rEPA
VPPAG	Vaccine Presentation and Packaging Advisory Group
VVM	vaccine vial monitor
WCB	working cell bank
WHOCC	WHO collaborating centre

1. Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 21 to 25 October 2013. The meeting was opened by Mr Kees de Joncheere, Director of the Department of Essential Medicines and Health Products (EMP).

Mr de Joncheere welcomed the Committee, meeting participants and observers, and highlighted the growing interest in biological medicines as evidenced by the record number of participants at this year's meeting. Such a level of participation reflected the strong need expressed by countries and regulators to WHO for support in appropriately regulating biological products. He also reminded the group that the standards established by the Committee were designed to be used as common global standards that could promote a process of regulatory convergence. The adoption and adaption of these WHO standards by national regulatory agencies, and their use by the WHO prequalification programme, represented practical approaches for achieving such convergence.

Mr de Joncheere also pointed out that the Committee has for several years recommended that WHO undertake activities specifically aimed at promoting the implementation of the standards it establishes. WHO has responded by coordinating a series of implementation workshops on selected standards that had proved to be both popular and useful. WHO has also been working on ways to strengthen its activities in the context of developing its Programme of Work for the coming years, and Mr de Joncheere presented a brief overview of a recent strategic reorganization process with particular relevance to EMP activities.

Mr de Joncheere then brought attention to the typically full agenda of the Committee which included consideration of three new written standards, and the need to reach decisions on 12 proposals to establish new international reference materials. The Committee would also be asked to consider 16 proposals to initiate new projects on new or replacement international reference materials. Mr de Joncheere expressed his thanks on behalf of WHO to the Committee, to WHO's collaborating centres, and to all the experts, institutions and professional societies working in this area whose efforts provide vital support to WHO programmes. Mr de Joncheere concluded by reminding the group that Committee members acted in their personal capacities as experts and not on behalf of their organizations or countries.

The Secretary to the Committee, Dr David Wood, then presented an overview of WHO Expert Committees and of the important and greatly valued role they played in providing assistance to Member States. Established by the World Health Assembly or Executive Board, WHO Expert Committees acted as official advisory bodies to the Director-General of WHO and were governed by formal rules and procedures. Dr Wood then outlined the organization of the meeting and the major issues to be discussed. Declarations of Interests made by two members of the Committee and by four Temporary Advisors were presented

to the group. Following an earlier evaluation, WHO had concluded that none of the declarations made constituted a significant conflict of interest, and the individuals concerned would be allowed to participate fully in the meeting.

Dr Elwyn Griffiths was elected as Chairman and Dr John Petricciani was elected as Rapporteur for the plenary sessions, and for the track considering vaccines and biotherapeutics. Dr Harvey Klein was elected as Chairman and Dr Anthony Hubbard and Dr Micha Nübling as Rapporteurs for the track considering blood products and in vitro diagnostic device reagents. Dr Klein was also elected as Vice-Chairman for the plenary sessions of the Committee. Following participant introductions, the Committee adopted the proposed agenda (WHO/BS/2013.2231).

Before proceeding with the agenda, the Committee took note of the many important contributions that Dr Jean-Marc Spieser, European Pharmacopoeia, Council of Europe, had made over many years to the field of biological standardization, and expressed its regret on learning of his recent death.

2. General

2.1 Current directions

2.1.1 Strategic directions in biological standardization: WHO priorities

Dr Wood pointed out that there were three major components of the current WHO norms and standards paradigm: (a) the production of global written standards; (b) the development of global measurement standards; and (c) the conducting of regulatory science in areas such as assay standardization, the further development and refinement of quality control tests, and establishing the scientific basis for setting specifications.

Key strategic drivers of standardization included the growing emphasis now being placed on the concept of universal health coverage (UHC). Dr Wood discussed major aspects of UHC in the context of WHO's work, and highlighted the role that medicines and health technologies played. Another key driver was increased recognition of the importance of strengthened international regulatory cooperation – the importance of which had been emphasized by a 2013 workshop organized by the Institute of Medicine which concluded that:

> *There is a need for globally harmonized, science-based standards for the development and evaluation of safety, quality, and efficacy of medical products.*

Regulatory "convergence" was highlighted as an emerging concept in cooperation that went beyond the development of common standards and processes to take into account implementation by regulatory authorities. Supporting the implementation of selected written standards was viewed as an increasingly important approach to achieving regulatory convergence and was a key WHO activity in this area. The aim of regulatory convergence was for regulatory decisions across economies or countries to become more aligned without requiring the harmonization of different national laws and regulations. Nevertheless, a number of challenges to strengthening convergence still needed to be addressed.

Regulatory science was highlighted as another driver of standardization and an essential element for the integration and application of research findings and innovation. Several examples were presented of how regulatory science had contributed to improved access to vaccines. However, as with regulatory convergence, significant challenges remained in the area of regulatory science that needed to be addressed.

Dr Wood then outlined a process of WHO reform that had resulted in a revised organizational structure and a set of leadership priorities aimed at providing a renewed focus and direction to WHO's role in improving global health. Within the six WHO leadership priorities, attention was drawn to the stated aim of increasing access to essential, high-quality and affordable

medical products – defined as medicines, vaccines, diagnostics and other health technologies. Meeting participants were reminded that the documents approved by the Committee are presented to the WHO Executive Board and eventually to the World Health Assembly with the intended effect of strengthening regulatory authorities and improving access to essential medicines.

The setting of norms and standards – and promoting and monitoring their implementation – is one of the six core functions of the 2014–2019 WHO General Programme of Work. One outcome of the reorganization of EMP had been the bringing together of the Expert Committee on Biological Standardization and the Expert Committee on Specifications for Pharmaceutical Preparations, along with the International Nonproprietary Names (INN) Committee, within a new WHO team called Technologies, Standards, and Norms. This reorganization would provide important opportunities to review the focus, priority-setting mechanisms and potential for collaboration of the three entities.

Dr Wood concluded by reminding the Committee that the next International Conference of Drug Regulatory Authorities (ICDRA) would be hosted by the Brazilian Government and organized by the Brazilian Health Surveillance Agency (ANVISA) in Rio De Janeiro, Brazil. An open pre-ICDRA meeting on 24–25 August 2014 would focus on similar biotherapeutic products ("biosimilars") and would be followed by a closed meeting for regulators only on 26–29 August 2014.

A number of discussion issues were then raised, including the potential utility of producing a WHO organogram to clarify the recent reorganization. Attention was also drawn to the increasing interest being shown in the area of biosimilars, which was itself partly driving recent developments. The importance of WHO efforts in supporting the parallel processes of regulatory harmonization and convergence was also reiterated. It was clarified that WHO played different roles in various ongoing harmonization and convergence efforts depending upon the context in which specific initiatives were being implemented. A broad range of WHO activities in this area were under way and would continue.

2.1.2 Vaccines and biotherapeutics: recent and planned activities in biological standardization

Dr Ivana Knezevic outlined activities in the area of vaccines and biotherapeutics that included the development and implementation of written standards for vaccines. Three such standards had been prepared for consideration by the Committee in 2013, three were under development for presentation to the Committee in 2014, and four were under consideration for 2015. In addition, five other documents were in the early stage of consideration with as yet unclear timelines for completion. The development process for guidelines and recommendations had been more extensive than in the past, and had involved

numerous groups of users and standard-setting bodies as well as a minimum of two rounds of public consultation. The range of different perspectives and inputs obtained from these groups had resulted in documents that were more suitable for implementation globally.

Activities in the key areas of biotherapeutic products and biosimilars had included a survey of national regulation trends involving regulators from the Pan American Network for Drug Regulatory Harmonization (PANDRH), Asia-Pacific Economic Cooperation (APEC), Association of Southeast Asian Nations (ASEAN) and African Vaccine Regulatory Forum (AVAREF) and from several Russian-speaking countries. The common objectives of all such networks included expertise and capacity building, regulatory convergence, and the sharing of information and knowledge.

In relation to WHO efforts to facilitate the implementation of standards, an overview was presented of a series of recent and planned workshops. Implementation workshops and case studies were particularly helpful in translating WHO guidance into practice, and workshops had been convened on either general issues or on issues related to specific vaccines or biotherapeutic products, with further events scheduled in both cases. The purpose of such workshops was to facilitate the implementation of recently adopted WHO standards by working with regulators on complex and/or difficult-to-implement issues. Manufacturers were also involved in a process of facilitating the implementation of guidance into their manufacturing practices. A need for assistance was often highlighted in consultations during the development of guidance documents, or by the Committee itself. Implementation workshops typically involved lectures on selected topics and case studies with small group discussions. In addition, meeting and case study reports, including case studies from implementation workshops, had been published in a special issue of *Biologicals* and elsewhere in order to increase the availability of materials to all those working in the field. A brief overview of a 2013 workshop on cell substrates was presented to illustrate the approach taken, highlight the scope of the issues discussed and set out the expectations for WHO as a lead agency.

In advance of a fuller report (see section 2.2.2) a brief outline was also provided of the role of WHO collaborating centres (WHOCCs) in the regulatory evaluation of vaccines. The crucial benefits provided by having a larger group of experts available to provide assistance to WHO and its Member States was clearly recognized.

Broader strategic aspects included recognition of the central role of WHO standards in facilitating regulatory convergence, which would involve the timely provision of well-balanced, scientifically based and agreed-upon standards. However, making standards available would not in itself be sufficient and would need to be supported by the regular exchanging of information with users, input from regulators, manufacturers and academia into the development

and implementation of standards, promoting synergies in standardization activities including through the involvement of WHOCCs, provision of support to regional and inter-country regulatory networks, and communication with other standard-setting bodies.

During discussion the recent establishment of regional initiatives to promote cooperation and mutual support among national regulatory authorities (NRAs) was highlighted. Comments were also made which emphasized that regulatory harmonization and convergence efforts were still at an early stage, and that limitations currently existed based in part on issues of national legislation and political considerations, in addition to technical constraints.

2.1.3 Blood products and related in vitro diagnostics: recent and planned activities in biological standardization

Dr Ana Padilla summarized the main activities carried out in the area of blood products and related in vitro diagnostics since the previous meeting of the Committee. Dr Padilla began by reminding the Committee that the widely endorsed concept of blood as an essential medicine, based upon voluntary non-remunerated donation and not-for-profit blood establishments, was of crucial importance in furthering the development of resolution WHA63.12. In 2010, this resolution requested that WHO provide additional support to Member States to help improve the availability, safety and quality of blood products; ensure the sustainable production of WHO biological reference preparations and their provision to those who need them; and improve access by developing countries to the scientific information obtained through their validation. Resolution 63.12 goes on to urge Member States:

> *...to update their national regulations...in order to ensure that regulatory control in the area of quality and safety of blood products across the entire transfusion chain meets internationally recognized standards.*

Resolution WHA63.12 also draws attention to the large volume of plasma that is separated from whole blood and discarded, rather than used as a starting material for the manufacture of essential plasma-derived medicinal products unavailable in many countries. It was explained that, through its Achilles project, WHO seeks to improve access to safe blood products in low- and middle-income countries by strengthening the local production of quality recovered plasma in blood establishments for the manufacture of plasma-derived medicinal products.

Two examples were outlined of WHO activities that aim to strengthen national and regional regulatory blood systems. At the national level, Indonesia had been selected as a pilot country for project implementation. A situation analysis had been carried out with regard to the national blood supply and blood regulatory system. As part of this analysis, a seminar on blood standards and regulation was

held involving 70 delegates representing the National Blood Committee, Indonesia Red Cross Blood Centre, central and regional offices of the National Agency for Drug and Food Control (NADFC), and Ministry of Health blood services. Strong governmental commitment was highlighted, including through the issuing of an upcoming decree by the Ministry of Health conferring sole regulatory authority for the entire system to the NADFC. Similarly, strong commitment was expressed by other interested parties. Agreement had been reached that a project using WHO standards and resources along with the regulatory expertise of relevant WHOCCs and the WHO Blood Regulators Network (BRN), supported by inspectors and auditors specializing in good manufacturing practice (GMP), could have a major beneficial impact on the blood system in Indonesia – and that harnessing the positive factors in place while recognizing the challenges was a realistic aim.

Dr Padilla reiterated the central role of the Committee in promoting the adoption of WHO guidelines and recommendations for effective blood regulatory systems, the establishment of relevant WHO biological reference preparations and the implementation of resolution WHA63.12 on the availability, safety and quality of blood products. A workplan was being developed to address: (a) training in GMP for blood establishments for NADFC inspectors and quality-assurance personnel in relevant blood establishments; and (b) training on the evaluation of blood safety and blood-testing technologies. Developments will take place in 2014 supported by the European Commission project on local production.

Regulatory oversight is advocated by WHO as an essential element of any blood system to ensure that blood standards are met. The African Society for Blood Transfusion (AfSBT) had requested at their meeting in 2012 that a WHO Workshop on Blood Regulatory Systems be held in the WHO African Region, recognizing that this activity would further the objectives of resolution WHA63.12. Such a workshop had been organized in September 2013 in South Africa under the initiative of the Renewed EU/ACP/WHO Partnership and hosted by the South Africa National Blood Service.

The workshop provided an introduction to blood regulation, quality-assurance systems and blood-safety testing strategies for blood products, and provided an unprecedented opportunity for participants from 11 sub-Saharan countries to discuss the needs and challenges in this area. It was recognized that adhering to GMP principles is necessary for blood establishments supplying plasma for fractionation, and it was thus necessary for blood operators and regulators to cooperate if they were to realize the goal of self-sufficiency. Countries reported that currently 80–90% of recovered plasma is wasted and sustained efforts and commitment will be needed to reach the standards necessary to satisfy the quality requirements for plasma fractionation. The "twinning" of well-resourced and less well-resourced blood establishments to support capacity building through the training of trainers and sharing of best practice needs to

be promoted. Strengthening regulatory systems for blood products and building up the technical capacities of national and regional blood regulatory authorities are fundamental to assuring the global availability of safe blood products.

Other recent activities had included a meeting of WHOCCs working in this area (see section 3.2.5) and the WHO Consultation on Commutability of WHO Biological Reference Preparations for in vitro Detection of Infectious Markers (see section 3.2.4).

Dr Padilla closed by highlighting the items in this area scheduled for submission to the Committee in 2013, which included the proposed establishment of eight new reference standards and panels for the quality control of diagnostic tests, and the submission of 10 new projects for endorsement.

2.2 Reports

2.2.1 Report from the WHO BRN

Dr Jay Epstein updated the Committee on the work of the BRN during 2013. Dr Epstein began by reminding the Committee of the objectives of the BRN, namely:

- to identify issues and share expertise and information;
- to promote the science-based convergence of regulatory policy, including by fostering the development of an international consensus on regulatory approaches;
- to propose solutions to specific issues, especially emerging public health challenges such as the vulnerability of countries to communicable disease threats.

A range of regulatory agencies from Australia, Canada, France, Germany, Japan, Switzerland and the United States are currently BRN members. Following on from its previous face-to-face meeting in 2012, the BRN had held three teleconferences with a face-to-face meeting scheduled during the current session of the Committee.

During 2013, BRN work products included a review of the scientific basis for donor exclusion for men who have sex with other men, and the preparation of a BRN Position Paper on Regulatory Considerations for Donor Screening for Men Who Have Sex with Other Men to be finalized for publication in 2013–14. In addition, technical and scientific support for the proposal to recognize blood components as essential medicines was provided through BRN endorsement and letters of support from BRN member countries.

The BRN Workplan 2013–14 had also been produced and reviewed, and a series of BRN-prepared materials and presentations on a range of topics had been identified for discussion during the current session of the Committee.

Dr Epstein concluded by informing the Committee that a request for BRN membership had been received from the Korean Ministry of Food and Drug Safety.

2.2.2 Report from the WHO collaborating centres for biological standards

An overview was presented by Dr Michael Pfleiderer of the network of WHOCCs for the standardization and evaluation of vaccines. This network currently consisted of eight WHOCCs:

- National Institute for Biological Standards and Control (NIBSC), Medicines and Healthcare Products Regulatory Agency, Potters Bar, England – re-designated in 2013;
- Center for Biologics Evaluation and Research (CBER), Food and Drug Administration, Silver Spring, MD, USA;
- Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases (NIID), Tokyo, Japan;
- Immunobiology and Biochemistry Group, Office of Laboratories & Scientific Services (OLSS), Therapeutic Goods Administration, Woden, Australia;
- National Institute of Food and Drug Safety Evaluation (NIFDS), Ministry of Food and Drug Safety (MFDS), Chungcheongbuk-do, Republic of Korea;
- Biologics and Genetic Therapies Directorate (BGTD), Health Canada, Ottawa, Canada;
- Institute for Biological Product Control (IBPC) of the National Institutes for Food and Drug Control (NIFDC), Beijing, China – designated in 2013;
- Division of Virology, Paul-Ehrlich-Institut (PEI), Langen, Germany – designated in 2013.

The first meeting of the network was held in April 2012 with agreement reached on the operational approach of the network and on the terms of reference (ToRs) for individual WHOCCs. In practice, the NIBSC plays a pivotal role in the development, establishment and distribution of international standards and reference materials with other WHOCCs contributing to these and other activities in accordance with their ToRs. It was felt that by working as a network a number of the challenges associated with working individually could be mitigated. For example, sharing responsibilities and expertise may be a more efficient way of supporting WHO activities, and of providing assistance in the areas of regulatory science and capacity building.

Further regulatory and scientific issues, including globally emerging regulatory issues and the provision of ad hoc regulatory and/or scientific support as requested by NRAs and other agencies could also be better addressed through a functional network. Important future activity areas in support of regulatory convergence included the translation of WHO standards and national regulatory and scientific requirements and practices into a common regulatory language, and the aligning of scientific and regulatory requirements in the setting of future standards.

The second meeting of the WHOCC network would be organized by WHO and hosted by PEI on 17–19 March 2014. The main focus would be on regulatory science and the role of the network in that context. Input from the Committee was invited as part of an upcoming process of agenda discussion and development.

The Committee welcomed the presentation given by Dr Pfleiderer and requested that it be kept updated at its future meetings of the progress of the network.

2.3 Feedback from custodian laboratories

2.3.1 Developments and scientific issues highlighted by custodians of WHO biological reference preparations

The Committee was informed of recent developments and issues identified by the following custodians of WHO biological reference preparations.

National Institute for Biological Standards and Control (NIBSC), Potters Bar, England

Dr Stephen Inglis informed the Committee that NIBSC had merged with the United Kingdom Medicines and Healthcare Products Regulatory Agency (MHRA) in 2013. He then went on to describe the changing landscape in biological standardization including the rapid growth in importance of biological substances (particularly novel and competitor products), and the increasingly globalized nature and growing complexity of biological production methods. These and other factors presented major challenges to biological standardization activities.

Two key challenges in ensuring success in standardization approaches were the need to responsibly increase access and the need to support innovation in the development of safe and effective new medicines. In terms of availability, the example was given of how recent growth in the number of new vaccine manufacturers and products relied in part on ensuring the wide availability of practical standards. The importance of multiple manufacturers and international initiatives in achieving increased vaccine access and affordability was stressed, along with the concomitant need for quality standards.

Corresponding opportunities to increase access to biosimilars were also highlighted and these too would need to be coupled to efforts to develop common

quality standards, and models of standardization. In order to reap the potential global health benefits of these and other developments, future standardization efforts needed to focus on key potency and safety characteristics, with a primary focus placed on the core determinants of the desired clinical outcome.

In relation to innovation, new and complex biological products, technologies and targeting approaches presented significant challenges for biological standardization. Although new standardization technologies themselves offered powerful new tools for analysis they would need to be applied appropriately and used intelligently so as not to stifle innovation.

In all these areas of development the Expert Committee had a critical role to play. In order to keep pace with such a daunting and rapidly changing field, there was a need to tap into the broader scientific infrastructure.

European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, France

Dr Karl-Heinz Buchheit outlined a range of recent EDQM activities in biological standardization, including the progressing of its Biological Standardisation Programme (BSP) in which WHO has Observer status. BSP goals included the establishment of European Pharmacopoeia biological reference preparations; the standardization of test methods for the quality control of biological substances; the elaboration of alternative methods in support of the 3Rs concept of "Replace Reduce Refine" to minimize the use of animals in research; and the provision of support to international harmonization efforts, including through collaboration with WHO and non-European partners. BSP achievements to date included the initiation or conclusion of 131 projects on reference standards and method development (including 20 projects on 3R methods).

Projects of potential interest to the Committee were highlighted and included the development and evaluation of alternative in vitro tests for both pertussis toxin and pertussis vaccine; a standardized in vitro assay for hepatitis A vaccine; and development of a potential serological assay as a replacement for the current in vivo batch potency test for rabies vaccine. In addition, a new standard category – biological reference reagent (BRR) – had been created to facilitate the use of in vitro assays, with, for example, BRRs now available from EDQM for the assay of hepatitis A vaccine. A number of other new and ongoing standardization projects of potential interest to the Committee were then outlined.

Dr Buchheit then reiterated that the development of alternatives to animal experiments remained a major commitment of EDQM in line with European Union directives, and WHO was requested to consider incorporation of the 3R initiative into its written standards and other guidance, where appropriate. The inclusion of 3R methods in WHO guidelines was viewed as being of paramount importance in promoting their global acceptance.

Dr Buchheit concluded by highlighting a number of key harmonization and other implementation issues for regional standard-setting bodies when no International Standard or other WHO guidance was available. In the absence of such guidance there was a potential risk of differences emerging in the direction or rate of implementation of approaches, including differences in the speed of implementation of the 3R approach.

Center for Biologics Evaluation and Research (CBER), Rockville, MD, USA

Dr Jay Epstein reported on the successful completion of the second year of the CBER-WHO Cooperative Agreement to enhance regulatory capacity to support influenza vaccine introduction in low-middle income countries. Funding had specifically been used to support NRA assessments, an international proficiency study of the single radial immunodiffusion (SRID) assay, a training course for regulators on influenza vaccine manufacturing and development of a WHO guideline document on the nonclinical evaluation of adjuvanted vaccines.

Activities supported during a successful first year of the CBER-WHO Cooperative Agreement to enhance global pharmacovigilance capacity had included the development of a Global Vaccine Safety Multi-country Collaboration for signal evaluation and hypothesis testing, which was a reflection of a growing recognition of the importance of strengthening pharmacovigilance capacities. Related activities had included the development of a narcolepsy case definition as an adverse event of special interest following influenza vaccination, the convening of a training course in pharmacovigilance, the development of data-management tools for adverse events following immunization (AEFI) reports and reporting on the work of the Global Advisory Committee on Vaccine Safety (GACVS) subgroup on influenza vaccines.

After outlining the working approach of the Division of Biological Standards and Quality Control (DBSQC) Dr Epstein informed the Committee of a wide range of ongoing or proposed activities in the further development of potency standards, reference preparations, international standards, reference panels and reagents.

A number of recent workshops sponsored by CBER were then highlighted as a key element of implementation and regulatory research promotion efforts. Dr Epstein then set out a range of new CBER regulatory activities that had been undertaken in areas such as increasing stakeholder involvement, monitoring drug shortages and enhancing the safety of the drug-supply chain. The Committee was also informed that a process of policy development in the area of biosimilars was under way.

Paul-Ehrlich-Institut (PEI), Langen, Germany

Dr Klaus Cichutek reminded the Committee that the PEI Division of Hematology/Transfusion Medicine had been designated as a WHOCC in 2005. In August

2013 the PEI Division of Virology was subsequently designated as a WHOCC for the standardization and evaluation of vaccines.

Reference preparations developed by PEI and put forward for establishment by the 2013 Expert Committee included the:

- First WHO International Standard for hepatitis D virus RNA for NAT-based assays;
- First WHO International Standard for anti-hepatitis B virus e antibodies;
- First WHO International Standard for hepatitis B virus e antigen;
- First WHO International Standard for mycoplasma DNA for NAT-based assays.

In addition, PEI was proposing for endorsement the development of the First WHO International Standard for anti-cytomegalovirus immunoglobulin G (plasma).

Numerous current scientific issues were then identified by Dr Cichutek, including the issue of biological products manufactured in non-European Economic Area (EEA) countries and the associated need for worldwide regulatory convergence, the need for NRA strengthening approaches, the emergence of new challenges such as the Middle East Respiratory Syndrome (MERS)-Coronavirus and developments in the pipeline of advanced therapy medicinal products (ATMPs). A range of recent activities in the area of vaccines had also been undertaken with a primary focus on the development of assays, including several alternative assays to lethal challenge assays in animals. In general, there was a need to ensure that the properties of individual products could be standardized, with products still in development, as well as highly unique products, presenting significant challenges.

Dr Cichutek then detailed various PEI activities related to standardization efforts in the areas of allergens, biosimilars, whole-cell therapeutic products and personalized biological medicines, along with work on companion diagnostics. Finally he pointed out a number of preconditions that would need to be met by proposed reference materials for biomarkers, including the need to be well characterized, equivalent to patient specimens and compatible with different types of diagnostic assays.

2.4 Cross-cutting activities of other WHO committees and groups

2.4.1 Updating of the WHO Essential Medicines List

A number of issues were highlighted arising from the Nineteenth Meeting of the WHO Expert Committee on Selection and Use of Essential Medicines held in April 2013. Among the new formulations of interest to the WHO Expert Committee on Biological Standardization were PEGylated interferon for the

treatment of chronic hepatitis C and the monoclonal antibody Bevacizumab for treating age-related macular degeneration. No changes had been made in the vaccines previously listed. Issues of future interest included the use of trastuzumab as an anticancer medicine.

An application to include whole blood and red blood cells had raised a broad range of issues with strong views expressed both for and against inclusion. After thorough consideration of the issues raised, the WHO Expert Committee on Selection and Use of Essential Medicines decided to restructure the relevant section and to explicitly highlight that:

> *WHO recognizes that achieving self-sufficiency, unless special circumstances preclude it, in the supply of safe blood components based on voluntary, non-remunerated blood donation, and the security of that supply are important national goals to prevent blood shortages and meet the transfusion requirements of the patient population. All preparations should comply with the WHO requirements.*

It was also noted that the WHO Expert Committee on Biological Standardization defines blood products as *any therapeutic substances derived from human blood, including whole blood, labile blood components and plasma-derived medicinal products*. Section 11 of the Essential Medicines List was therefore renamed "Blood products and plasma substitutes of human origin" and reorganized to include the following subsections:

- Blood and blood components
- Plasma-derived medicinal products
- Plasma substitutes.

A further review of this section would be undertaken at the next meeting of the WHO Expert Committee on Selection and Use of Essential Medicines in 2015.

Subsequent discussion centred on the role of the WHO Essential Medicines List in the context of the development of national lists and on emerging issues likely to require further consideration, including the development of advice on the use of similar biotherapeutic products. Broad recognition was expressed of the need for strengthened collaboration between the various WHO Expert Committees and groups working in this area.

2.4.2 Proposal for the naming of similar biotherapeutic products

The Committee was informed that a discussion on an INN proposal for similar biological products (SBPs) took place during the 56th INN Consultation as well during an INN ad hoc meeting attended by biological experts of the INN Expert Group and representatives of regulatory agencies worldwide. INNs for SBPs

follow general naming principles and there are no specific means of identifying them as SBPs within their INN. The current naming situation is such that non-glycosylated SBPs have the same INN as their reference product while glycosylated SBPs are likely to have a different name from their reference product due to potential differences in their glycoforms, this being achieved by the use of a Greek letter. In neither case is the reference product identified within the INN.

Although comparability studies were performed between an SBP and its reference product, studies between one SBP and another were not done – two separate SBPs may thus be compared to the same reference but not to each other. There was no consensus on the desirability of switching between SBPs, with some national authorities creating nomenclature qualifiers to distinguish between one SBP and another and between the SBP and its reference product.

As the mandate of the WHO INN Programme was to ensure the clear identification of pharmaceutical substances, both chemical and biological, it was felt that one way forward would be through developments in nomenclature with INN Programme involvement in developing unique global qualifiers. The aim would be to try to avoid non-unified qualifiers being assigned to SBPs by individual regulatory bodies.

The Committee heard that it was being proposed that an SBP should have a two-part name – the first part would be the INN of the reference product while the second part would be a qualifier that would both indicate that this was an SBP and identify it as a particular SBP or biological substance. To achieve this, WHO could assign the qualifier according to an agreed policy or could produce a policy document by which regulatory authorities could produce the required suffix or code. Alternatively, WHO could issue an advice document laying out a naming convention for use by regulatory authorities. It was stressed, however, that all regulatory authorities would need to support a global system.

Discussion topics included the possibility of any increase in the complexity of the current approach complicating related activities, for example the reporting of adverse events associated with specific products. The INN Expert Group was currently reviewing the range of issues to be considered and possible ways forward, and would keep the Committee informed of developments.

2.4.3 Development of technical supplements to WHO guidance on the storage and transport of time- and temperature-sensitive pharmaceutical products

The WHO *Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products* was developed in consultation with the WHO Task Force on Regulatory Oversight on Pharmaceutical Cold Chain Management and was published in 2011 as Annex 9 to the forty-fifth report of the WHO Expert Committee on Specifications for Pharmaceutical Preparations. The intention was that the guidance should be directly applicable in both

developed and less-developed countries since experience with vaccine supply chain assessments in the latter had demonstrated that the mandatory standards set out could be achieved, with some countries also being capable of meeting many of the optional requirements.

It was reported that the Committee Secretariat had worked with a number of experts in order to develop a set of 18 technical supplements, each of which followed the same structural format. Following external expert review the supplements were now being finalized prior to electronic publication and distribution to all regulatory agencies, ministries of health, relevant international organizations, public and private pharmaceutical industry sectors, and supply chain professionals.

The Forty-eighth meeting of the WHO Expert Committee on Specifications for Pharmaceutical Preparations had recommended that the documents be subjected to the usual consultation process prior to being submitted to the WHO Expert Committee on Biological Standardization and the WHO Expert Committee on Specifications for Pharmaceutical Preparations in 2014. The WHO Expert Committee on Biological Standardization agreed with this recommendation.

2.4.4 Proposed WHO Guideline on Good Regulatory Review Practice

The Committee was informed of a recent partnership between WHO and the APEC Regulatory Harmonization Steering Committee (RHSC) in the development of a draft document that was intended to evolve into a WHO Guideline on Good Regulatory Review Practice (GRevP). Such a guideline would be the first such global resource and would address an important gap previously identified at the 2012 ICDRA meeting.

It was intended that the final document would primarily focus on the provision of higher-level definitions, principles and other elements of good regulatory review practice applicable to both drugs and higher-risk medical devices. Envisioned as one building block in a set of tools, the guidelines would reference existing materials as appropriate while being sufficiently flexible to accommodate additional annexes or ancillary documents in the future.

It was anticipated that the guideline would be submitted to the WHO Expert Committee on Biological Standardization and the WHO Expert Committee on Specifications for Pharmaceutical Preparations in 2014. The WHO Expert Committee on Biological Standardization agreed that this would be a useful resource and looked forward to reviewing it in due course.

2.4.5 Scientific and regulatory considerations for the stability evaluation of vaccines under a controlled temperature chain

The Committee was informed of the progress that had been made on a WHO and Program for Appropriate Technology in Health (PATH) collaborative

approach to allow for the on-label use of vaccines in a controlled temperature chain (CTC). The approach would allow vaccines to be kept and administered at temperatures of up to 40 °C for a single or multiple periods of time immediately before administration. Over the course of two WHO consultations held in 2012 and 2013, CTC scientific and regulatory issues were considered, case studies reviewed and CTC data analysed.

The two options now under consideration for the development of WHO guidelines in this area were to revise existing vaccine stability guidelines to incorporate CTC study findings or to develop an independent document for use as an addendum or standalone guideline. A proposed structure for guideline development was then presented to the Committee. It was pointed out that the first vaccine going through a CTC process was the meningitis A conjugate vaccine, already licensed in India and prequalified by WHO for use in a CTC system with appropriate label amendments.

The Committee agreed with the proposed next steps for developing WHO guidelines on the evaluation of a vaccine in a CTC, provided a number of suggestions for consideration and looked forward to receiving a progress report at its next meeting.

2.4.6 Request for guidance on the harmonized labelling of vaccines

Requirements for vaccine labelling are currently provided in Annex 1 Good manufacturing practices for biological products, section 7 of the 1992 WHO Technical Report Series, No. 822. In 2011 the WHO Immunization Practices Advisory Committee (IPAC) had reported to the Committee on a proposal by the Vaccine Presentation and Packaging Advisory Group (VPPAG) to address a number of labelling legibility and harmonization issues previously identified through an open review process. These issues included text legibility, minimum requirements, multiple language requirements, package insert information, use of generic names on labels, ability to observe vial content by allowing a minimal clear area and the date format.

The Committee was informed that these and other labelling design issues had now been evaluated and a draft report produced. Following comments received by manufacturers and other parties through VPPAG, a final report had been produced in September 2013 and corresponding recommendations and proposed amendments to the 1992 guidance received by IPAC for improving vaccine label design and information content.

As part of an overall process of reviewing Annex 1 of the 1992 WHO Technical Report Series, No. 822, work was now under way on developing specific recommendations for changes to the current guidance on vaccine labelling. These recommendations were scheduled to be submitted to the Committee for its consideration in 2015.

3. International Recommendations, Guidelines and other matters related to the manufacture and quality control of biological substances

All Recommendations and Guidelines established at the meeting are listed in Annex 1, which provides an updated listing of all current WHO Recommendations, Guidelines and other documents related to the manufacture and quality control of biological substances used in medicine.

3.1 Vaccines and related substances

3.1.1 Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines

Over the past decades, strategies and approaches for the development and delivery of vaccine antigens have been expanded. Some of these antigens are weakly immunogenic and require the presence of adjuvants to induce or enhance an adequate immune response. As knowledge of the mechanisms of vaccine adjuvant action has expanded, the number of vaccines containing novel adjuvants being evaluated in clinical trials has increased. As a result, the development and evaluation of adjuvanted vaccines now presents a range of regulatory challenges. Vaccine manufacturers and regulators therefore have a need for guidance on the type of information and extent of data required to support a decision to proceed to clinical trials and to facilitate the eventual authorization of such vaccines.

Although existing WHO guidelines on the nonclinical evaluation of vaccines already provide valuable general guidance, they provide only limited information specifically related to new adjuvants and adjuvanted vaccines. The proposed draft Guidelines (WHO/BS/2013.2214) provide updated and more extensive guidance to NRAs and manufacturers on the nonclinical and initial clinical evaluation of vaccine adjuvants and adjuvanted vaccines by outlining international regulatory expectations in this important area of global public health.

Detailed discussion took place on a range of issues, with clarifications sought in a number of key areas and specific suggestions and other meeting inputs taken into consideration. After making a number of changes to the draft text, the Committee recommended that the revised Guidelines be adopted and annexed to its report (Annex 2).

3.1.2 Guidelines on the quality, safety and efficacy of typhoid conjugate vaccines

Based upon current evidence it would appear that the use of typhoid conjugate vaccines may overcome several of the limitations currently associated with unconjugated, plain Vi polysaccharide vaccines. Typhoid conjugate vaccines

are anticipated to demonstrate: (a) greater efficacy end effectiveness; (b) longer persistence of immunity; (c) immunogenicity across all age groups including infants and toddlers under 2 years of age; (d) advantages in conferring some degree of herd immunity; and (e) induction of immune memory with initial dosing, leading to anamnestic responses to subsequent doses(s).

The currently available guidelines for Vi polysaccharide typhoid vaccine and for live-attenuated Ty21a vaccines are not applicable to typhoid conjugate vaccines consisting of Vi polysaccharide conjugated with a carrier protein such as diphtheria toxoid, tetanus toxoid, recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA), non-toxic mutated or recombinant forms of diphtheria toxin (such as CRM197) or any suitable protein.

The proposed new Guidelines (WHO/BS/2013.2215) were intended to assist NRAs in evaluating the scientific issues associated with the quality, safety and efficacy of typhoid conjugate vaccines based on Vi polysaccharide covalently linked to a carrier protein. Following a presentation on general considerations in areas such as typhoid pathogenesis, transmission and disease burden and control, and a presentation by the Coalition Against Typhoid (CAT) on the epidemiological characteristics of typhoid and the status of the typhoid vaccine pipeline, a series of presentations was given on the need for the new guidance and on the content and development of each of the main sections of the proposed Guidelines.

The Committee expressed its thanks for all the work done in bringing the Guidelines to their current stage of development. Following discussion of all comments and submissions received, and after making a number of corresponding changes to the text, the Committee recommended that the revised Guidelines be adopted and annexed to its report (Annex 3).

3.1.3 Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology

Following a series of requests, a process of international consultation and review was undertaken as part of the revision of current WHO guidance in this area. The updated proposed Guideline document (WHO/BS/2013.2213) was developed in order to provide guidance to NRAs and manufacturers on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant deoxyribonucleic acid (rDNA) technology and intended for use in humans. The updated Guidelines were based upon experience gained over three decades in this technically demanding field, and are intended to replace previously developed guidance as they contain new sections on the clinical and nonclinical evaluation of rDNA-derived biotherapeutics that were lacking in the original document. Although some of the Guideline content may thus be useful for clinical trial application, it was very important to understand that the amount

and extent of data for clinical trials will be limited, and there would be a need to take into account the specific nature of the product and its stage of development.

In general, the updated Guidelines provide an overview of regulatory expectations for the licensing of all biologically active protein products used in the treatment of human diseases, and which are prepared by rDNA technology using prokaryotic or eukaryotic cells. They also apply to protein products used in diagnosis (for example for monoclonal antibody products including in vivo diagnosis and ex vivo treatment, but excluding in vitro diagnosis) and those intentionally modified – for example by PEGylation, conjugation with a cytotoxic drug or modification of an rDNA sequence.

It was further agreed that core elements of the Guidelines should apply to recombinant products derived from transgenic animals and plants, but that additional considerations applied to such products and the applicability of specific sections should be discussed with the NRA.

Part A of the proposed Guidelines sets out updated guidelines for the manufacture and quality control of rDNA-derived biotherapeutics, including consideration of the effects of manufacturing changes and of devices used in delivery on the product and its stability. The newly added sections B and C provided guidelines on nonclinical and clinical evaluation, respectively. Some of the aspects of manufacturing and quality control outlined may apply to protein-based vaccine antigens in the early stage of development and made by rDNA technology. However, more-detailed WHO guidance and recommendations on vaccine evaluation in terms of quality, safety and efficacy should be consulted.

Following discussion of all comments and submissions received, and after making a number of corresponding changes to the text, the Committee recommended that the revised Guidelines be adopted and annexed to its report (Annex 4).

3.1.4 Regulatory written standards pipeline

The Committee was informed that in addition to the three Guidelines outlined above for consideration by the Committee in 2013 three documents on the following subjects were scheduled for submission to the Committee in 2014:

- inactivated poliomyelitis vaccines (IPVs)
- regulatory evaluation of post-approval changes
- regulatory risk assessment in the case of adventitious agents in already licensed vaccines.

Inactivated poliomyelitis vaccines

A number of issues were raised in relation to the implementation of the various steps that will eventually lead to the conversion of production of oral poliomyelitis

vaccines (OPVs) to Sabin inactivated poliomyelitis vaccines (S-IPVs). Among the points of concern were: (a) the short timeline (by 2016) for conversion from OPV to S-IPV production; (b) S-IPV already being in production in some countries; (c) a need for NRA capacity-building in countries in which there will be new poliomyelitis vaccine manufacturers; (d) the need to identify the appropriate government agency responsible for environmental containment issues, and for coordination among agencies; and (e) the importance of having a final approved WHO Global Action Plan to minimize poliovirus facility-associated risk after eradication of wild polioviruses and cessation of routine OPV use (GAP III) document in order to proceed with the revision of appropriate WHO guidance documents for consideration by the Committee in 2014.

Following considerable discussion, the Committee agreed that a critical step requiring immediate attention was the completion and approval of the draft GAP III document. It also was pointed out that guidance on environmental assessment had already been provided in Annex 2 of the 2013 WHO Technical Report Series, No. 979.

Regulatory evaluation of post-approval changes

The Committee was reminded that WHO had published two documents on manufacturing changes.[1] However, regulators still faced difficulties in evaluating post-approval manufacturing and related changes, with different regulatory approaches having been applied to similar changes. As such changes were not uncommon, WHO had been requested to provide guidance in this important area and in November 2012 a drafting group was convened for this purpose with a subsequent consultation held in April 2013.

A detailed outline of the proposed Guidelines including appendices was then presented to the Committee and the various sections reviewed. A range of concerns – both previously expressed by industry and NRAs and raised by meeting participants – would now be taken into consideration in the further revision steps of the document.

The Committee made several further suggestions and agreed that further refinement of the current draft should continue so that a final draft can be considered at its next meeting.

[1] Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2; Regulation and licensing of biological products in countries with newly developing regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Forty-fifth report*. Geneva, World Health Organization, 1995 (WHO Technical Report Series, No. 858), Annex 1.

Regulatory risk evaluation in the case of adventitious agents in already licensed vaccines

The background and development history of a draft WHO guidance document on this issue was outlined. Following the development of new technologies capable of detecting previously undetectable agents, a number of requests had been made to WHO to assist countries in developing a risk-assessment process subsequent to marketing authorization. Although a broad pre-licensure regulatory framework existed, a number of aspects associated with the discovery of adventitious agents subsequent to marketing authorization were not well defined in terms of regulatory actions and decision-making.

In 2012 the Committee had reviewed the initial draft document and had recommended a number of changes. The subsequently revised draft document was discussed at several WHO meetings and was currently undergoing a second round of public consultation. The document would then be submitted to the Committee in 2014.

In addition, a manuscript was in preparation intended for publication in the scientific press. This paper will review four past cases in which an agent or signal of an agent was found in a licensed vaccine in order to illustrate how such situations were addressed and what lessons were learnt. In addition to supplementing the WHO guidance document the publication of the paper in the scientific literature will lead to wider access by all those interested in the field of biological substances. Discussion centred on a number of issues including the scope for the alignment of terminology with other sector initiatives on risk assessment and management, the need to balance general principles with the often highly specific nature of individual cases and the crucial role of well coordinated health communication in this area.

The Committee made a number of comments and suggestions, and encouraged the further development of the document for consideration at its next meeting.

Other written standards in the pipeline

In relation to the 2015 meeting of the Committee, written standards in the following four areas were scheduled for consideration: (a) good manufacturing practice (GMP) for biological substances; (b) human papillomavirus (HPV) vaccines; (c) regulatory risk assessment of biotherapeutic products; and (d) regulatory expectations in the context of CTC.

In addition, written standards for which there was currently no specific timelines included those on meningitis B, influenza vaccines for regulators in non-producing countries; vector-based vaccines; update of guidelines on the clinical evaluation of vaccines; and product-specific guidelines on SBPs.

3.1.5 Utility of deep sequencing in the manufacture of oral poliomyelitis vaccine

The Committee was informed that discussions had been held on the conducting of an international collaborative study involving national control authorities and vaccine manufacturers. The objectives of the proposed study (WHO/BS/2013.2216) were to assess the utility of massively parallel (deep) sequencing for monitoring the molecular consistency of OPV and to develop the common approaches, standards and acceptance criteria needed for introduction of the new method into regulatory decision-making. The study would aim to develop a database of the mutational composition of seed viruses and vaccine batches produced by different manufacturers that would facilitate the monitoring of production consistency and validation of new seed stocks.

Following a presentation on the study rationale and purpose, and associated practical considerations and intended next steps, the Committee endorsed its support for this study, made a number of suggestions for the study organizers to consider and requested that an update be presented at its next meeting.

The Committee was further informed that another collaborative study using the same techniques was under consideration. Because new-generation sequencing was in theory able to detect nucleic acid of unknown sequence – and therefore to identify unknown adventitious agents in cell banks or products made in them – it was conceivable that it could replace or supplement existing detection methods, including tests in animals or cell cultures.

Although recognizing that the proposed study was at an early stage of development, the Committee agreed that it could provide very useful information on the relative sensitivity of various methods and encouraged the development of a proposal for consideration at its next meeting.

3.2 Blood products and related substances

3.2.1 Strategic plan for assuring the quality and safety of blood products and related substances

The Committee reviewed and commented upon the following strategic goals that had been developed by the WHO Programme on Blood Products and Related Biologicals:

- Stabilize and provide sufficient resources for the WHO programme.
- Promote national and regional implementation of blood regulatory frameworks for blood products and related in vitro diagnostic devices.
- Enhance the global availability of essential blood-related medicines, including through local production.

- Complete ongoing projects for the development of written and physical standards for blood products and related in vitro diagnostic devices.
- Identify, prioritize and develop new standards to address unmet needs in ensuring the quality, safety, efficacy and availability of blood, blood products and related biologicals.

The Committee endorsed the goals and requested that the associated Strategic Plan 2014–2018 be submitted for subsequent review by the Committee.

3.2.2 Diagnostic needs for global malaria control and elimination

Malaria is a global health priority with more than 3 billion people estimated to be at risk. In addition, the proportion of suspected malaria cases confirmed by diagnostic testing has risen and resistance to artemisinin is increasing. In 2010 WHO guidelines were revised and a recommendation made for universal access to quality diagnosis and treatment for at-risk populations. Diagnosis based solely on clinical symptoms promotes misdiagnosis and the wasting of artemisinin-based combination therapies (ACTs) while eroding the credibility of health workers. Treatment based solely on clinical suspicion is therefore not recommended except as a temporary measure.

A new WHO initiative launched in 2012 promotes the scaling-up of diagnostic testing, treatment and surveillance (T3: Test. Treat. Track). Although light microscopy is preferable to rapid diagnostic tests (RDTs) for the calculation of parasite density, the identifying of malaria species and the monitoring of treatment, RDTs do provide rapid results for the presence of antigens and their use requires less training. The underlying mechanism of RDTs based upon the use of a nitrocellulose strip and labelled antibody which binds to target antigens was outlined to the Committee. The targeted malaria antigens are produced at various stages of the parasite lifecycle, with some allowing for the detection of a single species whereas others occurred in all malaria species.

A survey in 2005 indicated that over 200 different RDTs were available from over 60 manufacturers. However, optimal product selection has been hampered by reports of variable RDT performance in field trials in terms of sensitivity and/or specificity, a lack of reference standards and poor or nonexistent regulatory environments. A performance evaluation of RDTs was undertaken as part of an international collaboration based upon the WHO strategy for the quality assurance of RDTs which follows products from manufacture to end user, and covers product testing, lot testing and quality control at the point of use. Product testing at the United States Centers for Disease Control and Prevention (CDC) addressed issues of performance, heat stability and ease of use. The first

four rounds of RDT testing covered 124 unique products and have now been completed. In Round 5, 42 RDTs will be evaluated in 2014.

In 2007 WHO initiated the prequalification of malaria RDTs with WHO procurement criteria encompassing panel detection score, false-positive rate, invalidity rate and other considerations such as stability, ease of use and price. In the period 2007–2011 kit manufacturing was associated with improved panel detection scores based on WHO criteria, with the WHO lot-testing programme covering 50% of the public sector RDT market. Limitations of the current system include the need to reduce costs to ensure sustainability, the need to standardize panels and make them available to manufacturers, and the need to provide countries with standard and reliable materials for lot testing. Future diagnostic needs include a requirement for the detection of sub-microscopic infection, diagnostics to certify elimination and serology to detect recent infection in non-immune individuals, screening of blood donors in non-endemic regions and tools to manage non-malarial febrile illness.

The Committee noted the report.

3.2.3 DNA-based cancer diagnostics

The Committee was reminded of the stated WHO objective:

> *to reduce by 50% the mortality from all cancers that are amenable to early diagnosis and treatment*.[2]

The ability to identify the key "driver" mutation in a tumour and the existence of new drugs which target particular cancer mutations mean that some cancers are treated in a specific and targeted manner. Chronic myelogenous leukaemia (BCR-ABL fusion) was the paradigm for demonstrating the utility of genomic reference materials for cancer since the fusion gene/protein is the cause, drug target and biomarker of drug response. Quantification of the fusion gene-carrying clone is thus essential in the assessment of drug response. The development of the First WHO International Genetic Reference Panel for quantitation of BCR-ABL translocation (WHO Technical Report Series, No. 977, 2013) by real-time quantitative polymerase chain reaction (RQ-PCR) had facilitated the alignment of tests worldwide.

Given that mortality levels can be reduced by the use of standardized diagnostic approaches to curable cancers, a generic proposal was being made to develop standards for cancer diagnostics, in particular through the production of reference panels designed to help standardize bioassays for cancer diagnostics.

[2] *Cancer Control: Knowledge into Action. WHO Guide for Effective Programmes*. Module 4: *Diagnosis and Treatment*. Geneva, World Health Organization, 2008 (http://www.who.int/cancer/modules/en/, accessed 10 February 2014).

Panels for several gene mutations were now being proposed for production over the next 3 years (see section 6.2.5).

The Committee noted the report.

3.2.4 WHO Consultation on Commutability of WHO Biological Reference Preparations for in vitro Detection of Infectious Markers

The attention of the Committee was drawn to the outcomes of this 2013 WHO consultation on issues associated with the commutability of WHO reference materials. A reference was considered to be commutable if it behaved like clinical materials in all assays – a characteristic which is generally believed to be impossible to prove. However, there were degrees of commutability and ways of adjusting a non-commutable reference. The main issue was to decide on how poorly a given reference material had to perform before it was considered unacceptable.

A number of strategies had been discussed to assess commutability, including the inclusion of a number of clinical samples in the WHO collaborative study or in larger separate studies. However, this would raise ethical and supply issues, and would require decisions to be made on the scale of testing required. In addition, even once testing was concluded the issue of how well the material would perform in other systems would remain unresolved. It was therefore proposed that if a collaborative study were undertaken to assess commutability it should: (a) include all available methods; (b) include clinical samples spanning the range of values, excluding interfering substances; (c) avoid or check the effects of pre-treatments (such as freeze-thawing and pooling); and (d) be carried out only by laboratories prequalified for competence. The example of NAT-based assays for cytomegalovirus was provided to illustrate the range of problems associated with reference material commutability issues.

The primary conclusions reached by the consultation were:

- the commutability of a reference serum would depend upon the assays involved and the type of serum used as well as the specifics of the antigen;
- no serum could behave the same as all other sera in all assays;
- serological assays were difficult to control because of the many variables inherent in the procedure;
- reference materials could nonetheless have a major beneficial effect in improving comparisons between assay results.

Consultation participants urged WHO to consider revising its guidance in this area to give greater weight to commutability considerations. The Committee reviewed the issues raised and agreed upon the need to update the WHO Recommendations for the preparation, characterization and establishment

of international and other biological reference standards (revised 2004) which currently appears as Annex 2 of the 2005 WHO Technical Report Series, No. 932.

3.2.5 Fourth Meeting of WHO Collaborating Centres to support the development of WHO biological reference preparations for blood products and in vitro diagnostic devices

A summary report was presented to the Committee of the outcomes of this 2013 meeting of WHOCCs involved in the development of international standards for blood products and in vitro diagnostic devices. The meeting takes place biennially and involves participants from CBER, NIBSC and PEI.

A representative of the NIID, Japan, had been invited to this meeting as an observer, as part of the potential future involvement of the NIID in collaborations on the characterization of WHO biological reference preparations. Although WHO preparations are made in batches of several thousands, supplies are still limited and further efforts are needed in order to improve the accessing of these preparations by developing countries, as stated in the 2010 resolution WHA63.12 on the availability, safety and quality of blood products.

Updates were then presented to the Committee on the status of ongoing projects in the fields of blood products and diagnostic tests for infectious diseases. Significant efforts had been undertaken to ensure the development of new standard preparations, and the replacement of those previously established. This requires strong coordination and commitment on the part of WHO and WHOCCs towards the standardization of blood products and in vitro diagnostic devices. Agreement was reached that all standardization projects should be completed within 4 years of their initial endorsement.

4. International reference materials – vaccines and related substances

All reference materials established at the meeting are listed in Annex 5.

4.1 WHO International Standards and Reference Reagents – vaccines and related substances

4.1.1 Third WHO International Standard for inactivated poliomyelitis vaccine

An international collaborative study was conducted to establish the Third WHO International Standard for IPV. Three candidate samples were provided by three different European manufacturers and were analysed by 18 laboratories from 13 countries, each using their own in-house enzyme-linked immunosorbent assay (ELISA). Within- and between-laboratory repeatability was generally very good with very good agreement between laboratories whichever candidate was chosen as the standard for determining vaccine D-Antigen (D-Ag) content. All three candidate samples were thus found to be suitable for use as an international standard.

Based upon logistical and other practical considerations one of the candidate samples (NIBSC code 12/104 – 2820 vials) was proposed as the Third WHO International Standard for inactivated poliomyelitis vaccine. The two remaining candidates (NIBSC code 11/188 – 2201 vials and NIBSC code 08/143 – 10 000 vials) were proposed as candidates for a future replacement Fourth International Standard.

Stability studies had demonstrated that all candidate materials were stable at temperatures used for storage (−70 °C) and laboratory manipulation (4 °C to 20 °C) with real-time stability studies for the first year having been completed. The Committee suggested that further stability studies be undertaken to clarify previously noted issues and requested that a stability update be provided at its next meeting.

At present, the candidate standards had been validated for use only in in vitro assays. A second phase of the study was planned which would include evaluation of all three preparations for use in in vivo rat potency assays. The candidates may also be evaluated for their suitability for use in in vitro assays to measure the D-Ag content of IPV based on Sabin live-attenuated strains. In addition, an S-IPV candidate would be included in the study to see if it was a more appropriate material.

The Committee considered the report of the study (WHO/BS/2013.2217) and recommended that preparation 12/104 be established as the Third WHO International Standard for inactivated poliomyelitis vaccine with an assigned potency of 277 DU/ml for poliovirus type 1; 65 DU/ml for poliovirus type 2; and 248 DU/ml for poliovirus type 3. The committee also agreed that the two candidate preparations NIBSC code 11/188 and NIBSC code 08/143 should be

retained as possible replacements at a later date. The Committee further noted that, as stated in the current Instructions for Use, the established international standard had not yet been validated for use in in vivo rat assays, but that its intended use was likely to be modified in light of subsequent study results.

4.1.2 First WHO International Standard for anti-malaria (*Plasmodium falciparum*) human serum

The Committee was informed of an international collaborative study conducted to evaluate and establish the First WHO International Standard for anti-malaria (*Plasmodium falciparum*) human serum. Malaria is the world's largest parasitic disease and kills more people than any other communicable disease with the exception of tuberculosis. There was a recognized and urgent need for a reference serum preparation that contained antibodies against a number of malaria antigens to support the harmonization and standardization of immunological assays related to malaria vaccine development.

The material proposed as a candidate first international standard (NIBSC code 10/198) had been prepared from pooled plasma collected in Kenya and filled into 5400 glass ampoules. In addition, three anti-Pf-serum preparations with different titres had been filled. Sixteen laboratories from 12 different countries participated in the collaborative study with the objective of determining the total immunoglobulin G (IgG) content specific for three recombinant *P. falciparum* antigens. There was improved agreement between laboratories when the candidate standard was used for the calculation of relative potencies compared to estimates of the concentration required to achieve a 50% response (EC50) where no standard was used. The candidate was found to be extremely stable both in the freeze-dried state and after reconstitution.

The Committee considered the report of the study (WHO/BS/2013.2221) and discussed the utility of the candidate preparation in terms of vaccine development when the antigen(s) used in the vaccine may differ from the ones tested in the collaborative study. The Committee considered that establishment of an international standard was premature since the intended use was not sufficiently clear. The decision to establish this material as the first international standard was therefore deferred pending further information.

4.2 Proposed new projects and updates – vaccines and related substances

4.2.1 Proposed Third WHO International Standard for diphtheria toxoid for use in flocculation test

Diphtheria vaccines are among the most widely used and successful of human vaccines, and form an essential component of the primary immunization schedule of children, as well as being used for the reinforcement of immunity

in adults and adolescents. Standardizing the assays used to determine antigen content is crucial in allowing manufacturers to reliably measure antigenic purity and formulate the final bulk vaccine.

The Second WHO International Standard for diphtheria toxoid for use in flocculation test was established in 2007 and is widely used. As its current rate of use of around 200 ampoules per year was likely to continue – and may even increase – the 600 ampoules remaining represented only around 3 years supply. There was therefore a need to replace this standard before stocks are depleted. The Committee was informed that material had been obtained from one manufacturer sufficient to produce approximately 6000 ampoules. Preliminary studies suggested that this material was stable.

A proposed collaborative study was outlined in which the proposed standard would be calibrated in limit for flocculation (Lf) units using the WHO recommended flocculation method. The study design would follow that used to calibrate the second international standard and would involve laboratories from a number of different countries.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2223) to develop a Third WHO International Standard for diphtheria toxoid for use in flocculation test, and agreed that the collaborative study should proceed in 2014 with subsequent submission of its outcome to the Committee in 2015.

4.2.2 Proposed First WHO International Standard for typhoid Vi capsular polysaccharide

Several companies, including a number in developing countries, are starting to produce Vi polysaccharide (Vi PS) conjugate vaccines, with several currently undergoing clinical trials and/or regulatory licensing applications. As a variety of such vaccines become available a common global standard will be needed for the quantification and testing of selected vaccine quality parameters.

The Committee was informed of progress on the development of a First WHO International Standard for typhoid Vi capsular polysaccharide and of a proposed collaborative study involving a small number of manufacturers and national control laboratories (NCLs). Material had been obtained from one manufacturer sufficient for approximately 1000 ampoules. The proposed standard would be used to quantify the Vi polysaccharide antigen content as well as the *O*-acetyl content of the polysaccharide. A trial fill was planned to validate the process and formulation.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2223) to develop a First WHO International Standard for typhoid Vi capsular polysaccharide, and agreed that the collaborative study should proceed in 2014 with subsequent submission of its outcome to the Committee in 2015.

4.2.3 Update on the endorsed proposal for a First WHO International Standard for anti-typhoid Vi p capsular polysaccharide serum (human)

The Committee was informed that following its endorsement of the original proposal in 2009 an extension to the collaborative study had been initiated in June 2013. This study aimed to compare the reactivity of the candidate standard (NIBSC code 10/126 – approximately 1600 ampoules) with a United States National Institutes of Health (NIH) standard (Vi-IgGR1) serum already used in the evaluation of clinical materials. Study participants included vaccine manufacturers, NCLs and research institutes in seven countries.

The Committee noted the progress that had been made and looked forward to receiving the results of the extended collaborative study at its next meeting.

4.2.4 Proposed First WHO International Standard for meningococcal serogroup A polysaccharide

As vaccines containing meningococcal serogroup A polysaccharide (MenA pS) are now being manufactured by a wide range of manufacturers, including in developing countries, there is a recognized need to ensure that their potencies are standardized and equivalent to those of licensed vaccines. To ensure adequate protection against disease it is crucial that the MenA PS content in both monovalent and multivalent meningococcal conjugate vaccines is accurate.

The Committee was informed that the proposed First WHO International Standard for meningococcal serogroup A polysaccharide would be used by vaccine manufacturers and NCLs to determine the content of meningococcal serogroup A polysaccharide in monovalent and multivalent conjugate and polysaccharide vaccines. The use of nuclear magnetic resonance (NMR) techniques will be explored to assign unitage. Candidate material had been obtained from a manufacturer sufficient to produce approximately 3000 ampoules of the proposed international standard.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2223) to develop a First WHO International Standard for meningococcal serogroup A polysaccharide, and agreed that the collaborative study should proceed in 2014 with subsequent submission of its outcome to the Committee in 2015.

4.2.5 Proposed Second WHO International Standard for high and low mutant reference virus for MAPREC assay of poliovirus type 2

The potential consequences of polio infection are crippling and sometimes life-threatening, and infection and paralysis can occur in non-immune individuals of any age. Until the disease has been certified as eradicated globally, the risks of acquiring polio, and of the reintroduction of polio to currently free areas,

will remain. The safety testing of OPV is mandatory worldwide, and many manufacturers have adopted the mutant analysis by polymerase chain reaction and restriction enzyme cleavage (MAPREC) test as an alternative to in vivo testing for vaccine lot consistency. MAPREC is a WHO-recommended method for the quantification of mutations in poliomyelitis vaccines and a regulatory test for OPV batch release.

The Committee were informed that although at current rates of use there was sufficient stock of the type 2 high mutant reference virus (HMRV) and low mutant reference virus (LMRV) for 3 years, the introduction of the MAPREC test for type 2 as a regulatory test was likely to increase the level of demand and lead to early depletion. The MAPREC assay may also be used for the control of OPV seed virus destined for IPV production.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2223) to develop a Second WHO International Standard for HMRV and LMRV for MAPREC assay of poliovirus type 2, and agreed that the collaborative study should proceed in 2014 with subsequent submission of its outcome to the Committee in 2015.

4.2.6 Proposed First WHO International Standard for antiserum to respiratory syncytial virus

Respiratory syncytial virus (RSV) is a major cause of ill health in the first year of life and a serious and potentially fatal disease in the very young. RSV infections are also a problem in the elderly. Vaccines against RSV are now in development, with at least 40 organizations working in this field, at least three of which are conducting clinical trials. The development of a reference antiserum is therefore timely and needed to standardize clinical trials and allow for comparison of their outcomes.

The Committee was informed that potential sources of candidate materials were now being identified as part of a proposed collaborative study. Following discussion and further consideration, the Committee endorsed in principle the proposal (WHO/BS/2013.2223) to develop a First WHO International Standard for antiserum to RSV with the clear proviso that further consideration be given to the precise objectives, scope and final design – potentially with the assistance of the network of WHOCCs for the standardization and evaluation of vaccines. The Committee requested that an update of progress, including a revised study design, be presented at its next meeting.

5. International reference materials – blood products and related substances

All reference materials established at the meeting are listed in Annex 5.

5.1 Proposed new projects and updates – blood products and related substances

5.1.1 Update on the endorsed proposal for a First WHO International Standard for anti-rubella immunoglobulin

Rubella virus infection during pregnancy can cause birth defects and serological testing for antibodies against rubella is important for pregnant women as up to 50% of such infections are estimated to be asymptomatic. There are more than 100 manufacturers of anti-rubella test kits worldwide who calibrate their assays against the First WHO International Standard for anti-rubella immunoglobulin (NIBSC code RUBI-1-94). However, patient diagnosis often reveals test results that are discrepant between assays, possibly reflecting the use of different rubella antigens, test formats and technologies. In addition, traceability to the WHO international standard is frequently limited, bringing into doubt the current recommended cut-off point of 10 IU/ml used to determine whether or not to offer rubella vaccination. There has also been a shift in technology away from the previous neutralization and haemagglutination inhibition assays and towards the use of ELISA based upon recombinant antigens. To compound the current situation there is also a lack of certainty concerning the appropriate manufacture and calibration of secondary standards.

It was therefore concluded that dedicated investigations were now needed and scientific exchange with clinical virologists active in this field enhanced. It was agreed that a collaborative study be designed and conducted between NIBSC and a group of manufacturers to assess the feasibility of better harmonizing the calibration of various kits and assays and to interpret their results against a universal cut-off threshold.

Following discussion and further consideration, the Committee expressed its support for this proposed initiative (WHO/BS/2013.2230) and requested that it be kept informed of further developments.

5.1.2 Proposed Second WHO International Standard for Ancrod

Ancrod is a thrombin-like serine protease obtained from the venom of the Malayan pit viper (*Calloselasma rhodostoma*) that produces an anticoagulant and pro-fibrinolytic effect through a reduction of plasma fibrinogen levels. This is achieved by cleaving fibrinogen to form an unstable fibrin which is rapidly cleared from the circulation. Although Ancrod is not currently licensed in

any country, having been withdrawn in the 1980s, it is used in clinical trials for "sudden sensorineural hearing loss" and there is still interest in its clinical applications for stroke treatment.

The First WHO International Standard for Ancrod was established in 1977 and stocks were now low. As more than 100 ampoules are dispatched each year to laboratories in over 10 countries a replacement preparation is required. One product manufacturer had been identified and an agreement reached in principle for the donation of material sufficient for 5000 ampoules. The intention was to conduct a relatively small-scale but multi-centre study of the candidate material calibrated relative to the current WHO international standard.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop a Second WHO International Standard for Ancrod.

5.1.3 Proposed Fourth WHO International Standard for streptokinase

Streptokinase is a bacterial plasminogen activator and is used as a thrombolytic drug in the treatment of acute myocardial infarction (AMI). Streptokinase remains the most widely used thrombolytic worldwide, especially in developing countries. This has been reflected in sales of the Third WHO International Standard for streptokinase which was established in 2001 and remains the most popular thrombolytic standard. Following annual increases in sales over recent years, the level of demand had reached 283 ampoules per year by 2012.

Stocks of this WHO international standard were low and a replacement preparation is required. A therapeutic native streptokinase preparation, purified from culture filtrates of Group C *Streptococcus equisimilis* H46A would be sourced from one manufacturer. A multi-centre collaborative study was proposed of the candidate material calibrated relative to the Third WHO International Standard for streptokinase using chromogenic and fibrin-based methods. Recombinant products may also be sought for comparative analysis in different assay systems, which for some products are known to give different responses relative to the native international standard preparation.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop a Fourth WHO International Standard for streptokinase.

5.1.4 Proposal to assign factor IX antigen values to the current Fourth WHO International Standard for blood coagulation factors II, VII, IX, X (plasma) and to the proposed Fifth WHO International Standard for blood coagulation factor IX (concentrate)

These WHO international standards for factor IX (FIX) in plasma and concentrate currently have assigned values for FIX clotting activity but not for antigen. Assigning a value for FIX antigen is required for:

- characterization of FIX deficiency phenotype
- evaluation of recombinant and new-generation therapeutics (specific activity)
- determination of FIX expression in gene therapy.

The objective of this project was to assign values in International Units (IUs) for FIX antigen to both the plasma and concentrate international standards by assay relative to local normal plasma pools. This exercise would be combined with a collaborative study of a proposed replacement Fifth WHO International Standard for blood coagulation factor IX (concentrate).

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to assign FIX antigen values to both the Fourth WHO International Standard for blood coagulation factors II, VII, IX, X (plasma) and to the proposed Fifth WHO International Standard for blood coagulation factor IX (concentrate).

5.1.5 Proposed Second WHO International Standard for anti-tetanus immunoglobulin (human)

The clinical management of both suspected and confirmed cases of tetanus involves the use of tetanus antitoxin. Although equine preparations were still in use in some regions, the use of human tetanus immunoglobulin (TIG) was recommended and had completely replaced equine products in many countries.

With average sales of 200 ampoules per year, and less than 900 ampoules remaining, stocks of the current international standard were being depleted and a replacement was now required. The aim of the proposed collaborative study was to assign a tetanus antitoxin potency value in IUs to the selected candidate material. It was proposed that only the one candidate be included in the study given the comparability of the identified source material to the international standard it was to replace. Although suitable for the primary intended use, an assessment of commutability would also be needed to determine whether such material could also be used for the calibration of diagnostic kits and other immunoassays used for measuring tetanus antibody levels in human serum.

There was some discussion concerning the types of assays that would need to be included when calibrating the candidate material against the current international standard. It was agreed that both in vivo and in vitro assays would be included in the collaborative study. Following further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop a Second WHO International Standard for anti-tetanus immunoglobulin (human).

5.1.6 Proposed First WHO International Standard for activated coagulation factor XIa

Activated factor XIa (FXIa) has been implicated as a major factor in thromboembolic events associated with the infusion of intravenous immunoglobulins. In order to address an urgent need for a reference material to support the development of methods for FXIa estimation, allow comparison of FXIa levels in different therapeutic products and improve inter-laboratory harmonization the Committee established an interim reference reagent in 2012 (NIBSC code 11/236). Approximately half of the originally available 2700 ampoules of this reference reagent had now been issued with end users reporting excellent performance characteristics. A replacement international standard was now required.

The objective of the proposed collaborative study was the development of a First WHO International Standard for activated coagulation factor XIa. The source material had been purchased and filled into ampoules. Value assignment in IUs would be carried out relative to the current reference reagent. The bulk material would also undergo active site titration in a limited number of laboratories in order to estimate molar concentration for information purposes.

The Committee considered that the inclusion of thrombin-generation tests in the collaborative study would likely provide additional valuable characterization of the candidate material. Following further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop a First WHO International Standard for activated coagulation factor XIa.

6. International reference materials – in vitro diagnostic device reagents

All reference materials established at the meeting are listed in Annex 5.

6.1 WHO International Standards and Reference Reagents – in vitro diagnostic device reagents

6.1.1 First WHO International Reference Panel for HIV-1 circulating recombinant forms RNA for NAT-based assays

In 2003 NIBSC prepared an HIV-1 subtype reference panel to assist in the development of HIV nucleic acid amplification (NAT)-based assays across all subtypes. The replacement Second WHO Reference Panel for HIV-1 subtypes (NIBSC code 12/224) continues to fulfil expectations as an important tool in evaluating and assessing HIV NAT-based assays for subtype detection efficiency. In the meantime new circulating recombinant forms (CRFs) of HIV – in which the virus may exhibit different genotypes across genomic regions – have evolved. The development of an international reference panel containing a selection of more recent HIV recombinant forms would thus help manufacturers to assess any potential limitations within current assay systems.

Viruses were sourced from the NIBSC Centre for AIDS Reagents. Candidate materials were then grown via passage through human peripheral blood mononuclear cells and full-length sequences determined to the extent possible. Further manufacturing steps included dilution in human plasma (tested negative for anti-HIV-1/HIV-2; hepatitis B surface antigen (HBsAg); hepatitis C virus (HCV) RNA; and syphilis) and a lyophilization process. The lyophilized panel members included CRF 11 GJ, group O, CRF 02 AG, CRF01 AE, subtype J, CRF BG 24, subtype GUJ, subtype C, subtype G and CRF ADG.

The collaborative study involved 20 participant laboratories, with the Third WHO International Standard for HIV-1 RNA used in parallel. Users of quantitative assays were asked to report their quantitative results, with users of qualitative assays performing end-point dilutions. A total of 24 datasets were received and evaluated. The overall conclusion reached was that assays have improved since evaluation of the First WHO International Reference Panel for HIV-1 subtypes for NAT-based assays. It was therefore felt that the newly established CRF panel would provide an important supplementary resource in assessing detection efficiencies.

The Committee considered the report of the study (WHO/BS/2013.2226) and recommended that the panel be established as the First WHO International Reference Panel for HIV-1 circulating recombinant forms RNA for NAT-based assays – without the assignment of unitage to any individual panel member.

6.1.2 Second WHO International Standard for hepatitis A virus RNA for NAT-based assays

The First WHO International Standard for hepatitis A virus RNA for NAT-based assays (NIBSC code 00/560) has primarily been used by manufacturers of in vitro diagnostic devices, blood-product manufacturers, control authorities and clinical laboratories for hepatitis A virus (HAV) assay validation. There is an ongoing need to characterize the use of such assays for the detection of HAV contamination in plasma pools used in the manufacture of blood derivatives and in environmental and clinical samples. In Europe, for example, there is a requirement for assays to detect 100 IU/ml HAV RNA based upon the current WHO international standard.

As stocks of the this first international standard were low, a new lyophilized source material (human plasma, genotype 1A, NIBSC code 12/234) had been analysed in a WHO collaborative study, together with a lyophilized second bulk (NIBSC code 00/562) of the current standard. It was noted however that the latter material had been compromised by relatively high moisture (5.19%) and residual oxygen (14.7%) content. Two liquid-frozen positive plasma samples, along with the current standard were also included in the study.

Datasets of seven quantitative and 11 qualitative assays were assessed. For the candidate material 12/234 there was a 0.4 $\log_{10}$ discrepancy observed between quantitative and qualitative assays, while for 00/562 the overall difference in potency relative to 00/560 was similar when compared with the initial study in 2007, resulting now in a value of 54 000 IU/ml. However, simulation of transport conditions suggested degradation of the analyte at ambient temperature.

A proposal was therefore made to use 00/562 as the Second WHO International Standard for hepatitis A virus RNA for NAT-based assays for an interim period, and to manufacture a more-stable preparation in due course. In the meantime, the material should be shipped to users on dry ice, with specific instructions to store the material at −20 °C.

The Committee considered the report of the study (WHO/BS/2013.2225) and recommended that the candidate material 00/562 be established as the Second WHO International Standard for hepatitis A virus RNA for NAT-based assays with an assigned potency of 54 000 IU/ml (~4.73 $\log_{10}$ IU/ml). Shipment would take place on dry ice. It was further recommended that residual vials of the First WHO International Standard be retained for subsequent calibration studies of replacement standards to restrict potential drift of the unitage as far as possible. It was agreed that an update report on the preparation of a Third WHO International Standard would be provided to the Committee in 2015.

6.1.3 First WHO International Standard for hepatitis B virus e antigen

The hepatitis B envelope antigen (HBeAg) is a marker for high levels of viral replication and is therefore associated with the potentially high infectivity of

patients or materials infected with hepatitis B virus (HBV). HBeAg is also used to inform decisions on the initiation or continuation of antiviral therapy. Since 1982 an HBeAg reference preparation for the calibration of HBeAg tests has been distributed by PEI. This well characterized material is expressed in "PEI units" (100 PEI U/ml) and was chosen as the source material for the development of a candidate First WHO International Standard for hepatitis B envelope antigen (coded 129097/12). The candidate was characterized in relation to further viral markers (HBV DNA 20 000 IU/ml; positive for HBsAg, anti-HBc, anti-HCV and HCV RNA (< 30 IU/ml); and negative for anti-HBs, anti-HBe, HCV core Ag, anti-HIV-1/2 and anti-HDV). Lyophilization resulted in more than 2000 ampoules (containing 0.5 ml) with a fill coefficient of variation (CV) of 0.9% and a residual moisture content of 1.3%.

An international collaborative study of the candidate material involving 19 laboratories from 12 countries was conducted in 2012. In addition to the freeze-dried candidate material a number of other samples were also assessed. Relative potencies were determined by parallel line assay. The candidate material was assessed relative to the assigned unitage of the PEI standard material (100 PEI U/ml). The performance of the candidate material was similar to that of the samples studied in parallel and its commutability was assumed. An additional more extended commutability study using nine seroconversion panels resulted in the same ranking order for selected assays, reconfirming the commutability of the candidate material. The overall potency of the candidate material was 95.1 U/ml when determined by the geometric mean of all results or 95.8 U/ml when determined by parallel line assay. As the candidate was not a replacement for a previous standard it was considered reasonable to propose that it be assigned a unitage of 100 IU/ml.

The Committee considered the report of the study (WHO/BS/2013.2228) and recommended that the candidate material 129097/12 be established as the First WHO International Standard for hepatitis B virus e antigen with an assigned potency of 100 IU/ml.

6.1.4 First WHO International Standard for anti-hepatitis B virus e antibodies

The course of HBV infection is characterized by a transition from the presence of HBeAg to the production of antibodies against it (anti-HBe). While HBeAg reflects high levels of replication of HBV, seroconversion to anti-HBe is often used as the end-point for therapeutic treatment. There may be a small period of overlap of the two markers. Furthermore, detection of anti-HBe is useful evidence against a potential false-positive result for antibodies against hepatitis B core antigen (anti-HBc) in blood screening. Since 1982 a PEI reference preparation for anti-HBe (100 PEI U/ml) has been offered to kit manufacturers to calibrate assays in PEI units or to the limit of detection (sensitivity) by dilutional analysis.

During 2012–13 an international collaborative study was conducted involving 21 laboratories from 12 countries to develop a First WHO International Standard for anti-hepatitis B virus e antibodies. Among the different materials selected for study were a candidate international standard (coded 129095/12), the PEI reference preparation for anti-HBe and samples obtained by dilution of anti-HBe-positive plasma from three individuals.

The assays used in the collaborative study were of competitive (n = 14) or indirect (n = 2) test format, with 15 being qualitative and one quantitative. The mean potency of the candidate material relative to the PEI reference, as determined by parallel line assay, was 120 PEI U/ml after exclusion of two assays from the calculation. The results for the other samples were similar across the 16 test kits used, providing evidence for the commutability of the candidate material. Commutability was further assessed using seroconversion panels tested in five selected kits. Based upon the results obtained with these additional specimens, the sensitivity ranking of the kits followed the same order as that obtained when using the candidate material, reconfirming its commutability.

The Committee considered the report of the study (WHO/BS/2013.2229) and recommended that the candidate material 129095/12 be established as the First WHO International Standard for anti-hepatitis B virus e antibodies with an assigned potency of 120 IU/ml.

6.1.5 First WHO International Standard for hepatitis D virus RNA for NAT-based assays

Hepatitis D virus (HDV) is a defective virus dependent for its replication upon HBV acting as a "helper" virus. Approximately 5% of HBV carriers worldwide (10–15 million individuals) are co-infected with HDV with the implication of a 10-fold greater mortality rate in this group. The previously used serological assays for the detection of HDV are now being superseded by more-sensitive NAT-based assays. However, such assays are developed in-house and there is a need for standardization in this area – particularly given the reliance of treatment decisions on the results obtained.

Following a request to develop a First WHO International Standard for hepatitis D virus RNA for NAT-based assays, an international collaborative study was conducted involving 15 laboratories from 10 countries. A range of candidate materials harbouring HDV genotype 1 were characterized with one material of high HDV titre (coded 7657/12) chosen as a candidate for development of the WHO international standard. Freeze-drying was shown to have no impact on HDV detectability with stability studies indicating no degradation at the recommended storage temperature.

The candidate material was calibrated using a total of 19 globally representative qualitative and quantitative HDV NAT-based assays. Other materials tested in parallel included the liquid bulk and an HBV-positive patient

sample. Evaluation of study results revealed the harmonization of assay results when using the candidate material. The vast majority of the assays also showed more-consistent quantification of the clinical sample. Based on the mean value reported by the different assays used 575 000 PCR-detectable units/ml were calculated for the candidate material. It was concluded that this preparation appeared well suited for use as an international standard.

The Committee considered the report of the study (WHO/BS/2013.2227) and recommended that the candidate material 7657/12 be established as the First WHO International Standard for hepatitis D virus RNA for NAT-based assays, with an assigned potency of 575 000 IU/ml. The Committee also recommended that the commutability of the candidate material should be further evaluated and the results presented at its 2014 meeting.

6.1.6 Third WHO International Standard for parvovirus B19 DNA for NAT-based assays

Parvovirus B19 (B19V) is a pathogenic virus that is widely distributed in the human population, and is responsible for various pathologies and diverse clinical manifestations. Quantifying B19V contamination of plasma pools used in the manufacture of blood derivatives is a necessary measure in reducing the risk of infection transmission. In Europe, NAT-based assays are the recommended quantification method for B19V DNA in human plasma (pooled and treated for virus inactivation) and plasma pools used in the manufacture of human anti-D immunoglobulin. Plasma pools containing more than 10^4 IU/ml B19V DNA are excluded with similar guidance provided in other parts of the world. In addition, the detection and quantification of B19V in clinical samples frequently relies upon the WHO international standard. As a result there is demand for the international standard from blood-product manufacturers, control authorities, in vitro diagnostic device manufacturers and clinical laboratories.

Stocks of the current preparation (NIBSC code 99/802) used as the Second WHO International Standard for parvovirus B19 DNA for NAT-based assays were diminishing and needed to be replaced. An international collaborative study had therefore been conducted involving 17 laboratories from 12 countries. Two lyophilized batches (NIBSC codes 12/208 and 12/238) of a new material containing the same B19 genotype as 99/802 were produced as candidate preparations and assessed alongside 99/802 and two B19V-positive plasma specimens intended to provide information on commutability.

A total of 20 datasets were received, representing seven different commercial assays and six in-house assays. Based upon quantitative assays, the overall mean potency estimates for candidates 12/208 and 12/238 were approximately 6.15 $\log_{10}$ IU/ml relative to the current international standard. There was no evidence of non-commutability of the reference preparations when data from the plasma specimens were assessed. Accelerated thermal degradation

studies at 3 months indicated that both candidates were stable and suitable for long-term use.

The Committee considered the report of the study (WHO/BS/2013.2224) and recommended that the candidate material 12/208 be established as the Third WHO International Standard for B19V DNA for NAT-based assays with an assigned potency of 1 410 000 IU/ml (~6.15 $\log_{10}$ IU/ml).

6.1.7 First WHO International Standard for mycoplasma DNA for NAT-based assays

Mycoplasmas (trivial name for bacteria in the class Mollicutes) comprise a range of phylogenetically related bacteria species with a number of common biological features. Mycoplasmas are human or veterinary pathogens causing chronic infections. In addition, they may also contaminate cells or cell cultures used in the manufacture of biopharmaceuticals. To test for the potential mycoplasma contamination of such source materials or final products mycoplasma culture methods had been defined in detail in numerous national regulatory documents worldwide. With the development of NAT-based assays these laborious methods were being replaced by molecular mycoplasma testing, necessitating the development of an international standard in this area.

The development of a First WHO International Standard for mycoplasma DNA for NAT-based assays involved a feasibility study followed by a comparability study. The feasibility study investigated the detection efficiency for four distantly related mycoplasma species (*Acholeplasma laidlawii*, *Mycoplasma fermentans*, *M. orale* and *M. pneumoniae*) using a variety of (semi)-quantitative and qualitative NAT-based assays designed for generic mycoplasma detection and used worldwide. The results of the feasibility study led to the selection of a preparation of *M. fermentans* as the most promising candidate material for achieving a high level of harmonization between NAT-based assays.

In the comparability study phase involving 21 laboratories from nine countries the candidate preparation (strain NCTC 10117) was characterized against its counterpart from the feasibility study and an assigned unitage of 200 000 IU/ml (reflecting the PCR-detectable units) proposed.

The Committee considered the report of the study (WHO/BS/2013.2222) and recommended that the candidate material (strain NCTC 10117) be established as the First WHO International Standard for mycoplasma DNA for NAT-based assays with an assigned potency of 200 000 IU/ml.

6.1.8 Third WHO International Standard for human serum immunoglobulin E

Measurement of serum immunoglobulin E (IgE) is used for the diagnosis and management of atopic allergic disease and hyper-IgE immunodeficiency

syndromes. The results of external quality assurance schemes had indicated that the Second WHO International Reference Preparation of human serum immunoglobulin E (NIBSC code 75/502) was effective in maintaining good agreement between different kit methods. However, declining stocks of this material had necessitated its replacement.

The source material selected as a candidate Third WHO International Standard for human serum immunoglobulin E (NIBSC code 11/234) was a mixture of pooled human serum and defibrinated plasma from donors with elevated levels of IgE. An international collaborative study involving 18 laboratories from 11 countries was conducted to evaluate the lyophilized candidate material relative to 75/502. The mean estimate for 75/502, calculated relative to local standards, was found to accord extremely well with the value assigned over 30 years ago and provided some degree of reassurance that most methods for determining serum IgE were well calibrated and standardized.

A high level of agreement was observed in the results produced by the different laboratories (geometric coefficient of variation (GCV) = 3.8%) for the candidate material 11/234 relative to 75/502, with a mean potency value of 13 411 IU/ml obtained using parallel line analysis. Given a corresponding mean potency value based upon the calculations of individual laboratories of 13 627 IU/ml it was proposed that a value of 13 500 IU/ml should be assigned to the new standard. As the reconstitution volume for 11/234 was 0.5 ml this value corresponded to 6750 IU/ampoule.

During freeze-drying of the candidate material 11/234, an issue with the stoppering process had resulted in approximately 12% of the ampoules having a high oxygen content in the headspace. However, since no predictable loss of stability had been observed in accelerated degradation studies of high oxygen content ampoules, it was proposed that these ampoules simply be dispatched first, with annual stability checks carried out for 3 years for additional reassurance.

Discussion then took place on the length of time the ampoules may be kept at 20 °C without loss in relation to possible deterioration during shipment – with stability studies having indicated that the preparation would not be affected by normal shipping durations. The possibility of backfilling ampoules with argon was raised given that argon is heavier than nitrogen and might help reduce the incidence of air ingress before sealing. It was indicated that improvements in post-lyophilization vial-closure procedures were expected to reduce or eliminate this problem.

The Committee considered the report of the study (WHO/BS/2013.2220) and recommended that the candidate material 11/234 be established as the Third WHO International Standard for human serum immunoglobulin E with an assigned potency of 13 500 IU/ml.

6.2 Proposed new projects and updates – in vitro diagnostic device reagents

6.2.1 Proposal to develop a panel of recombinant antigens for the evaluation and quality control of malaria rapid diagnostic tests

Worldwide an increasing number of test systems for the rapid diagnosis of malaria infections are becoming available and are used for diagnosis. Efforts were under way to use recombinant parasite antigens to partially replace parasite samples in the evaluation and lot testing of devices. Such an approach could potentially lead to highly consistent and reproducible device testing, provided that the equivalence of recombinant antigens with natural proteins could be demonstrated. Dose–response curves obtained for different antigen/device combinations have already indicated proof of concept.

The Committee was informed that a process of collection and testing of recombinant candidates was now under way (WHO/ECBS 2013 Discussion doc.1). To date, two recombinant histidine-rich protein II (HRP2) antigens had shown good correlation for the detection of plasmodia – however, fLDH, vLDH and aldolase antigens have performed less well. Further activities include searching for further suitable antigens of both *Plasmodium vivax* and *P. falciparum*, and investigating the potential advantages of eukaryotic expression.

The Committee noted the developments that had been outlined.

6.2.2 Proposal to develop a malaria antibody reference panel

Malaria antibody reference panels manufactured from the sera of individuals infected with either *P. falciparum* or *P. vivax* would be potentially valuable tools for manufacturers of the respective detection kits, and might be very useful in vaccine development and standardization activities. Currently there is no United States Food and Drug Administration-licensed antibody detection test for malaria available. Such panels could be used by regulators, for example in the lot release of kits. The proposed study would use plasma from individuals from Ghana infected with *P. falciparum*, with antibody specificity (for *P. falciparum*) and antibody epitopes (asexual plasmodium stage) to be confirmed by the indirect fluorescent antibody test (IFAT).

An existing pool of 10 individual high-titre sera would be used as the primary source material. The design of the five panel members would include the dilution of suitable materials to target high, medium, low, indeterminate and absent reactivity in IFAT. Both liquid and lyophilized materials would be compared, and a stability study conducted covering different temperatures between −80 °C and 45 °C. The panel would be characterized by different laboratories using ELISA and IFAT. The panel would also be accompanied by 12-well toxoplasmosis slides containing *P. falciparum* asexual-stage parasites in each well.

Discussion was held on a number of different aspects, including the extent to which dilutional sensitivity reflected diagnostic sensitivity, and how predictable high and low reactivity was for different assays such as ELISAs potentially based upon different recombinant antigens. The intended field use of the panel in patient diagnosis or vaccine trials was also discussed.

Following further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop a malaria antibody reference panel with the strong recommendation that a consultation be held, with the involvement of vaccine experts, to clarify precisely the intended uses of the proposed panel.

6.2.3 Proposed Fifth WHO International Standard for hepatitis C virus RNA for NAT-based assays

It is estimated that over 200 million people worldwide are infected with HCV with an overall incidence of around 3.3% of the world's population. In many parts of the world NAT-based testing of blood and blood products for HCV RNA is mandated. The current Fourth WHO International Standard for hepatitis C virus RNA for NAT-based assays (NIBSC code 06/102) is used for the calibration of secondary reference preparations, the validation of HCV NAT-based assays used in the safety testing of blood and blood products and for the clinical management of HCV infections.

In contrast to the first and second WHO international standards (sourced from anti-HCV-positive material), the subsequent Third and Fourth International Standards have exhibited stability issues (analyte-concentration drops) under ambient conditions, including during transport. As a result the current international standard is now being shipped on dry ice. It was therefore decided, despite sufficient stocks remaining, to bring forward its replacement.

In 2013 a pilot study was conducted to investigate the stability of HCV RNA in lyophilized anti-HCV-positive and -negative materials, and the potential impact on stability of substances such as HEPES or trehalose. The outcome of this study would determine the choice of the candidate material for the Fifth WHO International Standard for hepatitis C virus RNA for NAT-based assays, which is planned to be filled in approximately 6000 vials.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop a Fifth WHO International Standard for hepatitis C virus RNA for NAT-based assays.

6.2.4 Proposed First WHO International Standard for anti-cytomegalovirus immunoglobulin G (plasma)

Human cytomegalovirus (HCMV) is associated with very high seroprevalence rates (for example in Europe: 33–73%; worldwide 60–100%) which increase with increasing age. HCMV infection poses increased risks of morbidity and

mortality following perinatal transmission to neonates or transmission to transplant recipients, premature infants and immunocompromised individuals in general. Despite a reduction of HCMV levels by leukodepletion, cellular blood components tested negative for HCMV are still required for specific patient groups (such as haemopoietic stem cell transplant patients, neonates, pregnant women and recipients of intrauterine transfusions). HCMV serology is further used for determining donor and recipient serostatus in transplantations, and for monitoring candidate HCMV vaccines.

An internationally accepted reference material for anti-HCMV immunoglobulin G (IgG) plasma is needed to determine the analytical sensitivity of anti-HCMV IgG assays in a range of calibration, validation and regulatory applications. The Committee was informed that three plasma donations with a high anti-HCMV IgG level and avidity and absence of detectable anti-HCMV IgM had been characterized during a PEI feasibility study. A pool of these materials might serve as a well characterized source for a candidate first WHO international standard, already providing the data basis for its potential replacement by an equivalent preparation. Further materials needed to address issues of commutability and specificity in the proposed collaborative study had also been identified. If considered to be useful following further evaluations, an anti-HCMV (IgG) plasma negative preparation could also be developed in parallel.

Following discussion and further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop a First WHO International Standard for anti-cytomegalovirus immunoglobulin G (plasma).

6.2.5 Proposal to develop reference panels for the standardization of bioassays for cancer diagnostics

The Committee was provided with a global health overview relating to cancer. There are over 12 million new cases of cancer each year and 7.6 million deaths with the global burden expected to increase significantly by 2030. Although there are similar levels of mortality in different geographical regions there are wide variations in cancer burden in terms of incidence. In particular, the incidence of the most-common cancers (breast, lung and colorectal) vary with geographical region, gender and by human development index (HDI) measures. Patterns of cancer are thus heterogeneous across the world and prevention efforts must be prioritized according to national and regional needs.

By 2030 the projected burden of cancer is expected to increase most in populations characterized by low scores in the three HDI components of human development – namely, long and healthy life; access to knowledge; and decent standard of living. The number of new cases is projected to almost double in the next two decades with a higher burden placed on the lower scoring HDI countries. Cancer will thus continue to make a major contribution to the global noncommunicable disease burden.

A proposal for the preparation of generic standards and/or reference panels for cancer diagnostics to standardize bioassays was put to the Committee for its endorsement. This would enable the harmonization of diagnostic test performance and reduce the incidence of false-negative and false-positive results. Reference panels would consist of a range of dilutions of mutant DNA in wild-type DNA extracted from cell lines, and may need to contain both minimum and maximum potency standards. Typically, assays were expected to be able to detect about 1% tumour cells in a background of normal cells.

In addition, several panels were being considered for production in the next 3 years and endorsement for one specific panel was also requested from the Committee. This panel would assist in the detection of JAK2 V617F-positive patients by quantitative polymerase chain reaction (qPCR) analysis of blood DNA. The JAK2 V617F mutation was frequently found in chronic myelogenous leukaemia patients negative for the BCR-ABL translocation. Although a CE-marked control panel was already available a global international reference panel was required, and would be made available to manufacturers of assays and companion diagnostics and to diagnostic laboratories for the assurance of method validity and sensitivity.

The Committee decided that the proposal in relation to the generic preparation of standards and/or reference panels for cancer diagnostics represented a large commitment that required urgent clarification from WHO in regard to the envisaged scope and resources of the Committee. The Committee endorsed the specific proposal for the development of an international reference panel for the JAK2 V617F mutation.

6.2.6 Proposed First WHO Reference Reagent for high-titre anti-A and anti-B

There is a need for plasma- or serum-based reference reagents for anti-A and anti-B estimation in plasma-rich blood components (such as platelet concentrates) and also in relation to kidney transplantation where mismatched transplants can be performed successfully if the recipient has sufficiently low levels of anti-A and anti-B. Such reference reagents are intended to support mismatched kidney transplants by improving the reliability of the estimated recipient anti-A and anti-B titres. The preparations would be plasma- or serum-based depending upon the outcome of a pilot study and would be assigned nominal anti-A and anti-B titres.

One main objective of developing suitable reference reagents would be to improve methodology standardization at the local, national and international level, and potentially to define cut-off limits. It was being proposed that both high- and low-titre preparations would be evaluated.

Discussion centred on the envisaged level of need for both high- and low-titre preparations and on the possibility that a fixed low "cut-off" level may not be globally applicable. Other issues raised included the use of either

plasma or serum in relation to the risk of coagulation, and the fact that test samples could be either plasma (donors) or serum (potential recipients). Following further consideration, the Committee endorsed the proposal (WHO/BS/2013.2230) to develop reference reagents for high-titre anti-A and anti-B in serum and/or plasma.

6.2.7 Update on the endorsed proposal for a First WHO International Reference Plasma Panel for lupus anticoagulant

The Committee was presented with a summary of ongoing and new standard projects in the areas of haemostasis and thrombosis (therapeutic products and in vitro diagnostic devices), together with an update on the development of a First WHO International Reference Plasma Panel for lupus anticoagulant (WHO/ECBS 2013 Discussion doc.2). This project had been initiated by the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) in 2002 and subsequently endorsed by the Expert Committee in 2006. Following completion of the collection of plasma samples from a large number of lupus-positive patients in 2010 and associated pilot studies, three candidate materials had been filled in 2012.

These materials comprised a lupus-negative sample, a moderate positive sample and a strong positive sample. The samples were evaluated in a collaborative study involving 19 laboratories from 11 countries. Analysis of the results had now been completed.

The study was currently being reviewed by SSC-associated experts and subject to their findings the candidate panel would be proposed for formal establishment by the Committee in 2014.

The Committee noted the developments that had been outlined.

6.2.8 Proposals for discontinuation or replacement of WHO biological reference preparations for blood products and related substances

The current list of WHO biological reference preparations comprises a total of 126 blood products and related substances. Although the majority (approximately 70%) of preparations had been established within the past 12 years, 19 standards had been established prior to 1990. Of these, 10 preparations had been identified as now being of low interest and/or having only limited information in respect of their origin, characteristics and/or calibration. A review had therefore been undertaken to evaluate the appropriateness of either discontinuing or replacing the following preparations:

- Anti-echinococcus serum – established in 1975 for the detection of the parasitic infection. Most of the test methods used in the original collaborative study had been superseded by more specific and

sensitive techniques such as ELISA and immunoblotting. An expert group had indicated that this reagent was no longer of use due to poor characterization.

- Anti-c incomplete, Anti-E complete, Anti-C complete blood typing sera – originally established for the detection of Rh antigens in red blood cells. However, current commercial test reagents for Rh phenotype detection are monoclonal preparations potentially explaining the low usage of these reference preparations.
- Anti-nuclear factor (homogeneous) serum, Anti-nuclear ribonucleoprotein serum, Anti-smooth muscle (anti-actin) serum – it was proposed that discontinuation or further testing of these three preparations now be considered because of: (a) very limited information and/or poor characterization in WHO documents; (b) methods for autoantibody testing had evolved since their establishment; and (c) the tests for these autoantibodies had limited impact on clinical decisions.
- Human serum complement components C1q, C4, C5 factor B and total complement CH50 – discontinuation was proposed on the basis of low usage and the limited information available on the preparation; commercial standards were also now available.
- β-thromboglobulin (β-TG) and Platelet factor 4 (PF4) – increased plasma levels have been used as a marker of abnormal platelet activation related to thrombotic disorders. However, current commercial ELISA kits provide recombinant protein standards for β-TG and PF4. There was now a need to review the utility of these standards and to consider the need for their replacement.

The Committee endorsed the proposal to discontinue the Anti-echinococcus serum standard. Anti-E complete and Anti-C complete blood typing sera would also be discontinued and potentially replaced by minimum potency standards based on monoclonal preparations for Anti-E and anti-C, together with a new standard developed for anti-e. For the other materials, it was concluded that further information on their utility should be sought from clinical experts and users before a decision was taken. The Committee further agreed that preparations which are discontinued should not be destroyed but instead stored at NIBSC and made available for issue, for example in relation to research applications, subject to an agreed policy.

7. International reference materials – biotherapeutics other than blood products

All reference materials established at the meeting are listed in Annex 5.

7.1 WHO International Standards and Reference Reagents – biotherapeutics other than blood products

7.1.1 Third WHO International Standard for tumour necrosis factor alpha (human, recombinant)

Human tumour necrosis factor alpha (TNF-α) is involved in the regulation of immune cells and is produced mainly by macrophages. Based on its ability to induce cytotoxic activity and inhibit tumorigenesis, it is used therapeutically as an adjunct to surgery for soft tissue sarcoma of the limbs. However, since TNF-α also promotes inflammatory responses that cause many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis and psoriasis there is also high interest in developing inhibitors of its activity.

In 2012 the Committee was informed that stocks of the Second WHO International Standard for tumour necrosis factor alpha (human, natural) had become almost entirely depleted. There was thus a recognized need to develop a replacement international standard suitable for the assignment of potency to preparations of therapeutic human TNF-α and of critical reagents used in the potency evaluation of several TNF-α antagonists.

An international collaborative study was carried out to evaluate a candidate preparation (NIBSC code 12/154) against the existing international standard (NIBSC code 88/786). Eighteen laboratories in 10 countries participated in the study, with results indicating that the candidate standard was sufficiently stable on the basis of a thermally accelerated degradation study to serve as an international standard for use in bioassay. Approximately 7800 ampoules were available which at the current rate of demand represented around 15 years' supply.

The Committee considered the report of the study (WHO/BS/2013.2219) and recommended that preparation 12/154 be established as the Third WHO International Standard for tumour necrosis factor alpha (human, recombinant) for use in bioassay, with an assigned bioactivity of 43 000 IU/ampoule. The Committee requested that the Information for Use contain a statement which made it clear that the standard was recommended for use only in bioassay.

7.1.2 First WHO International Standard for PEGylated granulocyte colony-stimulating factor

Human granulocyte colony-stimulating factor (G-CSF) is approved for use in indications relating to neutropenia. Due to its short half-life, a modified

(PEGylated) form of G-CSF, which has an enhanced half-life, is approved for use. Although some manufacturers have measured the activity of their PEG products in bioassays calibrated using the Second WHO International Standard for granulocyte colony-stimulating factor, the suitability of this approach has not been formally established. In addition, calibration practices were likely to vary between manufacturers potentially leading to the development of products with discrepant bioactivities. A reference standard was therefore required to determine the biological activity of such products.

An international collaborative study was carried out to evaluate a candidate preparation (NIBSC code 12/188) involving 23 laboratories in 11 countries. The biological activity of several PEG-G-CSF products was evaluated relative to the Second WHO International Standard for granulocyte colony-stimulating factor (the unmodified parent molecule) in an in vitro cell-based bioassay using a G-CSF responsive cell line. Although preliminary data derived from dose–response curves suggested that the current international standard for G-CSF could potentially serve as the international standard for PEG-G-CSF products, a degree of variability in potency estimates was observed. As result, the feasibility of establishing a PEG-G-CSF preparation as an international standard was explored.

About 4700 ampoules of the candidate preparation were available, with the material found to be stable for 7 months at 45 °C. Stability studies were ongoing. As the standard was only evaluated for use in in vitro bioassays, it could not be assumed to be suitable for evaluation in vivo or for pharmacokinetic studies without suitable validation.

The Committee considered the report of the study (WHO/BS/2013.2218) and recommended that preparation 12/188 be established as the First WHO International Standard for PEGylated granulocyte colony-stimulating factor with an assigned in vitro bioactivity of 10 000 IU/ampoule. The Committee requested that the Information for Use clearly state that the fitness for purpose of the reference material in calibrating products PEGylated differently to the standard had not yet been evaluated. When such data became available, they should be reported back to the Committee.

Annex 1

WHO Recommendations, Guidelines and other documents related to the manufacture and quality control of biological substances used in medicine

The Recommendations (previously called Requirements) and Guidelines published by WHO are scientific and advisory in nature but may be adopted by an NRA as national requirements or used as the basis of such requirements.

These international Recommendations are intended to provide guidance to those responsible for the production of biological substances as well as to others who may have to decide upon appropriate methods of assay and control to ensure that products are safe, reliable and potent.

Recommendations concerned with biological substances used in medicine are formulated by international groups of experts and are published in the WHO Technical Report Series[1] as listed below. A historical list of Requirements and other sets of Recommendations is available on request from the World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland.

Reports of the WHO Expert Committee on Biological Standardization published in the WHO Technical Report Series can be purchased from:

WHO Press
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland
Telephone: + 41 22 791 3246
Fax: +41 22 791 4857
Email: bookorders@who.int
Website: www.who.int/bookorders

Individual Recommendations and Guidelines may be obtained free of charge as offprints by writing to:

Technologies Standards and Norms
Department of Essential Medicines and Health Products
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland

[1] Abbreviated in the following pages to "TRS".

Recommendations, Guidelines and other documents	Reference
Animal cells, use of, as in vitro substrates for the production of biologicals	Revised 2010, TRS 978 (2013)
BCG vaccines (dried)	Revised 2011, TRS 979 (2013)
Biological products: good manufacturing practices	Adopted 1991, TRS 822 (1992)
Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (1997)	Unpublished document WHO/BLG/97.1
Biological substances: International Standards and Reference Reagents	Revised 2004, TRS 932 (2006)
Biotherapeutic protein products prepared by recombinant DNA technology	Revised 2013, TRS 987 (2014)
Biotherapeutic products, similar	Adopted 2009, TRS 977 (2013)
Blood, blood components and plasma derivatives: collection, processing and quality control	Revised 1992, TRS 840 (1994)
Blood establishments: good manufacturing practices	Adopted 2010, TRS 961 (2011)
Blood plasma (human) for fractionation	Adopted 2005, TRS 941 (2007)
Blood plasma products (human): viral inactivation and removal procedures	Adopted 2001, TRS 924 (2004)
Blood regulatory systems, assessment criteria for national	Adopted 2011, TRS 979 (2013)
Cholera vaccines (inactivated, oral)	Adopted 2001, TRS 924 (2004)
Dengue tetravalent vaccines (live, attenuated)	Revised 2011, TRS 979 (2013)
Diphtheria, tetanus, pertussis (whole cell), and combined (DTwP) vaccines	Revised 2012, TRS 980 (2014)
Diphtheria vaccines (adsorbed)	Revised 2012, TRS 980 (2014)
DNA vaccines: assuring quality and nonclinical safety	Revised 2005, TRS 941 (2007)
Haemophilus influenzae type b conjugate vaccines	Revised 1998, TRS 897 (2000)

Recommendations, Guidelines and other documents	Reference
Haemorrhagic fever with renal syndrome (HFRS) vaccines (inactivated)	Adopted 1993, TRS 848 (1994)
Hepatitis A vaccines (inactivated)	Adopted 1994, TRS 858 (1995)
Hepatitis B vaccines prepared from plasma	Revised 1987, TRS 771 (1988)
Hepatitis B vaccines made by recombinant DNA techniques	Revised 2010, TRS 978 (2013)
Human interferons prepared from lymphoblastoid cells	Adopted 1988, TRS 786 (1989)
Influenza, biosafety risk assessment and safe production and control for (human) pandemic vaccines	Adopted 2005, TRS 941 (2007)
Influenza vaccines (inactivated)	Revised 2003, TRS 927 (2005)
Influenza vaccines (live)	Revised 2009, TRS 977 (2013)
Influenza vaccines, human, pandemic, regulatory preparedness	Adopted 2007, TRS 963 (2011)
Japanese encephalitis vaccines (inactivated) for human use	Revised 2007, TRS 963 (2011)
Japanese encephalitis vaccines (live, attenuated) for human use	Revised 2012, TRS 980 (2014)
Louse-borne human typhus vaccines (live)	Adopted 1982, TRS 687 (1983)
Malaria vaccines (recombinant)	Adopted 2012, TRS 980 (2014)
Measles, mumps and rubella vaccines and combined vaccines (live)	Adopted 1992, TRS 848 (1994); Note TRS 848 (1994)
Meningococcal polysaccharide vaccines	Adopted 1975, TRS 594 (1976); Addendum 1980, TRS 658 (1981); Amendment 1999, TRS 904 (2002)
Meningococcal A conjugate vaccines	Adopted 2006, TRS 962 (2011)
Meningococcal C conjugate vaccines	Adopted 2001, TRS 924 (2004); Addendum (revised) 2007, TRS 963 (2011)
Monoclonal antibodies	Adopted 1991, TRS 822 (1992)
Papillomavirus vaccines (human)	Adopted 2006, TRS 962 (2011)
Pertussis vaccines (acellular)	Revised 2011, TRS 979 (2013)

Recommendations, Guidelines and other documents	Reference
Pertussis vaccines (whole-cell)	Revised 2005, TRS 941 (2007)
Pharmaceutical products, storage and transport of time- and temperature-sensitive	Adopted 2010, TRS 961 (2011)
Pneumococcal conjugate vaccines	Revised 2009, TRS 977 (2013)
Poliomyelitis vaccines (inactivated)	Revised 2000, TRS 910 (2002)
Poliomyelitis vaccines (inactivated): guidelines for the safe production and quality control of inactivated poliomyelitis vaccine manufactured from wild polioviruses	Adopted 2003, TRS 926 (2004)
Poliomyelitis vaccines (oral)	Revised 2012, TRS 980 (2014)
Quality assurance for biological products, guidelines for national authorities	Adopted 1991, TRS 822 (1992)
Rabies vaccines for human use (inactivated) produced in cell substrates and embryonated eggs	Revised 2005, TRS 941 (2007)
Regulation and licensing of biological products in countries with newly developing regulatory authorities	Adopted 1994, TRS 858 (1995)
Rotavirus vaccines (live-attenuated, oral)	Adopted 2005, TRS 941 (2007)
Smallpox vaccines	Revised 2003, TRS 926 (2004)
Snake antivenom immunoglobulins	Adopted 2008, TRS 964 (2012)
Sterility of biological substances	Revised 1973, TRS 530 (1973); Amendment 1995, TRS 872 (1998)
Synthetic peptide vaccines	Adopted 1997, TRS 889 (1999)
Tetanus vaccines (adsorbed)	Revised 2012, TRS 980 (2014)
Thiomersal for vaccines: regulatory expectations for elimination, reduction or removal	Adopted 2003, TRS 926 (2004)
Thromboplastins and plasma used to control oral anticoagulant therapy	Revised 2011, TRS 979 (2013)
Tick-borne encephalitis vaccines (inactivated)	Adopted 1997, TRS 889 (1999)
Transmissible spongiform encephalopathies in relation to biological and pharmaceutical products, guidelines	Revised 2005, WHO (2006) http://www.who.int/biologicals/publications/en/whotse2003.pdf

Recommendations, Guidelines and other documents	Reference
Tuberculins	Revised 1985, TRS 745 (1987)
Typhoid vaccines, conjugated	Adopted 2013, TRS 987 (2014)
Typhoid vaccines (live attenuated, Ty21a, oral)	Adopted 1983, TRS 700 (1984)
Typhoid vaccines, Vi polysaccharide	Adopted 1992, TRS 840 (1994)
Vaccines, clinical evaluation: regulatory expectations	Adopted 2001, TRS 924 (2004)
Vaccines, lot release	Adopted 2010, TRS 978 (2013)
Vaccines, nonclinical evaluation	Adopted 2003, TRS 926 (2004)
Vaccines, nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines	Adopted 2013, TRS 987 (2014)
Vaccines, prequalification procedure	Adopted 2010, TRS 978 (2013)
Vaccines, stability evaluation	Adopted 2006, TRS 962 (2011)
Varicella vaccines (live)	Revised 1993, TRS 848 (1994)
Yellow fever vaccines	Revised 2010, TRS 978 (2013)
Yellow fever vaccines, laboratories approved by WHO for the production of	Revised 1995, TRS 872 (1998)
Yellow fever virus, production and testing of WHO primary seed lot 213-77 and reference batch 168-736	Adopted 1985, TRS 745 (1987)

Annex 2

Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines

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Guidelines published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

Introduction

These Guidelines are intended to provide guidance to NRAs and manufacturers on the nonclinical and initial clinical evaluation of vaccine adjuvants and adjuvanted vaccines by outlining international regulatory expectations in this area. The Guidelines should be read in conjunction with existing WHO guidelines on nonclinical (*1*) and clinical (*2*) evaluation of vaccines. There is substantial diversity among vaccine adjuvants and adjuvanted vaccines and their nonclinical and clinical testing programmes will depend upon product-specific features and their clinical indications. Therefore, the following text is written in the form of WHO Guidelines instead of Recommendations. Guidelines allow greater flexibility than Recommendations with respect to specific issues related to particular adjuvanted vaccines.

Over the past decades, strategies and approaches for the development and delivery of vaccine antigens have been expanded. Some of these antigens are weakly immunogenic and require the presence of adjuvants for the induction or enhancement of an adequate immune response. Vaccines with aluminium-based adjuvants have been used extensively in immunization programmes worldwide and a significant body of safety information has accumulated for them (*3*, *4*). As the knowledge of immunology and the mechanisms of vaccine adjuvant action have developed, the number of vaccines containing novel adjuvants being evaluated in clinical trials has increased. Vaccines containing adjuvants other than aluminium-containing compounds have been authorized for use in many countries (e.g. human papillomavirus and hepatitis B vaccines), and a number of vaccines with novel adjuvants are currently under development, including, but not limited to, vaccines against human immunodeficiency virus (HIV), malaria and tuberculosis, as well as new-generation vaccines against influenza and other diseases. However, the development and evaluation of adjuvanted vaccines present regulatory challenges. Vaccine manufacturers and regulators have questions about the type of information and extent of data that would be required to support proceeding to clinical trials with adjuvanted vaccines and to eventual authorization.

Existing WHO guidelines on nonclinical evaluation of vaccines (*1*) provide valuable general guidance; however, they provide limited information specifically related to new adjuvants and adjuvanted vaccines. Some of the issues addressed here are also discussed in national or regional guidance documents (*5*, *6*). Given the importance and the complexity of the issues, this updated and more extensive guidance on the nonclinical and preclinical testing of adjuvants and adjuvanted vaccines should allow manufacturers and regulators to proceed in an efficient manner on the critical path towards development and licensure of adjuvanted vaccines indicated for the control of diseases with an important global public health impact.

Background

Over the past decades, there have been a number of international workshops and meetings in which the issues covered by these WHO Guidelines have been discussed (*7–12*). To address the need for additional international guidance on nonclinical evaluation of adjuvanted vaccines, a consultation was organized by WHO on 7–8 September 2011 in Rockville, Maryland, United States, to initiate the process of developing new WHO guidance on the subject. The consultation was attended by experts from academia, NRAs, national control laboratories and industry involved in the research, manufacture and approval of adjuvanted vaccines from countries around the world. The purpose was to review the scientific information and available data and to discuss and identify the issues to be considered for the development of such international guidance. On 27–28 November 2012, WHO organized an informal consultation at its headquarters in Geneva, Switzerland attended by academics, researchers, vaccine manufacturers and regulators involved in the evaluation of adjuvanted vaccines, to review draft WHO Guidelines prepared by the drafting group and to seek consensus on key regulatory issues. The approaches to nonclinical and initial clinical evaluation of vaccine adjuvants and adjuvanted vaccines discussed in this document are a result of the efforts of this and other international working groups.

Scope

This document addresses regulatory considerations related to the nonclinical and initial clinical evaluation of adjuvanted vaccines. The goal of this document is to provide consistent and harmonized guidance on nonclinical testing approaches to support the use of candidate adjuvanted vaccines in all stages of clinical development and ultimately for marketing authorization of the product. However, each NRA may determine the regulatory requirements applicable for adjuvanted vaccines to be marketed and used in their country.

Vaccine adjuvants are substances or combinations of substances that are used in conjunction with a vaccine antigen to enhance (e.g. increase, accelerate, prolong and/or possibly target) or modulate to a different type (e.g. switch a Th1 immune response to a Th2 response, or a humoral response to a cytotoxic T-cell response) the specific immune response to the vaccine antigen in order to enhance the clinical effectiveness of the vaccine (see "Terminology" section below). For the purposes of this document, the term "adjuvant" includes formulations that contain one individual adjuvant as well as adjuvant combinations that contain multiple adjuvants. These WHO Guidelines specifically address vaccine adjuvants that are either separate substances that are mixed with vaccine antigens and administered at the same time and location as the vaccine antigen, or immunostimulatory

moieties that are engineered by recombinant DNA technology to be an inherent part of the antigen molecule (e.g. fusion proteins) or the immunogen (e.g. vectored vaccines). In this context, it should be noted that no vaccine adjuvant is authorized in its own right, but only as a component of a particular adjuvanted vaccine. This document does not deal with the carrier proteins that are covalently linked to polysaccharide antigens in conjugate vaccines. Also, the immune enhancing properties that are intrinsic to certain vaccine antigen preparations, such as the naturally occurring adjuvant activity of whole-cell pertussis vaccines, are not considered "adjuvants" within this document.

This document covers adjuvanted vaccines used in both prophylactic and therapeutic indications against infectious diseases. Nevertheless, some of the principles outlined below may be applicable to the nonclinical and initial clinical testing of adjuvanted therapeutic vaccines for other indications as well (e.g. cancer).

Nonclinical evaluation, within the context of this document, refers to all in vivo (in animal) and in vitro testing performed before and during the clinical development of adjuvanted vaccines and includes product characterization, proof-of-concept and immunogenicity studies, as well as safety testing in animals. Preclinical testing specifically refers to the nonclinical testing done prior to initiation of any human testing and is a prerequisite to movement of a candidate adjuvanted vaccine from the laboratory to the clinic. Thus, for the remainder of this document, the term "preclinical" will be used only when referring specifically to the nonclinical evaluation done prior to the first-in-human clinical trials.

Many regulatory agencies, in addition to defining an adjuvant based on its immune-enhancing biological activity, provide a regulatory and/or legal classification for the adjuvant component of a vaccine (e.g. excipient, active ingredient or constituent material). It is possible that depending on the particular definition used by the regulatory authority, additional testing may be required. These regulatory and legal issues are specific for each regulatory authority and are beyond the scope of this document.

General considerations

Adjuvants have been used for decades to enhance the immune response to vaccine antigens (*7*). Possible benefits of administering antigens in conjunction with adjuvants include the induction of long-term protection, better targeting of effector responses, induction of long-term memory, reduction of the antigen amount and/or the number of vaccine doses needed for a successful immunization and optimization of the immune response for populations with poor responsiveness. For certain complex diseases, stimulation of cell-mediated immune responses appears to be critical, and adjuvants can be employed to

optimize a desired immune response, such as the induction of cytotoxic or helper T lymphocyte responses. In addition, certain adjuvants can be used to promote antibody responses in a relevant immunoglobulin class or at mucosal surfaces.

Successful preclinical evaluation of adjuvanted vaccines, including physicochemical characterization, proof-of-concept testing in animals, and toxicity testing, is an important step towards their clinical development. In addition, studies in animals are valuable tools to help select a safe dose, schedule and route of administration, and to identify unexpected or potential adverse effects for specific monitoring in clinical trials. Safety concerns include potential inherent toxicities of the vaccine antigen and/or adjuvant, potential toxicities of any impurities and contaminants, and potential toxicities due to interactions of the components present in the final formulation. The regulatory considerations for adjuvanted vaccines are similar to those for vaccines in general, with additional issues being considered that are unique to novel adjuvants. For the purposes of these WHO Guidelines, a novel adjuvant is defined as an adjuvant that has not been included in a licensed vaccine.

Throughout this document, guidance is provided related to the evaluation of new adjuvants and adjuvanted vaccines, to include:

- unlicensed adjuvanted vaccines;
- antigens and adjuvants that have been included in licensed vaccines, but for which the production process has undergone significant changes;
- previously licensed products that have undergone major formulation changes (e.g. a change in adjuvant or addition or removal of one of the components);
- previously licensed products given by a new route of administration.

Where appropriate, considerations specific to the evaluation of novel adjuvants will be provided.

The established benefits and increased availability of adjuvants have stimulated an interest in transferring adjuvant production technology from one adjuvant or adjuvanted vaccine manufacturer to another. As stated above, adjuvants are not approved in their own right. In the context of vaccines against infectious diseases, adjuvants may only exist as components in licensed vaccines that consist of specific antigen/adjuvant combinations. Thus, each new adjuvanted vaccine is considered a new entity that will require appropriate physicochemical characterization and nonclinical and clinical evaluations. However, in cases of technology transfer, existing data from similar antigen and adjuvant components and/or adjuvanted vaccines held by the original manufacturer can provide important information to guide and potentially accelerate the nonclinical and clinical studies (e.g. data from adjuvant-alone study arms). The need for and

extent of nonclinical testing will depend on the adjuvanted vaccine under consideration; manufacturers are encouraged to consult with the NRA regarding the nonclinical testing needed.

Vaccine adjuvants have been divided broadly into two main types – those known as vaccine delivery systems, which enhance the delivery of the antigen to the local lymph node, and those known as immunostimulators, although this division has become less clear since some delivery systems are now known to have direct immune stimulatory effects in addition to their ability to enhance the delivery of the antigen to the local lymph node. Delivery systems include, but are not limited to, particles, carriers, emulsions and liposomes. Immunostimulators in general include substances that enhance the immune response to vaccine antigens by activating the innate immune system, which usually sets off a cascade of events including, but not limited to, increased antigen uptake into antigen-presenting cells, increased release of stimulatory molecules such as cytokines and increased localization of the antigen in the local lymph node. Immunostimulators may include cytokines or other substances that are generally described as "immune potentiators" because they exert direct effects on immune cells.

Adjuvants also can be classified according to their source (e.g. synthetic or microbial-derived), mechanism of action and physical or chemical properties. A list of the most commonly described adjuvant classes, with specific examples, is provided in Appendix 1. It should be noted that a given vaccine adjuvant may be a combination adjuvant (see "Terminology" section below) that consists of multiple types of adjuvants and thus can fall into more than one of the listed categories.

Terminology

The definitions given below apply to the terms used in these WHO Guidelines. They may have different meanings in other contexts.

Adjuvanted vaccine: the complete formulation that includes one or more antigens, an adjuvant(s), and any additives (which may include, for example, excipients or preservatives), the administration of which is intended to stimulate the immune system to result in an immune response that leads to the prevention or treatment of an infection or infectious disease.

First-in-human trial: for the purposes of this document, this refers to the first evaluation in human subjects. Most commonly, the first-in-human clinical trials are carried out in small numbers of healthy and immunocompetent adults to test the properties of a vaccine, its tolerability and, if appropriate, clinical laboratory and pharmacological parameters. These trials are considered phase I trials (*2*) and are primarily concerned with safety.

Good laboratory practice (GLP): a quality system concerned with the organizational process and the conditions under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded,

archived and reported. GLP principles may be considered as a set of criteria to be satisfied as a basis for ensuring the quality, reliability and integrity of studies, the reporting of verifiable conclusions and the traceability of data (*1*, *13*).

Good manufacturing practice (GMP): a part of the pharmaceutical quality assurance which ensures that products are consistently produced and controlled according to the quality standards appropriate to their intended use and as required in the marketing authorization. In these Guidelines, GMP refers to the current GMP guidance published by WHO (*14*, *15*).

Immunogenicity: the capacity of a vaccine/adjuvanted vaccine to induce antibody-mediated immunity, cell-mediated immunity and/or immunological memory.

In vitro studies: refers to studies that are conducted in a laboratory environment using components (e.g. serum, cells or tissues) that were originally obtained from a living organism.

In vivo studies: refers to studies that are conducted with living organisms.

Nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines: nonclinical testing includes all in vivo and in vitro testing performed before and in parallel with the clinical development of adjuvanted vaccines. Nonclinical testing includes product characterization, proof-of-concept studies and animal in vivo/ in vitro toxicity testing. The potential toxicity of an adjuvanted vaccine should be defined not only prior to initiation of human trials, but throughout clinical development, if appropriate (see also the definition of preclinical evaluation of vaccine adjuvants and adjuvanted vaccines).

Novel adjuvant: a novel adjuvant is an adjuvant that has not been contained in a licensed vaccine.

Potency: a measure of biological activity, using a suitably quantitative biological assay, based on an attribute of the product (e.g. adjuvanted vaccine) that is believed to be linked to the relevant biological properties. Other measures of potency (e.g. physicochemical analyses) may be appropriate based on the nature of the products (e.g. polysaccharides).

Preclinical evaluation of vaccine adjuvants and adjuvanted vaccines: preclinical testing refers specifically to the nonclinical testing (see definition of nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines) done prior to the first-in-human clinical trials. Preclinical evaluation is a prerequisite to the initiation of clinical trials.

Process intermediates: the antigen(s) and the adjuvant(s) used to produce the formulated adjuvanted vaccine.

Product characterization: a full battery of physical, chemical and biological tests conducted for a particular product (e.g. adjuvanted vaccine). These tests include, but are not limited to, in-process control testing, testing for adventitious agents, testing of process additives and process intermediates, and lot-release testing (*1*).

Proof-of-concept studies: proof-of-concept studies as discussed in this document include the in vivo and in vitro nonclinical testing conducted to evaluate the immune response to the adjuvanted vaccine, the enhancement of the immune response to the antigen by the adjuvant and/or the demonstration of the resulting protection against challenge with the infectious agent targeted by the adjuvanted vaccine. For therapeutic vaccines, proof-of-concept studies would include, when possible, studies to evaluate the capacity to control or ameliorate disease and/or clear infection.

Protocol or study/trial plan: a document that states the background, rationale and objectives of the nonclinical study or clinical trial, and describes its design, methodology and organization, including statistical considerations, and the conditions under which it is to be performed and managed (*1*).

Raw materials: ingredients used to produce process intermediates.

Route of administration: the means by which the candidate adjuvanted vaccine is introduced to the recipient. Routes of administration for adjuvanted vaccines may include, for example, the intramuscular, subcutaneous, transcutaneous (with or without scarification), intradermal, oral, intranasal, inhaled (aerosol), intravenous, intranodal, intravaginal or intrarectal routes.

Safety: the relative freedom from direct or indirect harmful effect to animals or persons by a product when appropriately administered, taking into consideration the character of the product in relation to the condition of the recipient at the time.

Vaccine adjuvants: substances or combinations of substances that are used in conjunction with a vaccine antigen to enhance (e.g. increase, accelerate, prolong and/or possibly target) or modulate to a different type (e.g. switch a Th1 immune response to a Th2 response or a humoral response to a cytotoxic T-cell response) the specific immune response to the vaccine antigen in order to enhance the clinical effectiveness of the vaccine. It may be any of the types of substances identified as examples of adjuvants in Appendix 1. The term "adjuvant" is used throughout the document to include adjuvants that exist as one individual substance as well as combination adjuvants that consist of multiple adjuvants and sometimes other additives.

Vaccine and adjuvanted vaccine: the complete formulation that includes an antigen (or an immunogen, e.g. a plasmid DNA vaccine) and any additives such as adjuvants, excipients or preservatives, the administration of which is intended to stimulate the immune system to result in an immune response to the vaccine antigen leading to the prevention or treatment of an infection or infectious disease. When the vaccine contains an adjuvant, it may be referred to as an adjuvanted vaccine.

Vaccine antigen: the active ingredient in a vaccine (or generated by a vaccine) against which a specific immune response is raised. The vaccine antigen may be a live, attenuated preparation of bacteria, viruses or parasites; inactivated

(killed) whole organisms; crude cellular fractions or purified antigens, including recombinant proteins (i.e. those derived from recombinant DNA expressed in a host cell); polysaccharides and conjugates formed by covalent linkage of polysaccharides to components such as mutated or inactivated proteins and/or toxoids, synthetic antigens, or heterologous proteins expressed by plasmid DNA or viral or bacterial vectors. It may also be a combination of the antigens or immunogens listed above.

Part A. Manufacturing and quality considerations for the nonclinical and clinical evaluation of vaccine adjuvants and adjuvanted vaccines

Adjuvanted vaccine manufacturers are encouraged to discuss with the NRA the extent of the manufacturing and quality-related information necessary to support the intended use of the antigen, the adjuvant and the adjuvanted vaccine. The extent of information necessary to evaluate and assure the consistent safety and effectiveness of adjuvanted vaccines will vary with the phase of nonclinical and clinical investigation. Similarly, the nature and extent of the manufacturing controls needed to achieve, and testing needed to demonstrate, appropriate adjuvanted vaccine quality differ not only among the various phases of product development (that is, research, pilot, investigational and commercial manufacture) but also among the various phases of clinical evaluation.

A.1 Production, characterization and quality assurance of lots to be used in nonclinical pharmacology studies

It is generally accepted that nonclinical pharmacology studies (e.g. the proof-of-concept and mechanism-of-action studies) may be done as non-GLP studies, and that they are often conducted with research or pilot-scale lots of antigen, adjuvant and/or adjuvanted vaccine formulations. Also, these studies are often dose-optimization studies in which the antigen and adjuvant components may be provided in two separate containers to allow for the mixing of different amounts of each component prior to administration, and the generation of data that support the proposed dose of antigen and adjuvant to be used in the investigational adjuvanted vaccine. While the level of characterization of the lots of antigen and adjuvant used in these exploratory studies may be less extensive than those to be used in the nonclinical toxicology and clinical studies, the same raw materials should be used, where possible, in their preparation, and the source and any testing of the raw materials – for example, purity and assessment of levels of metal ions (such as copper) in aluminium-containing compounds – should be documented. Ideally, the lots of antigen and adjuvant used to formulate the final

product should be manufactured by the same process as the lots to be tested in the nonclinical toxicology studies. The general quality of the adjuvanted vaccine components (that is, antigen and adjuvant intermediates) used in the nonclinical pharmacology studies should be adequately characterized preliminarily. As the relationship between physical and chemical characteristics of the adjuvanted vaccine and its components and the immunogenicity and efficacy of the adjuvanted vaccine is not completely understood in many cases, biological characterization (i.e. through the use of biological assays) should complement the physical and chemical characterization of the intermediates and the adjuvanted vaccine (see section A.2 and Table 2.1).

A.2 Production, characterization and quality assurance of lots to be used in nonclinical toxicology studies and first-in-human clinical trials

Ideally, the lots of the antigen, the adjuvant, and the adjuvanted vaccine used in the nonclinical toxicology studies should be the same lots as those proposed for use in the first-in-human trials; these lots should be manufactured in compliance with the GMPs that are appropriate for phase I clinical trial materials (*16*, *17*). Additionally, the quality and stability of the antigen, adjuvant and final adjuvanted vaccine formulation should be characterized adequately prior to, if not in parallel with, their use in a toxicology study (see section A.2.1 and Table 2.1).

If use of the same lots is not feasible, the lots used for the nonclinical toxicology studies should be comparable to those proposed for use in the first-in-human trials with respect to manufacturing process, physicochemical data, formulation and stability. Where there are significant differences in the manufacture of the antigen or the adjuvant (or in the formulation of the adjuvanted vaccine) to be used in the nonclinical toxicology studies and the first-in-human clinical trial, a detailed description of the differences should be provided. This information will allow the NRA to evaluate the potential impact of such changes on the safety of the adjuvanted vaccine and to determine whether or not the differences are sufficient to warrant the conduct of additional toxicology studies to support the safety of the proposed clinical use.

With respect to the control and testing of adjuvanted vaccine lots manufactured for use in first-in-human clinical trials, emphasis should generally be placed on elements that assure the safety of subjects. This usually includes identification and control of the raw materials used to manufacture the antigen and the adjuvant. For this reason, Certificates of Analysis, with test specifications and results indicated, should be provided for ingredients that are acquired from contract suppliers for use in manufacturing the adjuvanted vaccine. For some adjuvanted vaccines, additional considerations related to the manufacturing and testing of the vaccine adjuvant and its individual components may be needed

to provide assurance that the adjuvant is manufactured consistently and has a consistent composition. This may apply particularly when one or more of the components of the adjuvant is biological in nature, when the vaccine contains a complex adjuvant mixture, or when the antigens are adsorbed to mineral salts or gels. Therefore, it is important to use established quality control procedures that ensure the consistent manufacture of adjuvants and antigens to be used in the preparation of adjuvanted vaccines. The antigen and adjuvant, or formulated adjuvanted vaccine, used in the first-in-human trial should be manufactured under GMPs that are appropriate for phase I clinical trial materials (*16*, *17*). Compliance with GMPs will ensure that the lots of antigen, adjuvant and adjuvanted vaccine are consistently manufactured and controlled to the quality standards appropriate to their intended use. Compliance with all aspects of GMPs will be required at the later stages of clinical development (*14*, *15*) as discussed below (see section A.3 and Table 2.1).

The clinical lot(s) of adjuvanted vaccine, or separate lots of antigen and adjuvant if provided in separate final containers, should be demonstrated to be stable for the duration of the clinical trial. Additionally, if the adjuvant is provided in a separate container (e.g. vial or syringe) to be used to reconstitute or be added to the antigen prior to vaccine administration, a detailed description of the procedure for mixing the components should be provided. A clear statement of the appropriate time and conditions for storage of the individual components and the final adjuvanted vaccine should be provided. Also, the appearance of the adjuvanted vaccine after mixing should be described, and stability data to support the storage of the adjuvanted vaccine up to the time of administration should be provided.

A.2.1 Analytical testing of adjuvant, antigen and adjuvanted vaccine

A detailed description of the adjuvant, antigen and adjuvanted vaccine should be provided and include information regarding the characterization conducted to assure the quality (e.g. identity, purity, sterility) and quantity of the antigen and adjuvant as well as the potency of the adjuvanted vaccine. It should be demonstrated that the adjuvant does not adversely affect the potency of the antigen upon mixing. In addition, information on the methods of manufacture and testing for the intermediates and final product, together with their preliminary release specifications, should be provided. Although it is not necessary to have validated methods for testing the lots of antigen and adjuvant or adjuvanted vaccine to be used in nonclinical toxicology studies and first-in-human clinical trials, the scientific background should justify the choice of the testing methods and the selected preliminary specifications. It is recommended that the NRA be consulted when designing analytical protocols appropriate for establishing the identity and quantity of the antigen(s), adjuvant(s) and any additives. It is important to

assess attributes of each of the antigen and the adjuvant components that may be relevant for adjuvant activity and adjuvanted vaccine potency. Additionally, the properties of the antigen and the adjuvant that are most indicative of stability, both when stored individually and as a formulated final adjuvanted vaccine, should be identified.

Assays used for characterization of the adjuvant may or may not be related to its mode of action, but should be adequate to ensure consistency of adjuvant production and to evaluate adjuvant stability. These may include, for example, assays for appearance, particle size distribution, presence of aggregates and pH for the adjuvant, and the amount of aluminium and degree of antigen adsorption for a vaccine adsorbed to an aluminium-containing compound. Analytical methods to evaluate the antigen and the adjuvant in an adjuvanted vaccine should be developed and validated as adjuvanted vaccine product development and clinical evaluation proceed. If relevant, the methods to be developed for characterization purposes should include, where possible, methods to assess compatibility and/or physical interactions between the antigen and adjuvant (and between the components of the adjuvant, if a combination adjuvant is used). Validation of these methods should be completed if they are intended for quality control batch release during later-stage clinical development or commercial distribution.

A quality-control test evaluating the potency of the final adjuvanted vaccine should be developed as one of the assays to assess consistency of manufacture. Depending on the type of potency assessment conducted on the adjuvanted vaccine and the requirements of the NRA, the assessment may or may not reflect the contribution of the adjuvant to the potency of the adjuvanted vaccine. If it does not, it will be important to conduct assessments of the identity and content of the adjuvant in the final adjuvanted vaccine. Also, the purity and sterility of the final adjuvanted vaccine will need to be assessed to ensure its safety. If the adjuvant or adjuvanted vaccine is tested for endotoxin via the Limulus amoebocyte lysate (LAL) test method, evidence that the adjuvant or adjuvanted vaccine does not interfere with the LAL test (e.g. data from lipopolysaccharide spiking experiments with and without adjuvant) should be provided, as certain adjuvants, such as cationic liposomes, may interfere with the LAL test method. If interference is observed, alternative tests (e.g. pyrogen test or macrophage-activation test) should be investigated.

If the final adjuvanted vaccine consists of co-packaged antigen and adjuvant, where each is provided in a separate container to be mixed prior to administration, both the antigen and the adjuvant should be evaluated prior to mixing for relevant parameters, such as identification, purity and sterility. In addition, the potency of the antigen and the content of the adjuvant per dose should be assessed. Also, where feasible, evidence should be provided as mentioned previously to demonstrate that the adjuvant does not adversely affect the potency of the final adjuvanted vaccine. Thus, the potency of the extemporaneously mixed,

adjuvanted vaccine formulation should be demonstrated. For some adjuvanted vaccines (e.g. aluminium-adsorbed vaccines), it may not be possible, depending on the nature of the potency assay, to evaluate the potency of the final formulated vaccine by certain assays. In this case, the determination of the potency of the antigen alone prior to adsorption may be recommended as well as the development of an in vivo method for potency assessment of the final formulation.

Consultation with the NRA is recommended to discuss both the need for and design of the quality control test known as the innocuity, general safety, or abnormal toxicity test for the adjuvanted vaccine. Additionally, if a particular NRA requires such a test for a formulated adjuvanted vaccine, it should be clarified whether only the antigen or both the antigen and adjuvant are to be tested when provided in separate final containers. While some regulatory authorities and WHO no longer require this test to be performed on a routine basis once the consistency of production has been established, some have further questioned the relevance of this test (*18–20*). In some countries there is a legal requirement to conduct an innocuity test with the objective of assessing the potential introduction of extraneous impurities into the final adjuvanted vaccine; however, this is not considered a toxicity test. If the innocuity test is required, and the investigational adjuvant or adjuvanted vaccine does not pass the innocuity test when administered according to the prescribed protocol, which is typically volume based and administered by the intraperitoneal route, it will be necessary to define the appropriate dose and route of administration for the adjuvanted vaccine. The manufacturer of the vaccine will need to provide justification for a modification of the innocuity test in regulatory submissions. Such modifications should be discussed with the NRAs. In the countries where the innocuity test is still necessary, once test data from many lots have been accumulated, and consistency of production has been well established to the satisfaction of the NRA, it may be possible to request an exemption from conduct of the innocuity test as part of routine lot-release testing.

A.3 Information required for later-stage clinical trials

In general, in the course of adjuvanted vaccine product development, the analytical technology and methodology is developed in parallel with the clinical investigations. As the adjuvanted vaccine product development and clinical evaluation proceed, the quality control and quality assurance of the antigen and adjuvant should be refined. When clinical trials to collect safety and efficacy data to support licensure are initiated, the manufacturing processes should be demonstrated to be consistent and validated, and a detailed description with appropriate validation information should be provided for all analytical procedures (except for those that are from an official pharmacopeial compendium) (*14*, *15*). If a national or international standard is not yet available for a particular

antigen, adjuvant or adjuvanted vaccine, the manufacturer should establish its own primary reference material during later-stage clinical trials.

A minimum of three consecutive lots of each of the antigen and the adjuvant intermediates (or final containers if provided separately) and formulated adjuvanted vaccine should be manufactured and tested for purposes of demonstrating consistency of manufacture of the vaccine antigen, the adjuvant and the formulated adjuvanted vaccine. Any changes in the manufacture or formulation should be carefully assessed to determine if such changes directly or indirectly affect the quality or safety of the adjuvanted vaccine. When analytical data from tests conducted on the adjuvanted vaccine demonstrate that the antigen, adjuvant or adjuvanted vaccine manufactured before and after such changes is not comparable, additional qualification and/or bridging studies should be undertaken to support the safety of the materials proposed for continued clinical evaluation.

To ensure that appropriate stability data are collected during later stage clinical trials of the adjuvanted vaccine, a stability protocol to be used for the formal stability studies should be developed for the antigen, the adjuvant and the adjuvanted vaccine. Stability programmes should be designed to monitor the chemical, physical, biological and microbiological stability of the antigen, the adjuvant, and the adjuvanted vaccine throughout the clinical testing programme. The properties of each antigen and adjuvant that are most indicative of stability, both when stored individually and as a mixed final adjuvanted vaccine, should be identified as stability evaluations proceed (as mentioned in section A.2.1). If it is determined that degradation products accumulate from either the antigen or the adjuvant over the shelf-life of the adjuvanted vaccine, these should be evaluated during stability testing of the final product. It is recommended that the NRA be consulted to determine whether additional suitable nonclinical toxicological testing should be undertaken to confirm their safety. Additional guidance on stability testing of vaccines can be found in WHO Guidelines on stability evaluation of vaccines (*21*).

Part B. Rationale for the use of the adjuvant

Adjuvant activity is a result of multiple factors and an adjuvant-mediated enhancement of the immune response to one vaccine antigen, as a rule, cannot be extrapolated to the enhancement of the immune response to another antigen. Individual antigens vary in their physical, biological and immunogenic properties and antigens may have different needs for immunological help from an adjuvant (*5*). Manufacturers should justify the choice of the adjuvant based on the immune response desired, which may include effects on the magnitude, the breadth and/or the type of immune response to specific antigens and on the safety profile. In addition, adjuvants are also used in antigen dose-sparing strategies with the aim of

increasing the availability and supply of vaccines – for example, under emergency situations of an influenza pandemic (*22*) or as a strategy to decrease the cost of the vaccine (e.g. use of inactivated poliovirus vaccine for polio eradication) (*23*).

Many advances in the understanding of innate immunity have begun to provide insights into the immunological mechanisms of adjuvant action. Many of the immunostimulatory adjuvants are recognized by various members of the toll-like receptor (TLR) family, a subclass of pathogen-recognition receptors, while other adjuvants may target other families of pathogen-recognition receptors that could prove to be important in shaping the adaptive immune response. Furthermore, there are complex regulatory interactions between the many families of innate receptors and other signalling pathways. Within this framework, the activities exerted by adjuvants include, but are not limited to, the facilitation of: (a) mobilization of antigen-presenting and/or polymorphonuclear cells; (b) antigen uptake and presentation of the antigen(s) in the vaccine by antigen-presenting cells; (c) secretion of proteins by antigen-presenting cells; (d) recruitment, targeting and activation of antigen-specific cells; (e) modulation of activities that regulate the ensuing immune responses; and/or (f) protection of the antigen from degradation and elimination.

The scientific rationale supporting the benefit of adding the adjuvant and the choice of specific adjuvant(s) should be provided by the adjuvanted vaccine manufacturer. Before evaluating a particular adjuvant in combination with an antigen in a clinical trial, it is recommended that data from in vitro and/or in vivo studies be generated to support the rationale for including the specific adjuvant in the vaccine formulation and for selecting the dose range of adjuvant to be tested. In the ideal case, the mode of action of the selected adjuvant as well as the mechanism of the enhanced immune response would be well understood prior to the initiation of later-stage clinical development. When the mode of adjuvant action is not well defined, supplemental in vivo or in vitro data (as discussed in sections B.1 and B.2, respectively) may be provided in addition to the pivotal toxicity study to support the added benefit of the adjuvant to the immune response induced by the adjuvanted vaccine as well as the safety of the adjuvanted vaccine.

B.1 In vivo proof-of-concept studies

Data from proof-of-concept studies, including data from early studies conducted to evaluate optimal antigen/adjuvant formulations, can provide important information with regard to the characteristics of the adjuvanted vaccine. These data include evidence for the need for the adjuvant, the type and magnitude of the immune responses induced (i.e. innate immunity, or humoral and cellular immunity), and the functional capacity of the immune response to either protect against disease (i.e. prophylactic vaccine) or ameliorate an existing infectious disease (i.e. therapeutic vaccine) when a relevant nonclinical disease model

is available. These pilot or exploratory studies designed to identify and screen adjuvanted vaccine formulations may be non-GLP-compliant; however, they may identify unknown or potential adverse effects, and provide crucial information for the design of GLP-compliant toxicity studies. In addition, in vivo proof-of-concept studies may provide the scientific justification for manufacturing changes and for optimization of adjuvanted vaccine formulation, dose and route of administration during the clinical development of the adjuvanted vaccine product.

It is recommended that proof-of-concept studies to support the use of an adjuvant be carried out to evaluate vaccine formulations with and without the adjuvant. Depending on the specific antigen and/or adjuvant being considered, possible examples of these types of studies include:

- evaluation of humoral immune responses with regard to magnitude (e.g. mean titre or concentration), quality (e.g. affinity or avidity), and functional activity (e.g. neutralizing activity);
- evaluation of cellular immune responses including assessment of the induction of specific types of cellular responses (e.g. examining Th1 or Th2 cytokine profiles, or testing for the induction of cytotoxic T cells);
- evaluation of protective or therapeutic responses against the relevant pathogen using appropriate animal or in vitro disease models and/ or evaluation of functional immune responses (e.g. neutralizing activity, serum bactericidal or opsonophagocytic antibody titres);
- evaluation of duration of (*24*) and extent of cross-protection provided by the induced immune response (*25*, *26*).

These studies will contribute to the elucidation of the adjuvant mode of action and may provide indication of the adjuvant-specific immune modulatory effects. In addition, these studies may assist in the interpretation of nonclinical safety studies and the identification of potential adverse effects to be monitored during clinical development. The development of in vitro model systems, particularly those using human cells, is recommended when possible, as they may provide additional relevant information to elucidate the mechanism of action of the adjuvant (see section B.2).

B.2 In vitro supporting studies

Functional in vitro bioassays may also provide helpful insight in understanding the mode of action of a particular adjuvant, and may provide valuable supplemental and complementary data to animal studies. This is particularly important when there are limitations to the animal models, such as species-specific differences (e.g. in TLRs). Antigen-presenting cells or other immune cells are widely used to assess and monitor the direct or indirect effects of adjuvants by measuring activation parameters (such as changes in the expression of cell

surface molecules and the pattern of cytokine secretion), and more recently such human cells have been used to develop in vitro assays that may be predictive of adjuvant safety in vivo (*27*). More complex tissue culture systems, containing a mixture of human immune cells mimicking lymphoid tissue, are being explored with the aim of evaluating human immune responses in vitro (*28*).

Part C. Considerations for selection of the animal species for nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines

Investigations of the properties that influence the safety and pharmacological activity of the adjuvant and the adjuvanted vaccine require the use of appropriate animal species. The animal species used for pharmacological and safety evaluations should be chosen carefully and justified. For ethical reasons, it is desirable to apply the 3Rs concept of "Replace Reduce Refine" to minimize the use of animals in research where scientifically appropriate (*29*). Both manufacturers and staff at the NRA or national control laboratory are encouraged to further develop in vitro assays and to evaluate their suitability for the control of vaccines (*30*).

C.1 Selection of animal species for nonclinical pharmacology studies

For the purpose of this document, the nonclinical pharmacological activity of an adjuvanted vaccine is defined as the ability of the adjuvanted vaccine to induce and/or modify an immune response in an animal species. Factors influencing the selection of a particular animal species include, but are not limited to, the vaccine antigen, the adjuvant chosen, the type of immunity (i.e. cell-mediated or humoral) to be induced and the route of administration. It is recommended that proof-of-concept studies be undertaken using an animal species in which: (a) an immune response to the vaccine antigen is developed; and (b) the immune response to the antigen is enhanced by the adjuvant through a mechanism similar to that expected in humans (e.g. TLRs known to be targeted by the adjuvant are present in the species, and enhanced humoral and/or cellular immunity is observed). However, it is acknowledged that species-specific differences in the immune responses induced in the animal species compared to the human are likely. Proof-of-concept studies most commonly are conducted in several animal species, including both naive and pre-exposed animals. In addition to evaluating the immune response induced by the vaccine antigen alone and in the presence of the adjuvant, the mechanism of action of the adjuvant in the absence of the vaccine antigen should also be evaluated.

If the adjuvanted vaccine is a therapeutic vaccine for an infectious disease indication, where feasible, disease animal models may need to be developed to

study the pharmacological activity of the adjuvanted vaccine and its effect on the disease. For preventive adjuvanted vaccines, the use, when available, of an animal species sensitive to the human pathogen may provide important insight into the mechanism of protection from the disease (e.g. the ferret model for human influenza).

Nonclinical pharmacology studies may be conducted under non-GLP compliant conditions. It is advisable to incorporate into the study design toxicological end-points to guide the design of GLP-compliant nonclinical safety studies. It is sufficient to conduct these studies in small animal species if it can be demonstrated that the animal species chosen is relevant and responsive to the vaccine antigen and the adjuvant when given by the intended route of administration. Nonhuman primates should be used only if no other relevant animal species is available.

C.2 Selection of animal species for nonclinical safety studies

When selecting the animal species for nonclinical safety studies, it is important to document the pharmacological activity of the vaccine in the presence and absence of adjuvant in that species. It is recommended that manufacturers conduct nonclinical safety studies in compliance with GLPs (see section D.2 and Table 2.1) and using an animal species in which an immune response to the vaccine antigen is developed and, ideally, the immune response to the antigen is enhanced by the adjuvant through a similar mechanism as expected in humans. It is not necessary, however, to conduct the nonclinical safety study in the same animal species used for proof-of-concept or nonclinical pharmacology studies (see sections B.1 and C.1). Nonhuman primates should be used only if no other relevant animal species is available. In situations where no animal species is available that is responsive to the adjuvanted vaccine, the choice of the animal species should be justified. In some circumstances, the use of in vitro model systems, particularly those using human cells, to evaluate the toxicity of the adjuvanted vaccine may provide additional supplementary information to assist in interpreting toxicity data (*27*).

It is highly recommended that the animal species chosen is one for which relevant and sufficient historical control data exist. Analysis and interpretation of data from the toxicity studies commonly includes a comparison with the inactive control (e.g. saline control) in the same study. However, historical control data from the same laboratory in which the study was conducted and for animals of comparable age and from the same species and/or strain may provide additional information. When historical control data are used, the data should be provided to the NRA.

The route of administration used in the toxicity study should correspond to that intended for use in the clinic. Also, when the adjuvanted vaccine is to be

administered in the clinic using a particular device, the same device should be used in the animal study, where feasible. For example, a small rodent species may not be an appropriate choice for nonclinical evaluation of a vaccine that is to be delivered intranasally because some of the inoculum could be delivered to the lungs. In this case, a larger animal or one with nasal surface area, anatomy and physiology similar to that of humans would be more appropriate.

Use of a single species is generally acceptable (see section D.2). This approach has commonly been accepted based primarily on pragmatic considerations – for example, the ability to predict the human immune response may be limited due to the species specificity of the response in animals to the antigen, the adjuvant or both.

C.3 Limitations of animal studies

The limitations of using animals to characterize the pharmacological and safety profile of an adjuvant or adjuvanted vaccine are acknowledged. The ability to predict the human immune response based on pharmacological studies in an animal may be limited due to the species specificity of the response to the antigen, the adjuvant, or both. Similarly, local and systemic adverse effects observed in a nonclinical safety study may not be directly translatable to the clinic. In addition, rare and/or late-onset adverse events that may occur in human subjects as a result of adjuvanted vaccine administration may not be observed in animal studies. Nevertheless, these studies offer the best currently available tools to evaluate the preclinical safety and pharmacology of adjuvanted vaccines.

D. Nonclinical safety assessment in animals

D.1 General remarks

Safety concerns for products such as vaccines include the potential inherent toxicities of the antigen and other vaccine components, as well as potential toxicities due to interactions of the components present in the final formulation. For adjuvanted vaccines, these concerns include the possibility that the immune-modulatory and/or inflammatory response induced may lead to undesired toxic side effects. Additionally, some adjuvants may elicit elevated levels of proinflammatory cytokines and other mediators of toxicity, irrespective of the immune response against the antigen.

Safety assessments in animal studies are valuable tools to help define an acceptable adjuvant/antigen ratio and a safe dose, as well as to identify unknown or potential adverse effects that should be taken into consideration for further product development or to be monitored in future clinical trials. The type of studies and the timing in relation to the clinical programme are presented in section D.2.

D.2 Toxicity studies of vaccine adjuvants and final adjuvanted vaccine formulations

The preclinical toxicity studies of the final adjuvanted vaccine formulation should be adequate to identify and characterize potential adverse effects of the vaccine in order to conclude that it is reasonably safe to proceed to first-in-human clinical investigation. As the mechanism of action of the adjuvant and/or adjuvanted vaccine formulation is often not fully understood, the toxicity studies should be designed to evaluate a broad spectrum of parameters due to the uncertainty of the in vivo effects and associated outcomes. Toxicity studies should be designed to mimic the intended route of administration in the clinic and to evaluate local reactogenicity (e.g. injection-site inflammation) and systemic toxicity (i.e. toxicity that occurs at sites distant from the site of initial administration). Pivotal toxicity studies should use the intended final formulation and dose of the adjuvanted vaccine (see section A.2) and should be conducted in compliance with GLPs.

When properly designed, conducted and interpreted, and when no major safety signals are revealed in the study results, one repeated-dose toxicity study in one relevant species should be sufficient. However, if there are significant manufacturing or formulation changes during product development, additional animal toxicity studies may be recommended to confirm that the safety profile of the product has not been changed. Also, throughout the clinical programme, additional animal toxicity studies (e.g. developmental and reproductive toxicity studies) may be necessary to investigate any adverse events observed in clinical trials or to support the use of the vaccine in a special population.

While comprehensive toxicity evaluations of the final adjuvanted vaccine formulation are considered essential, the advantages and limitations of toxicity studies with adjuvant alone have been discussed extensively in previous meetings and workshops (*7–11*). A comprehensive toxicity assessment of the adjuvant alone in animals (or of individual evaluations of its multiple components, if it is a combination adjuvant) may not be needed as a separate programme. However, to enable the interpretation of immunogenicity and safety studies of the adjuvanted vaccine, a study arm receiving adjuvant alone may be included in the repeated-dose toxicity studies (see section D.2.2) that are part of the comprehensive toxicity evaluations of the final adjuvanted vaccine formulation.

D.2.1 Safety pharmacology studies

The purpose of a safety pharmacology study is to investigate the effects of the candidate vaccine on vital functions. Although not usually required, safety pharmacology studies may be recommended by the NRA in some cases. For example, if data from nonclinical and/or human clinical studies suggest that the adjuvanted vaccine may affect physiological functions other than the immune

system (e.g. the central nervous system, respiratory or cardiovascular system, renal function or body temperature) then safety pharmacology studies should be incorporated into the safety assessment programme.

D.2.2 Repeated-dose toxicity studies

This section highlights important considerations regarding the study design for pivotal toxicity studies that should be conducted with the same vaccine formulation intended to be used in clinical trials (see section A.2). If more than one dose of an antigen or adjuvant is to be evaluated in the clinical study, the formulation containing the highest dose (i.e. the "worst case") should be included in the pivotal toxicity studies. Single-dose toxicity studies on the final formulated vaccine product, which are applicable to small-molecule chemical medicines, are usually not needed in accordance with *Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals: M3(R2)* (*31*). Acute effects of administering a vaccine can also be monitored in repeated-dose toxicity studies if they are adequately designed (e.g. an evaluation is conducted after the first administration). Alternatively, acute effects can be assessed in a single-dose design as part of a local tolerance study. For a study intended to support a first-in-human clinical trial, the number of animals studied per sex, group and time interval should be sufficient to allow meaningful scientific interpretation of the data generated. The size of the treatment group will depend on the animal species chosen; i.e. the number of animals included in studies using non-rodents (e.g. miniature pigs) would be expected to be fewer than the number included in studies using rodents. For mice and rats, it is recommended that at least 10 animals of each sex per group be used for the necropsy at the end of the treatment interval, and at least 5 animals of each sex per group be used for the necropsy at the end of the recovery period. For rabbits, it is recommended that at least five animals of each sex per group for each time interval be used. In general, the approximate age for rodents should be 6–8 weeks, and for rabbits, 3–4 months, at the start of the study.

D.2.2.1 Dose, dosing regimen and controls

Dose–response evaluation for the adjuvanted vaccine is generally not required as part of the basic toxicity assessment, given that, in most cases, dose–response assessment was explored in nonclinical pharmacology studies. For adjuvanted vaccines, the toxicity study should be performed using the highest anticipated human dose (in absolute terms) of the final adjuvanted vaccine to be used in the proposed clinical trial, where feasible. Ideally this dose provides optimal exposure of the animal to the candidate vaccine and the immune response induced. However, in the case of a novel adjuvant, it may be advisable to include additional (lower and higher) doses of the adjuvanted vaccine formulation or

adjuvant alone in order to identify a safe dose that could be used in a first-in-human clinical trial.

If the dose to be administered is limited by the total volume that can be administered in a single injection, guidelines for animal welfare should be followed (*32*). In such cases, the total volume may need to be administered at multiple sites using the same route of administration; however, it should be noted that the evaluation of local reactogenicity might be less reliable in such cases.

For adjuvanted vaccines intended to be given repeatedly, the number of doses administered to the animals in repeated-dose toxicity studies should equal or exceed the number of doses proposed in humans. However, in many cases, the studies are designed to include one dose more than planned for the clinical trial to allow for the possible inclusion of an additional dose in the clinical trial. To simulate the proposed clinical usage, vaccine doses should be given as episodic doses, but the dosing interval used in the toxicity study may be reduced (e.g. to 2 weeks or 3 weeks) compared with the proposed clinical dosing interval (which usually is greater than 2 weeks to 3 weeks). The nonclinical dosing interval should be based primarily on the kinetics of the primary and secondary antibody response observed in the animal study.

In general, the study design should include a negative control group that receives an inert placebo, such as saline, to evaluate a baseline level of treatment, and an adjuvant-alone arm to aid in the interpretation of safety data from the adjuvanted vaccine. Also, the treatment groups in the study should include a sufficient number of animals for evaluation (as described in section D.2.2.3) at later time points after treatment to evaluate the reversibility of adverse effects observed during the treatment period and to detect potentially delayed adverse effects.

D.2.2.2 Route of administration

The route of administration should correspond to that intended for use in the clinical trials. When the vaccine will be administered in human clinical trials using a particular device, the same device should be used in the animal study, where feasible.

D.2.2.3 End-points in toxicity studies

The following section discusses end-points that are especially relevant and important in the evaluation of adjuvanted vaccines in repeated-dose toxicity studies using the final vaccine formulation. In general, potential adverse effects of the adjuvanted vaccine should be evaluated in repeated-dose studies with regard to target organs (see Appendix 2), dose, route(s) of exposure, duration and frequency of exposure, and potential reversibility of observed toxic effects.

D.2.2.3.1 ***Parameters for monitoring of systemic toxicity***

Toxicity studies, repeated-dose toxicity studies in particular, should address the potential for systemic toxicity including, but not limited to, the systemic effects on the immune system. A broad spectrum of information should be obtained from the toxicity study, and both in-life and postmortem data should be collected. This routinely includes careful monitoring of body weight and food consumption, body temperature, histopathology, clinical chemistry, haematology, coagulation parameters and acute phase reactants. In addition, the immune response should be evaluated in a group of treated animals to confirm that the anticipated immune response occurred during the toxicity study. A detailed description of the assay(s) used should be provided with the toxicity study results.

While the standard in-life parameters routinely assessed for general pharmaceuticals (e.g. overall health, body weight and food consumption) are appropriate, it is important to note that for adjuvanted vaccines more frequent (e.g. daily) measurements of body weight and food consumption are recommended, especially during the first week after the administration of each dose as these parameters are very sensitive in detecting systemic toxicity effects. After the first week, body weights may be collected less frequently (e.g. 2–3 times each week). Body temperature should also be evaluated prior to, and 3–8 h and 24 h after each dose. If there is an increase in temperature, additional measurements should be taken every 24 h until the values return to baseline. Interim analyses of haematology and serum chemistry should be considered within approximately 1–3 days following the first and last dose administration, and at the end of the recovery period; in addition, the collection of a predosing sample is recommended. Coagulation parameters should be included routinely; in some cases, evaluation of urine samples and serum immunoglobulin classes may be of value. Additionally, it is recommended that species-appropriate acute phase reactants (e.g. C reactive protein) be measured in the toxicity study prior to immunization, at time points following the administration of the adjuvant or adjuvanted vaccine that have been demonstrated to reflect peak elevations in the acute phase reactants being evaluated (commonly 24–48 h), and after a recovery phase of 7 days. When measuring acute phase reactants, the choice of the animal species may determine which proteins can be measured as these reactants vary among species (*33*). The data discussed above should be collected not only prior to and during the treatment phase, but also following the treatment-free (recovery) phase (i.e. 2 or more weeks following the last dose) to determine persistence, exacerbation and/or reversibility of potential adverse effects.

Postmortem data, including data from gross necropsy (with tissue collection and preservation, including gross lesions and organ weights), should be collected within 3 days following the last dose and following the above-mentioned recovery period (e.g. 2 or more weeks following the last dose) (*1*). At study termination, final body weights (following overnight fasting) should be

obtained. Terminal blood collection and analysis should include serum chemistry, haematology, and coagulation parameters as well as an immune-response evaluation. Histopathological examinations should always include pivotal organs (brain, lung, heart, kidneys, liver, reproductive organs), and the site of adjuvant or adjuvanted vaccine administration. Special attention should be paid to the immune organs – i.e. lymph nodes (draining and distant to the application site), thymus, spleen, bone marrow, and Peyer's patches or bronchus-associated lymphoid tissue – as well as organs that may be primarily affected due to the particular route of administration. The extent of the list of tissues to be examined (i.e. the full tissue list as provided in Appendix 2 versus the reduced list mentioned above, which is limited to the immune system and pivotal organs) will depend on the adjuvant or adjuvanted vaccine in question, as well as on the experience and knowledge obtained through previous nonclinical and clinical testing of the vaccine's components. Additionally, any known target organs of the adjuvant or adjuvanted vaccine should be evaluated. For novel adjuvants and adjuvanted vaccines containing a novel adjuvant, it is recommended that the full tissue list be evaluated.

D.2.2.3.2 *Parameters for monitoring of local reactogenicity*

Local toxicities should be determined at the site(s) of adjuvant or adjuvanted vaccine administration and any other sites that come into contact with the adjuvant or adjuvanted vaccine components as a result of the method of administration. Local toxicity studies of intramuscularly administered vaccines should preferably be conducted in animals with sufficient muscle mass to test the full human dose of the final vaccine formulation.

Injection site reaction after inoculation should be scored using a prospectively defined system (e.g. the modified Draize test) (*34*) along with an assessment of any vesiculation, ulceration, severe eschar formation and other manifestations of significant toxicity (e.g. limb impairment).

The site of administration and any other site that comes in contact with the adjuvant or adjuvanted vaccine (e.g. eye exposure during aerosol administration, or digestive tract after oral administration) should also be evaluated histopathologically. In addition, a description of cellular infiltrates based on routine histological staining, if present, should be reported as part of the postmortem evaluation, as well as any manifestation of tissue damage at the site of injection and surrounding anatomic structures (e.g. sciatic nerves, nasal cavities or olfactory bulb).

D.2.3 Developmental and reproductive toxicity

Because vaccination programmes may include women of childbearing potential, it is important to consider the need for developmental and reproductive toxicity studies. As is the case for general toxicity, the use of a novel adjuvant may require

adding an adjuvant-alone arm to the reproductive toxicity studies. However, the study design is also dependent on the intended clinical use of the vaccine. For example, vaccination may be given early in pregnancy to protect the mother at risk, or might be given later in pregnancy to induce passive immunization to protect the infant directly from birth.

In general, the administration of one or several additional doses during organogenesis (i.e. implantation to closure of the hard palate) is recommended in order to evaluate the potential, direct embryotoxic effects of the components of the vaccine formulation, and, depending on the animal model, to allow maternal antibody to transfer to the progeny during pregnancy or the lactation period. Depending on the adjuvant, there may be concern about an adjuvant-induced systemic inflammatory response, such as fever, which may adversely affect early pregnancy (e.g. implantation or placental growth) (*35*). In these cases, it is recommended to include in the study design an additional treatment group to evaluate the effect of adjuvant on early pregnancy parameters. Rather than dosing this treatment arm prior to mating, it is recommended to dose animals post-mating and prior to implantation (e.g. post-mating day 1). Considering the short gestational period of the animal species that are most frequently used, it may be necessary to administer priming doses to the animals several days or weeks prior to mating in order to elicit a peak antibody response during the period of organogenesis.

End-points in embryo-fetal/perinatal-postnatal toxicity studies include, but are not limited to, viability, abortions, number of resorptions, fetal body weight, morphology, preweaning development and growth, as well as survival incidence and developmental landmarks. For details on such studies, please see the United States Food and Drug Administration's *Guidance for industry: considerations for developmental toxicity studies for preventive and therapeutic vaccines for infectious disease indications* (*36*) and WHO guidelines on nonclinical evaluation of vaccines (*1*).

In most cases, the developmental and reproductive toxicity studies can be performed in parallel to the clinical trials. However, some NRAs require that women of childbearing potential be excluded from large-scale late-stage clinical trials that are conducted prior to the completion of developmental and reproductive toxicity studies; other NRAs require the use of appropriate birth control methods for women of childbearing potential that are included in clinical trials. Further considerations can be found in *Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals: M3(R2)* (*31*).

D.2.4 Biodistribution studies

Adjuvants are expected to exert their action locally in close connection to the antigen. However, biodistribution studies can be helpful in understanding the

distribution of the adjuvant following injection. The feasibility of and need for such biodistribution studies should be evaluated on a case-by-case basis.

D.2.5 Genotoxicity and carcinogenicity studies

Genotoxicity studies are normally not needed for the final vaccine formulation (*1*). However, a standard battery of genotoxicity studies is generally recommended for most novel adjuvants that are (or contain) new chemical entities (*31*, *37*). Based on previous experience, carcinogenicity studies are generally not needed for adjuvants or adjuvanted vaccines.

D.2.6 Toxicity studies of adjuvant alone

As noted in the introduction to section D.2, comprehensive toxicity assessment of the adjuvant alone in animals may be included as part of the study design with the adjuvanted vaccine. However, evaluation of the adjuvant alone can be important for novel adjuvants that have not been studied previously or will be used in multiple different vaccine formulations. In the case of a novel adjuvant or combination adjuvant, it may be advisable to include additional (lower and higher) doses of the adjuvant component(s) in order to identify a safe dose that could be used in a first-in-human clinical trial, as well as safety signals that should be monitored in the proposed clinical trial.

Although not usually required, safety pharmacology studies may be recommended in some cases to demonstrate that a novel adjuvant has no adverse effects on physiological functions (e.g. on the central nervous system, or the respiratory or cardiovascular system, renal function, and body temperature). If needed, such evaluations could also be included as a specific arm with the adjuvant alone in the repeated-dose toxicity study of the intended final vaccine formulation (*1*, *38*). It is expected that these studies would be conducted before initiating first-in-human clinical trials.

D.2.7 Summary of recommendations regarding timing of studies

In general, the guidance provided in this document regarding the timing of studies in relation to clinical trials is consistent with that of other guidance documents (*31*). A repeated-dose toxicology study (including safety pharmacology end-points, if needed) should be conducted before the first-in-human clinical trial. It may be important to conduct some studies with adjuvant alone (e.g. systemic toxicity and genotoxicity, when needed as discussed in sections D.2.5 and D.2.6) prior to initiation of clinical trials (*31*). Developmental toxicology studies should be performed prior to initiation of any clinical study to be conducted in pregnant women – i.e. for those vaccines specifically developed for use in pregnancy. For vaccines indicated for females of childbearing potential, subjects can be enrolled in clinical trials provided that appropriate precautions are taken to

avoid vaccination during pregnancy, such as pregnancy testing and use of birth control. For these products, developmental toxicity studies (section D.2.3) may be performed in parallel to the clinical study.

D.3 Additional considerations

Additional studies for safety assessment have been considered for the specific situation in which the target population for a vaccine containing a novel adjuvant includes very young subjects – such as neonates. At this time, however, there is insufficient knowledge about suitable animal models to evaluate whether neonates with an immature immune system would adequately respond to adjuvanted vaccines or whether the adjuvant could modify the neonatal immune system in an undesirable way. Modified immune responses to vaccination also have been observed in elderly populations; however, there also is insufficient knowledge about animal models to evaluate the response to adjuvants and adjuvanted vaccines in the ageing population. Further research to improve methods that can be used for the nonclinical evaluation of adjuvanted vaccines that are targeted for neonatal and elderly populations is encouraged.

Thus far, there is no compelling clinical evidence that adjuvants are causally related to the induction of autoimmune phenomena (or autoimmune disease) or hypersensitivity in humans (*4*). Although there has been interest in developing animal models that could be used to screen adjuvants and adjuvanted vaccines for induction of autoimmunity or hypersensitivity, such models do not currently exist. Therefore, no recommendations can be made at this time regarding specific nonclinical studies that should be conducted. These are complex and multifactorial conditions; further research is needed to identify additional biomarkers related to autoimmunity and hypersensitivity phenomena.

Part E. Considerations for first-in-human clinical trials

As with the nonclinical safety assessment considerations, the first-in-human trial considerations for new adjuvanted vaccines are similar to those for non-adjuvanted vaccines (*2*); however, some issues unique to the clinical evaluation of vaccines with novel adjuvants may need to be considered. The initial clinical trials of adjuvanted vaccines are usually intended to: (a) determine the subjects' tolerability to the range of doses of antigen and adjuvant, and the dosing regimen that may be needed for later immunogenicity and clinical end-point trials; and (b) to aid in the collection of information on the nature of the adverse reactions that can be expected. This section provides guidance on the points to consider when transitioning from nonclinical to clinical testing of adjuvanted vaccines as signals observed in nonclinical studies can aid in the design of the first-in-human clinical trials. This section is intended to supplement the information

provided in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*2*).

Although there are limitations in the ability of animal and in vitro studies to predict safety in humans, all of the relevant nonclinical data, including the information on the pharmacologically active dose and the full toxicological profile of the adjuvanted vaccine, should be considered when designing the first-in-human trials. These data may aid in the selection of a safe starting dose, schedule, and route of administration, and in the identification of potential adverse effects for specific monitoring in the first-in-human clinical trial. A summary of such data from the nonclinical studies with the adjuvanted vaccine, and any available clinical data from similar or related adjuvanted vaccines, should be provided to support the acceptability of the proposed first-in-human clinical trial design. If, for example, dose-limiting toxicity was observed with the adjuvanted vaccine in the animal studies and the studies were repeated with lower doses to identify a dose that was without adverse effect in animals, it would be important to point that out and to summarize the specific adverse effects observed in the nonclinical studies.

Manufacturers should provide a rationale and scientific support for the use of an adjuvant in their vaccine. This could include information supporting the "added benefit" of the adjuvant derived from nonclinical studies (e.g. in vitro assays and/or proof-of-concept studies in animal models, including relevant challenge models when available) conducted prior to the initiation of clinical trials. In addition, it is recommended that the early clinical evaluations of an adjuvanted vaccine be designed to include the evaluation of both antigen-alone and adjuvanted vaccine arms to demonstrate the added benefit of the adjuvant; such data may include, for example, evidence of enhanced immune responses or antigen sparing.

If the safety of the adjuvanted vaccine was evaluated in appropriately designed toxicology studies that were conducted in line with the recommendations outlined above, and if there were no adverse effects observed in the toxicology studies conducted, the human dose tested in the toxicology studies may be acceptable as the starting dose in the first-in-human trials. However, such clinical trials are often designed as dose-escalating studies where the antigen and/or the adjuvant are given at escalating doses. With this in mind, given the limitations of the animal studies, it may be prudent to consider using a safety factor (a safety factor of 10 has been used historically) and to divide the human dose tested in the toxicology studies by the safety factor to find the recommended starting dose, and then escalate the dose from there. While it is anticipated that the adjuvant may have an antigen-sparing effect, the first-in-human trials should be designed to attempt to establish whether the adjuvant is needed and, if so, the minimum dose of adjuvant that is necessary to achieve adequate immunogenicity.

Although an inactive control (placebo) group may not be required in the first-in-human trial of an adjuvanted vaccine, the inclusion of a group receiving an inactive control, such as inert saline placebo, in early-phase clinical trials will enhance interpretation of the initial safety data through control for placebo effects and circulating community-acquired illnesses. It is recommended that the inclusion of an adjuvant-alone arm be discussed with the relevant NRA as some regulatory authorities recommend that such arms be avoided for ethical reasons; in those cases, an antigen-alone control arm may be preferred.

As with first-in-human trials of non-adjuvanted vaccines, those for adjuvanted vaccines are usually conducted in a limited number of healthy, adult volunteers (e.g. aged 18–50 years) with safety as the primary objective. The number of subjects enrolled in these first-in-human clinical trials typically ranges from 20 to 80 subjects; however, depending on the study design, the formulation of adjuvanted vaccine to be studied, and other relevant factors, a lower or higher number of subjects may be enrolled. To aid in the overall risk/benefit evaluation of the adjuvanted vaccine, the subject population should be clearly defined by inclusion and exclusion criteria, and the subjects should be closely monitored for safety. The clinical protocol should contain a safety monitoring plan with details of active post-vaccination monitoring, and predefined toxicity criteria for assessing the severity of clinical and laboratory parameters (*39*). In addition, the plan for increasing the dose of antigen and adjuvant, with predefined stepwise criteria for doing so, should be included in the clinical protocol. Also, it is recommended, especially when a novel adjuvant is used, that safety monitoring be extended through 12 months following the last vaccination (where the last follow-up may be accomplished by a telephone call). In this regard, it is recommended that serum specimens be banked where possible for potential future assessment in the event of a serious adverse event, a new-onset medical condition, or an adverse event of special interest that develops later in the course of the first-in-human clinical trial.

Any safety data based on experience with the same adjuvant formulated with other vaccine antigens, if available, may assist in developing the safety monitoring plan for the adjuvanted vaccine. However, since the mode of action in humans for the adjuvant in the specific adjuvanted vaccine to be evaluated in the first-in-human trial is usually unknown, and adjuvants may exhibit a range of properties that induce complex immune responses, it is recommended that subjects in first-in-human trials of adjuvanted vaccines be asked about specific adverse events. This may include, for example, inquiries on local reactions (e.g. pain, redness, swelling, granuloma formation, abscess, necrosis and regional lymphadenopathy), systemic reactions (e.g. fever, nausea, diarrhoea, and malaise), immune-mediated toxicity (e.g. cytokine release, immune suppression and autoimmune disease), and teratology. Examples of

adverse events of "special interest" may include neuroinflammatory disorders (e.g. optic neuritis and transverse myelitis), musculoskeletal and connective tissue diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus and Wegener granulomatosis), and gastrointestinal disorders (e.g. Crohn disease and ulcerative colitis). Additionally, targeted laboratory assessments (e.g. C reactive protein, fibrinogen, antinuclear antibody, antineutrophil cytoplasmic antibodies, and rheumatoid factor) may aid in the evaluation of adverse events and medical conditions.

Table 2.1

Points to consider for the manufacturing and quality information to be provided for pharmacology studies, toxicology studies[a] and first-in-human trials

Considerations	**Comment on information needed, by type of study**		
	Pharmacology	Toxicology[a]	First-in-human trials
Quality information regarding raw materials[b]	Information regarding purity and source of raw materials is important	Information regarding purity and source of raw materials is important	Information regarding purity and source of raw materials is important
Production of intermediates and adjuvanted vaccine	Production of intermediates and adjuvanted vaccine may be small scale	Production of intermediates and adjuvanted vaccine may be small scale; ideally, the lots used for the toxicology study should be the same as those that will be used in the first-in-human trials (or the lots should be comparable to the lots that will be used in the first-in-human trials in terms of the manufacturing process and the controls)	Production of intermediates and adjuvanted vaccine may be small scale, but control of manufacture is important; intermediates and adjuvanted vaccine should be manufactured in compliance with the appropriate good manufacturing practices

Table 2.1 *continued*

Considerations	Comment on information needed, by type of study		
	Pharmacology	Toxicology[a]	First-in-human trials
Presentation	Adjuvanted vaccine components (or antigen and adjuvant intermediates) often are provided in separate containers to be mixed prior to use	Adjuvanted vaccine may be provided as a premixed formulation or as two components (in separate containers) to be mixed prior to administration	Adjuvanted vaccine may be provided as a premixed formulation or as two components (in separate containers) to be mixed prior to administration
Characterization	Characterization of material may not be extensive; usually general quality information (e.g. composition, purity, potency[c, d]) is provided	Material should undergo considerable characterization to include, for example, information on purity, physicochemical characteristics and potency;[c, d] also, stability should be assessed	Material should undergo considerable characterization to include, for example, information on purity, physicochemical characteristics and potency;[c, d] also, stability should be assessed

[a] Toxicology studies should be compliant with GLPs (see "Terminology" section above).

[b] Ideally, the raw materials should be the same throughout all of the studies: pharmacology, toxicology and first-in-human trials.

[c] If a potency assay has been developed for the adjuvanted vaccine, such information should be provided. Alternatively, testing the antigen for potency, and the adjuvant for identity and content, is recommended.

[d] If the adjuvanted vaccine is provided premixed in one container, it should be tested for potency. However, in some cases, the potency assessment of the adjuvanted vaccine may require multiple types of tests (e.g. in the case of aluminium-adsorbed vaccines). In these cases, the determination of potency and amount of antigen present in the antigen intermediate preparation prior to adsorption (as well as the completeness of adsorption) may be recommended in addition to an in vivo method to assess the potency of the adjuvanted vaccine.

Authors and acknowledgements

Taking into account comments received during a WHO Consultation on the Nonclinical and Preclinical Evaluation of Adjuvanted Vaccines, held 7–8 September 2011 in Rockville, MD, USA, a first draft of these Guidelines was prepared by the following WHO Drafting Group: Dr M. Baca-Estrada, Health Canada, Canada; Dr G. Coleman, Health Canada, Canada; Dr M. Gruber, United States Food and Drug Administration Center for Biologics Evaluation

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The third draft was prepared by the WHO Drafting Group taking into account comments received during the WHO Informal Consultation on Guidelines for the Nonclinical Evaluation of Adjuvanted Vaccines, held 27–28 November 2012, in Geneva, Switzerland. The consultation was attended by: Dr M. Baca-Estrada, Health Canada, Canada; Dr D. Carter, Infectious Disease Research Institute, USA; Dr L.G. Castanheira, National Health Surveillance Agency, Brazil; Dr G. Coleman, Health Canada, Canada; Professor I. Feavers, National Institute for Biological Standards and Control, England; Dr E. Griffiths, Consultant, England; Dr M. Gruber, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Ms M. Iguchi, Pharmaceuticals and Medical Devices Agency, Japan; Dr K. Ishii, National Institute of Biomedical Innovation, Japan; Mrs T. Jivapaisarnpong, Ministry of Public Health, Thailand; Ms J. Dahlan, National Agency of Drug and Food Control, Indonesia; Dr M. Khaitov, Federal Medical and Biological Agency of the Russian Federation, Russia; Dr D. Masset, Agence nationale de sécurité du médicament et des produits de santé, France; Dr M. Matsumoto, Pharmaceuticals and Medical Devices Agency, Japan; Dr B. Meade, Meade Biologics, USA; Dr L. Martinez Munoz, Ministerio de Salud Pública, Cuba; Dr S.R. Pakzad, Food and Drug Control Laboratory, Islamic Republic of Iran; Dr M. Pallardy, Université Paris-Sud, France; Dr V.G. Somani, Ministry of Health and Family Welfare, India; Dr J. Southern, Ministry of Health, South Africa; Dr Y. Sun, Paul-Ehrlich-Institut, Germany; Dr E. Sutkowski, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr J.W. van der Laan, Medicines Evaluation Board, the Netherlands; Dr M. Xu, National Institutes for Food and Drug Control, China; Dr M. Bonelli, European Medicines Agency, England; Dr B. Fritzell (*International Alliance for Biological Standardization representative*), France. The following IFPMA representatives also attended: Dr D. Clarke (Pfizer), Dr N. Garçon (GlaxoSmithKline), Dr S. Gould (Sanofi Pasteur), Dr D. Novicki (Novartis Vaccines), Dr R. Zahn (Crucell). Representatives from the Developing Countries Vaccine Manufacturers Network (DCVMN) included: Dr S. Gairola, (Serum Institute of India), Dr M.A. Medeiros (Biomanguinhos), Dr R.I. Modi (Cadila Pharmaceuticals), Dr J. Petre (BioNet-Asia), Dr Q. Zhao (Xiamen Innovax) and Dr M. Reers (Biological E). Another industry representative who attended was Dr S. Dewasthaly (Intercell AG). WHO Secretariat members included: Dr J. Fournier-Caruana, Department of Essential Medicines and Health Products; Dr M.H. Friede, Information, Evidence and Research; Dr U. Fruth, Department of Immunization, Vaccines and Biologicals; Dr I. Knezevic, Department of Essential Medicines and Health Products; Dr S. Nishioka, Department of Essential Medicines and Health Products; Dr D.J. Wood, Department of Essential Medicines and

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A fourth draft document was subsequently prepared by the WHO Drafting Group taking into account comments submitted by the following reviewers: Dr E. Griffiths, England; Ms M. Iguchi, Pharmaceuticals and Medical Devices Agency, Japan; Dr K. Ishii, National Institute of Biomedical Innovation, Japan; Ms J. Dahlan, National Agency of Drug and Food Control, Indonesia; Dr M. Khaitov, Federal Medical and Biological Agency of the Russian Federation, Russia; Dr M. Matsumoto, Pharmaceuticals and Medical Devices Agency, Japan; Dr L. Martinez Munoz, Ministerio de Salud Pública, Cuba; Dr J. Southern, Ministry of Health, South Africa; Dr D. Clarke, Pfizer, USA; Dr N. Garçon, GlaxoSmithKline, Belgium; Dr S. Gould, Sanofi Pasteur, France; Dr D. Novicki, Novartis Vaccines, USA; Dr S. Gairola, Serum Institute of India, India. Contributors to combined comments received from the United States Food and Drug Administration Center for Biologics Evaluation and Research included: Dr M. Gruber, Dr C. Wrzesinski, Dr H. Golding, Dr M. Zaitseva, Dr B. Baldwin and Dr E. Sutkowski. Comments were also received from Dr J. Fournier-Caruana and Dr S. Nishioka, both from the Department of Essential Medicines and Health Products, World Health Organization, Switzerland.

During a round of public consultation organized through the WHO Biologicals website in April and early May 2013 comments were received from the following reviewers, and were taken into account by the WHO Drafting Group in the preparation of the document WHO/BS/2013.2214: Ms J. Dahlan, National Agency of Drug and Food Control, Indonesia; Dr M. Matsumoto, Pharmaceutical and Medical Devices Agency, Japan; Dr S. Løkstad, Brenntag Biosector A/S, Denmark; Dr M. Satoh, University of Florida, USA; Dr L. Martinez Munoz, Ministerio de Salud Pública, Cuba; Dr S. Sontakke, BGTD/Health Canada, Canada; Dr B. Keller-Stanislawski, Paul-Ehrlich-Institut, Germany; Dr S. Oh, Ministry of Food and Drug Safety, Republic of Korea; Dr N. Jain, Panacea Biotec, India; Dr G. Waxenecker, Austrian Agency for Health and Food Safety, Austria; Dr T. Morris, United States Pharmacopeia, USA; Dr K. Abraham, VaxInnate Corporation, USA. Comments from the IFPMA were coordinated by Dr L. Bigger, Vaccines Policy.

During a second round of public consultation on document WHO/BS/2013.2214 organized through the WHO Biologicals website from July to September 2013 further comments were provided by the following experts: Dr K. Zoon and F. Kaltovich, National Institutes of Health, USA; Dr M. Matsumoto, Pharmaceutical and Medical Devices Agency, Japan; Dr F. Cano, Dr D. Garcia and Dr S. Morgeaux, Agence nationale de sécurité du médicament et des produits de santé, France; Dr T. Nakano, Kyowa Hakko Bio Co., Japan; Dr S. Jadhav, Serum Institute of India, India; Dr M. Gruber, Dr E. Sutkowski, Dr C. Wrzesinski, Dr M. Green, Dr M. Serabian, and Dr G. Price, United States Food

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Appendix 1

Examples of classes of adjuvants

The following main classes of adjuvants (see section on "Scope" and section 2 above) are currently used in licensed vaccines or are being investigated. The list is an updated version of the list of adjuvants developed by the European Medicines Agency, Committee for Medicinal Products for Human Use (*1*). For each category, representative examples are provided.

Classification of adjuvants

- **Mineral salts or gels** – for example, aluminium hydroxide, aluminium phosphate gels or calcium phosphate gels.
- **Oil-in-water and water-in-oil emulsions, amphiphilic molecules and surfactant-based formulations** – for example, Novartis' MF59 (microfluidized detergent-stabilized oil-in-water emulsion); QS-21 (purified saponin, which is derived from plants); GlaxoSmithKline's AS03 adjuvant (an oil-in-water emulsion plus α-tocopherol); and SEPPIC's Montanide ISA 51 and Montanide ISA 720.
- **Particulate adjuvants** – for example, liposomes; virosomes (unilamellar liposomal vehicles incorporating influenza haemagglutinin); DC Chol (a lipoidal immunostimulator able to self-organize into liposomes); immune-stimulating complexes known as ISCOMS (structured complexes of saponins and lipids) and CSL's Iscomatrix (the iscom without the incorporated antigen); and biopolymers such as Poly(lactide-co-glycolide) (PLGA).
- **Pathogen-associated molecular patterns (natural and synthetic)** – for example, low-toxicity versions of LPS, including monophosphoryl lipid A (MPL or MPLA) and RC-529 (a synthetic acylated monosaccharide); Detox adjuvant (an oil drop emulsion of MPL plus *Mycobacterium phlei* cell-wall skeleton); OM-174 (lipid A derivative); CpG motifs (synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs); bacterial flagellin genetically fused with an antigen; bacterial toxins that have been genetically modified to provide nontoxic adjuvant effects such as modified heat-labile enterotoxin (LT) and cholera toxin (CT); and synthetic dsRNA such as Poly IC, Poly ICLC (also known as Hiltonol), and poly I:poly C12U (known as Ampligen).

- **Endogenous human immunostimulators** – for example, cytokines such as human granulocyte-macrophage colony-stimulating factor (hGM-CSF) or human interleukin-12 (hIL-12) that may be administered as proteins or as plasmid preparations (DNA sequences contained in DNA vaccine vectors that promote gene expression and are capable of inducing and/or promoting an immune response against an antigen in vaccine recipients).
- **Inert vehicles** – for example, gold particles.
- **Adjuvants derived from inulin** – for example, Vaxine's delta inulin (a plant-derived polysaccharide also known as Advax).
- **Combination adjuvants or adjuvant systems** consisting of combinations of vaccine-delivery systems and immunostimulatory agents that may result in more effective delivery of the immunostimulatory adjuvant as well as the antigen – for example, AS01 (liposomes, MPL and QS-21), AS02 (an oil-in-water emulsion plus MPL and QS-21), AS03 (an oil-in-water emulsion plus α-tocopherol), AS04 (MPL and aluminium hydroxide), AS15 (liposomes, MPL, QS-21 and a CpG oligodeoxynucleotide), glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) (a synthetic acylated monosaccharide in a stable oil-in-water emulsion) and CAF01 (liposomes, a quaternary ammonium lipid and a synthetic analogue of a mycobacterial lipid).

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Appendix 2

Tissue samples to be collected for a repeated-dose toxicity study

This is a comprehensive list of the tissues that should be evaluated for local and systemic toxicity in repeated-dose toxicity studies; some additional tissues have been included to represent those specifically targeted by adjuvanted vaccines. This is an updated version of a list developed initially by WHO for vaccines (*1*) that was broadened and harmonized by the European Medicines Agency, Committee for Medicinal Products for Human Use (*2*) and the Society of Toxicologic Pathology (*3*).

Samples should be collected from the following tissues. The type of tissue to be collected depends upon the species used for testing.

adrenal glands

aorta (thoracic)

bone (femur) with articulation

bone (sternum) with bone marrow

bone marrow smears[1]

brain

bronchi (main stem)

caecum

colon

diaphragm

duodenum

epididymides

eyes

gall bladder

Harderian glands

heart

ileum

injection site(s) (a sample should be taken from the area of injection)

jejunum

kidneys

lachrymal glands (from the main body and subconjunctival part)

larynx

liver

lungs

lymph nodes that drain the injection site

lymph nodes that do not drain the injection site (e.g. mandibular or mesenteric)

mammary gland

nasal–oropharyngeal cavity (depending on the vaccine and adjuvant)

[1] Bone marrow smears should be prepared for all animals at the time of necropsy, including from any moribund animals killed during the study. The smears should be fixed in methanol and then stained using the May-Grunwald-Giemsa method.

nasal tissue (skull/nasal cavity)
oesophagus
optic nerves
ovaries
oviducts
pancreas
parathyroid glands
Peyer's patches
pituitary gland
prostate
rectum
salivary glands (mandibular, parotid and sublingual)
sciatic nerves
seminal vesicles
skeletal muscle
skin
spinal cord (cervical, thoracic and lumbar)
spleen
stomach
testes
thymus
thyroid glands
tissues with macroscopic observations (a sample should be taken from any and all tissues with macroscopic observations)
tongue
trachea
ureters
urinary bladder
uterus (from the body, horns and cervix)
vagina

References

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Annex 3

Guidelines on the quality, safety and efficacy of typhoid conjugate vaccines

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Guidelines published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

Introduction

These Guidelines are intended to assist NRAs in evaluating the scientific issues connected with the quality, safety and efficacy of typhoid conjugate vaccines that use Vi polysaccharide covalently linked to a carrier protein. The available guidelines for Vi polysaccharide typhoid vaccine (*1*) and for live, attenuated Ty21a vaccines (*2*) are not applicable to typhoid conjugate vaccines consisting of Vi polysaccharide – derived from Salmonella enterica subspecies enterica serovar Typhi (*S.* Typhi), *Citrobacter freundii* sensu lato (*C. freundii* s.l.) or other bacterial sources – conjugated with a carrier protein, such as diphtheria toxoid (DT), tetanus toxoid (TT), recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA), the nontoxic mutated form of diphtheria toxin – such as cross-reactive material 197 (CRM197) – or another suitable protein.

These Guidelines are based on experience gained during the development of experimental typhoid conjugate vaccines as well as relevant information obtained from the evidence for other types of bacterial polysaccharide–protein conjugate vaccines, such as *Haemophilus influenzae* type b (Hib), meningococcal and pneumococcal conjugate vaccines. The evidence gathered thus far indicates that typhoid conjugate vaccines may overcome several limitations of unconjugated Vi polysaccharide vaccines. Conjugate vaccines may demonstrate: (a) greater efficacy and effectiveness; (b) longer persistence of immunity; (c) immunogenicity across all age groups, including infants and toddlers aged younger than 2 years; (d) perhaps some degree of herd immunity; and (e) induction of immune memory with initial dosing, leading to anamnestic responses to a subsequent dose or doses.

Part A of these Guidelines sets out guidance on manufacturing and quality control, while Part B addresses the nonclinical evaluation of these vaccines and Part C addresses their clinical evaluation. Part D provides guidance for NRAs.

General considerations

This section provides a brief overview of the scientific knowledge that underpins the guidance given in Parts A, B and C. A comprehensive review of the immunological basis for typhoid vaccines is also available from WHO (*3*).

Typhoid fever is an acute generalized infection of the mononuclear phagocyte system (previously known as the reticuloendothelial system), intestinal lymphoid tissue and gall bladder caused by *S.* Typhi. Paratyphoid fever is a clinically indistinguishable illness caused by *S. enterica* subspecies *enterica* serovar Paratyphi A or B (or, more rarely, C) (*4*, *5*). Typhoid and paratyphoid fevers are often referred to collectively as enteric fever. In most endemic areas, typhoid accounts for approximately 75–80% of cases of enteric fever. However,

in some regions, particularly in Asia, *S.* Paratyphi A accounts for a relatively larger proportion of all enteric fevers (*6*, *7*).

Pathogen

S. Typhi is a member of the family *Enterobacteriaceae*. It is a Gram-negative, non-lactose fermenting bacillus that produces trace amounts of hydrogen sulfide. Its antigens include an immunodominant lipopolysaccharide (LPS) O9, flagellar H and capsular polysaccharide Vi.

Vi acts as a virulence factor by preventing anti-O antibody from binding to the O antigen, and also inhibits the C3 component of the complement from fixing to the surface of *S.* Typhi. The Vi antigen is not unique to *S.* Typhi – it is also expressed by *S.* Paratyphi C, *C. freundii* s.l. and *S. enterica* subspecies *enterica* serovar Dublin. The genes responsible for the biosynthesis of Vi polysaccharide are located in a locus (*viaB*) within Salmonella pathogenicity island 7 (SPI-7) in the *S.* Typhi chromosome. Several other loci participate in the complex regulation of Vi expression. Almost all *S.* Typhi isolates from blood cultures can be shown to express Vi. Nevertheless, Vi-negative strains have been identified occasionally, both in sporadic cases as well as during outbreaks (*8*). Some of these strains are regulatory mutants that can revert to a Vi-positive state (*9*). However, some Vi-negative isolates from blood have been shown to harbour deletion mutations in critical genes (e.g. *tviB*) within the *viaB* locus that render the strains unable to synthesize Vi. This raises the theoretical concern that large-scale usage of Vi-containing vaccines (either polysaccharide or conjugate) could lead to selective pressure that creates a biological advantage for the emergence of Vi-negative strains (*10*).

Pathogenesis

Typhoid infection begins with ingestion of *S.* Typhi in contaminated food or water. In the small intestine, the bacteria penetrate the mucosal layer, and ultimately reach the lamina propria. Translocation from the intestinal lumen mainly occurs by *S.* Typhi targeting M cells overlying gut-associated lymphoid tissue. Within this lymphoid tissue and in the lamina propria, *S.* Typhi invokes an influx of macrophages and dendritic cells that ingest the bacteria but fail to destroy them. Thus some bacteria remain within macrophages in the lymphoid tissue of the small intestine and flow into the mesenteric lymph nodes where there is an inflammatory response mediated by the release of various cytokines. Bacteria enter the bloodstream via lymphatic drainage, thereby seeding organs of the mononuclear phagocyte system (such as the spleen, liver and bone marrow) and gall bladder by means of a silent primary bacteraemia. After a typical incubation period of 8–14 days the clinical illness begins, usually with the

onset of fever, abdominal discomfort and headache. An accompanying low-level secondary bacteraemia occurs.

Before the availability of fluoroquinolone antibiotics, clinical relapses were observed in 5–30% of patients treated with antibacterial agents such as chloramphenicol and sulfamethoxazole/trimethoprim. These post-treatment relapses occurred when typhoid bacilli re-emerged from their protected intracellular niches within the macrophages of the mononuclear phagocyte system, where the antibacterial agents could not penetrate.

In a small proportion of patients infected with *S.* Typhi who have premorbid abnormalities of the gall bladder mucosa, such as occurs consequent to gallstones, gall bladder infection becomes chronic (i.e. excretion lasts for longer than 12 months) (*11*). Such chronic carriers, who are not clinically affected by the presence of typhoid bacilli in their system, may excrete the pathogen in their faeces for decades (*12*). They serve as a long-term epidemiological reservoir in the community, and can transmit typhoid wherever there is inadequate sanitation, untreated water supplies or improper food handling.

Epidemiology

Typhoid is restricted to human hosts, and chronic carriers constitute the reservoir of infection. In the late nineteenth century and early twentieth century, typhoid was endemic in virtually all countries in Europe and the Americas. Subsequently, the widespread use of chlorination, sand filtration, and other means of water treatment drastically reduced the incidence of typhoid fever despite the high prevalence of chronic carriers (*11*). Typhoid remains endemic in most developing countries, mainly because large segments of the population lack access to safe water and basic sanitation services. In addition, there are limited programmes for detecting carriers and restricting them from handling food.

Disease burden

Variable estimates of typhoid fever have been published in the scientific literature. The true incidence of typhoid fever in most regions of developing countries is not known. A study published in 2004 estimated that 22 million cases occur each year, causing 216 000 deaths, predominantly in school-age children and young adults; the annual incidence was estimated to be 10–100 per 100 000 population (*13*). A systematic review of population-based studies from 1984 through 2005 reported an annual incidence of 13–976 per 100 000 population each year based on diagnosis by blood culture (*14*).

Several factors affect the calculation of the burden of typhoid disease. In the absence of a rapid, affordable and accurate diagnostic test, blood culture is recognized as the gold standard. However, blood culture alone identifies only 60–70% of the cases that are detectable using bone marrow culture or bile fluid

culture (*14*). Prior treatment with antibacterial agents also affects culture results. However, relying on clinical diagnosis alone may overestimate the burden because several febrile syndromes caused by other microorganisms, such as malaria, dengue and leptospirosis, can be confused with typhoid, particularly in children.

The incidence of typhoid, its age-specific distribution and the severity of clinical disease gleaned from passive surveillance implemented at health facilities often appears quite different from data acquired through active surveillance, during which households are visited systematically once or twice weekly to detect fever among their members. In 2008, a study by Ochiai and colleagues reported the incidence of typhoid detected through passive surveillance (and modified passive surveillance in two countries where additional health clinics were introduced into the community) in five Asian countries (*15*). The incidence of typhoid fever ranged from 15.3 per 100 000 person-years among people aged 5–60 years in China, to 451.7 per 100 000 person-years among children aged 2–15 years in Pakistan (*15*). Incidence data from the placebo control groups in vaccine trials have also provided information on the incidence of typhoid fever in multiple geographical areas and venues. However, because vaccine efficacy trials are typically carried out in areas with high endemicity, caution must be taken when extrapolating these incidence rates to other populations.

In general, there is less information on the burden of disease in children aged younger than 2 years than in older age groups. In the surveillance study conducted at sites in five Asian countries, two sites (Kolkata, India, and North Jakarta, Indonesia) included surveillance of children aged younger than 2 years (*15*). In Kolkata the recorded annual incidence among children aged younger than 2 years was 89 cases per 100 000 child-years (*15*); in North Jakarta the annual incidence was 0 cases per 100 000 child-years. In Kolkata only 1 of 145 blood cultures from febrile children in this age group was positive for *S.* Typhi (0.69%) (*16*); in Jakarta 0 of 404 blood cultures were positive for febrile children in this age group. By comparison, the incidence of culture-confirmed typhoid fever in Kolkata was 340.1 cases per 100 000 child-years in children aged 2–4 years; it was 493.5 cases per 100 000 child-years in children aged 5–15 years. In North Jakarta the annual incidence of typhoid was 148.7 cases per 100 000 child-years among 2- to 4-year-olds, and 180.3 cases per 100 000 child-years among children aged 5–15 years.

Prior to the availability of antibacterial agents, typhoid resulted in a case-fatality rate of approximately 10–20% (*17*). The 2008 WHO position paper on typhoid fever estimated that 216 000–600 000 deaths occurred annually (*18*). Most of the mortality occurs in developing countries, and 80% of deaths occur in Asia. A review by Crump and colleagues reported community-based mortality ranging from 0–1.8% across five studies in developing countries; hospital-based mortality ranged from 0–13.9% (across all ages in 12 studies); and in children younger than 15 years mortality ranged from 0–14.8% (across 13 studies) (*14*).

Few studies have estimated the prevalence of chronic carriers of typhoid and paratyphoid in developing countries. A survey in Santiago, Chile, conducted when typhoid fever was highly endemic there in the 1970s, estimated a crude prevalence of 694 typhoid carriers per 100 000 population (*19*). In Kathmandu, Nepal, among 404 patients (316 females and 88 males) with gall bladder disease undergoing cholecystectomy, *S.* Typhi was isolated from 3.0% of bile cultures and *S.* Paratyphi A from 2.2% (*20*). Since the overall prevalence of cholelithiasis in the population of Kathmandu was not known, the overall prevalence of chronic carriage in that population could not be calculated.

Clinical features

S. Typhi infection results in a broad spectrum of clinical features, most often characterized by persisting high-grade fever, abdominal discomfort, malaise and headache. Important clinical signs in hospitalized patients include hepatomegaly (41%), toxicity (33%), splenomegaly (20%), obtundation (2%) and ileus (1%) (*21*). Before antibacterial agents became available, gross bleeding from the gastrointestinal tract and perforations occurred in 1–3% of untreated patients, but these are now rarely observed except in settings with poor access to health care.

Typhoid fever has the potential to cause serious complications. Hospital-based reports suggest that more than 50% of patients may have complications. In 2005, Huang and colleagues analysed in which systems various complications were likely to occur – the central nervous system (3–55%), the hepatobiliary system (1–26%), the cardiovascular system (1–5%), the pulmonary system (1–6%), bones and joints (less than 1%), and haematological system (rarely) (*22*). Intestinal perforations leading to peritonitis and death continue to be reported in some settings today, although this is rare.

Immune responses to natural infection

Natural typhoid infection is usually associated with the detection of serum antibodies and mucosal secretory immunoglobulin (Ig) A intestinal antibody against various *S.* Typhi antigens; cell-mediated immune responses are also measurable (*23–27*). In areas where typhoid is endemic, there is an age-related increase in the prevalence and geometric mean titre of anti-Vi antibodies (*28*). Antiflagella (H antigen) serum IgG antibodies following natural infection are long-lived, and have been studied for seroepidemiological surveys (*29*).

While serological responses to LPS and flagella antigens tend to be fairly strong and are commonly found in patients with culture-confirmed acute typhoid fever, only about 20% of such patients exhibit significant levels of anti-Vi antibody (*30*, *31*). In contrast, high concentrations of anti-Vi serum IgG antibody are detected in 80–90% of chronic carriers (*30*, *31*).

Cell-mediated immunity also appears to play a part in protection – it has been observed that peripheral blood mononuclear leukocytes of otherwise healthy adults residing in typhoid-endemic areas, who have no history of typhoid, proliferate on exposure to *S.* Typhi antigens (*32*).

Disease control

Similar to other enteric and diarrhoeal diseases, typhoid fever exists predominantly in populations with inadequate access to safe water and basic sanitation. Effective typhoid control requires a comprehensive approach that combines immediate measures, such as accurate and rapid diagnostic confirmation of infection and timely administration of appropriate antibiotic treatment, as well as sustainable long-term solutions such as providing access to safe water and basic sanitation services.

Other interventions, such as treating household water, ensuring that food is handled properly, washing hands with soap, and discouraging defecation in the open may also be effective control measures (*33–35*). The most effective strategy for improving the health of typhoid-affected populations is to implement and maintain municipal water and sanitation systems.

Vaccination against typhoid has proved to be an effective preventive intervention, especially when coupled with hand washing, the treatment of household water, and the provision of adequate sanitation and other preventive measures. A detailed review of the immunological basis for typhoid vaccination has been published (*36*).

Typhoid vaccines

Inactivated whole cell vaccine

Inactivated *S.* Typhi bacteria (heat-inactivated and phenol-preserved) were first utilized to prepare parenteral vaccines more than 100 years ago. In the 1960s, WHO sponsored field trials that evaluated the efficacy of inactivated parenteral whole-cell vaccines in several countries (*37*, *38*), and documented a moderate level of efficacy lasting up to 7 years (*39*). Data from studies of human immune responses and immunogenicity studies in rabbits suggested that anti-H antibodies might represent an immune correlate of protection (*40*); later extrapolation from the results of mouse protection studies suggested that responses to Vi antigen may correlate with protection (*41*). However, these vaccines were associated with considerable rates of systemic adverse reactions (*42*) and are no longer used.

Live, attenuated Ty21a oral vaccine

In the early 1970s, an attenuated strain of *S.* Typhi was developed through chemical-induced mutagenesis of pathogenic *S.* Typhi strain Ty2 (*43*). The resultant mutant strain lost the activity of the epimerase enzyme encoded by the

galE gene, and was also no longer capable of expressing Vi polysaccharide. The vaccine was found to be stable, safe and efficacious in adults as well as children (*44–48*), but the level of protective immunity achieved varied according to the formulation of the vaccine, the number of doses administered and the interval between doses.

For example, three doses of a provisional formulation of vaccine or placebo administered to about 32 000 children (aged 6–7 years) in Alexandria, Egypt, gave a point estimate of efficacy of 95% (95% confidence interval (CI), 77–99%) during 3 years of follow-up (*49*). Three doses of enteric-coated capsules administered to Chilean schoolchildren aged 6–19 years using two different dose intervals (either alternate days or 21 days between doses) demonstrated a point estimate of efficacy of 67% (95% CI, 47–79%) during 3 years of follow-up; for the group receiving doses on alternate days, the point estimate of protection over 7 years was 62% (95% CI, 48–73%) (*44*, *50*). The estimate of protection was 49% (95% CI, 24–66%) with the 21-day interval between doses. Another trial used four doses administered within 7 days to Chilean schoolchildren and demonstrated even greater protection (*51*). Only 5% of children aged 6–7 years had difficulty swallowing the capsules (*51*). As of 2013, almost all countries where Ty21a is licensed utilize a three-dose course of enteric-coated capsules taken on alternate days, except the United States and Canada, which recommend a four-dose course.

Two other field trials in Chile (*48*) and Indonesia (*47*) compared the enteric-coated capsules with three doses of the liquid formulation. The liquid formulation conferred greater efficacy than the capsules in both trials. In Chile, where doses were given on alternate days, results with the liquid formulation were superior to Indonesia where the doses were administered 1 week apart (the point estimate of efficacy in Chile was 77%; in Indonesia it was 53%). In Chile, 78% protection was documented up to 5 years after vaccination with the liquid formulation (*50*). There is also indirect evidence that large-scale vaccination with Ty21a may provide some degree of protection against typhoid to people who have not been vaccinated through the mechanism of herd immunity.

Vi polysaccharide vaccine

Technological advances have made it feasible to purify Vi polysaccharide and to prepare vaccines that are almost totally free of contaminating LPS (*52*); these vaccines are associated with low rates of febrile reactions (1–2%).

The immunological basis of purified Vi polysaccharide parenteral vaccines is the generation of serum anti-Vi IgG antibodies in 85–90% of vaccine recipients older than 2 years.

Clinical trials with the vaccine showed a rise in anti-Vi antibody titres in adults and children (*53–55*). However, subsequent inoculations with Vi did not boost the antibody response. Although a single dose has been associated with the persistence of antibodies for up to 3 years in some recipients, many adult

recipients in non-endemic areas showed a marked drop in antibody levels after 2 years (*56*, *57*). An epidemic of typhoid fever among French soldiers deployed in Côte d'Ivoire showed that the risk of typhoid fever was significantly higher in persons vaccinated more than 3 years previously (*58*).

Field trials in children and adults in Nepal given a single (25-µg) dose showed 72% vaccine efficacy during 17 months of follow up (*53*); a field trial in schoolchildren in South Africa also using a single (25-µg) dose showed 60% protection during 21 months of follow-up (*54*). In South Africa, protection declined to 55% at 3 years (*59*). Another field trial in China in people aged 3–50 years given a single 30-µg dose showed 69% efficacy during 19 months of follow-up (*60*). Thus the main advantage of the Vi vaccine is that a single dose provides moderate protection. The disadvantage is that no data suggest that protective efficacy lasts beyond 3 years, so revaccination is necessary within that time.

Most data suggest that children who are younger than 5 years respond poorly to Vi polysaccharide vaccines (*61*). However, one cluster-randomized trial in Kolkata, India (*62*), showed that protective efficacy among young children (aged 2–4 years) was 80%, which was higher than that observed in children aged 5–14 years (56%) and in older persons (46%). In contrast, a cluster-randomized field trial of similar design and using the same Vi vaccine in Karachi, Pakistan, reported an adjusted total protective effectiveness of −38% (95% CI, −192% to 35%) for children aged 2–5 years compared with 57% (95% CI, 6% to 81%) for children aged 5–16 years (*61*).

Thus, a single dose of Vi vaccine can provide moderate protection for a limited duration, but the vaccines have the usual limitations associated with polysaccharide vaccines, including poor immunogenicity in infants and young children, short-lived immunity and lack of anamnestic immune responses to subsequent doses (*56*, *63*, *64*).

Vi polysaccharide–protein conjugate vaccine

Experience with several polysaccharide–protein conjugate vaccines (such as Hib, meningococcal and pneumococcal vaccines) has shown that conjugation overcomes many of the limitations associated with unconjugated bacterial polysaccharides. On the basis of this experience and to try to address the limitations of the various typhoid vaccines described above, several Vi polysaccharide–protein conjugate vaccines have been developed.

A preparation of Vi polysaccharide conjugated to rEPA (Vi-rEPA) was evaluated in a series of studies in endemic and other areas. Schoolchildren and preschool children from highly endemic areas who received the Vi conjugate vaccine achieved and maintained higher levels of anti-Vi IgG serum antibodies compared with those who received the Vi polysaccharide vaccine (*65–67*). The immunogenicity of this Vi conjugate vaccine was observed to be dose

dependent (*67*). Following the administration of a single dose, detectable antibody levels were maintained for as long as 10 years in adults and 8 years in children.

A placebo-controlled, randomized, double-blind study in Vietnamese children aged 2–5 years in the highly endemic area gave an estimated vaccine efficacy of 89% after nearly 4 years (*65*, *67*).

Vietnamese infants who received Vi-rEPA at age 2 months, 4 months and 6 months showed a rise in anti-Vi level from a geometric mean concentration (GMC) of 0.66 enzyme-linked immunosorbent assay (ELISA) units in cord blood to 17.4 ELISA units at 7 months (i.e. 1 month after the third dose) (*68*). By 12 months of age, the GMC had declined to 4.76 ELISA units. An additional dose at this age resulted in a boosting effect, with a GMC of 50.1 ELISA units 1 month later; 1 month after the booster dose more than 95% of infants had levels higher than 3.5 ELISA units, which is a putative antibody concentration associated with protection using the assay described in the study. Antibody responses to the routine vaccines used in the Expanded Programme on Immunization (administered simultaneously at age 2 months, 4 months and 6 months) were comparable in all groups.

A typhoid conjugate vaccine that uses Vi prepared from *C. freundii* s.l. and CRM197 as the carrier protein has been demonstrated to elicit a significantly higher level of anti-Vi IgG in European adults who had never been exposed to typhoid fever (*69*). Vi preparations from *C. freundii* s.l. have been shown to be immunologically indistinguishable from and structurally similar to those from *S.* Typhi (*70*, *71*), although size differences have been observed for Vi polysaccharide from *S.* Typhi and *C. freundii* s.l. by size-exclusion high-performance liquid chromatography (HPLC).

Animal challenge studies

In the 1950s and 1960s, WHO encouraged research to evaluate inactivated typhoid vaccines in various passive and active mouse-protection models to assess whether a model could be identified that predicted and correlated with the results of large-scale field trials of the vaccines in humans (*72–74*).

A more recent evaluation of Vi conjugate vaccines in bacterial challenge models has been reported (*71*). Hale and colleagues used a transformed Vi-producing *S. enterica* subspecies enterica serovar Typhimurium strain (C5.507) in a challenge model with BALB/c mice. Vaccination with Vi polysaccharide conjugated to the *Klebsiella pneumoniae* outer membrane 40-kD protein (rP40) provided partial protection from infection against C5.507. Opsonization assays demonstrated post-vaccination enhancement of Vi-positive bacterial uptake by macrophages derived from cultured murine bone marrow. Rondini and colleagues also showed protection in BALB/c mice against challenge with Vi-positive C5.507 subsequent to vaccination with Vi derived from *C. freundii* s.l. conjugated with CRM197 (*75*).

Historically, animal models could not closely mimic the disease process of human typhoid. In 2010, Libby and colleagues (*76*) reported results with engrafted human haematopoietic stem cells into (NOD)-SCID-IL12rγ^{null} diabetic mice. A 10-fold increase in liver bacterial burden was reported subsequent to intraperitoneal infection with *S.* Typhi. In other studies with engrafted immunocompromised Rag2$^{-/-}\gamma_c^{-/-}$ mice with human fetal liver haematopoietic stem cells and progenitor cells, or with human umbilical cord blood cells, a more human-like disease was observed that included dissemination and replication of bacteria in liver and spleen (*77–79*). Other murine typhoid models are in development, such as those in mice deficient in toll-like receptor 4 (TLR4) (*80*) and TLR11 (*81*, *82*). The TLR mouse models may provide an advantage over human immune system mice since variability due to engraftment is not present.

Part A. Guidelines on manufacture and control

A.1 Definitions

A.1.1 International name and proper name

The international name of the vaccine should be typhoid conjugate vaccine. The proper name should be the equivalent of the international name in the language of the country of origin. The use of the international name should be limited to the vaccines that satisfy the specifications formulated below.

A.1.2 Descriptive definition

A typhoid conjugate vaccine is a preparation of *S.* Typhi or *C. freundii* s.l. Vi polysaccharide covalently linked to a carrier protein. It may be formulated with a suitable adjuvant. It should be presented as a sterile, aqueous suspension or as freeze-dried material. The preparation should satisfy all of the specifications given below.

A.1.3 International reference materials

There are no international reference materials that can be used to measure the polysaccharide content, molecular mass or size distribution, or animal immunogenicity of the Vi polysaccharide-based typhoid conjugate vaccines being developed. Working standards for Vi polysaccharide, either from *S.* Typhi or *C. freundii* s.l., are being developed.

An international reference material to standardize antibody responses to Vi polysaccharide conjugate vaccines against typhoid is being developed, and is expected to be available in the future. A national reference preparation of purified human anti-Vi polysaccharide IgG is available for standardizing ELISAs to evaluate the immune response to experimental vaccines in clinical studies (*83*) (see section C.2.1).

A.1.4 Terminology

The definitions given below apply to the terms used in these Guidelines. They may have different meanings in other contexts.

Carrier protein: the protein to which the Vi polysaccharide is covalently linked for the purpose of eliciting a T cell-dependent immune response to the Vi polysaccharide.

Final bulk: the homogeneous preparation in a single container from which the final containers are filled, either directly or through one or more intermediate containers.

Final lot: a number of sealed, final containers that are equivalent with respect to the risk of contamination that may have occurred during filling and freeze-drying (if performed). Therefore, a final lot should have been filled from a single container and freeze-dried in one continuous working session.

Master-seed lot: bacterial suspensions for the production of Vi polysaccharide or the carrier protein should be derived from a strain that has been processed as a single lot and is of uniform composition. The master-seed lot is used to prepare the working-seed lots. Master-seed lots should be maintained in the freeze-dried form or be frozen below −45 °C.

Modified carrier protein: a carrier protein that has been chemically or physically modified and prepared for conjugation to the polysaccharide.

Modified polysaccharide: purified polysaccharide that has been modified by a chemical reaction or a physical process in preparation for conjugation to the modified carrier protein.

Purified bulk conjugate: a bulk conjugate prepared from a single lot or pool of lots of modified polysaccharide and a single lot or a pool of lots of carrier protein. This is the parent material from which the final bulk is prepared.

Purified polysaccharide: the material obtained after final purification of polysaccharide. The lot of purified polysaccharide may be derived from a single harvest or a pool of single harvests that have been processed together.

Single harvest: the material obtained from one batch of culture that has been inoculated with the working-seed lot (or with the inoculum derived from it), harvested and processed together during one production run.

Working-seed lot: a quantity of live strains for the production of Vi polysaccharide or the carrier protein that are of uniform composition and that have been derived from the master-seed lot by growing the organisms and maintaining them in freeze-dried aliquots or frozen at or below −45 °C. The working-seed lot is used to inoculate the production medium.

A.2 Guidelines on general manufacturing

The general manufacturing recommendations contained in Good manufacturing practices: main principles for pharmaceutical products (*84*) and Good

manufacturing practices for biological products (*85*) should be applied at establishments manufacturing Vi polysaccharide conjugate vaccines.

Details of the standard operating procedures for preparing and testing Vi polysaccharide conjugate vaccines that have been adopted by the manufacturer, together with evidence that each production step has been appropriately validated, should be submitted for approval to the NRA. All assay procedures used for quality control of the conjugate vaccine and vaccine intermediates should be validated. When they are required, proposals to modify the manufacturing process and quality control methods should also be submitted for approval to the NRA before they are implemented.

Production strains for Vi polysaccharide and the carrier proteins should be handled according the specifications for their biosafety level, and depend on the requirements of the NRA (*86*). Standard operating procedures should be developed to deal with emergencies arising from accidental spills, leaks or other accidents. Personnel employed by the production and control facilities should be adequately trained. Appropriate protective measures, including vaccination, should be implemented if available.

A.3 Control of starting material

A.3.1 Certification of bacterial strain

A.3.1.1 Bacterial strain for preparing Vi polysaccharide

The bacterial strain used for preparing Vi polysaccharide should be from single, well characterized stock that can be identified by a record of its history, including the source from which it was obtained and the tests used to determine the characteristics of the strain.

The strain should be capable of stably producing Vi polysaccharide. *S.* Typhi and *C. freundii* s.l. have been shown to be suitable sources for Vi polysaccharide. 1H nuclear magnetic resonance (NMR) spectroscopy and immunochemical tests are suitable methods for confirming the identity of the polysaccharide.

A.3.1.2 Bacterial strain for preparing the carrier protein

The bacterial strains used for preparing the carrier protein should be identified by their history, including the source from which they were obtained and the tests used to determine the characteristics of the strains.

A.3.2 Bacterial-seed lot system

The production of both Vi polysaccharide and the carrier protein should be based on a seed-lot system involving a master seed and working seed. Cultures derived from the working seed should have the same characteristics as the

cultures of the strain from which the master-seed lot was derived (see sections A.3.1.1 and A.3.1.2).

Each new seed lot prepared should be characterized for Vi production using appropriate methods. For example, if materials of animal origin are used in the medium – whether for seed-lot preparation, for the preservation of strain viability, for freeze-drying, or for frozen storage – they should comply with the *WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products* (*87*), and should be approved by the NRA.

Manufacturers are encouraged to avoid the use of materials of animal origin wherever possible.

A.3.3 Bacterial culture media

Basal medium must be sterilized, and manufacturers are encouraged to use semi-synthetic or chemically defined media that do not have ingredients of animal origin.

The liquid culture medium used to prepare bacterial-seed lots and to produce polysaccharide antigen should be free from ingredients that form a precipitate when hexadecyltrimethylammonium bromide (CTAB) is added at a concentration subsequently used in the manufacturing process.

Culture media should be free from substances likely to cause toxic or allergic reactions in humans. If materials of animal origin are used they should comply with the *WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products* (*87*) and should be approved by the NRA.

A.4 Control of vaccine production

A.4.1 Control of polysaccharide antigen production

The Vi polysaccharides that are used in licensed vaccines are defined chemical substances if they are prepared to similar specifications, for example as described in Requirements for Vi polysaccharide typhoid vaccine (Requirements for biological substances No. 48) (*1*). As a result, it is expected that they will have comparable potencies independent of the manufacturing process.

A.4.1.1 Single harvests for preparing Vi polysaccharide antigen

The consistency of the production process should be demonstrated by monitoring the growth of the organisms and the yield of Vi polysaccharide.

A.4.1.1.1 *Consistency of microbial growth for antigen production*

The consistency of the growth of production strains should be demonstrated by monitoring the growth rate, pH and the final yield of Vi polysaccharide, although monitoring should not be limited to these parameters.

A.4.1.1.2 ***Bacterial purity***

If required, samples of the culture should be taken before inactivation and examined for microbial contamination. The purity of the culture should be verified by using suitable methods; these should include inoculation on appropriate culture media. If contamination is found, the culture and any product derived from it should be discarded.

A.4.1.2 Bacterial inactivation and antigen purification

Generally, *S.* Typhi is inactivated by formaldehyde or by a suitable inactivating agent, or by alternative methods (e.g. heating). The inactivation process should be adequately validated.

The biomass of *S.* Typhi or *C. freundii* s.l. is removed by centrifugation or tangential flow filtration. The Vi polysaccharide is purified from the supernatant by precipitation with CTAB. All reagents should be pharmaceutical grade and sterile. Controls should be in place to monitor the bioburden during purification. Methods used for further purification of this intermediate should be agreed with the NRA. To ensure stability, purified Vi polysaccharide in powder form should be stored at 2–8 °C, and purified Vi polysaccharide in solution should be stored below –20 °C. The duration during which the polysaccharide will remain stable should be validated.

A.4.1.3 Control of purified Vi polysaccharide antigen

Each lot of purified Vi polysaccharide should be tested for identity and purity, as well as the additional parameters described below. All tests should be validated by and agreed with the NRA.

A.4.1.3.1 ***Identity***

Vi polysaccharide is a linear homopolymer composed of (1→4)-2-acetamido-2-deoxy-α-D-galacturonic acid that is *O*-acetylated at carbon-3 (*88*).

A test should be performed on the purified polysaccharide to verify its identity. NMR spectroscopy (*89*) or a suitable immunoassay is appropriate and convenient.

A.4.1.3.2 ***Molecular size or mass distribution***

The molecular size or mass distribution of each lot of purified polysaccharide should be estimated to assess the consistency of each batch. The distribution constant (KD) should be determined by measuring the molecular size distribution of the polysaccharide at the main peak of the elution curve obtained by a suitable chromatographic method. The KD value or the mass distribution limits, or both, should be established and shown to be consistent from lot to lot for a

given product. For gel filtration, typically at least 50% of the Vi polysaccharide should be eluted at a KD value lower than a predefined value, depending on the chromatographic method used.

An acceptable level of consistency should be agreed with the NRA. Alternatively, calculation of the peak width at the 50% level can be used to analyse the distribution of molecular weight (MW). Suitable methods for this purpose are gel filtration using: (a) a refractive index detector (*90*); (b) a colorimetric assay; or (c) a light scattering detector (*91*). Manufacturers are encouraged to produce Vi polysaccharide that has a consistent distribution of molecular size.

A.4.1.3.3 ***Polysaccharide content***

The concentration of the Vi polysaccharide in its fully *O*-acetylated, acid form in eluted fractions can be measured using Hestrin's method (*92*) or another suitable method, such as NMR (*89*). The acridine orange staining method (*88*, *93*) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD) (*93*) have been reported to produce comparable results for Vi polysaccharide in a range of 15–200 µg/ml. A suitable immunoassay, for example rocket immunoelectrophoresis or ELISA, may also be considered. A suitable reference preparation of Vi polysaccharide should be used. These methods should be validated, and agreed with the NRA.

A.4.1.3.4 ***O-acetyl content***

The *O*-acetyl content of the purified Vi polysaccharide is important for the immunogenicity of Vi; it should be at least 2.0 mmol/g polysaccharide (52% *O*-acetylation) (*88*, *90*, *94*).

Hestrin's method (*92*) or NMR (*89*, *95*) may also be used to quantitatively determine *O*-acetylation. The methods used and the acceptance criteria should be agreed with the NRA.

A.4.1.3.5 ***Moisture content***

If the purified polysaccharide is to be stored as a dried form, the moisture content should be determined using suitable, validated methods, and the results should be within agreed limits; the methods used and the acceptable limits should be agreed with the NRA.

A.4.1.3.6 ***Protein impurity***

Each lot of purified polysaccharide should contain no more than 1% (weight/weight) of protein as determined by a suitable, validated assay that uses bovine serum albumin as a reference (*96*).

Sufficient polysaccharide should be assayed to detect accurately 1% protein contamination.

A.4.1.3.7 *Nucleic acid impurity*

Each lot of purified polysaccharide should contain no more than 2% of nucleic acid by weight as determined by ultraviolet spectroscopy, on the assumption that the absorbance of a 10-g/l nucleic acid solution contained in a cell of 1 cm path length at 260 nm is 200 (*90*); other validated methods may be used.

Sufficient polysaccharide should be assayed to detect accurately 2% nucleic acid contamination.

A.4.1.3.8 *Phenol content*

If phenol has been used to prepare the Vi polysaccharide antigen, each lot should be tested for phenol content using a validated method that has been approved by the NRA. The phenol content should be expressed in µg per mg of purified Vi antigen, and shown to be consistent and within the limits approved by the NRA.

A.4.1.3.9 *Endotoxin*

To ensure an acceptable level of pyrogenic activity in the final product, the endotoxin content of each lot of purified Vi polysaccharide should be determined, and shown to be within limits agreed with the NRA.

A.4.1.3.10 *Residues of process-related contaminants*

The residues of process-related contaminants in the purified polysaccharide (e.g. CTAB, formaldehyde and antifoaming agents) should be determined, and shown to be within limits agreed with the NRA. The routine testing of each lot before release for residual process-related contaminants may be omitted once consistency has been demonstrated on a number of lots; this number should be agreed with the NRA.

A.4.1.4 Modified polysaccharide preparations

Several registered and candidate polysaccharide-conjugate vaccines use chains of modified polysaccharides. Subsequent modification or truncation of Vi may be considered for use if the strain has been adequately characterized.

A.4.1.4.1 *Chemical modification*

Several methods are satisfactory for the chemical modification of polysaccharides prior to conjugation. The method that is chosen should be approved by the NRA. As part of the in-process control procedures, the processed polysaccharide that will be used in the conjugation reaction may be assessed to determine the number of functional groups introduced.

A.4.1.4.2 *Molecular size or mass distribution*

The degree of reduction in the size of the polysaccharide depends upon the manufacturing process. The average size or mass distribution (i.e. the degree of polymerization) of the processed polysaccharide should be measured using a suitable method. The size or mass distribution should be specified for each type of conjugate vaccine; appropriate limits for consistency should be specified since the size may affect the reproducibility of the conjugation process.

A.4.2 Control of carrier-protein production

A.4.2.1 Consistency of microbial growth for the carrier protein

The consistency of the growth of the microorganisms used should be demonstrated using methods such as pH and the final yield of the appropriate protein or proteins; other methods may also be used.

A.4.2.2 Characterization and purity of the carrier protein

Proteins that have been used as carriers in licensed conjugate vaccines include TT, DT and CRM197, but carriers could also include other proteins if these are approved by the NRA, such as rEPA. Manufacturers may choose other carrier proteins for conjugation provided that the vaccine is safe and immunogenic.

The test methods used to characterize such proteins to ensure that they are nontoxic and to determine their purity and concentration should be agreed by the NRA.

TT and DT should be of high purity and satisfy the relevant recommendations published by WHO (*97*, *98*).

Either classical CRM197 or recombinant CRM197 produced by genetically modified micro-organisms may be used. CRM197 with a purity not less than 90% as determined by high-performance liquid chromatography (HPLC) should be prepared by column chromatographic methods.

For carrier proteins already in use, a higher level of purity may already have been specified and may be required. The content of residual host DNA should be determined, and results should be within the limits that have been approved for the particular product by the NRA. When CRM197 is produced in the same facility as DT, methods should be used to distinguish the CRM197 protein from the active toxin.

The identity of the carrier protein should be determined serologically, and characterized using a combination of the following physicochemical methods as appropriate: (a) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE); (b) isoelectric focusing; (c) HPLC; (d) amino acid analysis; (e) amino acid sequencing; (f) circular dichroism; (g) fluorescence spectroscopy; (h) peptide mapping; (i) or mass spectrometry (*99*). Outcomes should be consistent with the reference material.

A.4.2.3 Degree of activation of the modified carrier protein

Adipic acid dihydrazide (ADH) or other appropriate linkers, such as N-Succinimidyl 3-(2-pyridyldithio)-propionate, can be used to modify the carrier protein. The level of protein modification should be monitored, quantified and be consistent. The use of an in-process control may be required. The reproducibility of the method used for modification should be validated.

The level of modification of the carrier protein by ADH can be assessed by determining the amount of hydrazide; this is done by using colorimetric reactions with 2,4,6-trinitrobenzenesulfonic acid and with ADH as a standard (*100–102*). Other suitable methods include fluorescent tagging followed by HPLC, or quadrupole time-of-flight mass spectrometry.

A.4.3 Conjugation and purification of the conjugate

A number of methods of conjugation are in use; all involve multistep processes (*93*, *100–102*). Prior to demonstrating the immunogenicity of the Vi polysaccharide conjugate vaccine in clinical trials, both the methods of conjugation and the control procedures should be established to ensure the reproducibility, stability and safety of the conjugate.

The derivatization and conjugation processes should be monitored and analysed for unique reaction products. Residual unreacted functional groups or their derivatives are potentially capable of reacting in vivo, and may be present following the conjugation process. The manufacturing process should be validated, and the limits for unreacted activated functional groups (those that are known to be clinically relevant) at the conclusion of the conjugation process should be agreed with the NRA.

After the conjugate has been purified, the tests described below should be performed in order to assess the consistency of the manufacturing process. The tests are critical for ensuring consistency from lot to lot.

A.4.4 Control of the purified bulk conjugate

Tests for releasing purified bulk conjugate should be validated.

A.4.4.1 Identity

A suitable immunoassay should be performed on the purified bulk conjugate to verify its identity.

Depending on the buffer used, NMR spectroscopy may be used to confirm the identity and integrity of the polysaccharide in the purified bulk conjugate (*95*, *103–105*).

A.4.4.2 Endotoxin

The endotoxin content of the purified bulk conjugate should be determined unless otherwise justified, and shown to be within limits agreed with the NRA.

A.4.4.3 *O*-acetyl content

The *O*-acetyl content of the purified bulk conjugate should be determined by NMR or by other appropriate methods. The *O*-acetyl content of the purified bulk conjugate should be agreed with the NRA.

A.4.4.4 Residual reagents

The purification procedures for the conjugate should remove any residual reagents that were used for conjugation and capping. The removal of reagents, their derivatives and reaction by-products, such as ADH, phenol and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (known as EDC, EDAC or EDCI), should be confirmed using suitable tests or by validation of the purification process.

The specifications of the process and the quantifiable methods to be used should be agreed upon in consultation with the NRA.

The process should also demonstrate that no significant covalent modification of the Vi itself has occurred (e.g. less than 5% of the Vi monosaccharides should have been modified). For example, many common conjugation procedures use EDC, and a frequent side reaction can result in Vi carboxylates being covalently modified to form an N-acylurea. Such modification may alter the structure of the Vi, and this modification is known to be immunogenic, leading to antibodies that cross-react with other EDC-modified polysaccharides, such as those in Hib, pneumococcal and meningococcal conjugate vaccines; thus this modification may interfere with these vaccines. The N-acylurea content can be readily measured using NMR.

A.4.4.5 Polysaccharide content

The content of Vi polysaccharide should be determined using an appropriate validated assay. Methods that have been used to determine the Vi polysaccharide content include the colorimetric assay with acridine orange, or HPAEC–PAD (*93*), which has superior reproducibility.

A.4.4.6 Conjugated and unbound (free) polysaccharide

A limit for the presence of unbound (free) Vi polysaccharide relative to total Vi polysaccharide should be set for each purified bulk conjugate; this limit should be agreed with the NRA. The upper limit should be specific for the

polysaccharide conjugate formulation, and the limit should not be exceeded during the shelf-life of the batch. Methods that have been used to assay unbound polysaccharide include gel filtration; ultrafiltration and hydrophobic chromatography; ultracentrifugation followed by HPAEC–PAD, or colorimetric detection (*90*, *101*); other suitable methods may be developed and validated.

A.4.4.7 Protein content

The protein content of the purified bulk conjugate should be determined using an appropriate validated assay. Each batch should be tested for conjugated and unbound protein. The unconjugated protein content of the purified bulk conjugate should comply with the limit for the product that has been agreed with the NRA.

Appropriate methods for determining unbound protein include HPLC or capillary electrophoresis.

A.4.4.8 Conjugation markers

The success of the conjugation process can be assessed by characterizing the conjugate using suitable methods. For example, an increase in the MW of the protein component of the conjugate compared with the carrier protein should be determined using the Coomassie blue stain with SDS–PAGE; an increase in the MW of the conjugate compared with both the Vi polysaccharide and the protein components should be evidenced by the gel filtration profile. The conjugate should retain antigenicity for both Vi and the carrier protein as demonstrated by dot blot or western blot.

Where the chemistry of the conjugation reaction results in the creation of a unique linkage marker, such as a unique amino acid, the validation batch should be assessed to quantify the extent of the covalent reaction of the Vi polysaccharide with the carrier protein, so that the frequency of the covalent bond is given as a function of the number of polysaccharide repeating units or overall polysaccharide content.

A unique linkage marker could be assessed for the validation batch or, alternatively, the manufacturing process should be validated to demonstrate that it yields conjugate with a level of substitution that is consistent from batch to batch.

A.4.4.9 Absence of reactive functional groups

The validation batch should be shown to be free of reactive functional groups or their derivatives that are suspected to be clinically relevant on the polysaccharide and the carrier protein.

Where possible, the presence of reactive functional groups – for example, those derived by ADH treatment – should be assessed for each batch. Alternatively,

the product of the capping reaction may be monitored, or the capping reaction can be validated to show that reactive functional groups have been removed.

A.4.4.10 Ratio of polysaccharide to carrier protein

The ratio of polysaccharide to carrier protein in the purified bulk conjugate should be calculated. For this ratio to be a suitable marker of conjugation, the content of each of the conjugate components prior to their use should be known. For each purified bulk conjugate, the ratio should be within the range approved by the NRA for that particular conjugate, and should be consistent with the ratio in vaccine that has been shown to be effective in clinical trials.

A.4.4.11 Molecular size or mass distribution

It is important to evaluate the molecular size or mass of the polysaccharide–protein conjugate to establish the consistency of production, product homogeneity and stability during storage.

The relative molecular size of the polysaccharide–protein conjugate should be determined for each purified bulk conjugate using a gel matrix appropriate to the size of the conjugate (*106*). The method should be validated and should have the specificity to distinguish the polysaccharide–protein conjugate from other components that may be present (e.g. unbound protein or polysaccharide). The specification of molecular size or mass distribution should be vaccine-specific and consistent with that of lots shown to be immunogenic in clinical trials.

Typically the size of the polysaccharide–protein conjugate may be examined by methods such as gel filtration using high-performance size-exclusion chromatography (HPSEC) on an appropriate column. Since the ratio of polysaccharide to protein is an average value, characterization of this ratio over the molecular size or mass distribution (e.g. by using dual monitoring of the column eluent) can provide further proof of the consistency of manufacturing (*99*, *107*).

A.4.4.12 Bacterial and mycotic bioburden

The purified bulk conjugate should be tested for bacterial and mycotic bioburden according to the methods described in Part A, section 5.2, of the revised General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6, revised 1973) (*108*), or using methods approved by the NRA. If a preservative has been added to the product, appropriate measures should be taken to prevent it from interfering with the test.

A.4.4.13 Specific toxicity of the carrier protein

When appropriate, the bulk conjugate should be tested to confirm the absence of specific toxicity in the carrier protein.

A.4.4.14 pH

If the purified bulk conjugate is a liquid preparation, the pH of each batch should be tested, and the results should be within the range of values shown to be safe in clinical trials and stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

A.4.4.15 Appearance

The appearance of the bulk purified conjugate should be examined. It should be clear to moderately turbid, and colourless to pale yellow.

A.4.5 Preparation and control of the final bulk

A.4.5.1 Preparation

The final bulk is prepared by mixing a preservative or stabilizer (if used), or both, with a suitable quantity of the bulk conjugate to meet the specifications of vaccine lots that have been shown to be safe and efficacious in clinical trials. If an adjuvant is used, it should be mixed with the final bulk at this stage.

A.4.5.2 Test for bacterial and mycotic sterility

Each final bulk should be tested for bacterial and mycotic sterility according to the requirements of Part A, sections 5.1 and 5.2, of the revised General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6, revised 1973) (*108*), or using methods approved by the NRA. If a preservative has been added to the final bulk, appropriate measures should be taken to prevent it from interfering with the test.

A.4.5.3 Sterile filtration

The final bulk conjugate should be sterile-filtered just before the final bottling. The concentration of both Vi and carrier protein, and the integrity of the conjugate, should be verified in the final filtrate.

A.5 Filling and containers

The recommendations concerning filling and containers given in Good manufacturing practices for biological products (*85*) should be applied.

A.6 Control of the final product

A.6.1 Inspection of the final containers

Each container of a final lot should be inspected visually (manually or with automatic inspection systems), and those showing abnormalities – such as improper sealing, lack of integrity, clumping or the presence of particles – should be discarded.

A.6.2 Control tests on the final lot

The tests used before releasing the final lot should be validated.

A.6.2.1 Identity

Identity tests on the Vi polysaccharide and the carrier protein should be performed on each final lot. An immunological test or a physicochemical assay may be used for the Vi polysaccharide and the carrier protein.

A.6.2.2 Bacterial and mycotic sterility

The contents of the final containers should be tested for bacterial and mycotic sterility according to the requirements of Part A, sections 5.1 and 5.2, of the revised General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6, revised 1973) (*108*), or using a method approved by the NRA. If a preservative has been added, appropriate measures should be taken to prevent it from interfering with the sterility test.

A.6.2.3 Polysaccharide content

The amount of Vi polysaccharide conjugate in the final containers should be determined and shown to be within the limits agreed with the NRA.

The formulations of conjugate vaccines produced by different manufacturers may differ. A quantitative assay for the Vi polysaccharide should be carried out. The specification is likely to be product-specific. The following types of tests may be used: (a) colorimetric methods; (b) chromatographic methods (including HPLC or HPAEC–PAD); or (c) immunological methods (including rate nephelometry).

A.6.2.4 Unbound (free) polysaccharide

A limit for the presence of free Vi polysaccharide should be set for each type of conjugate vaccine. Assessing the level of unconjugated polysaccharide in the final lot may be technically demanding; as an alternative, the molecular size of the conjugate could be determined for the final lot to confirm the integrity of the conjugate. An acceptable value should be consistent with the value seen in batches used for clinical trials that showed adequate immunogenicity; the value should be approved by the NRA.

A.6.2.5 *O*-acetyl content

The *O*-acetyl content of the Vi polysaccharide conjugate in the final container should be determined for each final lot by NMR (*89*) or by other appropriate methods, such as Hestrin's method (*92*). Routine release testing of each lot for

O-acetyl content in the final product may be omitted if the NRA agrees and if the *O*-acetyl content is measured at the level of conjugate bulk, and data validating the process that were obtained during the product's development confirmed that formulation and filling do not alter the integrity of the functional groups. A limit for the *O*-acetyl content of the Vi polysaccharide conjugate should be approved by the NRA (*94*).

A.6.2.6 Molecular size or mass distribution

The molecular size or mass distribution of the polysaccharide conjugate should be determined for each final lot using a gel matrix appropriate to the size of the conjugate; for example, HPSEC multiple angle laser light scattering (MALLS) may be used (*106*). The analysis of molecular size or mass distribution for each final lot may be omitted provided that the NRA agrees and the test has been performed on the conjugate bulk (see section A.4.4.11).

A.6.2.7 Endotoxin or pyrogen content

The pyrogenic activity of the vaccine in the final container should be tested in rabbits. The endotoxin should be tested using a validated Limulus amoebocyte lysate test or a suitable in vitro assay. The pyrogen content and the endotoxin content should be within the limits agreed with the NRA.

A.6.2.8 Adjuvant content and degree of adsorption

If an adjuvant has been added to the vaccine, its content should be determined using a method approved by the NRA. The amount and nature of the adjuvant should also be agreed with the NRA. If aluminium compounds are used as adjuvants, the amount of aluminium should not exceed 1.25 mg per single human dose.

The consistency of adsorption of the antigen to the adjuvant is important; the degree of adsorption should be tested in each final lot and should be within the range of values measured in vaccine lots shown to be clinically effective. The methods used and the specifications should be approved by the NRA.

A.6.2.9 Preservative content

If a preservative has been added to the vaccine, its content should be determined using a method approved by the NRA.

The amount of preservative in each dose of the vaccine should be shown not to have any deleterious effect on the antigen, or to impair the safety of the product in humans. The efficacy of the preservative should be demonstrated. The preservative used and the concentration should be approved by the NRA.

A.6.2.10 General safety (innocuity)

The need to test the final lots of the Vi polysaccharide conjugate vaccine for unexpected toxicity (also known as abnormal toxicity) should be agreed with the NRA. This test may be omitted from routine lot release once the consistency of production has been established to the satisfaction of the NRA, and when reliable good manufacturing practices are in place.

A.6.2.11 pH

If the vaccine is a liquid preparation, the pH of each final lot should preferably be near 7.2; liquid preparations should be tested, and the results should be within the range of values shown to be safe and effective for vaccine lots in clinical trials and stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

A.6.2.12 Moisture content

If the conjugate is dried, the acceptable level of residual moisture should be established, and the limit should be agreed with the NRA.

A.6.2.13 Osmolality

The osmolality of the final lots should be determined and shown to be within the limits agreed with the NRA.

A.6.3 Control of diluents

The recommendations in Good manufacturing practices: main principles for pharmaceutical products (*84*) should apply to the manufacture and quality control of the diluents used to reconstitute conjugate typhoid vaccines. An expiry date should be established for the diluents based upon stability data. For lot release of the diluent, tests should be done to assess the appearance, identity, volume, sterility and content of key components.

A.7 Records

The recommendations in Good manufacturing practices for biological products (*85*) should be followed as appropriate for the level of development of the candidate vaccine.

A.8 Samples

A sufficient number of lot samples of the product should be retained for future studies and needs. Vaccine lots that are to be used for clinical trials may serve as reference materials in the future, and a sufficient number of vials should be reserved and stored appropriately for that purpose.

A.9 Labelling

The recommendations in Good manufacturing practices for biological products (*85*) that are appropriate for a candidate vaccine should be applied, and the following additional information should also be included.

The label on the cartons enclosing one or more final containers, or the leaflet accompanying each container, should include:

- a statement that the candidate vaccine fulfils Part A of these Guidelines;
- the information that if the vaccine is a lyophilized form it should be used immediately after reconstitution; if data have been provided to the licensing authority to indicate that the reconstituted vaccine may be stored for a limited time then the length of time should be specified;
- information on the volume and nature of the diluent to be added to reconstitute the lyophilized vaccine; this information should specify that the diluent approved by the NRA should be supplied by the manufacturer.

A.10 Distribution and shipping

The recommendations appropriate for candidate vaccines given in Good manufacturing practices for biological products (*85*) should be followed.

Shipments should be maintained within specified temperature ranges, and packages should contain cold-chain monitors (*109*).

A.11 Stability, storage and expiry date

The recommendations appropriate for candidate vaccines given in WHO Good manufacturing practices for biological products (*85*) and Guidelines on stability evaluation of vaccines (*110*) should be followed. The statements concerning storage temperature and expiry date that appear on primary or secondary packaging should be based on experimental evidence, and should be submitted to the NRA for approval.

A.11.1 Stability testing

Stability testing should be performed at different stages of production, namely on stored intermediates (such as the purified polysaccharide, the carrier protein and the purified bulk conjugate) and on the final product. Parameters that indicate stability should be defined or selected appropriately, according to the stage of production. A stability protocol should be established for intermediates and for the final product; the protocol should include release assays that have been agreed

with the NRA. During production, it is advisable to assign a shelf-life duration to all in-process materials, in particular to intermediates that are stored.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on at least three lots of the final product manufactured from different bulk conjugates.

In addition, a real-time real-condition stability study should be conducted on at least one final container lot produced each year.

The formulation of vaccine and adjuvant (if used) should be stable throughout the shelf-life. Acceptable limits for stability should be agreed with the NRA.

The polysaccharide component of conjugate vaccines may be subject to gradual hydrolysis at a rate that may vary depending upon the type of conjugate, the formulation or adjuvant, the excipient, and conditions of storage. The hydrolysis may result in a reduced molecular size of the Vi polysaccharide component, a reduction in *O*-acetyl content, a reduction in the amount of polysaccharide bound to the carrier protein or in a reduced molecular size of the conjugate, or some combination of these.

The *O*-acetyl content should be monitored quantitatively for stability testing and release testing. The quantity of free protein should be monitored for stability testing and release testing. The molecular size or mass distribution should be monitored for stability testing and release testing.

If applicable, the residual moisture should be monitored for stability testing and release testing.

Tests should be conducted before licensing to determine the extent to which the stability of the product is maintained throughout the proposed validity period. The free saccharide content should be determined as a percentage of total saccharide, and should meet recommendations for the final product until the expiry date as established by the manufacturer and defined in section A.6.2.

Where applicable, the level of adsorption of the conjugate to the adjuvant should be shown to be within the limits agreed with the NRA, unless data show that the immunogenicity of the final product does not depend on the adsorption of the antigen to the adjuvant.

Accelerated stability studies may provide additional supporting evidence of the stability of the product or consistency in manufacturing, or both, but are not recommended for establishing the shelf-life of the vaccine under a defined storage condition.

When any changes are made in the production process that may affect the stability of the product, the vaccine produced by the new method should be shown to be stable.

If manufacturers consider incorporating a vaccine vial monitor (VVM) into the label, they should provide appropriate data to justify a correlation between the stability kinetics of the vaccine and the selected VVM (*111*).

A.11.2 Storage conditions

Before being distributed by the manufacturer or before being issued from a storage site, the vaccine should be stored at a temperature that has been shown by the manufacturer to be compatible with a minimal loss of titre. The maximum duration of storage and the optimal storage conditions should be defined based on the findings of stability studies; these should be agreed with the NRA and should ensure that all quality specifications for the final product, including the minimum titre specified on the label of the container or package, will be maintained for the duration of the shelf-life.

A.11.3 Expiry date

Expiry dates should be based on the findings of stability studies and the determination of shelf-life, and approved by the NRA. The expiry dates for vaccines and diluents may be different from one another.

A.11.4 Expiry of reconstituted vaccine (if applicable)

For single-dose containers, the reconstituted vaccine should be used immediately. For multidose containers, the container should be stored in a dark place at 2–8 °C unless photostability studies have shown that this is unnecessary. The expiry time for an opened container should be defined by stability studies and approved by the NRA, but it should not exceed 6 h.

Part B. Nonclinical evaluation of new typhoid conjugate vaccines

B.1 General principles

Detailed guidelines from WHO on the design, conduct, analysis and evaluation of nonclinical studies of vaccines are available separately (*112*), and they should be read in connection with Part B of these Guidelines. Specific issues to be considered in relation to candidate Vi conjugate vaccines are considered in section B.3. Plans for nonclinical studies to be conducted during the development of the vaccine should be discussed with the NRA early in the review process.

B.2 Product characterization and process development

It is critical that vaccine production processes are appropriately standardized and controlled to ensure consistency in manufacturing and the collection of nonclinical data that may suggest safety and efficacy in humans.

Candidate formulations of Vi conjugate vaccines should be characterized to define the critical structural and chemical attributes that indicate the polysaccharide, the conjugating protein and the conjugate product are sufficiently

pure and stable, and their properties are consistent. The extent of product characterization may vary depending on the stage of development. Vaccine lots used in nonclinical studies should be adequately representative of those intended for use in clinical investigations – that is, the safety data should support the initiation of clinical studies in humans. Ideally, the lots should be the same as those used in the clinical studies. If this is not feasible, then the lots should be comparable with respect to physicochemical data, stability and formulation.

B.3 Nonclinical immunogenicity studies

Immunogenicity studies in animal models should be conducted because they provide valuable proof-of-concept information that can be used to support a clinical development plan. In addition, immunogenicity data derived from appropriate animal models are useful in establishing the immunological characteristics of the Vi polysaccharide conjugate product, and may guide the selection of doses, schedules and routes of administration that will be evaluated in clinical trials. To ensure immunogenicity in nonclinical testing weaning mice (younger than 6 weeks) should receive intramuscularly two injections 2 weeks apart of the conjugate vaccine and Vi should be used for a control group. Anti-Vi IgG should then be measured. The conjugate should induce a response that is at least four times higher than the response induced by Vi, and a booster response should occur after the second dose (*100*). Immunogenicity studies of Vi polysaccharide conjugates have been conducted in mice (*71*, *93*, *113–115*); in humans, correlation has been made between the level of anti-Vi IgG and protection against clinical disease (*53*, *116*). Therefore, the primary end-point for nonclinical studies of the immunogenicity of Vi conjugate vaccines should be the level of anti-Vi elicited.

Nonclinical studies of immunogenicity may include an evaluation of seroconversion rates or geometric mean antibody titres, or both. When possible, nonclinical studies may be designed to assess relevant immune responses, including the functional immune response (e.g. by evaluating serum bactericidal antibodies, opsonophagocytic activity and serum-dependent opsonophagocytic killing) (see section C.2.2). These studies may also address the interference that can occur among antigens when multiantigen vaccines are used (see section C.2.3). In such cases, the response to each antigen should be evaluated.

Although there have been advances in animal models (see the section on General considerations), no ideal animal model exists that establishes direct serological or immunological correlates of clinical protection. In the absence of such a model, it is important to ensure that the production batches have the same protective efficacy as those used and shown to be protective in clinical trials. Therefore, the emphasis is increasingly being placed on ensuring consistency in manufacture through the use of modern physical, chemical and immunological quality-control methods.

B.4 Nonclinical toxicity and safety

WHO guidelines on nonclinical evaluation of vaccines (*112*) should be followed when assessing toxicity and safety. Toxicity studies for Vi polysaccharide conjugate typhoid vaccines may be performed in an appropriate animal model. These studies should entail careful analysis of all major organs, as well as of tissues proximal to and distal from the site of administration, to detect unanticipated direct toxic effects; these effects should be assessed for a wide range of doses, including those exceeding the intended clinically relevant dose. If novel proteins are used to manufacture conjugate vaccines, toxicity studies should be performed on these proteins first. Nonclinical safety studies should be conducted in accordance with the GLPs that have been described elsewhere (*117*, *118*). For ethical reasons, it is desirable to apply the 3Rs concept of "Replace Reduce Refine" to minimize the use of animals in research where scientifically appropriate.

Part C. Clinical evaluation of new typhoid conjugate vaccines

C.1 General principles

C.1.1 General considerations for clinical studies

In general, clinical trials should adhere to the principles described in the WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (*119*).

The general principles described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*120*) apply to Vi polysaccharide conjugate vaccines, and should be followed. Some issues specific to conjugate vaccines or to the clinical development programme for Vi conjugate vaccines, or both, are discussed below and should be read in conjunction with the general guidance mentioned above.

In particular, the methodological and statistical considerations described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations, sections B.2 and B.3 (*120*) should be taken into account.

Additional data on the safety, immunogenicity, efficacy and effectiveness of Vi conjugate vaccines may emerge, including insights into correlates of protection, and the suggestions for clinical development programmes that are provided in this section should be read with this in mind. Clinical programmes are expected to change once licensed Vi polysaccharide conjugate vaccines become widely available for use in various age groups.

C.1.2 Outline of the clinical development programme

In accordance with the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*120*), early clinical development programmes should identify an appropriate dose of conjugated Vi antigen and suitable immunization schedules for the target age groups. These initial studies should also provide a preliminary assessment of the vaccine's safety. Studies to determine an adequate dose and regimen are necessary for each candidate Vi conjugate vaccine that is developed, since it is not possible to extrapolate the antigen content and schedule identified for one conjugate vaccine to another. This consideration applies even if the same carrier protein is used for two different Vi conjugate vaccines, since experience with other conjugated polysaccharide vaccines has indicated that differences in conjugation chemistry can affect immunogenicity.

It is recommended that the major part of the pre-licensure clinical development programme is conducted in subjects who are representative of the intended target population.

The minimum acceptable content of the pre-licensure clinical programme for each candidate conjugate vaccine, and the expectations for the data to be generated during the post-licensure period, should be discussed between sponsors and the relevant NRA. Factors expected to have an important influence on the pre-licensure programme include the intended target age range and the availability of licensed unconjugated Vi vaccines or conjugated Vi vaccines, or both, for each age group.

Although data on antibody persistence and responses to booster doses are considered important, the collection and submission of these data usually occur during the post-approval period. Therefore, sponsors and NRAs should agree on the minimum duration of follow-up that will be required before the initial application dossier is submitted.

C.1.3 Evidence to support efficacy

C.1.3.1 Subjects aged at least 2 years

Protective efficacy studies against typhoid can be conducted only in endemic areas with relatively high rates of disease. In endemic areas a prospective comparison of subjects aged 2 years or older immunized with a Vi conjugate vaccine and an unvaccinated control group is not appropriate because there are licensed vaccines that have documented efficacy against typhoid in certain age groups. Also, a study of the relative protective efficacy (e.g. comparing a candidate Vi conjugate vaccine with an unconjugated Vi vaccine) is not likely to be feasible due to the large number of subjects that would be required to derive robust statistical conclusions.

Taking these issues into account, as well as evidence supporting the role of anti-Vi IgG antibody in protecting against Vi-expressing *S.* Typhi, it is not considered necessary to estimate the protective efficacy of candidate Vi conjugate vaccines against typhoid in subjects who are aged 2 years or older. In this age group the pre-licensure assessment of the likely protective efficacy of conjugated Vi vaccines could be based on appropriate studies of comparative immunogenicity (see section C.3).

Nevertheless, successful typhoid challenge studies conducted in healthy adults using an appropriate and validated model (i.e. one in which some protective efficacy of unconjugated Vi vaccines is detectable) could provide considerable supporting evidence of the efficacy of a Vi conjugate vaccine. Human challenge studies may also provide at least limited information on the relationship between the immune response and various efficacy parameters. If, in consultation with the NRA, sponsors decide to conduct typhoid challenge studies in humans, they should be undertaken only by physicians with appropriate expertise, and in a carefully controlled setting, to ensure the safety of the volunteers. Healthy adults should be screened to detect underlying pre-existing conditions and to exclude risk factors for complications, including gall bladder disease. The challenge strain should be well characterized and there should be complete information on its susceptibility to antibacterial agents.

C.1.3.2 Subjects younger than 2 years

There is no information on the protective efficacy or effectiveness against typhoid of any Vi conjugate vaccine in children who are younger than 2 years old when first vaccinated. Therefore, there is a need to carefully consider the potential value and feasibility of conducting a prospective, randomized study of protective efficacy in a region where background rates of proven typhoid disease have been documented in this age group. Whether such a study is required, or whether it can be replaced by an appropriate assessment of immunogenicity that is followed by post-approval effectiveness studies, can only be decided on a case-by-case basis after discussions between sponsors and NRAs. Since conducting a pre-licensure study of protective efficacy would likely prolong the time until the vaccine is approved, the decision regarding the requirement for a protective efficacy study should take into account factors such as the regional burden of typhoid disease in this age group.

If a pre-licensure protective efficacy study is conducted, it should compare rates of febrile illnesses associated with a positive blood culture for *S.* Typhi between a group that receives the candidate Vi conjugate vaccine and an appropriate control group. A double-blind design is recommended but this would require that the control group is randomized to a non-typhoid vaccine from which they may derive some benefit that is indistinguishable in appearance from the candidate conjugate vaccine and is administered in the same way (i.e.

route and schedule). If a suitable non-typhoid vaccine cannot be identified then the control subjects could be unvaccinated (i.e. avoiding the use of a placebo injection). In this case a double-blind design would not be possible but it would be important to make every effort to ensure that investigators are unaware of the treatment assignment.

Further information on designing and conducting studies of protective efficacy, and on assessing the effectiveness of vaccines is provided in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*120*).

C.1.3.3 Vaccine effectiveness

Whether or not a pre-licensure study of protective efficacy against typhoid is performed, it is recommended that efforts be made to estimate the vaccine's effectiveness during the post-licensure period (see section C.5).

C.2 Assessment of the immune response

C.2.1 Total anti-Vi IgG in serum

The primary parameter for assessing the humoral immune response to a vaccine is usually based on a measure of functional antibody. However, there are no well established or standardized assays for assessing functional antibody responses to Vi-containing vaccines, and it is not known how the results of such assays correlate with vaccine efficacy. A correlation between total serum antibody (*59*) or total anti-Vi IgG in serum (*61*, *65*, *67*, *68*, *121*) and protection against typhoid has been described, although there is no established cut-off value that clearly predicts prevention of clinical disease. Thus, it may be acceptable to base the primary assessment of the immunogenicity of candidate Vi conjugate vaccines on the total concentration of anti-Vi IgG.

In recent years, the assessment of immune response to licensed unconjugated Vi vaccines has predominantly used ELISA to measure total anti-Vi IgG in serum (*62*, *66*, *122*). Older assays, such as radioimmunoassay (*53*) and passive haemagglutination (*55*), are now rarely used (*57*). However, several ELISAs have been used in studies of different vaccines (*69*, *123*). In 2013, when these Guidelines were prepared, there was no international standard available. However, reagents and a software analysis tool for a Vi antibody ELISA are available free of charge from the United States Food and Drug Administration and the United States Centers for Disease Control and Prevention (contact information is listed in Table 3.1).

It is essential that the assays used to report data from the clinical studies that are considered to be pivotal for an application dossier should be fully validated. Once an international standard becomes available, all sponsors should use the standard to calibrate the assays used to determine concentrations of anti-Vi IgG.

Table 3.1
Contact details for materials for Vi antibody ELISA

Name	Provider address	Website
S. typhi anti-Vi (human) and *S.* typhi Vi polysaccharide, lot 05	Center for Biologics Evaluation and Research, United States Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD, 20903, USA	http://www.fda.gov/BiologicsBloodVaccines/ScienceResearch/BiologicsResearchAreas/default.htm
ELISA calculation programme	United States Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA	http://www.cdc.gov/ncird/software/elisa/index.html

C.2.2 Other immune-response parameters

As part of the overall characterization of the immune response to candidate Vi conjugate vaccines, sponsors may consider evaluating one or more of the following parameters, at least for subsets of serum obtained from different age groups and at different time points:

- serum bactericidal antibody (SBA);
- opsonophagocytic antibody (OPA);
- antibody avidity; following an initial T cell-dependent immune response in individuals naive for Vi antigen it would be expected that antibody avidity would increase over time, and there should also be differences between postprimary doses and postbooster doses;
- IgG subclass responses;
- evidence of T cell-dependent immune response with memory B-cell recruitment – e.g. an anamnestic response to a booster dose of vaccine, or detection of memory B cells using an in vitro cultured enzyme-linked immunosorbent spot assay (ELISPOT).

C.2.3 Characterization of the immune response

C.2.3.1 Antibody kinetics

The anti-Vi antibody kinetic should be assessed in recipients of the candidate Vi conjugate vaccine group and in subjects who receive any control Vi-containing vaccine (licensed unconjugated or conjugated) after the primary series and following booster doses.

Following the primary series (which may consist of one or several doses), serum samples may be collected at approximately day 14 and 28, at 6 months and then at 1 year and 3 years as a minimum.

Following a booster dose, a rapid rise in anti-Vi is expected if there has been efficient priming of the immune system. Therefore it is suggested that sera should be obtained at approximately 6 days, 10 days and 28 days after the booster dose, and then at preplanned intervals.

To reduce the number of samples taken from each participant, groups could be further randomized to provide samples at different time points. It is suggested that all subjects should at least provide samples before vaccination and on day 28 post-vaccination. Longer-term assessments of postprimary immunization and postbooster levels should be planned at least for subgroups of vaccinated subjects.

C.2.3.2 Immune memory

Due to concerns that vaccination with unconjugated Vi polysaccharide can lead to hyporesponsiveness to sequential doses, and may potentially blunt the immune response to a conjugated Vi vaccine, unconjugated Vi polysaccharide should not be administered to subjects primed with a candidate Vi conjugate vaccine in order to demonstrate that the initial dose(s) of the conjugate elicited a T cell-dependent immune response.

Whether a T cell-dependent initial immune response has been elicited by the initial dose or doses can be assessed by administering a further dose of the Vi conjugate vaccine after an interval of approximately 6–12 months. The immune response observed (ideally by measuring not only anti-Vi IgG but also functional antibody, antibody avidity and cell-mediated immunity using ELISPOT) following a single dose of Vi conjugate vaccine administered to subjects who completed a primary series of the same vaccine can be compared with the response to a first dose administered to previously unvaccinated subjects of the same age. The immune response to a single dose of the Vi conjugate vaccine in primed subjects should be superior to that in subjects who are Vi naive (see section C.3.4 for additional information regarding the administration of booster doses, including the administration of Vi conjugate vaccine to subjects who previously received conjugated or unconjugated Vi vaccines).

C.2.4 Analyses of immune responses

Although elicitation of anti-Vi IgG by vaccination has been shown to correlate with protection, the minimum concentration of anti-Vi IgG required for protection against typhoid remains uncertain (*61*, *65*, *67*, *68*, *121*).

The assessment of anti-Vi IgG concentrations should take into account all of the following factors:

- Proportions of vaccinees who achieve concentrations above one or more predefined threshold concentrations – analyses of protective efficacy observed over time in a prospective, randomized and placebo-controlled study with one Vi conjugate vaccine in children who were first vaccinated when aged 2–5 years have suggested a benchmark (or threshold) value that could be applied to the interpretation of anti-Vi IgG concentrations (*65*, *67*, *68*, *83*, *121*). Based on the assay used in these studies to assess stored serum samples, a threshold value of 4.3 µg/ml anti-Vi antibody measured by ELISA (*83*) appears to be associated with a high level of sustained protection lasting approximately 4 years after vaccination. If the antibody decay curve for a candidate conjugate vaccine resembles that of the vaccine that was used in the study, then the antibody concentrations at earlier time points after vaccination should considerably exceed this threshold value. Until an international standard becomes available, sponsors who wish to apply this threshold value to the results of their own assays need to perform a calibration against the assay used in the study of efficacy mentioned above (*83*).
- Seroconversion rates – seroconversion may be defined as a change from seronegative before vaccination to seropositive after vaccination (e.g. based on the assay cut-off or based on achieving a defined threshold value), or as at least a four-fold increase from pre-vaccination concentrations to post-vaccination concentrations in subjects who were seropositive at baseline.
- Reverse cumulative distributions (RCDs).
- Geometric mean concentrations (GMCs).
- When selecting the most appropriate immune-response parameter to use for the primary assessment of immunogenicity in any one study, researchers should take into account the population selected for investigation, the anticipated pre-existing antibody concentrations that may reflect prior vaccinations or natural exposure, and whether the assessment relates to postprimary series immunizations or postboosting. Regardless of which parameter is selected for the predefined primary analysis (see section C.3), between-group comparisons based on other parameters should be presented.

C.3 Clinical study designs

C.3.1 Studies that compare conjugated Vi vaccines with unconjugated Vi vaccines

Studies that compare candidate Vi conjugate vaccines with licensed unconjugated Vi vaccines can only be conducted in subjects who are aged at least 2 years. Data

should be generated across the entire age range for which a claim for use will be sought. Studies should stratify subjects by appropriate age subgroups, or separate studies should be conducted in different age groups.

It is recommended that these studies are randomized and double blind. If the sponsor proposes to administer more than one dose of Vi conjugate in any age subgroup there will be a need to consider matching of the schedule in the unconjugated Vi vaccine control group. Sponsors should identify suitable non-typhoid vaccines that could be administered to the control group in order to avoid or at least to minimize the need for placebo injections. The selection of the unconjugated Vi control vaccine for each study should take into account the available evidence on safety and immunogenicity and should be discussed with the relevant NRA.

The primary comparison of immune responses could be based on:

- percentages that achieve anti-Vi IgG levels above predefined threshold values (e.g. as suggested in section C.2.4);
- seroconversion rates.

The immune responses should be measured in samples collected at day 28 after the initial vaccination series has been completed (i.e. after a single dose or after the last assigned dose of the primary series), or in samples collected at an alternative time point if this is justified by data on the antibody kinetic.

The primary analysis should demonstrate that the immune response to the Vi conjugate vaccine is at least non-inferior when compared with the immune response to the control vaccine. The predefined margin of non-inferiority should be carefully justified. Protocols may also plan for sequential analyses to assess whether there is superiority in immune responses to the Vi conjugate vaccine if the predefined criterion for concluding non-inferiority has been met.

C.3.2 Studies that compare vaccinated groups with unvaccinated groups

These studies should employ random allocation to the Vi conjugate candidate vaccine (i.e. the vaccinated group) or to a licensed non-typhoid vaccine from which study subjects may derive some benefit (i.e. the unvaccinated group).

This study design is most likely to be used for subjects who are younger than 2 years. There are no Vi-containing vaccines known to be efficacious in this age group, which means that data on the immune response cannot provide a direct bridge to vaccine efficacy. Therefore, other options need to be considered for interpreting the anti-Vi IgG immune response to a candidate Vi conjugate vaccine.

The immune responses in the group receiving the candidate Vi conjugate vaccine should be superior to those in the unvaccinated group. In addition, the immune responses observed after the last assigned dose has been administered may be compared with:

- the immune response to unconjugated Vi vaccine in one or more older age groups; or
- the immune response to the same candidate Vi conjugate vaccine in one or more older age groups; or
- both of these responses.

The comparative data could be derived from subjects (e.g. children aged 2–5 years) enrolled in a randomized study of candidate Vi conjugate vaccine versus unconjugated Vi vaccine that has successfully demonstrated non-inferiority as described in section C.3.1.

The primary analysis should be based on demonstrating that the immune response to the candidate Vi conjugate vaccine is at least non-inferior when compared with the immune response to the control vaccine in another age group. However, comparing immune responses among age groups (and among regimens) is not straightforward. For example, seroconversion rates may be impacted by pre-existing antibodies, and final GMCs may vary by age. Therefore, it may be appropriate to place more weight on comparing the proportions of subjects that achieve post-vaccination anti-Vi IgG concentrations that rise above a threshold value (as discussed in section C.2.4).

C.3.3 Studies that compare conjugated Vi vaccines

The availability of licensed Vi conjugate vaccines will have implications for the design of clinical studies in all age groups. Some of the issues that will need to be taken into account include:

- whether the protective efficacy of any licensed Vi conjugate vaccine has been documented in a pre-licensure study of protective efficacy or by post-approval data on effectiveness, or both – if so, then conducting comparative immunogenicity studies against such a vaccine would allow for direct bridging between anti-Vi IgG concentrations and protection;
- whether data on efficacy or effectiveness point to a specific anti-Vi antibody concentration that strongly correlates with efficacy;
- whether and where Vi conjugate vaccines have been introduced into routine immunization programmes and in which age groups;
- investigators' and subjects' willingness to take part in studies that use a control group that receives an unconjugated Vi vaccine or an unvaccinated control group.

As more Vi conjugate vaccines become licensed, it is expected that there will be a transition towards conducting comparative studies between candidate and control Vi conjugate vaccines. The selection of the most appropriate licensed

Vi conjugate vaccine for a comparative study must be agreed between the sponsor and the NRA. However, the optimal candidate would be a Vi conjugate vaccine for which protective efficacy has been demonstrated; these data may come from post-approval studies of effectiveness. If no licensed conjugates have documented efficacy, then the extent of the data on comparative immunogenicity for each age group of interest for licensed Vi conjugate vaccines should be taken into account.

The aim of these studies is to demonstrate the non-inferiority of the immune response to the candidate vaccine when compared with the licensed Vi conjugate vaccine. If efficacy data have supported derivation of an anti-Vi antibody concentration that strongly correlates with protection, then the proportions of subjects that achieve at least this concentration after vaccination should be compared.

C.3.4 Antibody persistence and booster doses

Longer-term assessment of antibody persistence is considered essential. At the time when a Vi conjugate vaccine is initially approved there should be adequate documentation of anti-Vi concentrations for at least 1 year after the initial dose has been administered. The collection of further data on antibody persistence should be planned but, subject to agreement with the NRA, may be reported at intervals after the initial approval.

In studies that compare conjugated Vi vaccines with unconjugated Vi vaccines, data on antibody persistence should be analysed among the randomized groups. Using antibody decay curves observed following administration of unconjugated Vi vaccines, data for up to 1 year can indicate whether there is any difference between vaccines in the initial rate of decrease of anti-Vi antibody. While there is no established immunological correlate of protection, antibody persistence data may be viewed in terms of the percentages of vaccinees that have anti-Vi IgG concentrations above a predefined threshold for a specified period of time.

Determining the need for and the appropriate timing of a booster dose of Vi conjugate vaccine is not straightforward, and needs and timing may differ among age groups and populations – there may, for example, be a considerable natural boosting effect in highly endemic regions. Bacteraemia can be detected shortly after oral inoculation with *S.* Typhi, and several days before the onset of clinical symptoms. This suggests that it may not be appropriate to rely on immune memory responses to achieve a sufficiently rapid rise in anti-Vi antibody to protect individual subjects. In addition, data on one Vi conjugate vaccine suggest that protection against typhoid depends on maintaining a certain concentration of anti-Vi antibody (*65*, *67*, *68*, *83*, *121*).

Extensive post-approval data on antibody persistence and vaccine effectiveness are needed to support decisions on boosting. Nevertheless, in order to facilitate decisions regarding the introduction of booster doses,

it is recommended that studies in all age groups should plan to document at predetermined intervals immune responses to booster doses. Subjects may be sub-randomized to different schedules for booster doses after the initial dose or doses. As mentioned in section C.2.3, by including an unvaccinated control group, these data can also be used to demonstrate that the initial dose or doses elicited a T cell-dependent immune response.

In studies that compare a candidate Vi conjugate vaccine with an unconjugated Vi vaccine, it is important to analyse immune responses to a sequential (booster) dose of the Vi conjugate vaccine in different groups. These data can be used to determine whether prior exposure to unconjugated Vi polysaccharide may blunt the immune response to a conjugate vaccine as a result of inducing hyporesponsiveness. The data may indicate whether more than one dose of the conjugate vaccine is needed in these subjects, which would be important information for planning the introduction of Vi conjugate vaccines into regions where there has been extensive use of unconjugated Vi vaccines in the past.

The assessment of immune responses to a booster dose should be based on antibody concentrations found immediately before and after the booster dose. The rate of change in immune parameters after the booster dose, as well as the magnitude of the response observed, should be compared among groups primed with the same Vi conjugate vaccine, unprimed subjects and subjects that previously received unconjugated Vi vaccine. If the Vi conjugate vaccine is found to have efficiently primed the immune system, then the onset of the response after the booster dose should be faster, and the antibody concentrations achieved should be higher, than in the other groups (see section C.2.3).

C.3.5 Immune responses to, and effects of, the carrier protein

Proteins such as CRM197, DT, TT and rEPA have been used in the production of various Vi conjugate vaccines. Based on experience with other types of conjugate vaccines that use CRM197, DT or TT as the carrier protein, there is some potential that the immune response to the conjugated antigen may be reduced in subjects who have high levels of tetanus or diphtheria antitoxin before vaccination. This phenomenon should be explored during the development of Vi conjugate vaccines; this may be accomplished by analysing post-vaccination responses and comparing these with pre-vaccination antibody concentrations. The potential clinical significance of any effect requires careful consideration.

C.3.6 Co-administration with other vaccines

Concomitant administration of some types of conjugates with other vaccines already in routine use, including other conjugated vaccines, may give rise to detectable immune interference – which may be a depression or an enhancement

of antibody levels – although the magnitude of the effect may not necessarily be of clinical significance. The possible effects of co-administration on immune responses cannot be predicted simply by considering the vaccine content. Therefore, clinical studies are needed in which candidate Vi conjugate vaccines are co-administered with other vaccines that are representative of the types that for convenience and reasons related to a vaccine programme are likely to be given at the same time. These studies could examine co-administration with vaccines routinely used in infants and toddlers in endemic areas or co-administration with vaccines commonly used by travellers resident in non-endemic areas.

Co-administration studies may be conducted before or after initial licensure, or both, depending on the importance of being able to recommend co-administration with specific types of vaccines to facilitate use within existing vaccination programmes targeting specific age groups.

In co-administration studies the immune response to the Vi conjugate and to all other co-administered antigens should be evaluated. The approach to these studies is based primarily on demonstrating the non-inferiority of immune responses to antigens when vaccines are co-administered compared with each vaccine given alone, with careful justification of predefined non-inferiority margins.

C.4 Pre-licensure assessment of safety

There is no evidence that points to anticipation of specific safety issues for Vi conjugate vaccines. At present it is possible only to recommend that the assessment of safety in pre-licensure studies should follow the usual approaches to ensure comprehensive monitoring and data collection.

C.5 Postmarketing studies and surveillance

The information in the application dossier is likely to be restricted to studies of safety and immunogenicity that have been conducted in certain geographical areas and in populations with particular demographic features. In addition, the total population evaluated for safety in pre-licensure clinical studies may be limited such that only those adverse events that occur at a frequency of at least 1 per 1000 persons vaccinated can be described with any degree of confidence (*120*). Therefore, it is considered critically important that well developed plans are put in place prior to licensure to ensure that vaccine safety and effectiveness are assessed during routine use in the post-approval period. In particular:

- Studies of vaccine effectiveness and impact should include a careful evaluation of any herd immunity effect of Vi conjugate vaccines. It may not be possible to collect vaccine-specific effectiveness data if more than one Vi conjugate is introduced concurrently in the same region, but the overall effectiveness of a programme that includes

specific vaccines is still informative regardless of whether vaccines are delivered routinely or as an outbreak intervention.

- Further attempts should be made to identify an immunological correlate of protection. This requires that factors be considered in addition to the usual issues surrounding the approach selected to assess effectiveness.
- If the pre-licensure safety database is limited in size, or if any particular safety issues are observed during clinical studies or after approval, a dedicated post-authorization safety study may be necessary in addition to routine passive surveillance.

Sound and comprehensive data on safety and effectiveness cannot be collected by the sponsors alone. Therefore, collaborations should be planned between sponsors and public health bodies to ensure that adequate and reliable data are collected in areas where there is routine and widespread use of a Vi conjugate vaccine. Protocols should be developed before the initial approval, and should be included in the application dossier. These protocols can be refined once it is known where and how a vaccine will actually be used.

Other issues to be addressed after initial licensure include:

- Assessing longer-term antibody concentrations in selected cohorts, including antibody concentrations after booster doses are administered (see section C.3.4).
- Conducting safety and immunogenicity studies in populations that were not included in pre-licensure studies and in which there are good reasons to expect that immune responses may differ (e.g. in immunosuppressed people, or age groups not previously studied). Additional safety and immunogenicity studies may also be considered if there is a good scientific rationale for anticipating that the immune response to the Vi conjugate vaccine in the pre-licensure study population (e.g. residents in endemic areas) may not predict that in populations that have not been studied (e.g. residents in non-endemic areas who are travelling to endemic areas).
- Assessing the possibility that widespread use of a vaccine and high immunization coverage in a population where typhoid fever is endemic may lead to the emergence of otherwise rare Vi-negative variants of *S*. Typhi (*124–127*); these variants exist and can cause typhoid fever, albeit they have lower attack rates (*128*, *129*).

All data collected should be regularly submitted to the responsible regulatory authorities so that any implications for the marketing authorization can be assessed, and appropriate actions can be taken.

Part D. Guidelines for NRAs

D.1 General guidelines

The general recommendations for NRAs and national control laboratories given in the Guidelines for national authorities on quality assurance for biological products (*130*) and the Guidelines for independent lot release of vaccines by regulatory authorities (*131*) should be followed.

These Guidelines specify that no new biological substance should be released until consistency in manufacturing and quality has been demonstrated by regularly releasing consistent batches.

The detailed procedures for production and quality control, and any significant changes in these that may affect the quality, safety or efficacy of a Vi polysaccharide conjugate typhoid vaccine, should be discussed with and approved by the NRA. The NRA may obtain the product-specific working reference from the manufacturer and use this for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in ensuring the quality of vaccines. In particular, the NRA should carefully monitor production records and the results of quality-control tests for clinical lots, as well as results for a series of consecutive lots of the final bulk and final product.

D.2 Official release and certification

A vaccine lot should be released only if it fulfils the national requirements and Part A of these Guidelines.

A model protocol for the manufacturing and control of typhoid conjugate vaccines is shown in Appendix 1; this protocol should be signed by the responsible official of the manufacturing establishment, and should be prepared and submitted to the NRA in support of a request to release the vaccine for use.

A certificate signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine meets all national requirements as well as Part A of these Guidelines. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory determination of critical quality parameters (such as the ratio of free Vi polysaccharide to bound Vi polysaccharide) as well as the expiry date assigned on the basis of the shelf-life of the vaccine should be stated. A model NRA Lot-release Certificate is given in Appendix 2. A copy of the model protocol should be attached to the lot-release certificate – the purpose of which is to facilitate the exchange of typhoid conjugate vaccines between countries.

Authors and acknowledgments

The first draft of these Guidelines was prepared by Dr J. Mathew, Post Graduate Institute of Medical Education and Research, India and Dr M. Levine, University of Maryland School of Medicine, USA (General considerations); Dr S. Rijpkema, National Institute for Biological Standards and Control, England (Part A); Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA (Part B); Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England (Part C); Dr J. Shin, Department of Essential Medicines and Health Products, World Health Organization, Switzerland. Consolidation of each part of the Guidelines then took place following three expert consultations: (a) joint Korea Food and Drug Administration (KFDA) and WHO working group held in Jeju, Republic of Korea, 5–7 September 2012; (b) WHO clinical working group held in London, 7–8 January 2013, for which financial and technical support was provided by Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; and (c) WHO informal consultation held in Geneva, 29–30 April 2013.

Acknowledgments are due to the following experts who helped to improve earlier versions by providing written comments and proposed changes: Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Dr I. Feavers, National Institute for Biological Standards and Control, England; Dr M. Levine, University of Maryland School of Medicine, USA; Dr A. Pollard, Oxford University, England.

Acknowledgments are extended to the following individuals who participated in discussions at the joint KFDA/WHO working group: Dr C. Ahn, National Institute of Food and Drug Safety Evaluation, Republic of Korea; Dr M. Bonnet (*IFPMA representative*), Sanofi Pasteur, France; Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr R. Carbis, International Vaccine Institute, Republic of Korea; Dr D. Cardoso-Gonzalez (*DCVMN representative*), Finlay Institute, Cuba; Dr D. Garcia, National Drug and Health Products Safety Agency, France; Dr H. Izumiya, National Institute of Infectious Diseases, Japan; Ms W. Jaroenkunathum, Ministry of Public Health, Thailand; Dr C. Jones (*Rapporteur*), National Institute for Biological Standards and Control, England; Dr B.G. Kim, Korea Food and Drug Administration, Republic of Korea; Dr C.K. Lee (*Chair*), Korea Food and Drug Administration, Republic of Korea; Dr T. King Jr, Food and Drug Administration, Philippines; Dr I. Knezevic, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Ms D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Dr M. Levine, University of Maryland School of Medicine, USA; Dr L. Martin, Novartis Vaccines Institute for Global Health, Italy; Dr J. Mathew, Post Graduate Institute

of Medical Education and Research, India; Dr G. Meller (*Observer*), Bill & Melinda Gates Foundation, USA; Dr M. Morita, National Institute of Infectious Diseases, Japan; Dr P. Namgyal, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Ms N. Nurainy (*DCVMN representative*), Biofarma, Indonesia; Dr H.J. Oh, Korea Food and Drug Administration, Republic of Korea; Dr M. Paste (*IFPMA representative*), GlaxoSmithKline Biologicals, Belgium; Dr A. Ramkishan, Ministry of Health and Family Welfare, India; Dr S. Rijpkema, National Institute for Biological Standards and Control, England; Dr S. Sahastrabuddhe, International Vaccine Institute, Republic of Korea; Dr J. Shin, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Dr S. Szu, National Institutes of Health, USA; Ms J. Tresnabudi (*Observer*), Biofarma, Indonesia; Dr M. Zeng, National Institutes of Food and Drug Control, China. The following KFDA senior managers and staff members are also acknowledged: Ms Y. Choi, Dr S.T. Chung, Ms S.Y. Eum, Dr S.H. Hong, Dr S.J. Kang, Dr S.Y. Kang, Dr K.H. Lee, Dr K.T. Nam, Dr I.S. Shin and Dr Y. Sohn.

Acknowledgments are due to the following experts who attended the WHO clinical working group and who reviewed clinical data and agreed upon the key points to be considered in the clinical guidelines: Dr I. Feavers, National Institute for Biological Standards and Control, England; Dr E. Griffiths (*Chair*), England; Dr I. Knezevic, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Dr M. Levine, University of Maryland School of Medicine, USA; Dr C. Nelson (*Rapporteur*), Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Dr A. Pollard, Oxford University, England; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency; Dr S. Rijpkema, National Institute for Biological Standards and Control, England.

The second draft of these Guidelines was prepared by the following individuals taking into consideration: (a) written comments from a first public consultation from March to April 2013 following the uploading of the first draft document onto the WHO Biologicals website; and (b) discussion during the 2013 WHO informal consultation: Dr J. Mathew, Post Graduate Institute of Medical Education and Research, India (General considerations); Dr S. Rijpkema, National Institute for Biological Standards and Control, England (Part A); Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA (Part B); Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England (Part C); Dr J. Shin, Department of Essential Medicines and Health Products, World Health Organization, Switzerland.

Acknowledgments are due to the following experts who provided written comments during the first public consultation: Dr M. Bonnet, Sanofi Pasteur, France; Dr P. Chagnaud, National Drug and Health Products Safety Agency,

France; Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr S. Dutta, National Institute of Cholera and Enteric Diseases, India; Ms M.J. Escoto-López, Center for State Control on the Quality of Drugs, Cuba; Dr E. Griffiths, England; Dr A. Goel, Biological E, India; Ms W. Jaroenkunathum, Ministry of Public Health, Thailand; Dr O. Le Doledec, National Drug and Health Products Safety Agency, France; Professor M. Levine, University of Maryland School of Medicine, USA; Dr L. Martin, Novartis Vaccines Institute for Global Health, Italy; Dr A. Merkle, Paul-Ehrlich-Institut, Germany; Dr K. Meunier, National Drug and Health Products Safety Agency, France; Dr T. Mongeau, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr S. Morgeaux, National Drug and Health Products Safety Agency, France; Dr T. Morris, United States Pharmacopoeial Convention, USA; Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Dr H.J. Oh, Ministry of Food and Drug Safety, Republic of Korea; Dr S. Park, Ministry of Food and Drug Safety, Republic of Korea; Dr M. Paste, GlaxoSmithKline Biologicals, Belgium; Dr B. Patnaik, Bharat Biotech International, India; Dr S. Rijpkema, National Institute for Biological Standards and Control, England; Dr J. Robbins, USA; Dr R. Schneerson, USA; Dr S. Sontakke, Health Canada, Canada; Dr S. Szu, Eunice Kennedy Shriver National Institute of Child Health and Human Development, USA; Dr W. Van Molle, Scientific Institute of Public Health, Belgium; Dr A. Worobec, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Ms D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Dr G. Xie, China National Biotec Group, China; Dr M. Zeng, National Institutes of Food and Drug Control, China; Japan Paediatric Society (anonymous contribution); State Food and Drug Administration and Center for Drug Evaluation of China (anonymous contribution).

Acknowledgments are due to the following experts who participated in the 2013 WHO informal consultation: Dr A. Bentsi-Enchill, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Dr M. Bonnet (*IFPMA representative*), Sanofi Pasteur, France; Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr J. Cirunay, Food and Drug Administration, Philippines; Dr R. Carbis, International Vaccine Institute, Republic of Korea; Dr D. Cardoso-Gonzalez (*DCVMN representative*), Finlay Institute, Cuba; Ms J. Dahlan, National Agency of Drug and Food Control, Indonesia; Ms M.J. Escoto-López, Center for State Control on the Quality of Drugs, Cuba; Dr I. Feavers, National Institute for Biological Standards and Control, England; Dr D. Garcia, National Drug and Health Products Safety Agency, France; Mr K. Gopinathan, Bharat Biotech International, India; Dr E. Griffiths (*Chair*), England; Ms W. Jaroenkunathum, Ministry of Public Health, Thailand; Dr C. Jones, National Institute for Biological Standards and Control, England; Dr C.K. Lee (*Co-Chair*), Korea Food and Drug

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A further improved draft was prepared following written submissions on the second draft, and the contributions of the following experts are acknowledged: Ms J. Dahlan, National Agency of Drug and Food Control, Indonesia; Mr K. Gopinathan, Bharat Biotech International, India; Ms W. Jaroenkunathum, Ministry of Public Health, Thailand; Dr M. Levine, University of Maryland School of Medicine, USA; Dr L. Martin, Novartis Vaccines Institute for Global Health, Italy; Dr S. Szu, National Institutes of Health, USA; Ms G. Trisnasari, Biofarma, Indonesia.

During a second round of public consultation on document WHO/BS/2013.2215 organized through the WHO Biologicals website from July to September 2013 further comments were provided by the following experts: Dr F. Cano, Dr D. Garcia, Dr S. Morgeaux and Dr M. Surgot, National Drug and Health Products Safety Agency, France; Dr J. Cipollo and Dr T. Mongeau, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr H.J. Oh, Ministry of Food and Drug Safety, Republic of Korea; Dr S. Rijpkema, National Institute for Biological Standards and Control, England.

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Appendix 1

Model protocol for the manufacturing and control of typhoid conjugate vaccines

The following protocol is intended for guidance and indicates the minimum information that should be provided by the manufacturer to an NRA. Information and tests may be added or omitted as required by an NRA.

It is thus possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that accompanies the vaccine container. If the protocol is being submitted in support of a request to permit importation, it must also be accompanied by a lot-release certificate from the NRA of the country where the vaccine was produced, stating that the product meets national requirements as well as the recommendations in Part A of this document.

Summary information on the final lots

International name of product: ______________________________

Commercial name: ______________________________

Product licence (marketing authorization) number: ______________________________

Country: ______________________________

Name and address of manufacturer: ______________________________

Final packing lot number: ______________________________

Type of containers: ______________________________

Number of containers in this packing lot: ______________________________

Final container lot number: ______________________________

Number of filled containers in the final lot: ______________________________

Date of manufacture: ______________________________

Nature of final product: ______________________________

Preservative used and nominal concentration: ______________________________

Volume of each recommended single human dose: ______________________________

Number of doses per final container: ______________________________

Summary of composition: ______________________________

(*Include a summary of the qualitative and quantitative composition of the vaccine per single human dose; include the conjugate, any adjuvant used and other excipients*)

Shelf-life approved (months): ______________________________
Expiry date: ______________________________
Storage conditions: ______________________________

The following sections are intended for reporting the results of the tests performed during the production of the vaccine, so that the complete document will provide evidence of consistency in production; thus, if any test had to be repeated, this information must be indicated. Any abnormal results should be recorded on a separate sheet.

Detailed information on manufacture and control

SUMMARY OF STARTING MATERIALS

It is possible that a number of bulk lots may be used to produce a single final lot. A summary of the bulk polysaccharide, activated saccharide, bulk carrier protein and bulk conjugate lots that contributed to the final lot should be provided.

CONTROL OF TYPHOID Vi POLYSACCHARIDE

Bacterial strain

Identity of *Salmonella* Typhi Ty2 or
Citrobacter freundii: ______________________________
Origin and short history: ______________________________
Authority that approved the strain: ______________________________
Date approved: ______________________________

Bacterial culture media for seed-lot preparation and Vi production

Free from ingredients that form precipitate when
CTAB is added: ______________________________
Free from toxic or allergenic substances: ______________________________
Any components of animal origin (list): ______________________________
Certified as TSE-free: ______________________________

Master-seed lot

Lot number: ______________________________
Date master-seed lot established: ______________________________

Working-seed lot

Lot number: ______________________________
Date working-seed lot established: ______________________________
Type of control tests used on working-seed lot: ______________________________
Date seed lot reconstituted: ______________________________

Control of single harvests

For each single harvest, indicate the medium used; the dates of inoculation; the temperature of incubation; the dates of harvests and harvest volumes; the results of tests for bacterial growth rate, pH, purity and identity; the method and date of inactivation; the method of purification; and the yield of purified polysaccharide.

Control of purified typhoid Vi polysaccharide

Lot number: ______________________________
Date of manufacture: ______________________________
Volume: ______________________________

Identity

Date tested: ______________________________
Method used: ______________________________
Specification: ______________________________
Result: ______________________________

Molecular size or mass distribution

Date tested: ______________________________
Method used: ______________________________
Specification: ______________________________
Result: ______________________________

Polysaccharide content

Date tested: ______________________________
Method used: ______________________________
Specification: ______________________________
Result: ______________________________

O-*acetyl content*

Date tested: ______________________________
Method used: ______________________________
Specification: ______________________________
Result: ______________________________

Moisture content

Date tested: ______________________________
Method used: ______________________________
Specification: ______________________________
Result: ______________________________

Protein impurity

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Nucleic acid impurity

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Phenol content

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Endotoxin content

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Residues of process-related contaminants

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Control of modified polysaccharide

Lot number: ______________________________

Method of chemical modification: ______________________________

Extent of activation for conjugation

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Molecular size or mass distribution

Date tested: __________

Method used: __________

Specification: __________

Result: __________

CONTROL OF CARRIER PROTEIN

Microorganisms used

Identity of strain used to produce carrier protein: __________

Origin and short history: __________

Authority that approved the strain: __________

Date approved: __________

Bacterial culture media for seed-lot preparation and carrier-protein production

Free from ingredients that form precipitate when CTAB is added: __________

Free from toxic or allergenic substances: __________

Any components of animal origin (list): __________

Certified as TSE-free: __________

Master-seed lot

Lot number: __________

Date master-seed lot established: __________

Working-seed lot

Lot number: __________

Date established: __________

Type of control tests used on working-seed lot: __________

Date seed lot reconstituted: __________

Control of carrier-protein production

List the lot numbers of harvests: indicate the medium used; the dates of inoculation; the temperature of incubation; the dates of harvests and harvest volumes; the results of tests for bacterial growth rate, pH, purity and identity; the method and date of inactivation; the method of purification; and the yield of purified carrier protein. Provide evidence that the carrier protein is nontoxic.

Purified carrier protein

Lot number: ______________________________

Date produced: ______________________________

Identity

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Protein impurity

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Nucleic acid impurity

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Modified carrier protein

Lot number: ______________________________

Date produced: ______________________________

Method of modification: ______________________________

Extent of activation

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

CONTROL OF PURIFIED BULK CONJUGATE

Production details of bulk conjugate

List the lot numbers of the saccharide and carrier protein used to manufacture the conjugate vaccines, the production procedure used, the date of manufacture and the yield.

Tests on purified bulk conjugate

Identity

Date tested: ____________________
Method used: ____________________
Specification: ____________________
Result: ____________________

Endotoxin content

Date tested: ____________________
Method used: ____________________
Specification: ____________________
Result: ____________________

O-*acetyl content*

Date tested: ____________________
Method used: ____________________
Specification: ____________________
Result: ____________________

Residual reagents

Date tested: ____________________
Method used: ____________________
Specification: ____________________
Result: ____________________

Vi polysaccharide content

Date tested: ____________________
Method used: ____________________
Specification: ____________________
Result: ____________________

Conjugated and unbound (free) polysaccharide

Date tested: ____________________
Method used: ____________________
Specification: ____________________
Result: ____________________

Protein content

Date tested: ____________________
Method used: ____________________

Specification: ______________________
Result: ______________________

Conjugation markers
Date tested: ______________________
Method used: ______________________
Specification: ______________________
Result: ______________________

Absence of reactive functional groups (capping markers)
Date tested: ______________________
Method used: ______________________
Specification: ______________________
Result: ______________________

Ratio of polysaccharide to protein
Date tested: ______________________
Method used: ______________________
Specification: ______________________
Result: ______________________

Molecular size or mass distribution
Date tested: ______________________
Method used: ______________________
Specification: ______________________
Result: ______________________

Bacterial and mycotic bioburden
Method used: ______________________
Media: ______________________
Volume tested: ______________________
Date of inoculation: ______________________
Date of end of test: ______________________
Specification: ______________________
Result: ______________________

Specific toxicity of carrier protein (where appropriate)
Method used: ______________________
Strain and type of animals: ______________________
Number of animals: ______________________
Route of injection: ______________________

Volume of injection: ___
Quantity of protein injected: ___
Date of start of test: ___
Date of end of test: ___
Specification: ___
Result: ___

pH

Date tested: ___
Method used: ___
Specification: ___
Result: ___

Appearance

Date tested: ___
Method used: ___
Specification: ___
Result: ___

Depending on the conjugation chemistry used to produce the vaccine, tests should also be included to demonstrate that amounts of residual reagents and reaction by-products are below a specified level.

CONTROL OF FINAL BULK

Lot number: ___
Date prepared: ___

Preservative (if used)

Name and nature: ___
Lot number: ___
Final concentration in the final bulk: ___

Stabilizer (if used)

Name and nature: ___
Lot number: ___
Final concentration in the final bulk: ___

Adjuvant (if used)

Name and nature: ___
Lot number: ___
Final concentration in the final bulk: ___

Tests on final bulk

Bacterial and mycotic sterility

Method used: ______________________
Media: ______________________
Volume tested: ______________________
Date of inoculation: ______________________
Date of end of test: ______________________
Specification: ______________________
Result: ______________________

FILLING AND CONTAINERS

Lot number: ______________________
Date of sterile filtration: ______________________
Date of filling: ______________________
Volume of final bulk: ______________________
Volume per container: ______________________
Number of containers filled (gross): ______________________
Date of lyophilization (if applicable): ______________________
Number of containers rejected during inspection: ______________________
Number of containers sampled: ______________________
Total number of containers (net): ______________________
Maximum duration approved for storage: ______________________
Storage temperature and duration: ______________________

CONTROL TESTS ON FINAL LOT

Inspection of final containers

Date tested: ______________________
Method used: ______________________
Specification: ______________________
Results: ______________________
Appearance before reconstitution:[1] ______________________
____
Appearance after reconstitution:[1] ______________________
____
Diluent used: ______________________
Lot number of diluent used: ______________________

[1] This applies only to lyophilized vaccines.

Tests on final lot

Identity

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Sterility

Method used: ______________________________

Media: ______________________________

Number of containers tested: ______________________________

Date of inoculation: ______________________________

Date of end of test: ______________________________

Specification: ______________________________

Result: ______________________________

Polysaccharide content

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Unbound (free) polysaccharide

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

O-*acetyl content*

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Molecular size or mass distribution

Date tested: ______________________________

Method used: ______________________________

Specification: ______________________________

Result: ______________________________

Endotoxin or pyrogen content

Date tested: ____
Method used: _______________________________________
Specification: _______________________________________
Result: ____

Adjuvant content and degree of adsorption (if applicable)

Date tested: ____
Nature and concentration of adjuvant per single human dose: _____________________________________
Method used: _______________________________________
Specification: _______________________________________
Result: ____

Preservative content (if applicable)

Date tested: ____
Method used: _______________________________________
Specification: _______________________________________
Result: ____

General safety

Date tested: ____
Method used: _______________________________________
Specification: _______________________________________
Result: ____

pH

Date tested: ____
Method used: _______________________________________
Specification: _______________________________________
Result: ____

Moisture content[2]

Date tested: ____
Method used: _______________________________________
Specification: _______________________________________
Result: ____

[2] This applies only to lyophilized vaccines.

Osmolality

Date tested: __________
Method used: __________
Specification: __________
Result: __________

Control of diluent (if applicable)

Name and composition of diluent: __________
Lot number: __________
Date of filling: __________
Type of diluent container: __________
Appearance: __________
Filling volume per container: __________
Maximum duration approved for storage: __________
Storage temperature and duration: __________
Other specifications: __________

CONTROL OF ADJUVANT[3]

Summary of production details for the adjuvant

When an adjuvant suspension is provided to reconstitute a lyophilized vaccine, a summary of the production and control processes should be provided. The information provided and the tests performed depend on the adjuvant used.

Summary information for the adjuvant

Name and address of manufacturer: __________
Nature of the adjuvant: __________
Lot number: __________
Date of manufacture: __________
Expiry date: __________

Tests on the adjuvant

Adjuvant content

Date tested: __________
Method used: __________
Specification: __________
Result: __________

[3] This section is required only when an adjuvant is provided separately to reconstitute a lyophilized vaccine.

Appearance

Date tested: ______

Method used: ______

Specification: ______

Result: ______

Purity or impurity

Date tested: ______

Method used: ______

Specification ______

Result: ______

pH

Date tested: ______

Method used: ______

Specification: ______

Result: ______

Pyrogenicity[4]

Date tested: ______

Method used: ______

Specification: ______

Result: ______

Sterility

Method used: ______

Media: ______

Number of containers used: ______

Date of inoculation: ______

Date of end of test: ______

Specification: ______

Result: ______

[4] A pyrogen test of the adjuvant is not needed if a pyrogen test was performed on the adjuvanted reconstituted vaccine.

CERTIFICATION BY THE MANUFACTURER

Name of head of quality control (typed) ______________________________

Certification by the person from the control laboratory of the manufacturing company taking overall responsibility for the production and quality control of the vaccine

I certify that lot no. ____________________ of typhoid conjugate vaccine, whose number appears on the label of the final containers, meets all national requirements and/or satisfies Part A[5] of WHO Guidelines on the quality, safety and efficacy of typhoid conjugate vaccines (2014).[6]

Signature ______________________________
Name (typed) ______________________________
Date ______________________________

10. Certification by the NRA

If the vaccine is to be exported, attach a Lot-release Certificate from the NRA (as shown in Appendix 2), a label from a final container and an instruction leaflet for users.

[5] With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

[6] WHO Technical Report Series, No. 987, Annex 3.

Appendix 2

Model NRA Lot-release Certificate for typhoid conjugate vaccines

Certificate no. ____________________

The following lot(s) of typhoid conjugate vaccine produced by ______________ ________________________[1] in ____________________________,[2] whose numbers appear on the labels of the final containers, meet all national requirements[3] and Part A[4] of the WHO Guidelines on the quality, safety and efficacy of typhoid conjugate vaccines (2014)[5] and complies with WHO Good manufacturing practices: main principles for pharmaceutical products;[6] Good manufacturing practices for biological products;[7] and Guidelines for independent lot release of vaccines by regulatory authorities.[8]

The release decision is based on ____[9]

Final lot number: ____
Number of human doses released in this final lot: ____________________
Expiry date: ____

The Director of the NRA (or other appropriate authority)

Name (typed) ____
Signature ____
Date ____

---

1 Name of manufacturer.

2 Country of origin.

3 If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

4 With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

5 WHO Technical Report Series, No. 987, Annex 3.

6 WHO Technical Report Series, No. 961, Annex 3.

7 WHO Technical Report Series, No. 822, Annex 1.

8 WHO Technical Report Series, No. 978, Annex 2.

9 Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

Annex 4

Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology

Replacement of Annex 3 of WHO Technical Report Series, No. 814

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Guidelines published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

Introduction

These Guidelines are intended to provide guidance to national regulatory authorities (NRAs) and manufacturers on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant deoxyribonucleic acid (DNA) technology (rDNA-derived biotherapeutics) and intended for use in humans. The Guidelines are based on experience gained over three decades in this technically demanding field and replace Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology (*1*).

Part A of this annex sets out updated guidelines for the manufacture and quality control of rDNA-derived biotherapeutics, including consideration of the effects of manufacturing changes and of devices used in the delivery of the product and in its stability. Part B is new and provides guidelines on nonclinical evaluation, while Part C, also new, provides guidance on clinical evaluation. The nature and extent of characterization and testing (Part A) required for a product undergoing nonclinical and clinical studies will vary according to the nature of the product and its stage of development. The legal status of investigational products varies from country to country. The need for and extent of studies (e.g. on characterization) will depend on the product under consideration. Early communication between the manufacturer and the responsible NRA to agree on the requirements for, and the type of, studies is recommended. Some aspects of manufacturing and quality control in these Guidelines may apply to protein-based vaccine antigens made by rDNA technology. However, more detailed guidelines/recommendations on vaccine evaluation in terms of quality, safety and efficacy should be consulted (*2*, *3*). Other product-specific vaccine-related WHO Recommendations and Guidelines are also available elsewhere.[1] Additional considerations for similar biotherapeutic products are addressed elsewhere (*4*).

Background

Developments in molecular genetics and nucleic acid chemistry have enabled genes encoding natural biologically active proteins to be identified, modified and transferred from one organism to another in order to obtain highly efficient synthesis of their products. This has led to the production of new rDNA-derived biological medicines using a range of different expression systems such as bacteria, yeast, transformed cell lines of mammalian origin (including human

[1] See: http://www.who.int/biologicals/vaccines/en/

origin), insect and plant cells, as well as transgenic animals and plants. rDNA technology is also used to produce biologically active proteins that do not exist in nature, such as chimeric, humanized or fully human monoclonal antibodies, or antibody-related proteins or other engineered biological medicines such as fusion proteins.

There has been great progress in the ability to purify biologically active macromolecules. In addition, analytical technologies have improved tremendously since the early days of biotechnology, allowing the detailed characterization of many biological macromolecules, including their protein, lipid and oligosaccharide components.

Together these technologies have enabled the production of large quantities of medicinal products that are difficult to prepare from natural sources or were previously unavailable. Nevertheless, it is still not possible fully to predict the biological properties and clinical performance of these macromolecules on the basis of their physicochemical characteristics alone. In addition, the production processes are biological systems which are known to be inherently variable – a feature which has important consequences for the safety and efficacy of the resulting product. Therefore a prerequisite for introducing such biological substances into routine clinical use is to ensure consistency of quality from lot to lot, and for this purpose robust manufacturing processes are developed on the basis of process understanding and characterization, including appropriate in-process controls. Process understanding and consistency are critical since slight changes can occasionally lead to major adverse effects, such as immunogenicity, with potentially serious safety implications.

As with many other new technologies, a new set of safety issues for consideration by both industry and NRAs has been generated by these biotechnologies. Potential safety concerns arose from the novel processes used in manufacture, from product- and process-related impurities, and from the complex structural and biological properties of the products themselves. Factors that have received particular attention include the possible presence of contaminating oncogenic host-cell DNA in products derived from transformed mammalian cells (*5*), and the presence of adventitious viruses (*5*). Since the nature and production of these products are highly sophisticated, they require similarly sophisticated laboratory techniques to ensure their proper standardization and control. Although comprehensive analytical characterization of the drug substance and/or drug product is expected, considerable emphasis must also be given to the manufacturing process – i.e. process validation and in-process control. Adequate control measures relating to the starting materials and manufacturing process are, therefore, as important as analysis of the drug product. Thus, data on the host-cell quality, purity, freedom from adventitious agents, adequate in-process testing during production, and effectiveness of test methods are required for licensing.

At a very early stage in the development of rDNA-derived medicines, the European Medicines Agency (EMA) and the United States Food and Drug Administration produced guidelines and points to consider, respectively, for the development and evaluation of these new products (*6*, *7*). Such guidelines, based as they were on long experience with traditional biological substances, set the scene for regulatory expectations both for clinical trials and for licensing. At the global level, WHO produced a series of guidance documents on the quality, safety and efficacy of rDNA-derived products, including specific guidance for products such as interferons and monoclonal antibodies (*1*, *8–10*). These regulatory concepts have been instrumental in establishing expectations for the quality, safety and efficacy of rDNA-derived biotherapeutics which play a major role in today's medical practice.

As patents and data protection measures on biotechnology products have expired, or have neared expiry, considerable attention has turned to producing copies of the innovator products with a view to making more affordable products that may improve global access to these medicines. Since by definition it is not possible to produce identical biological substances, the normal method of licensing generic medicines – which relies primarily on bioequivalence data – is not appropriate for licensing such products. Consequently, the terms "similar biological product" and "biosimilar product" came into existence (*4*, *11*). The concept of similar biological medicinal products was introduced first by the EMA (*11*) and subsequently by other NRAs (although the actual term used has varied slightly from agency to agency). The WHO Guidelines on evaluation of similar biotherapeutic products were produced in 2010 (*4*) and provided a set of globally acceptable principles regarding the regulatory evaluation of biosimilars, although it was recognized that these would not by themselves resolve all issues. During international consultations on the development of the biosimilar WHO Guidelines, and also during their implementation, it became clear that there was a need to update WHO guidance on the quality, safety and efficacy of rDNA-derived medicines and biotechnology products in general (*12*). In 2010, the International Conference of Drug Regulatory Authorities noted that WHO should supplement its guidance on the evaluation of similar biotherapeutic products by providing up-to-date Guidelines for the evaluation of biotherapeutic products in general.

The present Guidelines have been developed through international consultation and are intended as a replacement of those in Annex 3 of WHO Technical Report Series, No. 814 (*1*). They are considered to be a replacement and not a revision of the earlier Guidelines because they contain new sections on nonclinical and clinical evaluation of rDNA-derived biotherapeutics which were lacking in the original document. In addition, a section on issues related to manufacturing changes, both during development and once the product is

on the market, has also been introduced because considerable improvements to the production process and to the product itself can take place during all stages of development and post-licensure, especially in the immediate post-licensing years. These changes may unintentionally have an impact on the clinical performance of the product and therefore need to be handled carefully from a regulatory perspective.

Guidance on various aspects of rDNA-derived medicines is also available from several other bodies such as the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the EMA, and the United States Food and Drug Administration. These WHO Guidelines are not intended to conflict with, but rather to complement, these other documents.

Scope

These WHO Guidelines provide guidance to NRAs and manufacturers on the quality, nonclinical and clinical aspects of rDNA-derived biotherapeutic protein products for the purpose of licensing. Relevant sections of the Guidelines may also be useful with regard to rDNA-derived biotherapeutic protein products intended for clinical trials; however, the amount and extent of data submitted for such products will be limited and will vary according to the nature of each product and its stage of development. In addition, the legal status of investigational products varies from country to country.

The Guidelines apply, in principle, to all biologically active protein products which are used in the treatment of human diseases and which are prepared by recombinant DNA technology using prokaryotic or eukaryotic cells. The Guidelines also apply to protein products used for in vivo diagnosis (e.g. monoclonal antibody products used for imaging), products used for ex vivo treatment, and those intentionally modified by, for example, PEGylation, conjugation with a cytotoxic drug, or modification of rDNA sequences. Some aspects of these Guidelines may apply to products produced in transgenic animals and plants. However, specific issues for such products can be found in the relevant documents published by WHO – for example, on products from transgenic plants (*13*) – and the responsible NRA should be consulted for specific advice on this.

Additional considerations for quality, safety, and efficacy of biosimilar products are available in the WHO Guidelines on evaluation of similar biotherapeutic products (*4*).

Some aspects of manufacturing and quality control in these Guidelines may apply to protein-based vaccine antigens made by rDNA technology. However, more-detailed guidelines/recommendations on vaccine evaluation in terms of quality, safety and efficacy should be consulted (*2*, *3*). For example, WHO

guidance on vaccines such as yeast-derived hepatitis B vaccine or malaria vaccine produced by rDNA technology (*14*, *15*). WHO guidance on DNA vaccines for therapeutic as well as prophylactic use, adopted by the WHO Expert Committee on Biological Standardization in 2005, is also available (*16*).

Protein products used for in vitro diagnosis are excluded.

Terminology

The definitions given below apply to the terms used in this document. They may have different meaning in other contexts.

Acceptance criteria: numerical limits, ranges or other suitable measures for acceptance of the results of analytical procedures which the drug substance or drug product or materials at other stages of their manufacture should meet.

Anti-drug antibody: an antibody that binds to the active substance of a biotherapeutic product.

Anti-product antibody: an antibody that binds to the active substance, impurities or excipients of a biotherapeutic product.

Biomarkers: a laboratory measurement that reflects the activity of a disease process, correlates (either directly or inversely) with disease progression, and may also be an indicator of a therapeutic response. A genomic biomarker is a measurable DNA and/or RNA marker that measures the expression, function or regulation of a gene.

Biotherapeutic: a biological medicinal product with the indication of treating human diseases.

Comparability exercise: the activities – including study design, conduct of studies, and evaluation of data – that are designed to investigate whether a pre-change product and a post-change product are highly similar.

Critical quality attribute: a physical, chemical, biological or microbiological property or characteristic that is selected for its ability to help indicate the consistent quality of the product within an appropriate limit, range or distribution to ensure the desired product quality.

Drug product: a pharmaceutical product type in a defined container closure system that contains a drug substance, generally in association with excipients.

Drug substance: the active pharmaceutical ingredient and associated molecules that may be subsequently formulated, with excipients, to produce the drug product.

Expiry date: the date given on the individual container (usually on the label) of a product up to and including which the drug substance and drug product are expected to remain within specifications, if stored as recommended. The expiry date is established for each batch by adding the shelf-life period to the date of manufacture.

Good clinical practice (GCP): an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety and well-being of trial subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki, and that the clinical trial data are credible.

Good laboratory practice (GLP): a quality system concerned with the organizational process and conditions under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported.

Good manufacturing practice (GMP): that part of the pharmaceutical quality assurance process which ensures that products are consistently produced and meet the quality standards appropriate to their intended use as required by the marketing authorization. In these Guidelines, GMP refers to the current GMP guidelines published by WHO.

Immunogenicity: the ability of a substance to trigger an immune response or reaction (e.g. development of specific antibodies, T-cell response, or allergic or anaphylactic reaction).

Impurity: any component present in the drug substance or drug product that is not the desired product, a product-related substance, or excipient including buffer components. An impurity may be either process- or product-related.

In-process control: checks performed during production in order to monitor and, if necessary, to adjust the process to ensure that the intermediate or product conforms to its specifications. The control of the environment or equipment may also be regarded as a part of in-process control.

In-silico modelling: a computer-simulated model.

Master cell bank (MCB): an aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions.

Non-human primates (NHPs): primates used as models for the study of the effects of drugs in humans prior to clinical studies.

P450 (CYP) enzymes: indicates the family of metabolizing enzymes which is the most common group.

Pharmacodynamics (PD): the study of the biochemical and physiological effects of drugs on the body and the mechanisms of drug action and the relationship between drug concentration and effect. One dominant example is drug–receptor interactions. PD is often summarized as the study of what a drug does to the body, as opposed to pharmacokinetics, which is the study of what the body does to a drug.

Pharmacogenomics: the study of the pharmacological correlation between drug response and variations in genetic elements has become of increasing importance for drug development. Such variations can have effects on

the risk of developing adverse drug reactions (ADRs) as well as on the response to treatment. Variations in drug pharmacokinetics and metabolic pathways can cause higher drug concentrations in some patients, resulting in increased drug toxicity, and/or lower drug concentrations in some patients, resulting in decreased drug effects.

Pharmacokinetics (PK): the study and characterization of the time course of drug absorption, distribution, metabolism and elimination. Pharmacokinetics is a quantitative analysis of how living systems handle foreign compounds.

Pharmacovigilance: the activities that are carried out after a medicinal product is marketed in order to observe and manage in a continuous manner the safety and the efficacy of the products.

QT/QTc: QT interval is a measure of the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle on the electrocardiogram. It measures the conduction speed between the atria and the ventricles. A genetic predisposition to the prolongation of the QT interval can be triggered by several factors, including various medicinal products by themselves or as a result of their metabolic interaction. It is critical to understand whether a particular drug or a biological triggers the prolongation, because any prolongation of the QT interval outside of the normal limits determined for electrocardiograms indicates a potential for arrhythmia (disturbed heart rhythm), which is a serious adverse event during drug therapy. In extreme cases, this can lead to sudden death. Since the QT interval is affected by the heart rate, "corrected" QT (QTc) should also be used.

rDNA-derived biotherapeutics: biotherapeutics prepared by recombinant DNA technology, i.e. all biologically active protein products which are used in the treatment of human diseases and which are prepared by rDNA technology.

Recombinant DNA technology: technology that joins together (i.e. recombines) DNA segments from two or more different DNA molecules that are inserted into a host organism to produce new genetic combinations. It is also referred to as gene manipulation or genetic engineering because the original gene is artificially altered and changed. These new genes, when inserted into the expression system, form the basis for the production of rDNA-derived protein(s).

Risk management plan: a detailed description of the activities that continuously ensure patients' safety and their benefit from a medicinal ingredient. A risk management plan includes pharmacovigilance and many other elements.

Shelf-life: the period of time during which a drug substance or drug product, if stored correctly, is expected to comply with the specification, as determined by stability studies on a number of batches of the product. The shelf-life is used to establish the expiry date of each batch.

Source material/starting material: any substance of a defined quality used in the production of a biological medicinal product, but excluding packaging materials.

Specification: a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges or other criteria for the tests described. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities.

Working cell bank (WCB): the working cell bank is prepared from aliquots of a homogeneous suspension of cell obtained from culturing the master cell bank under defined culture conditions.

Part A. Manufacturing and quality control

A.1 Definitions

A.1.1 International name and proper name

Where an International Nonproprietary Name (INN) is available for an rDNA-derived biotherapeutic, the INN should be used (*17*). The proper name should be the equivalent of the INN in the language of the country of origin.

A.1.2 Descriptive definition

The description of an rDNA-derived biotherapeutic should indicate the biological system in which it is produced (e.g. bacterial, fungal or mammalian cells) as well as the presentation of the drug product.

A.1.3 International standards and reference materials

International standards and reference preparations have been established for a wide range of biological substances prepared by rDNA technology. These standards and materials are used either to calibrate assays directly or to calibrate secondary standards or manufacturers' working standards. A list of such materials is available on the WHO website.[2] Each standard or reference preparation is held by one of the WHO custodian laboratories (e.g. the National Institute for Biological Standards and Control, Potters Bar, England).

A.2 General manufacturing guidelines

The present Guidelines cover the following three main areas:

- control of starting/source materials, including data both on the host cell and on the source, nature and sequence of the gene used in production;

[2] See: http://www.who.int/bloodproducts/catalogue/AlphFeb2013.pdf

- control of the manufacturing process;
- control of the drug substance and the drug product.

The quality, safety and efficacy of rDNA-derived products rely heavily on adequate control of the starting/source materials and on the manufacturing process, in addition to control tests on the drug substance and drug products themselves. These Guidelines therefore place considerable emphasis on the characterization and testing of host cell lines and other materials used during manufacturing and on validating the ability of the purification processes to remove or inactivate unwanted materials – especially possible viral contaminants and process-related impurities such as host-cell-derived proteins and DNA. The Guidelines also cover in-process controls in manufacturing and comprehensive characterization of the drug substance and the drug product.

Information should therefore be provided to describe adequately the starting/source materials, the manufacturing process and in-process controls. The description of the manufacturing process should be provided in the form of a flow diagram and sequential procedural narrative, and the in-process controls for each step or stage of the process should be indicated in this description. In addition, an explanation should be provided of how batches of the drug substance and drug product are defined (e.g. splitting and pooling of harvests or intermediates). Details of batch size or scale should also be included.

The manufacturing process should be validated before licensing. Process validation studies should include appropriate evaluation of the process and process steps (e.g. cell culture, harvest, purification, mixing, sterilization, filling) and the provision of evidence that they are capable of consistently delivering quality product and intermediates (i.e. meeting their predetermined specifications and quality attributes). The capacity of the purification procedures to remove product- and process-related impurities (e.g. unwanted variants, host-cell proteins, nucleic acids, resin leachates) should be investigated thoroughly (also see section A.4.2 and Appendix 1).

The general recommendations for manufacturing establishments contained in the WHO Good manufacturing practices: main principles for pharmaceutical products (*18*) and Good manufacturing practices for biological products (*19*), as well as those in the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*5*), should apply to establishments manufacturing rDNA-derived biotherapeutics. Not all of the requirements outlined in this Part are expected for each phase of clinical development (*20*).

A.3 Control of starting/source materials

A.3.1 Expression vector and host cell

A description of the host cell, its source and history, and of the expression vector used in production, including source and history, should be provided in detail. The description should include details of the origin and identity of the gene being cloned as well as the construction, genetic elements contained and structure of the expression vector. An explanation of the source and function of the component parts of the vector, such as the origins of replication, promoters, or antibiotic markers, should be provided in addition to a restriction-enzyme map indicating at least those sites used in construction.

Methods used to amplify the expression constructs and to transform expression constructs into host cells, and the rationale used to select the cell clone for production, should be fully described. The vector within the cell, whether integrated or extrachromosomal, and the copy number, should be analysed. A host cell containing an expression vector should be cloned and used to establish a master cell bank (MCB) and the correct identity of the vector construct in the cell bank should be established. The genetic stability of the host-vector combination should be documented.

The nucleotide sequence of the cloned gene insert, including any codon optimization, and of the flanking control regions of the expression vector should be indicated and all relevant expressed sequences clearly delineated.

Any measures used to promote and control the expression of the cloned gene in the host cell during production should be described in detail.

A.3.2 Cell bank system

Typically, rDNA-derived biotherapeutics are produced using a cell bank system which involves a manufacturer's working cell bank (WCB) derived from an MCB. It is acknowledged that a WCB may not always be established in the early phases of development.

The type of cell bank system used, the size of the cell bank(s), the container (vials, ampoules, or other appropriate vessels) and closure system used, the methods for preparation of the cell bank(s) including the cryoprotectants and media used, and the conditions employed for cryopreservation or long-term storage should all be documented and described in detail.

Evidence should be provided for banked cell stability under defined storage conditions. Such evidence can be generated during the production of material from the banked cells and can be supported by a programme for stability monitoring that indicates attributes over time (e.g. data on cell viability upon thawing, stability of the host–vector expression system in the cell bank). Available data should be clearly documented and the proposed stability monitoring programme should be described in the marketing application. Evidence should

be provided of the stability of the host-vector expression in the cell bank both under storage and under recovery conditions.

For animal cells and animal-derived cell banks, reference should be made to the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*5*).

A.3.2.1 Control of cell banks

The characterization and testing of banked eukaryotic or prokaryotic cell substrates is a critical component of the control of rDNA-derived biotherapeutics. Cell banks should be tested to confirm the identity, purity and suitability of the cell substrate for the intended manufacturing use. The MCB should be characterized for relevant phenotypic and genotypic markers which should include the expression of the recombinant protein and/or presence of the expression construct. The testing programme chosen for a given cell substrate will vary according to the nature and biological properties of the cells (e.g. growth requirements) and its cultivation history (including use of human-derived or animal-derived biological reagents). The extent of characterization of a cell substrate may influence the type or level of routine testing needed at later stages of manufacturing. Molecular methods should be used to analyse the expression construct for copy number, insertions or deletions, and the number of integration sites. Requirements for bacterial systems expressing the protein from a plasmid or mammalian epigenetic expression should be distinguished from mammalian cell systems. The nucleic acid sequence should be shown to be identical to that determined for the expression construct and should correspond to that expected for the protein sequence. If any differences in nucleic acid sequences are identified, these should be clearly delineated and shown to be stable and capable of expressing the expected product consistently (see also section A.4.1.1).

Animal cell substrates are subject to contamination and have the capacity to propagate extraneous, adventitious organisms, such as mycoplasma and viruses. In addition, animal cells contain endogenous agents such as retroviruses that may raise safety concerns. Testing of cell substrates for both endogenous (e.g. retroviruses) and adventitious agents is critical. A strategy for testing cell banks for adventitious agents should be developed. This strategy should also involve an assessment of specific viruses and the families of viruses that may potentially contaminate the cell substrate. Such testing is described in detail in WHO's Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*5*) and the ICH guidelines Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin (*21*).

Although cell substrates contaminated with microbial agents are generally not suitable for production, there are exceptions. For example, some murine cell lines that are widely used for the production of rDNA-derived biotherapeutics express endogenous retroviral particles. In such circumstances, risk mitigating strategies should be implemented, including the removal of such agents and/or their inactivation by physical, enzymatic and/or chemical treatment during processing of the rDNA-derived biotherapeutics.

In addition, tests of purity and identity should be performed once on each WCB. A specification that includes test methods and acceptance criteria should be established for the WCB.

A.3.2.2 Cell substrate genetic stability

The limit of in vitro cell age for production should be defined by the time of registration; it should be based on data derived from production cells expanded under pilot plant-scale or commercial-scale conditions to the proposed limit of in vitro cell age for production use or beyond. The production cells are generally obtained by expansion of cells from the WCB (*22*).

Specific traits of cells – which may include, for example, morphological or growth characteristics, biochemical or immunological markers, productivity of the desired product, or other relevant genotypic or phenotypic markers – may be useful for the assessment of cell substrate stability during the culture phase. The nucleotide sequence of the insert encoding the rDNA-derived biotherapeutic should be determined at least once after a full-scale culture for each MCB.

The molecular integrity of the gene being expressed and the phenotypic and genotypic characteristics of the host cell after long-term cultivation (i.e. end of production testing) should be established and defined by the time of registration.

A.3.3 Cell culture medium/other materials

Materials used in the manufacture of the drug substance (e.g. solvents, reagents, enzymes) should be listed, indicating where each material is used in the process. Information should be provided on the source, quality and control of these materials. There should also be information demonstrating that the materials (including biologically sourced materials, such as media components, monoclonal antibodies and enzymes) meet standards appropriate for their intended use (including the clearance or control of adventitious agents).

Media and other components should comply with the *WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products* (*23*). The latest version of the *WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies* (*24*) should also be consulted. Corresponding tables in this area are periodically updated as new data become available – see, for example, reference (*25*).

A.4 Control of the manufacturing process

Adequate design of a process and knowledge of its capability are part of the strategy used to develop a manufacturing process which is controlled and reproducible, yielding a drug substance and drug product that consistently meet specifications. In this respect, limits are justified on the basis of information gathered from the entire process from early development through to commercial-scale production.

In-process controls are performed both at critical decision-making steps and at other steps where data serve not only to ensure the appropriate performance of the manufacturing process but also to demonstrate adequate quality during the production of both the drug substance and the drug product. Process parameters that are found to have an impact on the quality attributes of the drug substance or drug product should be controlled by suitable acceptance limits. Where appropriate, in-process controls may alleviate the need for routine testing of some quality attribute(s) at the level of the drug substance and/or drug product.

A.4.1 Cell culture

A 4.1.1 Production at finite passage

Procedures and materials used both for cell growth and for the induction of the product should be described in detail. Acceptable limits for potential contamination should be set and the sensitivity of the methods used to detect it should be indicated. In case of contamination, the nature of the microbial contamination needs to be identified. Microbial and fungal contamination should be monitored according to Part A, section 5.2 of General requirements for the sterility of biological substances (*26*) or by methods approved by the NRA.

Data should be presented on the consistency of culture conditions and culture growth and on the maintenance of product yield. Criteria for the rejection of culture lots should be established. The maximum number of cell doublings or passage levels to be permitted during production should be specified taking into account the limit of in vitro cell age. For a process demonstrating consistent growth characteristics over the proposed cell age range for production, it may also be acceptable to define the cell age limit on the basis of the maximum number of permitted days in culture from thaw to the end of production.

Host-cell/vector characteristics at the end of production cycles should be monitored to establish consistency. For this purpose, information on the plasmid copy number or the degree of retention of the expression vector within the host cell may be of value, as may restriction enzyme mapping of the vector containing the gene insert. If the vector is present in multiple copies integrated into the host-cell genome, it may be difficult to confirm the rDNA sequence directly. In such cases, alternative approaches to confirming the sequence of insert-encoding

the rDNA-derived biotherapeutics should be considered and defined by the time of registration – e.g. restriction fragment length polymorphism (RFLP), fluorescence in situ hybridization (FISH), polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP), Southern blot). For example, confirmation of protein sequence by peptide mapping might be an appropriate alternative to rDNA sequencing.

A.4.1.2 Continuous culture production

As recommended above, all procedures and materials used for cell culture and induction of the product should be described in detail and validated. In addition, particular consideration should be given to the procedures used in production control. Monitoring is necessary throughout the life of the culture, although the frequency and type of monitoring required depend on the nature of both the production system and the product.

Evidence should be produced to show that variations in yield or other culture parameters do not exceed specified limits. The acceptance of harvests for further processing should be clearly linked to the monitoring schedule being used, and a clear definition of "batch" of product should be established for further processing. Criteria for the rejection of harvests or termination of the culture should also be established. Tests for microbial contamination should be performed as appropriate to the harvesting strategy. In the case of continuous processing, multiple harvests from long fermentations could lead to a drift in some quality attributes, such as glycosylation, with the appearance of "new" variants with possible impacts on the quality, safety and efficacy of the product. Such drift should be appropriately addressed in process evaluation/validation studies.

The maximum period of continuous culture should be specified on the basis of information on the stability of the system and consistency of the product during and after this period. In long-term continuous culture, the cell line and product should be fully re-evaluated at intervals determined by information on the stability of the host-vector system and the characteristics of the product.

A.4.2 Purification

The methods used for the harvesting, extraction and purification of the product and related in-process controls, including acceptance criteria, should be described in detail and should be validated. Special attention should be given to the removal of viruses, nucleic acid, host-cell proteins and impurities considered to pose a risk of immunogenicity.

The ability of the purification procedure to remove unwanted product-related or process-related impurities (e.g. host-cell-derived proteins, nucleic acid, viruses and other impurities, including media-derived compounds and

undesirable chemicals introduced by the purification process itself) should be investigated thoroughly, as should the reproducibility of the process. Particular attention should be given to demonstrating the removal and/or inactivation of possible contaminating viruses and residual DNA from products manufactured using continuous cell lines.

A.4.2.1 Residual cellular DNA from continuous cell lines

The ability of the manufacturing process to reduce the amount of residual cellular DNA (rcDNA) to an acceptable level, to reduce the size of the rcDNA or to chemically inactivate the biological activity of this DNA should be demonstrated.

Acceptable limits on the amount of rcDNA, as well as points to be considered concerning the size of rcDNA in an rDNA-derived biotherapeutic, are discussed in WHO's Recommendations for the evaluation of animal cell substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*5*). In setting these limits, there should be consideration of the characteristics of the cell substrate, the intended use and route of administration of the rDNA-derived biotherapeutics and, most importantly, the effect of the manufacturing process on the size, quantity and biological activity of the residual host-cell DNA fragments. In general, it has been possible to reduce rcDNA levels in rDNA-derived biotherapeutics to < 10 ng per dose. Alternatively, once validation studies (e.g. spiking studies using an adequate size distribution of DNA) have been performed, and once the reproducibility of the production process in reducing residual DNA to the level expected has been demonstrated, rcDNA testing may be omitted.

A.4.2.2 Virus clearance

For cell substrates of human or animal origin, virus removal or inactivation processes, individually and overall, should be shown to be able to remove/inactivate any contaminating viruses and to ensure viral safety in the drug substance.

Where appropriate, validation studies (see Appendix 1) should be undertaken using small-scale studies with carefully selected model viruses in order to evaluate the virus clearance/inactivation capability of selected process steps and of the overall process, aiming at a significant safety margins. The results will indicate the extent to which these contaminants can theoretically be inactivated and removed during purification.

The overall manufacturing process – including the testing and selection of the cells and source materials, as well as the validation of the ability of the purification process to adequately remove possible contaminants – should ensure the absence of infectious agents in the drug product. Nevertheless, to complement such approaches, routine testing of the fermentation process for the absence of contamination by infectious viruses is also recommended. A sample of the

unprocessed bulk following fermentation constitutes one of the most suitable levels at which adventitious virus contamination can be determined with a high probability of detection. A programme of ongoing assessment of adventitious viruses in fermentation should be undertaken. The scope, extent and frequency of virus testing on the unprocessed bulk should take into account the nature of the cell lines used, the results and extent of virus testing performed during the qualification of the MCB and WCB, the cultivation method, the source materials used, and the results of virus clearance studies. In vitro screening tests using one or more cell lines are generally used to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

If contamination by adventitious viruses is detected in the unprocessed bulk, the manufacturing process should be carefully checked to determine the cause of the contamination and to decide on appropriate action. Typically, adventitious virus contamination leads to the batch being discarded.

Further considerations of the detection, elimination and inactivation of viruses in animal cell substrates used in the production of rDNA-derived biotherapeutics, as well as the problem of rcDNA, can be found in the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*5*) as well as in the ICH guidelines Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin (*21*).

A.5 Control of drug substance and drug product

A.5.1 Characterization

Rigorous characterization of the rDNA-derived biotherapeutics by chemical, physicochemical and biological methods is essential. Characterization is typically performed in the development phase to determine the physicochemical properties, biological activity, immunochemical properties, purity and impurities of the product, and – following significant process changes and/or for periodic monitoring – to confirm the quality of the product. Characterization allows appropriate release specifications to be established.

Particular attention should be given to using a wide range of analytical techniques that exploit different physicochemical properties of the molecule (e.g. size, charge, isoelectric point, amino acid sequence, hydrophobicity). Post-translational modifications such as glycosylation should be identified and adequately characterized. It may also be necessary to include suitable tests to establish that the product has the desired conformation and higher order structure. In addition to evaluation of purity, there should also be investigation of impurities (e.g. aggregates including dimers and higher multiples of the desired product). The rationale for selecting the methods used for characterization should

be provided and their suitability should be justified since the characterization of the product is intended to identify attributes that may be important to the overall safety and efficacy of the product. Details of the expected characterization of an rDNA-derived biotherapeutic and techniques suitable for such purposes are set out in Appendix 2. The specific technical approach employed will vary from product to product; alternative approaches, other than those included in Appendix 2, will be appropriate in many cases. New analytical technologies and modifications to existing technologies are continually being developed and should be utilized when appropriate.

Where relevant and possible, characteristics of the properties of the product should be compared with those of its natural counterpart. For example, post-translational modifications such as glycosylation are likely to differ from those found in the natural counterpart and may influence the biological, pharmacological and immunological properties of the rDNA-derived biotherapeutics.

A.5.2 Routine control

Not all the characterization and testing described in section A.5.1 and in Appendix 2 needs to be carried out on each batch of drug substance and drug product prior to release for licensing or clinical use. Some tests may need to be performed only initially and/or periodically to establish or verify the validity or acceptability of a product and of its manufacturing process. Other tests may be required on a routine basis. A comprehensive analysis of the initial production batches is expected in order to establish consistency with regard to identity, purity and potency. A more limited series of tests is appropriate for routine control, as outlined below and in more detail in Appendix 3. Tests for use in routine control should be chosen to confirm quality. The rationale and justification for including and/or excluding testing for specific quality attributes should be provided.

An acceptable number of consecutive batches should be characterized to determine the consistency of analytical parameters at the time of licensing, unless otherwise justified. Any differences between one batch and another should be noted. Data obtained from such studies, as well as knowledge gained from clinical and nonclinical development and during stability studies, should be used as the basis for establishing product specifications.

The selection of tests to be included in the routine control programme will be product-specific and should take into account the quality attributes (e.g. potential influence on safety, efficacy or stability), the process performance (e.g. clearance capability, content), the controls in place through the manufacturing process (e.g. multiple testing points), and the material used in relevant nonclinical and clinical studies. These tests should include criteria such as potency, the nature and quantity of product-related substances, product-related impurities, process-related impurities, and absence of contaminants.

A.6 Filling and containers

The general requirements for filling and containers given in WHO Good manufacturing practices for biological products (*19*) should apply.

A description of the container closure systems for the drug substance and the drug product should be provided, including a specification for their component materials.

Evidence shows that formulated proteins can interact chemically or physically with the formulation excipients and/or the container closure system, and could therefore influence the quality, safety or pharmacological properties of the product. Some products have been shown to form aggregates with excipients, and such aggregates may lead to the formation of potentially immunogenic complexes – see, for example references (*27–29*). The suitability of the container closure system should be evaluated and described for its intended use. This should cover evaluation of the compatibility of the container construction materials with the formulated product, including adsorption to the container, leaching, and other chemical or physical interactions between the product and the materials in contact with it. The integrity of the closure and its ability to protect the formulation from contamination and to maintain sterility needs to be ensured.

When a delivery device is presented as part of the drug product (e.g. prefilled syringe, single-use autoinjector), it is important to demonstrate the functionality of such a combination – such as the reproducibility and accuracy of the dispensed dose under testing conditions which should simulate the use of the drug product as closely as possible. For multi-use containers such as vials or cartridges for a pen injector, proper in-use stability studies should be performed to evaluate the impact of the in-use period of the vial or the assembled device on the formulation and the functionality of the pen injector. Dose accuracy should be demonstrated for the first and last dose delivered. In addition, the effect of multiple injections/withdrawals on the closure system should be evaluated.

A.7 Records, retained samples, labelling, distribution and transport

The requirements given in the WHO Good manufacturing practices for biological products (*19*) should apply.

The conditions of shipping should be such as to ensure that the products are maintained in appropriate conditions.

A.8 Stability, storage and expiry date

A.8.1 Stability studies

While the expectations outlined in this section are primarily applicable to the marketing application stage, products in clinical development should be tested for stability concurrently with clinical trials. For proteins, maintenance of biological

activity is generally dependent on maintaining molecular conformation. Such products can be particularly sensitive to environmental factors such as temperature changes, oxidizing factors, and light exposure. In order to ensure the maintenance of biological activity and to avoid degradation, appropriate conditions for storage are necessary.

A detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods should be developed. This should include all information necessary to demonstrate the stability of the rDNA-derived biotherapeutics throughout the proposed shelf-life, including, for example, well-defined specifications and test intervals.

Each product should remain within its specification for stability-indicating attributes, including potency throughout its proposed shelf-life. Specifications should be derived from all available information using appropriate statistical methods at the time of licensing. There is no single stability-indicating assay or parameter that profiles the stability characteristics of an rDNA-derived biotherapeutic. Consequently, the manufacturer should develop a stability-indicating programme that provides assurance that changes in the quality and potency of the product will be detected.

Primary data to support a requested storage period for both drug substance and drug product should be based on long-term, real-time, and real-condition stability studies, and these should be further supported by accelerated- and stress-condition stability data, as available, to justify the claimed shelf-life. In cases where the stability of the product is influenced by the storage of intermediates (e.g. a significant degradation trend is observed during storage of an intermediate), a cumulative stability study should be considered. This study should include all intermediates stored at the longest storage time claimed, or a selection of the most storage-sensitive intermediates, as appropriate. In view of the time necessary to generate the data, inclusion of study results may not be feasible at the time of licensing. The absence of such a cumulative study could be justified on the basis of a proposed stability programme that will include such monitoring. In addition, stability studies should include an evaluation of the impact of the container closure system on the formulated rDNA-derived biotherapeutics throughout the shelf-life. In order to ensure that the formulated product is in contact with all material of the container closure system, stability studies should include samples maintained in the inverted or horizontal position (i.e. in contact with the closure). Data should be supplied for all different container closure combinations that will be marketed.

Stability information should be provided on at least three batches for which manufacture and storage are representative of the commercial process.

When shelf-lives of 1 year or less are proposed, real-time stability studies should be conducted monthly for the first 3 months and at 3-month intervals

thereafter. For products with proposed shelf-lives greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter. A minimum of 6 months' data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested, unless otherwise justified. For storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis.

It is recommended that stability studies under accelerated and stress conditions, including the impact of the container closure system (see section A.6), should also be conducted on the drug product. Studies under accelerated conditions may: (a) provide useful supportive data for establishing the expiry date; (b) provide product stability information for future product development (e.g. preliminary assessment of proposed manufacturing changes such as changes in formulation or scale-up); (c) assist in validation of analytical methods for the stability programme; or (d) generate information which may help elucidate the degradation profile of the rDNA-derived biotherapeutics. Studies under stress conditions may also be useful for determining whether accidental exposures to conditions other than those proposed (e.g. during transportation) are deleterious to the product and for evaluating which specific test parameters may be the best indicators of product stability.

Further guidance on both general and specific aspects of stability testing of an rDNA-derived biotherapeutic can be obtained by consulting the WHO guidelines on the stability testing of active pharmaceutical ingredients and finished pharmaceutical products (*30*), as well as the WHO Guidelines on stability evaluation of vaccines (*31*).

A.8.2 Drug product requirements

Stability information should be provided on at least three batches of drug product that are representative of that which will be used in commercial manufacture, and presented in the final container. Where possible, the drug product batches included in stability testing should be derived from different batches of drug substance.

Where one product is distributed in multiple presentations, the samples to be entered into the stability programme may be selected on the basis of a matrix system and/or by bracketing. Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability programme (i.e. bracketing). The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples is represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

Matrixing (i.e. the statistical design of a stability study in which account is taken of factors such as the tests, process characteristics, presentation characteristics and different testing time-points) should be applied only when appropriate documentation is provided confirming that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure and, possibly in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

For preparations intended for use after reconstitution, dilution or mixing, in-use stability data should be obtained. The stability should be demonstrated up to and beyond the storage conditions and the maximum storage period claimed.

In addition to the standard data necessary for a conventional single-use vial, it should be shown that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its identity, strength, potency, purity and quality for the maximum period specified in the instructions for use on containers, packages and/or package inserts.

A.9 Manufacturing process changes

Changes to the manufacturing process of an rDNA-derived biotherapeutic often occur both during development and after approval. The reasons for such changes include, for example, improvement of the manufacturing process, increase in scale, a site change, improvement of product stability, or compliance with changes in regulatory requirements. When substantial changes are made to the manufacturing process, a comparability exercise to evaluate the impact of the change(s) on the quality, safety and efficacy of the rDNA-derived biotherapeutics should be considered. The extent of such an exercise depends on the potential impact of the process change(s) as well as on the manufacturer's experience in the process and knowledge of the product. The demonstration of comparability does not necessarily mean that the quality attributes of the pre-change and post-change product are identical, but rather that they are highly similar and that the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact on the safety or efficacy of the rDNA-derived biotherapeutics. The reason for each significant change should be explained, together with an assessment of its potential to impact on quality, safety and efficacy.

The extent of a comparability exercise depends on the potential impact of the process change(s) on the quality, safety and efficacy of the product.

A comparability exercise can range from analytical testing alone (e.g. where process changes lead to no changes in any quality attribute) to a comprehensive exercise requiring nonclinical and clinical bridging studies (e.g. the establishment of a new host cell line with altered properties resulting in more pronounced changes in quality attributes). If assurance of comparability can be shown through analytical studies alone, nonclinical or clinical studies with the post-change product may not be necessary. However, where the relationship between specific quality attributes and safety and efficacy has not been established, and differences between quality attributes of the pre-change and post-change product are observed, it may be appropriate to include a combination of quality, nonclinical and/or clinical studies in the comparability exercise.

Further considerations of manufacturing changes can be found in guidelines provided by the ICH (*32*), the EMA (*33*), the United States Food and Drug Administration (*34*) and other major NRAs.

Part B. Nonclinical evaluation

B.1 Introduction

The general aim of nonclinical evaluation is to determine whether new medicinal products possess the desired pharmacodynamic (PD) activity and whether they have the potential to cause unexpected and undesirable effects. However, classic PD, safety or toxicological testing, as recommended for chemical drugs, may be of only limited relevance for rDNA-derived biotherapeutics due to the latter's unique and diverse structural and biological properties, including species specificity, immunogenicity, and unpredicted pleiotropic activities. These properties pose particular problems in relation to nonclinical testing in animals, and their pharmacological and safety evaluation will have to take a large number of factors into account. Thus, a flexible approach is necessary for the nonclinical evaluation of rDNA-derived biotherapeutics. For example, certain proteins (e.g. interferons) are highly species-specific, so that the human protein is pharmacologically much more active in humans than in any animal species. Furthermore, human proteins frequently produce immunological responses in animal species which may ultimately modify their biological effects and may result in toxicity (e.g. due to immune complex formation). Such toxicity has little bearing on the safety of the product in the intended human host.

Although some safety testing will be required for most products, the range of tests that need to be carried out should be decided on a case-by-case basis (see Appendix 4) in consultation with the NRA or national control laboratory. A wide range of pharmacological, biochemical, immunological, toxicological and histopathological investigative techniques should be used, where appropriate, to assess a product's effect over an appropriate range of doses and, in accordance

with the desired clinical indication(s), during both acute and chronic exposure. However, the points made above concerning species specificity and antibody formation should always be taken into consideration.

Additional information on specific safety issues – such as, for example, carcinogenic potential, reproductive toxicity or safety pharmacology – is provided in respective ICH safety guidelines (*35–37*). Relevant sections of this part may be useful with regard to products intended for clinical trials; however, the amount and the extent of data submitted for a product will be limited and will need to take into account the nature of the product and its stage of development. Recommendations concerning timing and interplay of nonclinical and clinical studies in drug development are given in the ICH Guidance on nonclinical safety studies for the conduct for human clinical trials and marketing authorization for pharmaceuticals (*38*) and in the ICH guideline Preclinical safety evaluation of biotechnology-derived pharmaceuticals (*39*).

B.1.1 Objectives of the nonclinical evaluation

The objectives of nonclinical studies are to define pharmacological and toxicological effects throughout clinical development, not only prior to initiation of human studies.

The primary goals are:

- to identify an initial safe dose and subsequent dose escalation schemes in humans;
- to identify potential target organs for toxicity and for the study of whether such toxicity is reversible;
- to identify safety parameters for clinical monitoring.

Nonclinical evaluation should consider:

- selection of the pharmacologically or toxicologically relevant animal species;
- the age of the animals;
- the physiological state of the animals (e.g. whether healthy/diseased animals are used, whether treatment-naïve animals are used);
- the weight of the animals;
- the manner of delivery, including relevant dose or amount, route of administration, and treatment regimen;
- stability of the test material under the conditions of use;
- interpretation of results.

Both in vitro and in vivo studies can contribute to this characterization.

rDNA-derived biotherapeutics that belong structurally and pharmacologically to a product class for which there is wide experience in clinical practice may need less-extensive toxicity testing.

B.1.2 Product development and characterization

In general, the product that is used in the definitive pharmacology and toxicology studies should be representative of the product proposed for the initial clinical studies. However, it is appreciated that during the course of development programs, changes normally occur in the manufacturing process in order to improve product quality and yields. The potential impact of such changes for extrapolation of the animal findings to humans should be considered, including the impact of post-translational modifications.

The comparability of the test material should be demonstrated when a new or modified manufacturing process or other significant changes in the product or formulation are made in an ongoing development program. Comparability can be evaluated on the basis of biochemical and biological characterization (i.e. identity, purity, stability and potency). In some cases, additional studies may be needed (i.e. PK, PD and/or safety). The scientific rationale for the approach taken should be provided.

B.1.3 Good laboratory practice

Pivotal (toxicity) studies should be performed in compliance with good laboratory practice (GLP) (*40*, *41*). However, it is recognized that some studies employing specialized test systems which are often needed for rDNA-derived biotherapeutics may not comply fully with GLP. Areas of non-compliance should be identified and their significance evaluated relative to the overall nonclinical assessment. In some cases, lack of full GLP compliance does not necessarily mean that the data from these studies cannot be used to support clinical trials and marketing authorization. However, justification which is supported with data, such as method validation should be provided for the data quality assurance.

B.2 Pharmacodynamics

B.2.1 Primary and secondary pharmacodynamics/biological activity

Biological activity may be evaluated by the use of in vitro assays to determine which effects of the product may be related to clinical activity. The use of cell lines and/or primary cell cultures can be useful to examine the direct effects on cellular phenotype and proliferation. Due to the species specificity of many rDNA-derived biotherapeutics, it is important to select relevant animal species for testing (see Appendix 5). Non-human primates (NHPs) are often the only pharmacologically or toxicologically relevant species; however, other species should also be evaluated for relevant biological activity. In vitro cell lines derived

from mammalian cells can be used to predict specific aspects of in vivo activity and to assess quantitatively the relative sensitivity of various species, including humans, to the biotherapeutics. Such studies may be designed to determine, for example, receptor occupancy, receptor affinity, and/or pharmacological effects, and to assist in the selection of an appropriate animal species for further in vivo pharmacology and toxicology studies. The combined results from in vitro and in vivo studies assist in the extrapolation of the findings to humans. In vivo studies to assess pharmacological activity, including defining mechanism(s) of action, are often used to support the rationale for the proposed use of a product in clinical studies. When feasible, PD end-points can be incorporated into general toxicity studies (e.g. haemoglobin blood concentration in repeated dose toxicity studies with erythropoietins).

B.2.2 Safety pharmacology

According to the target or mechanism of action of the product, it is important to investigate the potential for undesirable pharmacological activity in appropriate animal models. The aim of the safety pharmacology studies is to reveal any functional effects on the major physiological systems (e.g. cardiovascular, respiratory, central nervous system). These functional indices may be investigated in separate studies or incorporated in the design of toxicity studies and/or clinical studies. Investigations may include the use of isolated organs or other test systems not involving intact animals. All of these studies may allow for a mechanistically based explanation of specific organ effects/toxicities, which should be considered carefully with respect to applicability for human use and indication(s).

B.3 Pharmacokinetics/toxicokinetics

B.3.1 General principles

It is difficult to establish uniform guidelines for PK studies for rDNA-derived biotherapeutics. Single-dose and multiple-dose PK, toxicokinetics (TK) and tissue distribution studies in relevant species are useful; however, routine studies that attempt to assess mass balance are not useful. Differences in PK between animal species may have a significant impact on the predictiveness of animal studies or on the assessment of dose–response relationships in toxicity studies. Scientific justification should be provided for the selection of the animal species used for PK/TK evaluation, taking into account that the PK profile in the chosen animal species should ideally reflect the PK profile in humans. Alterations in the PK profile due to immune-mediated clearance mechanisms may affect the kinetic profiles and the interpretation of the toxicity data (see also section B.4.8.1). For some products there may also be significant inherent delays in the expression of PD effects relative to the PK profile (e.g. cytokines) or there may be prolonged expression of PD effects relative to plasma levels.

PK studies should, whenever possible, utilize preparations that are representative of that intended for toxicity testing and clinical use, and should employ a route of administration that is relevant to the anticipated clinical studies. Patterns of absorption may be influenced by formulation, active substance concentration, application site, and/or application volume. Whenever possible, systemic exposure should be monitored during the toxicity studies. When feasible, PK/TK evaluations can be incorporated into general toxicity studies.

Some information on absorption, disposition and clearance in relevant animal models should be available prior to clinical studies in order to predict margins of safety based on exposure and dose. Understanding the behaviour of the biotherapeutic in the biological matrix (e.g. plasma, serum, cerebral spinal fluid) and the possible influence of binding proteins is important for understanding the PD effect.

B.3.2 Assays

The use of one or more assay methods should be addressed on a case-by-case basis and the scientific rationale should be provided. One validated method is usually considered sufficient. For example, quantitation of trichloracetic acid (TCA)-precipitable radioactivity following administration of a radiolabelled protein may provide adequate information, but a specific assay for the analyte is preferred. Ideally, the assay methods should be the same for animal and human studies. The possible influence of plasma-binding proteins and/or antibodies in plasma/serum on the performance of the assay should be determined.

B.3.3 Distribution

Unlike small chemical drugs that readily diffuse, rDNA-derived biotherapeutics, due to their molecular weight, usually do not readily do so but, following intravenous application, are initially confined to the vascular system. However, with time they may distribute to the extravascular space as a result of various factors, including bulk flow and active transport.

As a supplement to standard tissue distribution studies, complementary information about the tissue distribution of molecular targets for rDNA-derived biotherapeutics may be obtained from tissue cross-reactivity (TCR) studies, if appropriate (see section B.4.8.3).

Tissue concentrations of radioactivity and/or autoradiography data using radiolabelled proteins may be difficult to interpret due to rapid protein metabolism in vivo or unstable radiolabelled linkage. Care should be taken in interpreting studies using radioactive tracers incorporated into specific amino acids because of the possibility of recycling of amino acids into non-drug-related proteins/peptides.

B.3.4 Metabolism

The expected consequence of metabolism of rDNA-derived biotherapeutics is degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood. Classic biotransformation studies, as performed for pharmaceuticals, are not needed.

B.4 Toxicity studies

B.4.1 General principles

B.4.1.1 Number/gender of animals

For ethical reasons, it is desirable to apply the 3Rs concept of "Replace Reduce Refine" to minimize the use of animals in research, and consideration should be given to the use of appropriate in vitro alternative methods for safety evaluation (*42*).

The number of animals used per dose has a direct bearing on the ability to detect toxicity. A small sample size may lead to failure to observe toxic events due to observed frequency alone, regardless of severity. The limitations that are imposed by sample size, as often is the case for NHP studies, may in part be compensated by increasing the frequency and duration of monitoring. Both genders should generally be used or justification given for specific omissions. As an example, the minimum sample size for a pivotal GLP toxicity study in NHPs is considered to be three animals per sex and, if a recovery group is included in the study, an additional minimum of two animals per sex would be included.

B.4.1.2 Administration/dose selection and application of PK/PD principles

The route and frequency of administration should be as close as possible to that proposed for clinical use. Consideration should be given to the pharmacokinetics and bioavailability of the product in the species being used, as well as the volume that can be safely and humanely administered to the test animals. For example, the frequency of administration in laboratory animals may be increased compared to the proposed schedule for the human clinical studies in order to compensate for faster clearance rates or low solubility of the active ingredient. In these cases, the level of exposure of the test animal should be defined relative to the clinical exposure. Consideration should also be given to the effects of application volume, active substance concentration, formulation, and site of administration. The use of routes of administration other than those used clinically may be acceptable if the route must be modified due to limited bioavailability, limitations due to the route of administration, or to size/physiology of the animal species used.

If feasible, dosage levels should be selected in order to provide information on a dose–response relationship, including a toxic dose and a "no observed adverse effect level" (NOAEL). These data may be used for estimating the maximum

recommended starting dose in initial clinical trials (*43*). In addition, for selection of a safe starting dose for first-in-human clinical trials (*38*), the identification of the "minimum anticipated biological effect level" (MABEL) (*44*) should be considered. For oncology products, see Appendix 4.

The toxicity of most rDNA-derived biotherapeutics is related to their targeted mechanism of action; therefore, relatively high doses can elicit adverse effects which are apparent as exaggerated pharmacology. For some classes of product which show little or no toxicity it may not be possible to define a specific maximum dose. In these cases, a scientific justification of the rationale for the dose selection and projected multiples of human exposure should be provided. To justify selection of a high dose, consideration should be given to the expected pharmacological/physiological effects and the intended clinical use. Where a product has a lower affinity for, or potency in, the cells of the selected species than for human cells, testing of higher doses may be important. The multiples of the human dose that are needed to determine adequate safety margins may vary with each class of rDNA-derived biotherapeutics and its clinical indication(s).

A rationale should be provided for dose selection, taking into account the characteristics of the dose–response relationship. PK-PD approaches (e.g. simple exposure–response relationships or more complex modelling and simulation approaches) can assist in high-dose selection by identifying: (a) a dose which provides the maximum intended pharmacological effect in the selected animal species; and (b) a dose which provides an approximately 10-fold exposure multiple over the maximum exposure to be achieved in the clinic. The higher of these two doses should be chosen for the high-dose group in nonclinical toxicity studies unless there is a justification for using a lower dose (e.g. maximum feasible dose).

Where in vivo/ex vivo PD end-points are not available, the high-dose selection can be based on PK data and on available in vitro binding and/or pharmacology data. Corrections for differences in target binding and in vitro pharmacological activity between the nonclinical species and humans should be taken into account to adjust the exposure margin over the highest anticipated clinical exposure. For example, a large relative difference in binding affinity and/or in vitro potency might suggest that testing higher doses in the nonclinical studies is appropriate. In the event that toxicity cannot be demonstrated at the doses selected using this approach, then additional toxicity studies at higher multiples of human dosing are unlikely to provide additional useful information.

B.4.1.3 Use of one or two species

With regard to the use of one or two species for toxicity studies, see Appendix 5.

B.4.1.4 Study duration

For chronic-use products, repeat dose toxicity studies of 6 months' duration in rodents or non-rodents are usually considered sufficient so long as the high dose is

selected in accordance with the principles above. Studies of longer duration have not generally provided useful information that has changed the clinical course of development (see also section B.4.3). Performance of (6-month) chronic toxicity studies may not always be feasible (e.g. if an induction of anti-drug antibodies prevents a meaningful study interpretation). For chronic use of rDNA-derived biotherapeutics developed for patients with advanced cancer, see Appendix 4.

B.4.1.5 Evaluation of immunogenicity

Many rDNA-derived biotherapeutics intended for human use are immunogenic in animals. Therefore, plasma samples from animals subjected to repeated dose toxicity studies should be stored at an appropriate temperature and analysed for the presence of anti-drug antibody when considered necessary for study interpretation (see section B.4.8.1).

B.4.2 Single-dose toxicity studies

In general, single-dose toxicity studies should be pursued only in cases where significant toxicity is anticipated and the information is needed to select doses for repeated dose studies (*38*, *39*). Single-dose studies may generate useful data to describe the relationship of dose to systemic and/or local toxicity. These data can be used to select doses for repeated dose toxicity studies. Information on dose–response relationships may be gathered through the conduct of a single-dose toxicity study as a component of pharmacology or animal-model efficacy studies. The incorporation of safety pharmacology parameters in the design of these studies should be considered.

B.4.3 Repeated dose toxicity studies

For consideration of the selection of animal species for repeated dose studies, see section B.4.1. The route and dosing regimen (e.g. daily versus intermittent dosing) should reflect the intended clinical use or exposure. When feasible, these studies should include TK measurements, but interpretation should consider the formation of possible anti-drug antibodies (see section B.4.8.1).

B.4.3.1 Study duration

The duration of repeated dose studies should be based on the intended duration of clinical exposure and disease indication. Duration of animal dosing has generally been 1–3 months for most rDNA-derived biotherapeutics. For rDNA-derived biotherapeutics intended for short-term use (e.g. < 7 days) and for acute life-threatening diseases, repeated dose studies of up to 2 weeks' duration have been considered adequate to support clinical studies as well as marketing authorization. For those rDNA-derived biotherapeutics intended for chronic indications, studies of 6 months' duration have generally been appropriate,

although in some cases shorter or longer durations have supported marketing authorizations. For rDNA-derived biotherapeutics intended for chronic use, the duration of long-term toxicity studies should be scientifically justified.

B.4.3.2 Recovery period

Recovery from pharmacological and toxicological effects with potential adverse clinical impact should be understood when they occur at clinically relevant exposures. This information can be obtained by understanding that the particular effect observed is generally reversible/nonreversible, or by including a non-dosing period in at least one study, at least at one dose level, to be justified by the sponsor. The purpose of the non-dosing period is to examine reversibility of these effects and not to assess delayed toxicity. The demonstration of complete recovery is not considered essential. The addition of a recovery period for the sole purpose of assessing the potential for immunogenicity is not required.

B.4.4 Genotoxicity studies

The range and type of genotoxicity studies routinely conducted for pharmaceuticals are not applicable to rDNA-derived biotherapeutics and are therefore not needed. Moreover, the administration of large quantities of peptides/proteins may yield un-interpretable results. It is not expected that these substances will interact directly with DNA or other chromosomal material.

With some rDNA-derived biotherapeutics there is a potential concern about accumulation of spontaneously mutated cells (e.g. via facilitating a selective advantage of proliferation), leading to carcinogenicity. The standard battery of genotoxicity tests is not designed to detect these conditions. Alternative in vitro or in vivo models to address such concerns may have to be developed and evaluated (see section B.4.5).

Studies in available and relevant systems, including newly developed systems, should be performed in those cases where there is cause for concern about the product (e.g. because of the presence of an organic linker molecule in a conjugated protein product).

The use of standard genotoxicity studies for assessing the genotoxic potential of process contaminants is usually not considered appropriate. If performed for this purpose, however, the rationale should be provided.

B.4.5 Carcinogenicity studies

B.4.5.1 General principles

Carcinogenicity is, in the strict sense, increased probability of development of new tumours. However, activation of proliferation and progression of existing tumour cells/tumours should also be considered.

The need for a product-specific assessment of the carcinogenic potential of rDNA-derived biotherapeutics should be determined with regard to the intended clinical population and treatment duration – see, for example, reference (*36*). When an assessment is warranted, the sponsor should design a strategy to address the potential hazard. This strategy could be based on a review of relevant data from a variety of sources. The data sources can include published data (e.g. information from transgenic, knock-out or animal disease models, and human genetic diseases), information on class effects, detailed information on target biology and mechanism of action, in vitro data, and data from chronic toxicity studies and clinical data. In some cases, the available information can be sufficient to address carcinogenic potential and inform clinical risk without additional nonclinical studies.

The mechanism of action of some rDNA-derived biotherapeutics may raise concern regarding potential for carcinogenicity (e.g. immunosuppressives and growth factors). If the review of all available data (see above) supports this concern, rodent bioassays are not warranted. In this case, potential hazard can be best addressed by product labelling and risk management practices. When a review of all available data suggests that there is no carcinogenic concern, no additional testing is needed. However, if the potential for carcinogenicity remains unclear after a review of all available data, the sponsor can propose additional studies that could mitigate the mechanism-based concern – see, for example, reference (*39*). When a review of all available data, including the additional study data, supports concern regarding carcinogenic potential, this is best addressed by product labelling and risk management practices. Correspondingly, if the potential for carcinogenicity remains unclear after the extended data review, this should also be addressed by product labelling and risk management practices. In case the concern regarding carcinogenicity is mitigated by the additional study data, this should be reflected in the product information.

For products where there is insufficient knowledge about specific product characteristics and mode of action in relation to carcinogenic potential, a more extensive assessment might be appropriate (e.g. understanding of target biology related to potential carcinogenic concern, and inclusion of additional end-points in toxicity studies). If the review of all data from this more extensive assessment does not suggest a carcinogenic potential, no additional nonclinical testing is recommended. Alternatively, if the review of all data available suggests a concern about carcinogenic potential, then the sponsor can propose additional nonclinical studies that could mitigate the concern (see above), or the label should reflect the concern.

The selection of animal models for the assessment of tumour growth potential should take into account that rDNA-derived biotherapeutics may have secondary, unspecific effects on tumour growth which would be clinically irrelevant. Careful design and choice of controls should be used to avoid misinterpretations.

B.4.5.2 Use of homologous proteins

A homologous protein is defined as a protein of animal origin (e.g. from mouse, rat, dog, rabbit or non-human primates) that recognizes the appropriate target(s) in the respective species with similar potency as the clinical candidate recognizes the corresponding human target(s) (*45*). Rodent bioassays (or short-term carcinogenicity studies) with homologous products are generally of limited value for assessing the carcinogenic potential of the clinical candidate. Since the production process, range of impurities/contaminants, pharmacokinetics, and exact pharmacological mechanism(s) may differ between the homologous form and the product intended for clinical use, studies with homologous proteins are generally not useful for quantitative risk assessment (see Appendix 5).

B.4.5.3 Risk communication

The product-specific assessment of carcinogenic potential is used to communicate risk and provide input to the risk management plan along with labelling proposals, clinical monitoring, post-marketing surveillance, or a combination of these approaches.

B.4.6 Reproductive performance and developmental toxicity studies

B.4.6.1 General principles

The need for reproductive/developmental toxicity studies is dependent upon the product, the clinical indication and the intended patient population. The specific study design and dosing schedule may be modified on the basis of issues related to species specificity, immunogenicity, biological activity and/or a long elimination half-life. For example, concerns regarding potential developmental immunotoxicity, which may apply particularly to certain monoclonal antibodies with prolonged immunological effects, could be addressed in a study design modified to assess immune function of the neonate.

B.4.6.1.1 *Products with expected/probable adverse effects on fertility/pregnancy outcome*

When the available data (e.g. mechanism of action, phenotypic data from genetically modified animals, class effects) clearly suggest that there will be an adverse effect on fertility or pregnancy outcome, these data can provide adequate information to communicate risk to reproduction and, under appropriate circumstances, additional nonclinical studies might not be warranted. There may be extensive public information available regarding the potential reproductive and/or developmental effects of a particular class of compounds (e.g. interferons) where the only relevant species is the non-human primate. In such cases, mechanistic studies indicating that similar effects are likely to be caused by a new but related molecule may obviate the need for formal reproductive/developmental

toxicity studies. In each case, the scientific basis for assessing the potential for possible effects on reproduction/development should be provided.

B.4.6.1.2 *Products with unclear potential for adverse effects on fertility/pregnancy outcome*

The specific study design and dosing schedule can be modified on the basis of an understanding of species specificity, the nature of the product and its mechanism of action, immunogenicity and/or PK behaviour, and embryo-fetal exposure.

Species selection – an assessment of reproductive toxicity of the clinical candidate should usually be conducted only in pharmacologically relevant species. When the clinical candidate is pharmacologically active in rodents and rabbits, both species should be used for embryo-fetal development (EFD) studies, unless embryo-fetal lethality or teratogenicity has been identified in one species. Developmental toxicity studies should be conducted in NHPs only when they are the only relevant species. When the clinical candidate is pharmacologically active only in NHPs, there is still a preference to test the clinical candidate. However, an alternative model can be used in place of NHPs if appropriate scientific justification is provided.

Alternative evaluation in the absence of a relevant species – when no relevant animal species exist(s) for testing the clinical candidate, the use of transgenic mice expressing the human target or homologous protein in a species expressing an orthologue of the human target can be considered, assuming that sufficient background knowledge (e.g. historical background data) exists for the model.

B.4.6.1.3 *Products for which adverse effects on fertility/pregnancy outcome are not expected*

For products that are directed at a foreign target such as bacteria and viruses, in general no reproductive toxicity studies would be expected.

B.4.6.2 Fertility

For products where mice and rats are pharmacologically relevant species, an assessment of fertility can be made in one of these rodent species (*35*). Study designs can be adapted for other species provided they are pharmacologically relevant. In such cases the designs should be amended as appropriate – for example, to address the nature of the product and the potential for immunogenicity.

It is recognized that mating studies are not practical for NHPs. However, when the NHP is the only relevant species, the potential for effects on male and female fertility can be assessed by evaluation of the reproductive tract (organ weights and histopathological evaluation) in repeat-dose toxicity studies of at least 3 months' duration, using sexually mature NHPs. If there is a specific cause for concern based on pharmacological activity or previous findings, specialized

assessments such as menstrual cyclicity, sperm count, sperm morphology/motility, and male or female reproductive hormone levels can be evaluated in a repeat-dose toxicity study.

If the pharmacological activity leads to a specific concern about potential effects on conception/implantation and the NHP is the only relevant species, the concern should be addressed experimentally. A homologous product or transgenic model could be the only practical means to assess potential effects on conception or implantation when those are of specific concern. However, it is not recommended to produce a homologous product or transgenic model solely to conduct mating studies in rodents. In the absence of nonclinical information, the risk to patients should be mitigated through clinical trial management procedures, informed consent and appropriate product labelling.

B.4.6.3 EFD and pre/postnatal development

B.4.6.3.1 *Selection of study design*

Potential differences in placental transfer of rDNA-derived biotherapeutics should be considered in the design and interpretation of developmental toxicity studies (see Appendix 6, Note 1).

For products that are pharmacologically active only in NHPs, several study designs can be considered according to intended clinical use and expected pharmacology. Separate EFD and/or pre/postnatal development (PPND) studies, or other study designs (justified by the sponsor) can be appropriate, particularly when there is some concern that the mechanism of action may lead to an adverse effect on EFD or pregnancy loss. However, one well-designed study in NHPs which includes dosing from day 20 of gestation to birth – "enhanced PPND" (ePPND) – can be considered rather than separate EFD and/or PPND studies.

B.4.6.3.2 *ePPND studies*

For the single ePPND study design described above, no caesarean section group is warranted, but assessment of pregnancy outcome at natural delivery should be performed. This study should also evaluate offspring viability, external malformations, skeletal effects (e.g. by X-ray) and, ultimately, visceral morphology at necropsy. Ultrasound is useful for tracking the maintenance of pregnancy but is not appropriate for detecting malformations. These latter data are derived from postpartum observations. Because of potential adverse effects of treatment on maternal care of offspring, dosing of the mother postpartum is generally not recommended. Other end-points in the offspring can also be evaluated if relevant to the pharmacological activity. The duration of the postnatal phase will depend on which additional end-points are considered relevant in view of the mechanism of action (see Appendix 6, Note 2).

Developmental toxicity studies in NHPs can provide only hazard identification. The number of animals per group should be sufficient to allow meaningful interpretation of the data (see Appendix 6, Note 3).

The study design should be justified if species other than the cynomolgus monkey are used. The developmental toxicity studies in NHPs, as outlined above, are hazard identification studies; therefore, it may be possible to conduct these studies using a control group and one dose group, provided there is a scientific justification for the dose level selected (see Appendix 6, Note 4).

B.4.6.4 Timing of studies

If women of childbearing potential are included in clinical trials prior to acquiring information on the effects on EFD, suitable clinical risk management is appropriate – such as the use of highly effective methods of contraception (*38*). For rDNA-derived biotherapeutics pharmacologically active only in NHPs, where there are sufficient precautions to prevent pregnancy an EFD or ePPND study can be conducted during phase III and the report submitted at the time of marketing application. When a sponsor cannot take sufficient precautions to prevent pregnancy in clinical trials, either a complete report of an EFD study or an interim report of an ePPND study should be submitted before initiation of phase III (see Appendix 6, Note 5). Where the product is pharmacologically active only in NHPs and its mechanism of action raises serious concern about embryo-fetal development, the label should reflect the concern without warranting a developmental toxicity study in NHPs and the administration to women of childbearing potential should be avoided.

If the rodent or rabbit is a relevant species, timing of reproductive toxicity/fertility studies should follow the recommendations given – see, for example, reference (*38*).

For oncology products, see Appendix 4.

B.4.7 Local tolerance studies

Local tolerance should be evaluated. Ideally, the formulation intended for marketing should be tested; however, in certain justified cases, the testing of representative formulations may be acceptable. If feasible, the potential adverse effects of the product can be evaluated in single- or repeated-dose toxicity studies, thus obviating the need for separate local tolerance studies.

B.4.8 Other toxicity studies

B.4.8.1 Antibody formation

Immunogenicity assessments in animals should be conducted only to assist in the interpretation of the study results and to improve the design of subsequent studies. Such analyses in animal studies are usually not relevant in terms of

predicting potential immunogenicity of human or humanized proteins in humans. Since antibody formation to human proteins in animal studies is usually not predictive of the clinical situation, concerns regarding antibody formation to the endogenous hormones (as in the case of erythropoietin or somatropin) will have to be addressed on a clinical safety level.

Measurement of anti-drug antibodies in nonclinical studies should be evaluated when there is: (a) evidence of altered PD activity; (b) unexpected change in exposure in the absence of a PD marker; or (c) evidence of immune-mediated reactions (immune complex disease, vasculitis, anaphylaxis, etc.). Since it is difficult to predict prior to study completion whether such analysis will be necessary, it is often useful to obtain appropriate samples during the course of the study so that these can subsequently be analysed when warranted to aid in interpretation of the study results.

When anti-drug antibodies are detected, their impact on the interpretation of the study results should be assessed. Antibody responses should be characterized (e.g. titre, number of responding animals, neutralizing or non-neutralizing activity), and their appearance should be correlated with any pharmacological and/or toxicological changes. Specifically, the effects of antibody formation on PK/PD parameters, incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data. Attention should also be paid to the evaluation of possible pathological changes related to immune complex formation and deposition.

Characterization of neutralizing potential is warranted when anti-drug antibodies are detected and there is no PD marker to demonstrate sustained activity in the in vivo toxicology studies. Neutralizing antibody activity can be assessed indirectly with an ex vivo bioactivity assay or an appropriate combination of assay formats for PK-PD, or directly in a specific neutralizing antibody assay.

The detection of antibodies should not be the sole criterion for the early termination of a nonclinical safety study or modification in the duration of the study design, unless the immune response neutralizes the pharmacological and/or toxicological effects of the rDNA-derived biotherapeutics in a large proportion of the animals. In most cases, the immune response to rDNA-derived biotherapeutics is variable, similar to that observed in humans. If the interpretation of the data from the safety study is not compromised by these issues, then no special significance should be ascribed to the antibody response.

B.4.8.1.1 *Anaphylaxis tests*

The occurrence of severe anaphylactic responses to rDNA-derived biotherapeutics is uncommon in humans. In this regard, the results of guinea pig anaphylaxis tests, which are generally positive for protein products, are usually not predictive for reactions in humans and are usually not conducted.

B.4.8.2 Immunotoxicity studies

One aspect of immunotoxicological evaluation is the assessment of potential immunogenicity (see sections B.4.1 and B.4.8.1). Many rDNA-derived biotherapeutics are intended to stimulate or suppress the immune system and, therefore, may affect humoral as well as cell-mediated immunity. Inflammatory reactions at the injection site may be indicative of a stimulatory response. It is important to recognize, however, that simple injection trauma and/or specific toxic effects caused by the formulation vehicle may result in toxic changes at the injection site. The expression of surface antigens on target cells may be altered, with implications for autoimmune potential. Immunotoxicological testing strategies may require screening studies followed by mechanistic studies to clarify such issues. Routine tiered testing approaches or standard testing batteries, however, are not recommended for rDNA-derived biotherapeutics.

The following modes of action may require special attention (*44*):

- A mode of action that involves a target which is connected to multiple signalling pathways (a target with pleiotropic effects), e.g. leading to various physiological effects, or targets that are ubiquitously expressed, as often seen in the immune system.
- A biological cascade or cytokine release, including one leading to an amplification of an effect that might not be sufficiently controlled by a physiological feedback mechanism (as in the immune system or blood coagulation system). The so-called cytokine release syndrome (CRS) is characterized by the uncontrolled release of cytokines (such as interleukin-6, tumour necrosis factor or interferon gamma). CD3 or CD28 (super-)agonists may serve as an example. In severe cases, a "cytokine storm" (hypercytokinaemia) with potentially fatal consequences might be induced (*46*).

Currently available tests for prediction of the potential of an rDNA-derived biotherapeutic with immunomodulatory properties to induce a CRS could, for example, include on a case-by-case basis whole blood assays, peripheral blood mononuclear cell (PBMC)-based assays and biomimetic cell models (*47*).

B.4.8.3 Tissue cross-reactivity studies

Tissue cross-reactivity (TCR) studies are in vitro tissue-binding assays employing immunohistochemical (IHC) techniques that are conducted to characterize the binding of monoclonal antibodies and related antibody-like products to antigenic determinants in tissues. Other technologies can be employed in place of IHC techniques to demonstrate distribution to the target/binding site.

A TCR study with a panel of human tissues is a recommended component of the safety assessment package supporting initial clinical dosing

of these products – see, for example, references (*48*, *49*). However, in some cases the clinical candidate is not a good IHC reagent and a TCR study may not be technically feasible.

TCR studies can provide useful information to supplement knowledge of target distribution and can provide information on potential unexpected binding. Tissue binding does not as such indicate biological activity in vivo. In addition, binding to areas not typically accessible to the active substance in vivo (i.e. cytoplasm) is generally not therapeutically relevant. Findings should be evaluated and interpreted in the context of the overall pharmacology and safety assessment data package. When there is unexpected binding (i.e. cross-reactivity) to human tissues, a TCR evaluation of selected tissues for the animal species chosen for the nonclinical toxicity studies can provide supplementary information on potential correlations or the lack thereof, with preclinical toxicity. TCR using a full panel of animal tissues is not recommended.

When a bi-specific antibody product is to be evaluated in a TCR study using a panel of human tissues, there is no need to study the individual binding components. Evaluating the tissue binding of homologous products does not provide additional value when TCR studies have been conducted with the clinical candidate in a human tissue panel, and is not recommended. TCR studies are not expected to detect subtle changes in critical quality attributes. Therefore TCR studies are not recommended for assessing the comparability of the test article as a result of process changes over the course of a development programme.

B.4.8.4 Impurities

Safety concerns may arise as a result of the presence of impurities or contaminants. There are potential risks associated with host-cell contaminants, whether derived from bacteria, yeast, insect, plant or mammalian cells. The presence of cellular host contaminants can result in allergic reactions and other immunopathological effects. The adverse effects associated with nucleic acid contaminants are theoretical but include potential integration into the host genome (*5*). For products derived from insect, plant and mammalian cells, or transgenic plants and animals, there may be an additional risk of viral infections. However, it is preferable to rely on manufacturing and quality control processes to deal with these issues (Part A) rather than to establish a preclinical testing programme for their qualification.

Part C. Clinical evaluation

C.1 Good clinical practice

All clinical trials should be conducted under the principles described in the WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (*50*).

C.2 Clinical pharmacology (Phase I)

C.2.1 Initial safety and tolerability studies

Initial safety and tolerability studies are the first-in-human studies of drugs after the completion of essential nonclinical studies (*35–39*, *51*). The safety of clinical study participants is the paramount consideration when proceeding to first-in-human studies. Decisions on strategies for the development of a new medicine and the experimental approaches used to assemble information relevant to the safety of first-in-human studies must be science-based and ethically acceptable. Such studies should be closely monitored and are generally conducted with small numbers of subjects who may be healthy volunteers or patients. However, products that are designed to bind a target or receptor present only in patients are normally studied in the intended target population. Study protocols should define stopping rules for individual subjects, for cohorts and for the trial itself. Initial safety and tolerability studies are designed to detect common adverse reactions, the tolerated dose range and the potential drug effect. The ultimate goal of the studies is to obtain adequate safety and pharmacokinetic data to permit the design of sufficiently valid phase II studies.

Initial safety and tolerability studies should preferably be randomized, placebo-controlled studies but may also be single-arm studies with no comparator; they may range from single-dose studies to studies involving multiple doses and lasting for an extended period of time. Drug doses usually start at low levels, and study participants are monitored very carefully as the dose is escalated. In some settings, and depending on the study protocol, individual participants receive only one dose (see sections C.2.3 and C.2.4).

From a clinical perspective, rDNA-derived biotherapeutics present particular challenges compared with chemically derived small molecule drugs, and special safety issues should be addressed in the initial safety and tolerability studies, as follows:

- Currently, the nonclinical data are not completely predictive of safety in humans. In particular, since rDNA-derived biotherapeutics typically contain non-host proteins and polysaccharides, nonclinical studies are usually not predictive for immunogenicity (i.e. a test species may not react to an rDNA-derived biotherapeutic, which could cause serious adverse reactions in humans, or a test species may react when humans do not).
- Data from healthy volunteers may also not be fully predictive of safety/efficacy in patients, especially in the case of monoclonal antibodies which exhibit a target-mediated effect.

Predicting the potential for severe ADRs for first-in-human use of an investigational medicinal product, involves the identification of risk factors,

which may be related to: (a) the mode of action; (b) the nature of the target; and/or (c) the relevance of animal models. High-risk biological substances (e.g. TGN1412, an anti-CD28 superagonist which caused an acute cytokine storm in humans that was not predicted from animal studies) require extended safety measures, which may include strict sequential inclusion of trial participants with clear stopping rules and extremely careful calculation of the first dose in man (*51*).

The toxicity of most rDNA-derived biotherapeutics is related to their targeted mechanism of action; therefore, relatively high doses can elicit adverse effects which are apparent as exaggerated pharmacology. A rationale should be provided for dose selection, taking into account the characteristics of the dose–response relationship in non-human (in vitro and/or in vivo) PK/PD studies in a relevant animal model. PK-PD approaches (e.g. simple exposure–response relationships or more complex modelling and simulation approaches) can assist in high-dose selection. Where in vivo/ex vivo PD end-points are not available, the high-dose selection can be based on PK data and on available in vitro binding and/or pharmacology data.

C.2.2 Pharmacogenomics

Pharmacogenomic studies performed early during drug development can provide useful information for the design of robust phase III trials – such as identifying receptor, genetic or phenotypic characteristics and drug response in populations; using biomarkers to identify dose response in individuals; and identifying patients with genetic polymorphisms whose drug dosages should be adjusted for improved safety and/or efficacy or for whom a particular treatment should not be used (*52*, *53*). However, pharmacogenomic effects are not commonly seen with rDNA-derived biotherapeutics. The most recent guidance documents on this topic from appropriate regulatory agencies should be consulted.

C.2.3 Pharmacokinetics

The PK profile is an essential part of the basic description of a medicinal product and should always be investigated. PK studies should be performed for the intended dose range and routes of administration (*4*). In general, the PKs (absorption, distribution and elimination) of rDNA-derived biotherapeutics should be characterized during single-dose and steady-state conditions in relevant populations. However, historically, the PK evaluation of peptide or protein products has suffered from limitations in the assay methodology, thus limiting the usefulness of such studies. Immunoassays and bioassays are most frequently used for assaying therapeutic proteins in biological matrices. Special emphasis should, therefore, be given to the analytical method selected and its capability to detect and follow the time course of the protein (the parent

molecule and/or degradation and/or metabolic products) in a complex biological matrix that contains many other proteins. The method should be optimized for satisfactory specificity, sensitivity and a range of quantification with adequate accuracy and precision (*4*).

The choice of the study population as well as the choice of single-dose and/or multiple-dose studies should be justified (*4*). If part of the PK information is gathered in healthy volunteers, the validity of extrapolation of that information to the target population needs to be addressed (*54*). A prospective plan for defining the dosing schedule on the basis of observed/calculated PK parameters should be developed and should be included in the PK study protocol (*55*). It should be kept in mind that changes in the manufacturing process may alter the quality attributes, thereby potentially altering the PK profiles of rDNA-derived biotherapeutics. In such cases a comparison of the pre-change and post-change products is indicated and it may be necessary to repeat PK studies with the post-change product.

C.2.3.1 Absorption

Most biological products are administered parenterally through intravenous, subcutaneous or intramuscular administration. Alternative routes proposed for delivery of proteins may be considered (e.g. nasal and pulmonary administration) which bypass the interstitial subcutaneous or intramuscular environment. Oral delivery of proteins for systemic effects is still rare due to low bioavailability (*54*).

Unless the intravenous route is used exclusively, appropriate in vivo studies should be conducted in healthy volunteers or patients to describe the absorption characteristics of the rDNA-derived biotherapeutics – i.e. the rate and extent of absorption. Single-dose studies are generally sufficient to characterize absorption and to compare different administration routes (*56*). It should be noted that the rate of absorption following intramuscular or subcutaneous administration may vary according to the site and depth of the injection, and the concentration and volume of the solution injected, and may also be influenced by patient-specific factors (*54*, *56*). These factors which have an influence on the PK/PD parameters should be identified, described and controlled for through established methodologies as far as is possible in order to allow for a better interpretation of the observed outcomes.

Protein therapeutics administered by the subcutaneous route exhibit limited transport into blood capillaries and enter the systemic circulation indirectly through the lymphatics. Passage through the lymphatic system usually results in presystemic elimination, and consequently a bioavailability of less than 100% is obtained. In addition, small proteins may undergo proteolytic degradation in tissues as a first-pass mechanism (*54*). Since proteases can be affected by disease states and are reported to be upregulated with disease progression, consideration should be given to patient-specific circumstances (*56*).

C.2.3.2 Distribution

Tissue distribution studies should be undertaken unless otherwise justified. The volume of distribution of a drug is determined largely by its physicochemical properties (e.g. charge, lipophilicity) and its dependency on active transport processes. Because most rDNA-derived biotherapeutics are large in size, their volume of distribution is usually small and is limited to the volume of the extracellular space due to their limited mobility resulting from impaired passage through biomembranes. Site-specific and target-oriented receptor-mediated tissue uptake and binding to intravascular and extravascular proteins, however, can substantially increase the volume of distribution of rDNA-derived biotherapeutics (*57*).

The binding capacity to plasma proteins (albumin, α-acid glycoprotein) should be studied when considered relevant (*57*).

PK calculations of steady-state volume of distribution may be problematic for some rDNA-derived biotherapeutics. Noncompartmental determination using statistical moment theory assumes first-order disposition processes with elimination occurring from the rapidly equilibrating or central compartment. This basic assumption, however, is not fulfilled for numerous recombinant peptide and protein products, as proteolysis in peripheral tissues may constitute a substantial fraction of the overall elimination process for such rDNA-derived biotherapeutics (*57*). There is an inverse correlation between the steady-state volume of distribution and molecular weights, and a similar relationship is also seen between permeability and molecular weight. Unlike in the case of small-molecule chemical drugs, distribution to tissues (i.e. cellular uptake) is often part of the elimination process and not part of the distribution process as such, thus contributing to the small distribution volumes. Thus, a small steady-state volume of distribution should not necessarily be interpreted as indicating low tissue penetration, and adequate concentrations may be reached in a single target organ due to receptor-mediated uptake (*54*).

C.2.3.3 Elimination

The main elimination pathway, including the major organs of elimination, should be identified. Radiolabelled proteins can be used for this purpose (*57*). However, for therapeutic proteins, the main elimination pathway in vivo can be predicted to a large extent by the molecular size; consequently, specific studies may not be necessary.

Breakdown products may have different PK profiles when compared with the parent rDNA-derived biotherapeutics. However, in cases where measurement of separate active peptide fragments is not technically feasible, the PKs of the active moiety could be determined (*54*).

Catabolism of small proteins and peptides (molecular weight (MW) < 50 000 Da) appears to occur mainly in the kidneys. The liver may also play a major role in the metabolism of peptides and proteins, mediated by substance-specific enzymes such as for insulin, glucagon, epidermal growth factor, antibodies, and tissue plasminogen activators (*57*). If biliary excretion of peptides and proteins occurs, it generally results in subsequent breakdown and metabolism of these compounds in the gastrointestinal tract (*57*).

Catabolism of proteins usually occurs by proteolysis via the same catabolic pathways as for endogenous or dietary proteins. Proteolytic enzymes such as proteases and peptidases are ubiquitously available throughout the body. Thus, locations of intensive peptide and protein metabolism also include blood and various body tissues (*57*).

If elimination of the protein is largely dependent on target receptor uptake, differences in receptor density between healthy volunteers and target populations, such as over expression of receptors in tumours or inflamed tissues, can create important pharmacokinetic differences in half-life. These differences should be considered when using healthy volunteer data for predictions to the target population (*54*). After subcutaneous administration of proteins with relatively rapid elimination, the rate of absorption can be slower than the rate of elimination, leading to longer apparent half-lives (flip-flop kinetics) and prolonged exposure when compared to intravenous administration. As a consequence, dosing frequency may have to be reduced (*58*).

C.2.3.4 Subpopulations

The clinical development programme should involve studies to support the approval in subpopulations such as patients with organ dysfunction. Whether such studies are necessary depends on the elimination characteristics of the compound. If no study is conducted, this should be justified by the applicant. An understanding of the influence of intrinsic factors, such as age and body weight, should be provided. Such information might arise from dedicated studies in the respective population or from population PK analyses of phase II/III data (54).

C.2.3.4.1 *Renal impairment*

For proteins with MW lower than 50 000 Da, renal excretion is important for elimination (increasing in importance with lower MW) and consequently for the half-life of the protein. Thus, for these products, PK studies in patients with renal impairment are recommended. It is also conceivable that renal impairment itself may affect functioning of other organs and tissues (e.g. by up- or down-regulation of enzymes or receptors), thereby influencing the PKs and/or PDs of the experimental compound. This should be taken into account in the planning of the clinical pharmacology programme (*54*).

C.2.3.4.2 *Hepatic impairment*

Reduced hepatic function may decrease the elimination of a protein for which hepatic degradation is an important elimination pathway. Where relevant, PK studies in patients with different degrees of hepatic impairment are recommended (*54*).

C.2.3.5 Interaction studies

Therapeutic proteins may influence the pharmacokinetics of conventional drugs metabolized by cytochrome P450 enzymes (CYPs) even if the proteins are not metabolized by CYPs (*59*). Therefore it is important that drug interaction studies are also conducted with therapeutic proteins, unless sufficient evidence is provided from published data or sufficient scientific rationale is provided on the basis of biological plausibility. Additionally, since elimination of proteins may involve capacity-limited steps such as receptor-binding, the inhibition or induction of receptors may have an impact on pharmacokinetics. However, there is currently a lack of knowledge about suitable tools to explore such interactions.

C.2.3.5.1 *Dose-dependency and time-dependency*

The dose–concentration relationship may be nonproportional, depending on the relative impact of capacity-limited barriers on distribution and elimination of the product. The dose proportionality should be evaluated in single-dose or multiple-dose studies and the clinical consequences should be discussed. Time-dependent changes in PK parameters may occur during multiple-dose treatment (e.g. due to down- or up-regulation of receptors responsible for (part of) the elimination of the rDNA-derived biotherapeutics or due to formation of anti-drug/product antibodies). Using appropriate methods, soluble receptors may be measured before and during treatment, differentiating between free and bound receptors. The effect on the PKs should be evaluated and the clinical relevance discussed (*39*).

It is recommended that PKs should be determined at several dose levels on several occasions during long-term studies. Population PK analysis of data from long-term trials could be considered (*54*).

C.2.3.6 Pharmacokinetic data analysis

As in the case of small-molecule products, the pharmacokinetics may be analysed using compartment or noncompartment methods. The choice of the PK model used to derive PK parameters should be justified. Mean (or median) and individual results should always be included in a licensure submission. The inter-subject variability should be estimated and, if possible, the important sources of the variability (e.g. demographic factors such as weight and age) should be identified. Potential additional sources of inter-subject variability specific to

therapeutic proteins are the formation of antibodies, absorption variability (e.g. differences in site of injection), variable levels of binding components in blood, variability in target burden (e.g. tumour load), and variability in degradation rate (e.g. of de-PEGylation) or in degradation pattern. Based on the results, individualized dosing should be considered if necessary from safety and/or efficacy perspectives. For products intended for multiple-dose administration, the variability within an individual should also be quantified, since knowledge about the variability between occasions is especially valuable for products for which titration is recommended. Population PK analysis of phase II/III data using a sparse sample approach is recommended for characterizing the PKs, the variability of the PK parameters and possible covariate relationships (*54*). Population analyses may thus support the individualization of doses.

C.2.4 Pharmacodynamics

In many cases, PD parameters are investigated in the context of combined PK/PD studies. Such studies may provide useful information on the relationship between dose/exposure and effect, particularly if performed at different dose levels. PD markers should be selected according to their clinical relevance.

Studies in relevant animal models, if available, provide important information on the PD properties of a biological medicinal product and may guide PD studies in humans. If no animal model is available, a suitable human population must be chosen. In any case, relevant PD effects should always be confirmed in human subjects, either in patients with the disease that is being targeted by the biological medicinal product or in healthy volunteers when the mechanism of action/receptor(s) is the same as in patients. Human PD studies are usually carried out during phase I or phase II studies. Phase II studies can also be called proof-of-concept clinical studies and are important for the subsequent development of the product by helping to determine the dose to be used in further confirmatory trials, and by providing some level of confidence that the biotherapeutic is pharmacologically active and can do what it is intended to do.

C.2.5 Pharmacokinetic/pharmacodynamic relationship

The relationship between drug concentration and PD response (PK/PD relationship) should be evaluated as part of drug development. If feasible, markers for both efficacy and safety should be measured, preferably in the same study. It should be noted that PK and PD for a biological medicinal product may not necessarily be entirely and fully correlated (e.g. ceiling effect due to saturation of target receptors) and both may be altered by modifications to the molecule, binding to blood components, or formation of anti-drug/product antibodies. Early preclinical and clinical data can be evaluated using appropriate models for a mechanistic understanding of the disease and the PK/PD relationship. PK/PD models may be developed to account for the time delay between plasma

concentrations and measured effect. Models may also need to take into account the presence or absence of the therapeutic target (e.g. presence of antigen in the case of anticancer monoclonal antibodies). PK/PD models may allow extrapolation from volunteers to the target population if suitable assumptions have been made (e.g. regarding the influence of disease-related factors). These models may provide guidance for dose selection and are helpful when interpreting changes in the PKs in important subpopulations or when evaluating comparability in the context of a change in the manufacturing process. Efforts to explore relevant biomarkers and their link (surrogacy) to safety and efficacy end-points are encouraged (*54*).

C.2.6 Modifications of pharmacokinetic and pharmacodynamic profiles of therapeutic proteins

Many protein drugs display suboptimal therapeutic efficacies due to their inherent poor molecular stability, low systemic bioavailability and, as a consequence of their innate susceptibility to various clearance mechanisms, short circulatory lifetimes. Higher protein concentrations and increased dosing frequencies are therefore often employed to achieve favourable therapeutic responses. Approaches to improve these factors, and thus in vivo efficacy, include targeted mutations, the generation of fusion proteins and conjugates, glycosylation engineering, and PEGylation (*60*).

Glycosylation may influence a variety of physiological processes at both the cellular level (e.g. intracellular targeting) and the protein level (e.g. protein–protein binding, protein molecular stability, plasma persistence lifetimes). Since the glycosylation pattern of a biological medicinal product may be influenced even by subtle changes in the manufacturing process, the potential effects on PK and PD profiles need to be considered when evaluating comparability of pre-change and post-change product in the context of a change in the manufacturing process. PEGylation increases the size of a protein, which prolongs its half-life by reducing renal clearance. PEGylation can also provide water solubility to hydrophobic drugs and proteins.

C.3 Efficacy

C.3.1 Phase II

Phase II studies provide the first test of efficacy in patients with the disease targeted by the rDNA-derived biotherapeutics. The studies aim to determine the correct dosage, identify common short-term side-effects and determine the best regimen to be used in pivotal clinical trials.

Conventionally, the first step (frequently called phase IIa) is focused on an initial proof of concept. This step aims to demonstrate that the rDNA-derived biotherapeutics interacts correctly with its molecular target and, in turn, alters the disease or its symptoms. Subsequent trials (frequently called phase IIb trials)

are larger and may use placebo, and/or active comparator agents and a broader dosage range to obtain a much more robust proof of concept and additional guidance on dose selection.

For initial proof of concept, single-arm trials may be used with their results interpreted relative to historical control subjects. However, this design could introduce bias since, for example, current study participants may be different from historical control subjects in ways that affect the outcome of interest or because changes in supportive care may limit the validity of the conclusions. Therefore, comparative randomized phase II trials are generally preferred.

Phase II trials usually explore a variety of possible end-points (e.g. time-to-event end-points, change in a continuous end-point of tumour size) and provide opportunities for biomarker discovery. A variety of study designs can be used, including the randomized parallel-group design, randomized discontinuation design, single-stage and two-stage designs, delayed-start design and adaptive (Bayesian) designs. In all cases, clear decision rules should be in place.

Standard study designs for assessing dose–response have been described (*61*), such as randomized parallel dose–response studies. However, the approaches to selecting the optimal dose may differ for rDNA-derived biotherapeutics compared to small chemical molecules. For example, biological agents developed in oncology are usually cytostatic and their maximal activities may occur at doses lower than their maximum tolerated doses.

Combination therapy is an important treatment modality in many disease settings such as cancer. Increased understanding of the pathophysiological processes that underlie complex diseases has provided further impetus for therapeutic approaches using combinations of (new) products directed at multiple therapeutic targets to improve treatment response, minimize development of resistance or improve tolerability. This requires the use of flexible designs and new modelling approaches for the design of clinical trials.

As observed for small-molecule chemical drugs, rDNA-derived biotherapeutics may affect cardiac electrical activity either directly or indirectly. The amount and type of electrocardiogram data considered appropriate should be individualized according to the type of product and the nonclinical findings regarding its cardiotoxic potential. A thorough QT/QTc (TQT) study (*62*), or a study that incorporates many of the key components of a TQT study, should be considered (*62*). However, this may not be necessary if electrocardiogram data are collected in at least a subset of patients during clinical development and reviewed by respective experts, preferably in a blinded manner.

C.3.2 Confirmatory phase III

Phase III clinical trials are designed to evaluate the benefit of the rDNA-derived biotherapeutics in a carefully selected patient population with the disease.

These trials are carried out to confirm efficacy at the chosen dose(s) and dosing regimen(s), to further evaluate safety and monitor side-effects, and sometimes to compare the candidate product to commonly used treatments. Confirmatory phase III clinical trials should be adequately sized and powered to meet the primary objectives.

Confirmatory trials should be prospective randomized trials comparing the test agent against placebo (in addition to the best supportive care) or an active comparator, usually the best available evidence-based current standard. If no such comparator is available (e.g. in patients who have failed several lines of therapies), the comparator may be the investigator's best choice. Ideally, trials should be double-blinded, where neither the patient nor the investigator knows the nature of the product received by the patient. Blinding or masking is intended to limit the occurrence of conscious or unconscious bias in the conduct and interpretation of a clinical trial (*63*).

The design of the trials depends on the hypothesis to be tested – superiority to placebo or active comparator, or equivalence or non-inferiority to an active comparator (*64*).

The choice of end-points depends on the therapeutic indication; there should be sufficient evidence that the primary end-point can provide a valid and reliable measure of clinically relevant and important treatment benefit in the targeted patient population. If a single primary variable cannot be selected, a composite end-point integrating or combining multiple measurements into a single variable, using a predefined algorithm, can also be used. Such validated end points are commonly used in inflammatory diseases (e.g. ACR20 in rheumatoid arthritis, ASAS20 in ankylosing spondylitis, CDAI in Crohn disease, PASI in psoriasis) or in oncology (disease progression, disease-free survival, or overall survival). Patient-reported outcomes and quality-of-life scales are also important end-points and may already be included in some of these composite end-points.

When direct assessment of the clinical benefit to the patient is not practical, a surrogate end-point can be considered. The strength of the evidence for surrogacy depends on: (a) the biological plausibility of the relationship; (b) the demonstration of the prognostic value of the surrogate for the clinical outcome in epidemiological studies; and (c) evidence from clinical trials that treatment effects on the surrogate correspond to effects on the clinical outcome. Most surrogate end-points are not formally validated, but such end-points can be used if they are reasonably likely to predict the desired clinical benefit (e.g. the effect on tumour size, as assessed by imaging, in patients refractory to available treatments). In some cases, particularly for rare diseases, a biomarker could be considered acceptable as the primary study end-point on the basis of biological plausibility and mechanism of action of the product.

Specific decisions about the size of the study group will depend on factors such as the magnitude of the effect of interest, characteristics of the study population, and study design (see section C.4).

Preferably, two confirmatory trials should be performed in order to show that the results can be replicated. However, one controlled study with statistically compelling and clinically relevant results may be sufficient, especially with regard to life-threatening conditions or rare disorders. If the biological medicinal product shows promising efficacy for a serious or life-threatening condition where no other treatment option exists, licensing based on a limited amount of data may be possible with further confirmatory efficacy data being provided post-marketing. Because most rare diseases have a more homogeneous genetic pattern than common diseases and because they are often characterized by similar or identical genetic or epigenetic defects, patients with these diseases could be expected to have a more uniform therapeutic response. This should reduce the size of phase III studies required to demonstrate efficacy. The use of historical controls (or possibly no controls) may also be justified if the rare disease has a defined course in the absence of treatment that will permit comparisons with the results for the investigational rDNA-derived biotherapeutics.

C.3.3 Biomarkers for patient selection

Biomarkers have the potential to enhance the benefit–risk profile of rDNA-derived biotherapeutics by enabling the selection of patients who are more likely to respond, especially with molecules that target serum or cell markers. In such a case, the treatment may benefit only a subset of patients defined by the biomarker (e.g. those with tumours overexpressing HER-2 or negative for KRAS mutations). The biomarker evaluation process should consist of the following three steps: (a) analytical validation; (b) qualification (i.e. assessment of available evidence on associations between the biomarker and disease states, including data showing effects of interventions on both the biomarker and clinical outcomes); and (c) utilization (i.e. contextual analysis based on the specific use proposed and the applicability of available evidence to this use) (*64–66*). In principle, biomarker qualification should occur prior to its use as the inclusion and exclusion criteria for patient selections in confirmatory phase III trials. However, these trials can also be used for qualification or identification of other (new) biomarkers.

C.3.4 Manufacturing and formulation changes

While manufacturing and formulation changes may be expected during product development, the phase III trials should be conducted with the test rDNA-derived biotherapeutics manufactured according to the final manufacturing (commercial) process. If this is not the case, a comparability exercise between the clinical and commercial products is necessary to ensure that the change would

not have an adverse impact on the clinical performance of the product (*32*, *33*). This comparability exercise should normally follow a stepwise approach, starting with a comparison of quality attributes of the active substance and relevant intermediates. A comparability exercise should not be limited to release testing but should include more extensive characterization, using a range of suitable analytical methods as appropriate to the product and process changes in question (see section A.9). If differences are detected that may have an impact on the clinical properties of the product, nonclinical and/or clinical bridging studies, such as PK/PD studies and possibly immunogenicity studies, will generally be needed.

C.3.5 Special populations

As in any clinical development programme, studies in special populations would be expected where relevant to the indications (e.g. in the elderly and in paediatric patients). The elderly population is arbitrarily defined as those patients aged 65 years or older. However, patients 75 years and above should also be considered to the extent possible (*67*). Recommended age categories for the paediatric population include preterm and term newborn infants, infants to toddlers, children, and adolescents (*68*).

Some rDNA-derived biotherapeutics that may be of particular importance to elderly patients are those developed for cancer, Parkinson's disease, Alzheimer's disease, coronary heart disease and diabetes mellitus. It is important to determine whether the PK profile of an rDNA-derived biotherapeutic is different in elderly as compared to younger subjects since impairment of organ function such as renal or hepatic function is more frequent in an aged population. The elderly subpopulation should also be represented sufficiently in the clinical trials to permit the comparison of treatment effects, dose response and safety between older and younger patients. Where the disease to be treated is characteristically associated with ageing, it is expected that elderly patients will constitute the major portion of the clinical database (*67*).

The extent of the studies needed in children depends on the possibility of extrapolation from adults and children of other age groups. Some rDNA-derived biotherapeutics may be used in children from the early stages of drug development, especially those targeting genetic diseases where manifestations occur early in life. Evaluation should be made in the appropriate age group and it is usually recommended to begin with older children before extending the trial to younger children and then infants (*68*). Where justified, extrapolation of efficacy data from adult to paediatric patients may be based on PK and/or PD data (e.g. when a similar effect can be expected with similar exposure). However, safety data usually cannot be extrapolated and need to be generated in children (see section C.5).

C.3.6 Post-marketing: Phase IV

Phase IV trials may be required to evaluate further an approved rDNA-derived biotherapeutic and to obtain more information about safety or effectiveness, or both, especially if the biotherapeutic has been approved on the basis of a surrogate end-point.

C.4 Statistical considerations

C.4.1 General considerations

The application of sound statistical principles to the design, conduct, analysis and interpretation of clinical trials should be considered an important and integral component of the overall development of an rDNA-derived biotherapeutic. The success of a trial depends on the appropriateness of the study design, the conduct of the trial and the analysis of trial results. Statistical principles are relevant to all three aspects of the clinical trial. In general, details on these aspects should be specified in the trial protocol which should be written and finalized prior to the start of the trial. Any subsequent amendments to the protocol should be clearly justified, should be documented in a formal amendment to the protocol, and should include the statistical consequences of the proposed changes.

The scientific integrity of the trial and the credibility of the data from the trial depend substantially on the trial design (*69*). The study protocol should include a clear description of the specific design selected for a particular trial. Additional details regarding the primary end-point, which is directly related to the primary objective of the trial, should also be included. If multiple primary end-points are defined, the criteria for achieving study success should be clearly laid out in order to avoid potential problems with the interpretation of the trial results. The protocol should also clearly define secondary end-points, and their role in the interpretation of the trial results should be stated. Details on measures that have been put in place to avoid or minimize bias in the trial (e.g. randomization and blinding) should also be provided.

With regard to the type of hypothesis to be tested in a specific trial, it should be clear in the protocol whether the trial is designed to show superiority, non-inferiority, or equivalence. The statistical issues involved in the design, conduct, analysis and interpretation of equivalence and non-inferiority trials are complex and subtle, and they require that all aspects of these trials are carefully evaluated. Sample size and power are important for the success of a clinical trial and should be given careful consideration at the trial design stage. In determining sample size, the specific hypothesis being tested should be taken into consideration.

It is important to ensure that the protocol will provide good quality data that permit an adequate evaluation of the efficacy (and safety) of the product

under development. In addition, if formal interim analyses are planned, then the details governing such analyses should be pre-specified in the protocol.

In an era when it is recognized that improvements in the drug development process are needed in order to increase the likelihood of trial success, decrease costs and increase the efficiency with which efficacious and safe medicines are brought to market, adaptive clinical trial designs are increasingly considered as one tool through which these improvements can be achieved. Adaptive design refers to a clinical study design that uses the accumulation of data as a basis for modifying aspects of the study as it continues, without undermining the validity and integrity of the trial (*70*, *71*). A key statistical issue for adaptive designs is the preservation of the Type I error rate. The methods used to control the Type I error rate properly should be described in the study protocol, with additional details provided in the Statistical Analysis Plan (SAP).

Details regarding the statistical methodology to be applied to the clinical trial should be provided in the protocol, with the more technical details being captured in the SAP. The SAP should be prepared and finalized prior to unblinding the clinical study. Any amendments to the SAP must also be finalized prior to unblinding.

C.4.2 Specific considerations for rDNA-derived biotherapeutics

Since rDNA-derived biotherapeutics are often indicated to treat severe and/or life-threatening diseases and chronic diseases, trials for rDNA-derived biotherapeutics present unique statistical challenges.

C.4.2.1 Trials in small populations and single-arm studies

Some rDNA-derived biotherapeutics are intended for the treatment of rare diseases for which the target population is very small. Consequently, trials that are considered confirmatory for rare disease indications are often based on a limited number of subjects. While such studies must still be designed with the rigour of traditional trials, and should be conducted with high quality in order to provide reliable and valid data for assessing efficacy and safety, some flexibility is needed with regard to the statistical methods that will be utilized in these trials. Single-arm studies with comparisons made to an external control can sometimes be justified.

C.4.2.2 Tumour-based end-points in oncology trials and composite end-points

In confirmatory oncology trials for rDNA-derived biotherapeutics, the use of tumour-based end-points such as disease-free survival and progression-free survival as the primary end-point is not uncommon (*72*). The use of a tumour-based end-point as the primary end-point creates several statistical challenges, and considerations for the collection and analysis of such end-points have been

discussed – see, for example, reference (*73*). Clinical trials may involve the use of a composite primary end-point arising from the combination of multiple clinical measurements or outcomes (e.g. major adverse cardiac events (MACE), which is the most commonly used composite end-point in cardiovascular studies). For such a composite end-point, it is important that the individual components are analysed separately (usually as secondary end-points) in order to ensure that the treatment effect is shown across all components and is of similar magnitude.

C.4.2.3 Missing data

Missing data is a common problem in long-term trials of rDNA-derived biotherapeutics targeting chronic diseases such as diabetes and rheumatoid arthritis, although it is usually not a problem in short-term trials. The impact of missing data on the validity of trial results should be carefully assessed using sensitivity analyses with appropriate underlying assumptions.

C.5 Safety

Pre-licensing safety data should be obtained in a sufficient number of patients in order to characterize and quantify the safety profile – including type, frequency and severity of ADRs – of the rDNA-derived biotherapeutics. The safety evaluation should cover a reasonable duration of time, taking into account the intended duration of use of the drug, so as to assess potential changes in the ADR profile over time and to capture delayed ADRs.

For drugs intended for long-term treatment of non-life-threatening conditions, a 12-month exposure of at least 100 patients to the investigational medicinal product at the intended clinical dosage should be considered (*74*). When no serious ADR is observed in a 1-year exposure period, this number of patients can provide reasonable assurance that the true cumulative 1-year incidence is no greater than 3%. This estimate is based on the statistical "rule of three" which states that if no major ADR occurred in a group of n people, there can be 95% confidence that the chance of a major ADR is less than one in n/3 (or equivalently, less than 3 in n). This estimate is considered a good approximation for n > 30.

The safety database may need to be larger or may require longer patient observation if a safety signal is identified, if the drug is expected to cause late-developing ADRs, or if ADRs increase in severity or frequency over time. Concerns requiring a larger safety database may arise from nonclinical or early clinical data, or from experience with other products of the same or related pharmacological class. A smaller safety database may be acceptable if the intended treatment population is small. Safety data should be obtained from prospective, and preferably controlled, studies including a placebo or active comparator arm since comparison with an external control group (e.g. with

published data) is usually hampered by differences in the investigated patient population, concomitant therapy, observation period and/or reporting. Causality assessment – i.e. whether the observed adverse event is causally related to the investigational drug – is usually easiest in placebo-controlled studies. Generally accepted definitions and terminology, as well as procedures, are important for harmonizing the way to gather and, if necessary, to take action on important clinical safety information arising during clinical development (*75*). The term "adverse event" describes any untoward medical occurrence developing with administration of a pharmaceutical product irrespective of a causal relationship. The term "adverse drug reaction", on the other hand, should be used only for adverse events that have at least a reasonably possible causal relationship to the pharmaceutical agent.

Standardized reporting is important for the transmission of pre- or post-marketing safety information – for example, between the reporting source or pharmaceutical industry and regulatory authorities, or between regulatory authorities and the WHO Collaborating Centre for International Drug Monitoring (*76*). Data elements to be included in individual case safety reports should comprise all important information on the primary source, date, sender and receiver of the information, the type, seriousness, duration and outcome of the adverse event or ADR, detailed patient characteristics and drug information, actions taken with the drug (e.g. dose reduction, discontinuation), and an assessment of the degree of suspected relatedness of the drug to the adverse event (*76*).

To facilitate international sharing of regulatory safety information for medical products used by humans, specific MedDRA terminology has been developed. This is a rich and highly specific standardized medical terminology for accurate and consistent safety information that allows for the aggregation of reported terms in medically meaningful groupings (*77*). Products covered by the scope of MedDRA include pharmaceuticals, vaccines and drug device combination products.

Since safety data obtained from pre-marketing clinical trials can be expected to detect mainly common and shorter-term ADRs, further monitoring of clinical safety of the biological product to detect rare but sometimes serious adverse effects and an ongoing benefit–risk evaluation are necessary in the post-marketing phase (see section C.7).

C.5.1 Special populations

C.5.1.1 Elderly population

The safety of rDNA-derived biotherapeutics should be investigated in elderly patients during clinical drug development (*67*, *78*), except where there is no intention to use these biotherapeutics in this age group. Elderly patients are

more prone to adverse effects since they often have comorbidities and are taking concomitant medication that could interact with the investigational drug. The adverse effects can be more severe, or less tolerated, and may have more serious consequences than in the non-elderly population. Depending on the mechanism of action of the drug and/or the characteristics of the disease, specific effects on cognitive function, balance and falls, urinary incontinence or retention, weight loss and sarcopenia should be investigated.

Elderly patients may be included in the main phase III or phase II/III studies, or in separate studies. Inclusion of younger and elderly patients in the same studies has the advantage of allowing direct comparisons using data collected in similar ways. Certain assessments, however, such as studies of cognitive function, require special planning and can be best accomplished in separate studies.

Where enrolment of elderly patients has been insufficient despite the efforts of the applicant, a specific plan to collect post-marketing data should be presented in the marketing application.

C.5.1.2 Paediatric population

Data on the safety of medicinal products in the pediatric population should be generated unless their use is clearly inappropriate (*68*). During clinical development, the timing of paediatric studies will depend on the medicinal product, the type of disease being treated, safety considerations, and the efficacy and safety of alternative treatments. Justification for the timing and approach to the clinical programme needs to be clearly addressed with the regulatory authorities.

Medicinal products may affect physical and cognitive growth and development, and the adverse event profile may differ in paediatric compared to adult patients. In addition, adverse effects may not be seen immediately but may become apparent only at a later stage of development. Long-term studies, or surveillance data while patients are on chronic therapy and/or during the post-therapy period, may be needed to determine possible effects on skeletal, behavioural, cognitive, sexual and immune maturation and development.

C.6 Immunogenicity

rDNA-derived biotherapeutics may induce unwanted humoral and/or cellular immune responses in recipients. Immunogenicity of rDNA-derived biotherapeutics should therefore always be investigated prior to authorization (*79*). Since animal data are usually not predictive of the immune response in humans, immunogenicity needs to be investigated in the target population. Although in-silico modelling may help in identifying T-cell epitopes related to immunogenicity (i.e. T-helper epitopes), it does not predict whether immunogenicity will occur. The frequency and type of product antibodies induced against the active substance, impurity

or excipient, as well as possible clinical consequences of the immune response, should be thoroughly assessed.

The immune response against a biotherapeutic is influenced by many factors – such as the nature of the drug substance, product- and process-related impurities (e.g. host-cell proteins, aggregates), excipients and stability of the product, the route of administration (subcutaneous administration is usually more immunogenic than intravenous administration), the dosing regimen (intermittent use is usually more immunogenic than continuous use), and patient-related, disease-related and/or therapy-related factors (e.g. antibody development is more likely in an immune-competent than in an immunosuppressed state and is potentially enhanced in the presence of autoimmune disease). The consequences of unwanted immunogenicity on safety may vary considerably, ranging from clinically irrelevant to serious and life-threatening (e.g. serious infusion/anaphylactic) reactions. Neutralizing antibodies may directly alter the PD effect of a product (i.e. by blocking the active site of the protein), leading to reduction or loss of efficacy. Binding antibodies often affect pharmacokinetics and may indirectly influence pharmacodynamics. Thus, an altered effect of the product over time due to anti-drug antibody formation might be a composite of pharmacokinetic, PD and safety effects.

The proposed antibody testing strategy – including the selection, assessment, and characterization of assays, the identification of appropriate sampling time points (including baseline samples), sample storage and processing, and selection of statistical methods for analysis of data – should be appropriately justified (*79*). The studies to be considered for immunogenicity testing (e.g. short-term and/or long-term clinical trials or even single-dose studies) and the sampling time points depend on the expected appearance of antibodies and the clinical consequences of such antibodies. For example, some rDNA-derived biotherapeutics are highly immunogenic and may elicit an immune response after the first dose, others may require prolonged or intermittent exposure to mount an immune response, and some may have a very low immunogenic potential. Anti-product antibody screening and subsequent characterization for confirmation, titre, neutralizing activity, isotype, subclass, etc. should be determined early as the sponsor performs an immunogenicity risk assessment, mitigation and management strategy. The assessment should consider the immunogenic factors listed above and the potential clinical consequences if antibodies develop.

Antibody assays (screening, confirmation, and neutralizing) should be validated for their intended purpose. Validation studies need to establish appropriately linear responses to relevant analytes as well as appropriate accuracy, precision, sensitivity, specificity and robustness of the assay(s) (*79–81*). Possible interference of the circulating antigen with the antibody assay(s) should be taken

into account. A highly sensitive screening assay should be used for antibody detection and a confirmatory assay should be used to confirm the presence of antibodies and eliminate false-positive results. To achieve confirmation of specificity, it is necessary to include an assay which evaluates specificity. A neutralization assay should be available for further characterization of antibodies. The determination of the phase of clinical testing at which the need for characterization (e.g. neutralizing, isotype, subclass, etc.) of detected anti-drug antibodies is warranted, is commensurate with the potential safety risk to patients, and may be based on knowledge and experience with the substance class.

If the rDNA-derived biotherapeutic is a monoclonal antibody (mAb), the development of assays to detect antibodies against this mAb can be technically challenging (*80*). Many standard assay formats involve the use of anti-immunoglobulin reagents such as antibodies against immunoglobulins, protein A or protein G, but these are inappropriate for use in detecting antibodies against mAbs as they often bind to the product itself. Different assay approaches have been developed to overcome this problem, such as the bridging enzyme-linked immunosorbent assay (ELISA) format or surface plasmon resonance (SPR) procedures which do not require anti-immunoglobulin reagents but may be less sensitive than other immunoassay methods (*2*).

Detected antibodies should be further characterized with regard to antibody content (concentration/titre) and possibly, depending on case-by-case considerations, other criteria such as antibody class and subclass (isotype), affinity and specificity. For example, the isotype of the antibodies could be determined if this may be predictive of safety (such as the development of IgE antibodies causing allergic and anaphylactic responses). Potential clinical implications of detected antibodies regarding safety, efficacy and pharmacokinetics should always be evaluated. Special attention should be paid to the possibility that the immune response seriously affects the endogenous protein and its unique biological function (e.g. neutralizing anti-erythropoietin antibodies cross-reacting with endogenous erythropoietin and causing pure red cell aplasia).

The required observation/monitoring period for immunogenicity testing will depend on the intended duration of therapy and the expected time of antibody development, if known, and should be justified. In the case of chronic administration, 1-year data will usually be appropriate prior to licensing to assess antibody incidence and possible clinical implications. If considered clinically relevant, development of antibody titres, their persistence over time, potential changes in the character of the antibody response and the possible clinical implications should be assessed pre- and post-marketing.

Since pre-licensing immunogenicity data are often limited, further characterization of the immunogenicity profile may be necessary post-marketing,

particularly if rare but clinically meaningful, or even serious, antibody-related ADRs have been encountered with biological agents of the same or related substance class that are not likely to be detected in the pre-marketing phase.

C.7 Pharmacovigilance and risk management planning

NRAs should be vigilant to ensure that the health of the public is protected. The aim is to ensure that the risks associated with rDNA-derived biotherapeutics are actively minimized. Patient safety is a key concern for all medicinal products that are on the market, and rDNA-derived biotherapeutics are no exception. Due to the specific characteristics of rDNA-derived biotherapeutics already discussed in these Guidelines, pharmacovigilance activities required for rDNA-derived biotherapeutics may differ in some respects from those required for small-molecule drugs. For example, biotherapeutic use may lead to antibody formation with consequences for clinical efficacy and/or safety.

A risk management plan should be submitted and agreed to by the NRA. The key components of a risk management plan may include:

- safety specifications, which summarize the known and potential safety issues and missing information about the rDNA-derived biotherapeutic;
- a pharmacovigilance plan to further evaluate important known or potential safety concerns and to provide post-marketing data where relevant information is missing;
- a risk minimization plan, which provides proposals on how to minimize any identified or potential safety risk.

In the risk management plan, the known or potential risks may be described with pharmacovigilance, and risk minimization activities may be proposed to identify, characterize, prevent or minimize risks related to the use of the rDNA-derived biotherapeutics, to assess the effectiveness of those interventions, and to communicate those risks to both patients and health-care providers.

Pharmacovigilance and risk minimization activities that might be included in a risk management plan usually fall into two categories: (a) routine activities, which would generally be conducted for any medicine where no special safety concerns have arisen; and (b) additional activities designed to address identified and potential safety concerns that could have an impact on the benefit–risk balance of a product. Routine pharmacovigilance activities would include the monitoring and reporting of spontaneous adverse events post-approval and any safety evaluations incorporated in clinical trials that may be initiated by the marketing authorization holder following marketing authorization for a wide variety of reasons. In case there are relevant safety issues,

NRAs may request additional pharmacovigilance activities in the form of active surveillance (e.g. registries), epidemiology studies, further clinical studies, and drug utilization studies. Routine risk minimization activities would ensure that suitable contraindications and warnings are included in the product information and that this information is updated on an ongoing basis. A risk minimization plan can further specify other risk minimization activities, as appropriate, which could include: (a) specific educational material about the product and its use; (b) patient-oriented or physician-oriented training programmes; (c) restricted use of the product; and (d) registration programmes for patients, physicians and/or pharmacists.

Once on the market, manufacturers should monitor the effectiveness of their risk minimization plans and revise them if new safety and effectiveness concerns are identified. Changes in the manufacturing processes introduced post-marketing could also influence the safety profile (e.g. by enhancing immunogenicity) of rDNA-derived biotherapeutics and may necessitate enhanced safety monitoring.

In case a relevant or even serious potentially drug-related adverse event occurs, it is important to be able to identify the specific biological causing this event. Therefore, all ADR reports should carry information unique to the product, including the proprietary (brand) name, the INN, the identification code (if there is one), and the lot information of the respective biological to help trace an ADR to a specific product and ascertain any relation to causality.

A risk management plan will not reduce the scientific and clinical standards or the data requirements for the market authorization of rDNA-derived biotherapeutics, nor will it replace the precautionary approach that is taken to managing the risks associated with those products. On the contrary, implementation of a risk management plan will further strengthen the rigour of post-marketing surveillance, allowing for earlier identification of risks associated with rDNA-derived biotherapeutics and earlier interventions to minimize those risks.

C.8 Additional guidance

Further guidance on various aspects of clinical trials is available from several other bodies such as the ICH, the EMA and the United States Food and Drug Administration, as well as from several other NRAs. These WHO Guidelines are not intended to conflict with, but rather to complement, these other documents with respect to medicinal products prepared by rDNA technology. Relevant sections of this part may be useful with regard to products intended for clinical trials; however, the amount and extent of data submitted for a product will be limited and should take into account the nature of the product and its stage of development.

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The preliminary draft of these Guidelines was prepared by the following WHO Drafting Group members after a meeting held at the National Institute for Biological Standards and Control, England on 19–20 March 2012: Dr M-C. Bielsky, Medicines and Healthcare Products Regulatory Agency, England; Dr E. Griffiths, Consultant, England; Dr H-K. Heim, Federal Institute for Drugs and Medical Devices, Germany; Dr H-N. Kang, World Health Organization, Switzerland; Dr R. Thorpe, National Institute for Biological Standards and Control, England; Dr M. Wadhwa, National Institute for Biological Standards and Control, England; Dr M. Weise, Federal Institute for Drugs and Medical Devices, Germany.

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Commission, China; Dr N. Hassannia, Biological Office Food and Drug Organization, Islamic Republic of Iran; Dr K. Ho, Agence nationale de sécurité du médicament et des produits de santé, France; Dr S. Hufton, National Institute for Biological Standards and Control, England; Mrs W. Jariyapan, Ministry of Public Health, Thailand; Mr R. Jian, Health Sciences Authority, Singapore; Dr J. Joung, Korea Food and Drug Administration, Republic of Korea; Dr H-K. Heim, Federal Institute for Drugs and Medical Devices, Germany; Dr H-N. Kang, World Health Organization, Switzerland; Dr Y. Kishioka (*Japanese Pharmacopoeia representative*), Pharmaceutical and Medical Devices Agency, Japan; Dr I. Knezevic, World Health Organization, Switzerland; Mr J. Leong, Health Sciences Authority, Singapore; Dr J. Li, Shanghai CP-Guojian Pharmaceutical Co. Ltd., China; Dr J. Luo, State Food and Drug Administration, China; Mrs V. Madrigal, Recepta Biopharma, Brazil; Dr C. Njue, Health Canada, Canada; Mrs Y. Hechavarria Nunez, Centro para el Control Estatal de la Calidad de los Medicamentos, Cuba; Dr P.H. Pan (*DCVMN representative*), Innovax Biotech Co. Ltd., China; Dr S. Pluschkell (*IFPMA representative*), Pfizer Inc., USA; Professor C. Rao, National Institutes for Food and Drug Control, China; Dr M. Schiestl (*International Generic Pharmaceutical Alliance representative*), Sandoz GmbH, Austria; Dr T. Schreitmueller (*IFPMA representative*), F. Hoffmann-La Roche Ltd., Switzerland; Dr S. Shani, Ministry of Health and Social Welfare, India; Dr X. Shen, China Bio-Tech Group, China; Dr L. Sun, Xiamen Amoytop Biotech Co. Ltd., China; Dr R. Thorpe, National Institute for Biological Standards and Control, England; Mrs C. Ulm (*European Generic Medicines Association representative*), Mylan GmbH, Switzerland; Dr A. Vallin, Centre of Molecular Immunology, Cuba; Dr J. Wang, Health Canada, Canada; Dr J. Wang, National Institutes for Food and Drug Control, China; Dr M. Weise, Federal Institute for Drugs and Medical Devices, Germany; Dr M. Xu, National Institutes for Food and Drug Control, China.

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The draft Guidelines were then posted on the WHO website for the first round of public consultation from 20 March to 19 April 2013.

The document WHO/BS/2013.2213 was prepared by Dr M-C. Bielsky, Medicines and Healthcare Products Regulatory Agency, England; Dr E. Griffiths, Consultant, England; Dr H-K. Heim, Federal Institute for Drugs and Medical Devices, Germany; Dr K. Ho, Agence nationale de sécurité du médicament et des produits de santé, France; Dr J. Joung, Ministry of Food and Drug Safety (formerly Korea Food and Drug Administration), Republic of Korea; Dr H-N. Kang, World Health Organization, Switzerland; Dr A. Klein, Health Canada, Canada; Dr I. Knezevic, World Health Organization, Switzerland; Dr F. Lentz, Federal Institute for Drugs and Medical Devices, Germany; Dr R. Thorpe, National Institute for Biological Standards and Control, England; Dr M. Wadhwa, National Institute for Biological Standards and Control, England; Dr M. Weise, Federal Institute for Drugs and Medical Devices, Germany, taking into account comments received from the following reviewers: Ms P. Agsiri, Ministry of Public Health, Thailand; Mrs J. Bernat on behalf of the IFPMA, Switzerland (reviewed by Dr C. Phillips, Eli Lilly and Company, and Dr S. Ramanan, Amgen); Dr B. Brake, Federal Institute for Drugs and Medical Devices, Germany; Mrs J. Dahlan, National Agency of Drug and Food Control, Indonesia; Ms C. Dubeaux on behalf of GlaxoSmithKline, Belgium (reviewed by Dr C. Lecomte, Dr F. Mortiaux and Dr C. Saillez); Dr T. Go, Health Sciences Authority, Singapore; Dr S. Jadhav (*DCVMN representative*), Serum Institute of India, India; Dr Y. Kishioka, Pharmaceutical and Medical Devices Agency, Japan; Dr B. Lan (*DCVMN representative*), China National Biotec Group, China; Dr T. Morris, United States Pharmacopoeial Convention, USA; Dr C. Njue, Health Canada, Canada; Dr G. Raychaudhuri on behalf of the United States Food and Drug Administration Center for Biologics Evaluation and Research (reviewed by Dr I. Mahmood, Dr C. Kimchi-Sarfaty and Dr M. Serabian); Dr Y. Ren, Chinese Pharmacopoeia, China; Dr I. Shin on behalf of the Ministry of Food and Drug Safety, Republic of Korea (reviewed by Mr D. Baek, Dr J. Joung, Mrs Y. Kim and Mr O. Kwon); Dr S. Sontakke, Health Canada, Canada; Dr J. Southern (*Developing Country Vaccine Regulators' Network representative*), South Africa; Dr R. Thorpe, National Institute for Biological Standards and Control,

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The document WHO/BS/2013.2213 was then posted on the WHO website for the second round of public consultation from 1 July to 20 September 2013, and comments were received from the following reviewers: Mrs J. Bernat and Dr A. Womack on behalf of the IFPMA, Switzerland; Mrs M. Bevzyuk on behalf of Apotex Inc., Canada; Dr G. Castillero, Dirección Nacional de Farmacia y Drogas, Panama; Dr C. Dubeaux on behalf of GlaxoSmithKline, Belguim (reviewed by Dr C. Lecomte, Dr F. Mortiaux, Dr C. Saillez and Dr M. Duchêne); Dr P. Duffy, National Institutes of Health, USA; Dr K. Heidenreich, Novatis, Switzerland; Dr Y. Huang, China Food and Drug Administration, China; Dr S. Jadhav, Serum Institute of India, India; Dr Y. Kishioka, Pharmaceutical and Medical Devices Agency, Japan; Mrs D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Mrs D. Lucía Mesa Lautero on behalf of the Instituto Nacional de Vigilancia de Medicamentos y Alimentos, Colombia (reviewed by Mr A. Alarcón, Mr D. Duarte, Mrs A. Fula, Mrs J. García, Mrs F. Gil, Mrs L. Hidalgo and Mrs C. Niño); Dr R. Mody, Lupin Ltd., India; Dr T. Morris, United States Pharmacopeial Convention, USA; Dr J. Petricciani, Consultant, USA; Dr G. Raychaudhuri on behalf of the United States Food and Drug Administration Center for Biologics Evaluation and Research (reviewed by Dr I. Mahmood and Dr C. Kimchi-Sarfaty); Dr J. Schletter on behalf of the European Biosimilars Group (a sector group of the European Generic Medicines Association), Belgium; Dr G. Unger, Paul-Ehrlich-Institut, Germany; Dr K. Zoon, National Institutes of Health, USA.

Acknowledgement is also given to Dr M. Schiestl, Sandoz GmbH, Austria and to Dr T. Schreitmueller, F. Hoffmann-La Roche Ltd., Switzerland for providing expertise on the section on drug product container closure system and delivery devices.

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Appendix 1

Manufacturing process validation

- Process validation is the documented evidence that the process, operated within established parameters, can perform effectively and reproducibly to produce a drug product, drug substance or intermediate that meets its predetermined specifications and quality attributes.
- Process validation should include the collection and evaluation of data throughout production in order to establish scientific evidence that a process is capable of consistently delivering a quality drug substance. It generally includes collection of data on an appropriate number of production batches. The number of batches can depend on several factors that include, but are not limited to: (a) the complexity of the process being validated; (b) the level of process variability; and (c) the amount of experimental data and/or process knowledge available on the specific process.
- Process conditions (e.g. column loading capacity, column regeneration and sanitization, height) should be appropriately evaluated. Columns should also be evaluated throughout their expected lifespan with regard to their purification ability (e.g. impurity clearance, collection of intended variants), leaching of ligands (e.g. dye, affinity ligand) and/or chromatographic material (e.g. resin). Process validation activities should normally include the evaluation of resin lifetime, including maximum cycles and/or maximum time duration, using small-scale studies to ensure proper performance and integrity of the columns. In addition, the results should normally be verified at full scale through the life-cycle of the product. These studies should also confirm the suitability of the column cleaning, storage and regeneration procedures.
- Where hold times are applied to intermediates (e.g. harvest, column eluate), the impact of hold times and hold conditions on the product quality (e.g. degradation) should be appropriately evaluated.
- Evaluation of selected steps (e.g. steps for which high impurity or viral clearance are claimed) operating in worst-case and/or challenging conditions (e.g. maximum hold times, spiking challenge) could be performed to demonstrate the robustness of the process. Depending on the relevance of the experimental model

with regard to the final process (e.g. scale, materials, equipment, operating conditions), these studies could be leveraged in support of process validation and/or quality control data requirements.

- The information provided in the dossier in support of process validation usually contains both commercial-scale process validation studies and small-scale studies. Process validation batches should be representative of the commercial process, taking into account the batch definition as detailed in the process description.
- Process changes at the level of fermentation and/or purification during progression to full-scale commercial production may have considerable consequences for the quality of the product, the yield and/or quantitative and qualitative differences in impurities. Consequently the contribution of data from small-scale studies to the overall validation package will depend on demonstration that the small-scale model is an appropriate representation of the proposed commercial scale. Data demonstrating that the model is scalable and representative of the proposed commercial process should be provided. Successful demonstration of the suitability of the small-scale model can enable manufacturers to propose process validation with reduced dependence on testing of commercial-scale batches. Data derived from commercial-scale batches should confirm results obtained from small-scale studies used to generate data in support of process validation. Scientific rationale or reference to guidelines can be an appropriate justification to conduct certain studies (e.g. viral removal) only at small scale.
- In order to demonstrate viral safety of purification processes used to manufacture drug substance for clinical trials, in-house data from previous validation studies may be used. If in-house experience with highly robust and well understood process steps is available, it may be justified to reduce the product-specific validation effort (*1*).

Reference

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Appendix 2

Characterization of rDNA-derived biotherapeutics

This appendix provides details of suggested approaches that can be applied to the characterization of an rDNA-derived biotherapeutic. It also provides examples of technical approaches which may be considered for structural characterization and confirmation, and for evaluation of physicochemical and biological properties of the desired product, drug substance and/or drug product. The methods should provide an understanding of the product with a sufficient level of detail (e.g. complete primary structure, properties for the higher order structure, qualitative and quantitative analysis of product-related substances and product- and process-related impurities, assessment of biological functions).

A subset of the methods described in this appendix can be used for routine batch release testing. Others are subject to extended characterization of the desired product during product and process development and are also often used to support process evaluation/validation and/or comparability studies (e.g. after making significant process changes). The selection of release testing methods depends on the overall design of quality control for which release testing is only one element among others. For example, if a certain quality attribute can be controlled by in-process tests, parametric controls and/or demonstrated manufacturing process capability (e.g. high impurity clearance), that attribute may not need to be tested routinely on every batch.

1. Physicochemical characterization

1.1 Primary structure

The primary structure – i.e. amino acid sequence, including the disulfide linkages – of the desired product can be determined as far as possible using combined approaches such as those described in items (a) and (b) below and then compared with the sequence of the amino acids deduced from the gene sequence of the desired product. Attention should be paid to the possible presence of N-terminal methionine (e.g. in *Escherichia coli*-derived products), signal or leader sequences, other possible N-terminal and C-terminal modifications (such as acetylation, amidation or partial degradation by exopeptidases), and any heterogeneity (e.g. C-terminal processing, N-terminal pyroglutamation, deamidation, oxidation, isomerization, fragmentation, disulfide bond mismatch, N-linked and O-linked oligosaccharide, glycation, aggregation). The variability of N-terminal and C-terminal amino acid sequences should be analysed (e.g. C-terminal lysine(s)).

Free sulphydryl groups and disulfide bridges should be determined. Disulfide bridge integrity and mismatch should be analysed. Experimentally

determined disulfide bonding patterns should be compared to the predicted structure based on the class of the molecule.

(a) Peptide map – selective fragmentation of the product into discrete peptides is performed by using suitable enzymes or chemicals. The resulting peptide fragments are analysed by high-performance liquid chromatography (HPLC) or other appropriate analytical procedures. The peptide fragments should be identified as far as possible using appropriate techniques such as mass spectrometry (MS) methods (e.g. electrospray ionization MS, matrix-assisted laser-desorption ionization time-of-flight MS). The use of MS/MS coupling should also be considered as it could reveal more detailed sequence information about the analysed peptide fragment. If one fragmentation method does not deliver the complete amino acid sequence, the use of an orthogonal enzyme or chemical cleavage method can increase the sequence coverage. The correct formation of the disulfide bridges may be characterized by the use of peptide mapping under reducing and non-reducing conditions.

(b) Molecular weight determination by mass spectrometry – the molecular weight of the intact molecule, as determined by MS, serves as an additional confirmation of the primary structure. For smaller peptides, MS/MS sequencing can provide the complete amino acid sequence. MS can be performed under reduced and non-reduced conditions and under deglycosylated and intact conditions for multi-subunit and glycosylated protein molecules such as monoclonal antibodies.

1.2 Glycan structure

Glycosylation should be identified and adequately characterized. The glycan content (neutral sugars, amino sugars and sialic acids) should be determined if it is linked to clearance or activity. In addition, the structure of the glycan chains, the glycan pattern (antennary profile native glycan profile and site-specific glycan analysis), and the glycosylation site(s) of the polypeptide chain are analysed as far as possible. This task can be achieved by the combination of enzymatic or chemical hydrolytic cleavage with a variety of separation methods (HPLC, electrophoresis) and detection/identification methods (MS including MS/MS, ultraviolet, fluorescence detection, electrochemical detection). The quantitative oligosaccharide analysis (chemical or enzymatic cleavage followed by HPLC) provides additional useful qualitative and quantitative information on the glycan structure.

Measurement of the quantitative charge patterns of the intact glycoprotein, such as by measuring the charge-based isoforms using an appropriate method (e.g. capillary electrophoresis, isoelectric focusing), may be useful as an overall

measure of the degree of sialylation and antennary profile. Particular attention should be paid to glycan structures that may be associated with adverse effects, such as non-human structures or residues. Further tests to be conducted include analysis of charge heterogeneity.

1.3 Higher-order structure

Higher-order structure should be characterized by appropriate physicochemical methodologies and confirmed by biological function. The analysis of PEGylated proteins should include, though should not be limited to, the average rate of modification, the location of modification and the analysis of site occupancy.

The complete assessment of the three-dimensional chemical structure in the context of product characterization is rarely achieved because absolute methods such as X-ray crystallography or nuclear magnetic resonance (NMR) with isotope-labelled amino acids deliver only an approximation to the structure of the product of interest. They measure the product either in a nonrelevant state or require a separate production of the isotope-labelled sample. However, the use of applicable but relative orthogonal methods as described below enables the determination and characterization of discrete folding and the assessment of changes in the higher-order structure (e.g. in the case of comparability studies).

The higher-order structure of the product should be examined using appropriate procedures such as circular dichroism, Fourier transform infrared spectroscopy (FT-IR), fluorescence, differential scanning calorimetry, proton nuclear magnetic resonance (1H-NMR) and/or other suitable techniques such as hydrogen-deuterium exchange MS. FT-IR and CD in the far ultraviolet range deliver information on the secondary structure, whereas CD in the near ultraviolet reflects to some extent the tertiary and quaternary structure. When using these methods, their capabilities and limitations need to be considered (e.g. impact of protein concentration).

In vitro or in vivo assays that illustrate the functional activity of the therapeutic may also serve as additional confirmation of the higher-order structure in addition to demonstrating biological function.

2. Biological activity

Assessment of the biological properties of a product constitutes an essential step in establishing a complete characterization profile. The biological activity describes the specific ability or capacity of a product to achieve a defined biological effect. Description of a relevant biological assay to measure the biological activity should be provided by the manufacturer.

The biological activity should be assessed by in vitro, in vivo, biochemical (including immunochemical assays) and/or physicochemical assays as appropriate.

For antibody products, where effector function may play a role in the mechanism of action and/or have an impact on the product safety and efficacy, a detailed analysis of biological activity demonstrating the mechanism of action (e.g. antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, apoptosis), ability for complement-binding and activation, and other effector functions – including Fc gamma receptor-binding activity and neonatal Fc receptor-binding activity – should be provided as appropriate.

The mechanism of action should be discussed and, where relevant, the importance (or consequences) of other functions (e.g. effector functions) with regard to the safety and efficacy of the product should be included.

Potency (expressed, for example, in units or international units (IU)) is the quantitative measure of biological activity based on the attribute of the product which is linked to the relevant biological properties, whereas quantity (expressed in mass) is a physicochemical measure of product content. For assessing potency, use of bioassays that reflect the biological activity in the clinical situation is preferable but is not always possible or necessary for lot release. For example, bioassays which assess some functional aspect of the protein or mechanism of action (rather than the intended clinical effect) can also be used as the basis for a potency assay.

Examples of procedures used to measure biological activity include:

- animal-based biological assays, which measure an organism's biological response to the product;
- cell-based biological assays, which measure biochemical or physiological response at the cellular level;
- biochemical assays, which measure biological activities such as receptor- or ligand-binding, enzymatic reaction rates or biological responses induced by immunological interactions.

3. Immunochemical properties

Where relevant (e.g. for monoclonal antibody products), the immunochemical properties should be extensively characterized. Binding assays using purified antigens and defined regions of antigens should be performed, where feasible, to determine affinity, avidity and immunoreactivity (including cross-reactivity with other structurally homologous proteins).

The part of the target molecule bearing the relevant epitope should be characterized to the extent that this is possible. This should include biochemical identification of these structures (e.g. protein, oligosaccharide, glycoprotein, glycolipid) and relevant characterization studies (amino acid sequence, carbohydrate structure) as appropriate.

Since glycosylation and PEGylation may have an impact on the pharmacological properties of the product and may modulate its immunogenic properties, appropriate characterization studies should be conducted.

Unless otherwise justified, the ability for complement-binding and activation, and/or other effector functions, should be evaluated even if the intended biological activity does not require such functions.

4. Purity, impurity and contaminant

Biotechnological products commonly display several sources of heterogeneity (e.g. C-terminal processing, N-terminal pyroglutamation, deamidation, oxidation, isomerization, fragmentation, disulfide bond mismatch, N-linked and O-linked oligosaccharide, glycation, aggregation), which lead to a complex purity/impurity profile comprising several molecular entities or variants. This purity/impurity profile should be assessed by a combination of methods, and individual and/or collective acceptance criteria should be established for relevant product-related substances and impurities. These methods generally include the determination of physicochemical properties such as molecular weight or size, isoform pattern, determination of hydrophobicity, electrophoretic profiles, chromatographic data including peptide mapping and spectroscopic profiles including mass spectroscopy. Multimers and aggregates should also be appropriately characterized using a combination of methods. Unless otherwise justified, the formation of aggregates and subvisible and visible particulates in the drug product is important and should be investigated and closely monitored at the time of release and during stability studies.

Impurities may be either process-related or product-related. These materials should be characterized as far as is possible and their impact on biological activity should be evaluated if appropriate.

Potential process-related impurities (e.g. host-cell protein, host-cell DNA, cell culture residues, downstream processing residues) should be identified and evaluated qualitatively and/or quantitatively, as appropriate.

Contaminants, which include all adventitiously introduced materials not intended to be part of the manufacturing process (e.g. microbial species, endotoxins) should be strictly avoided and/or suitably controlled. Where non-endotoxin pro-inflammatory contaminants, such as peptidoglycan, are suspected, the use of additional testing should be considered.

4.1 Process-related impurities and contaminants

Process-related impurities are derived from the manufacturing process itself and can be classified in three major categories: (a) cell substrate-derived; (b) cell culture-derived; and (c) downstream-derived. Contaminants, on the other hand,

are unwanted materials, such as adventitious viruses, that are introduced by unintentional means into the manufacturing process.

(a) Cell substrate-derived impurities – include, but are not limited to, proteins derived from the host organism, and nucleic acid (host-cell genomic, vector, or total DNA). For host-cell proteins, a sensitive assay (e.g. immunoassay) capable of detecting a wide range of protein impurities is generally utilized. In the case of an immunoassay, polyclonal antibodies used in the test are typically generated by the immunization of animals with an appropriate preparation derived from the production cell minus the product-coding gene, which have been cultured in conditions representative of the intended culture and appropriately collected (e.g. filtered harvest, partial purification).

The level of DNA from the host cells can be detected by direct analysis on the product (e.g. qPCR, immunoenzymatic techniques). Clearance studies, which could include spiking experiments conducted at small scale, to demonstrate the removal of cell substrate-derived impurities such as nucleic acids and host-cell proteins, may sometimes be used to eliminate the need for establishing acceptance criteria for these impurities.

(b) Cell culture-derived impurities – include, but are not limited to, inducers, antibiotics, serum and other media components. These impurities need to be tested and evaluated on a case-by-case basis using a risk-assessment and risk-management approach. In the case of a potential impact on the safety of the product, the removal of such impurities to acceptably low levels during downstream purification may need to be validated or end-product testing and specification limits established.

(c) Downstream-derived impurities – include, but are not limited to, enzymes, chemical and biochemical processing reagents (e.g. guanidine, dyes, oxidizing and reducing agents), inorganic salts (e.g. heavy metals, non-metallic ions), solvents, carriers, ligands (e.g. protein A) and other leachables. As for cell culture-derived impurities, these impurities should be evaluated on a case-by-case basis using a risk-assessment and risk-management approach. Where appropriate, development of analytical methods for these impurities and validation of their removal could be considered.

4.2 Product-related substances and impurities, including degradation products

Molecular variants of the desired product may need considerable effort in isolation and characterization in order to identify the type of modification(s). When the activity of these variants is comparable to the desired product, the variants should be included in the product purity profile. Degradation products arising in significant amounts during manufacture and/or storage

should be appropriately considered. The most frequently encountered molecular variants of the desired product, and relevant technology for their assessment, are listed below.

(a) Truncated forms – hydrolytic enzymes or chemicals may catalyse the cleavage of peptide bonds. This may lead to terminal heterogeneity (e.g. for C-terminal Lys in monoclonal antibodies). These may be detected by HPLC and/or electrophoretic methods and verified by mass spectrometry. Peptide mapping may also be useful, depending on the property of the variant.

(b) Amino acid modifications – individual amino acid modification may include deamidation (Asp/Gln to Asp, Glu), oxidation (e.g. Met to Met-sulfoxide), spontaneous formation of pyroglutamate out of N-terminal Glu or Gln residues, glycation of Lys residues and others. These forms may be detected and characterized by relevant analytical methods (e.g. HPLC, capillary electrophoresis, mass spectrometry). In some cases peptide mapping is important to clearly identify and localize the site and nature of the amino acid modification.

(c) High molecular weight species and particles – high molecular weight species (HMWS) includes dimers and higher oligomers of the desired product. Particles include intrinsic visible particles of the desired product. HMWS are generally resolved from the desired product and product-related substances, and are quantitated by appropriate separation procedures (e.g. size exclusion chromatography, field flow fractionation, analytical ultracentrifugation) coupled with sensitive detection methods (e.g. ultraviolet, fluorescence, light scattering). Using orthogonal methods and/or procedures with overlapping analytical windows (e.g. light obscuration testing, micro-flow imaging for testing of subvisible particles) can greatly enhance the characterization of aggregates and particles. Foreign particles are not intended to be part of the product and should be minimized.

5. Quantity

Quantity should be determined by use of an appropriate physicochemical and/or immunochemical assay. The protein content (expressed in mass units) can be determined by measuring the sample against an appropriate reference standard using a suitable method (e.g. HPLC). The protein content can also be measured in an absolute way – such as by ultraviolet photometry using an extinction coefficient (e.g. at 280 nm). If the deviation is too large, redetermination by another method can be considered.

Appendix 3

Routine control of rDNA-derived biotherapeutics

This appendix discusses approaches to routine control of an rDNA-derived biotherapeutic.

1. Specification

A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. A specification establishes the set of criteria to which a drug substance and drug product – or materials at other stages of the manufacture – should conform in order to be considered acceptable for its intended use. “Conformance to specification” means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. The justification of specification should take into account relevant development data and data from nonclinical, clinical and stability studies. The setting of acceptance ranges should also take into account the sensitivity of the analytical method used.

The selection of tests to be included in the specifications is product-specific and should take into account the quality attributes (e.g. potential influence on safety, efficacy or stability), the process performance (e.g. clearance capability, content), the controls in place through the manufacturing process (e.g. multiple testing points), and the material used in relevant nonclinical and clinical studies. These tests could include criteria such as potency, the nature and quantity of product-related substances, product-related impurities, process-related impurities, and absence of contaminants. Such attributes can be assessed by multiple analytical procedures, each yielding different results. Since specifications are chosen to confirm quality rather than to characterize the product, the rationale and justification for including and/or excluding testing for specific quality attributes should be provided.

The rationale used to establish the acceptable range of acceptance criteria should be described. Acceptance criteria should be established and justified on the basis of data obtained from lots used in nonclinical and/or clinical studies. Nevertheless, where appropriately justified, data from lots used for stability studies, or relevant development data, could support limits beyond ranges used in clinical studies.

2. Identity

The identity test(s) should be highly specific and should be based on unique aspects of the product's molecular structure and/or other specific properties (e.g. peptide map, anti-idiotype immunoassay, or other appropriate method). Depending on the product, more than one test (physicochemical, biological and/or immunochemical) may be necessary to establish identity, and such test(s) should possess sufficient specificity to discriminate other products that may be manufactured in the same facility.

3. Purity and impurities

As noted in the characterization section, recombinant proteins may display a complex purity/impurity profile that should be assessed by a combination of orthogonal methods, and for which individual and/or collective acceptance criteria should be established for relevant product-related variants. Chromatographic and/or electrophoretic methods capable of detecting product truncation, dissociation and aggregation should be included, and quantitative limits should be proposed for these, as appropriate. In addition, as appropriate, such control could further confirm the consistency of the product.

The control of relevant process-related impurities should be included in the plan for quality control. Control of process-related impurities (e.g. protein A, host-cell protein, DNA, and other potential culture or purification residues) is typically part of the drug substance specification, as appropriate. In some situations, and where appropriately demonstrated, their control may be performed on an intermediate product at an appropriate process step. Routine testing may not be necessary for some impurities for which the process has been demonstrated to achieve high reduction levels.

4. Potency

Potency is the quantitative measure of biological activity based on the attribute of the product which is linked to the relevant biological properties. A relevant potency assay should be part of the specifications for drug substance and/or drug product, and should reflect the presumed mechanism of action whenever possible. Specific activity (units of biological activity per mg of product) is of considerable value in demonstrating consistency of production.

The potency of each batch of the drug substance and the final dosage form should be established using, wherever possible, an appropriate national or international reference material – see, for example, section A.1.3 – which is normally calibrated in units of biological activity such as IU. In the absence of

such preparations, an approved in-house reference preparation may be used for assay standardization.

For biological substances with antagonist activity, it may be appropriate to calibrate the potency assay using the standard/reference preparation for the agonist and to express activity of the antagonist in terms of inhibition of biological activity – i.e. units of the agonist. For example, for tumour necrosis factor (TNF) antagonists, bioassays can be calibrated using the international standard for TNF-α and activity expressed as the number of IUs of TNF neutralized by the amount of the antagonist.

5. Quantity

The quantity of the drug substance and drug product, usually based on protein content, should be determined using an appropriate assay.

6. General tests

General tests should be performed in accordance with relevant monographs, which could include appearance (e.g. form, colour), solubility, pH, osmolality, extractable volume, sterility, bacterial endotoxins, stabilizer and water, and visible and subvisible particulate, as appropriate.

Appendix 4

Product-/indication-specific guidance in nonclinical evaluation (examples)

1. Anticancer rDNA-derived biotherapeutics

For anticancer rDNA-derived biotherapeutics, nonclinical evaluations are intended to identify the pharmacological properties, establish a safe initial dose level for the first human exposure and understand the toxicological profile (e.g. identification of the target organ, estimation of the safety margin and reversibility). In the development of anticancer drugs, most clinical studies involve cancer patients whose disease condition is often progressive and fatal. In addition, the clinical dose levels are often close to or at the adverse effect dose levels. For these reasons, the type and timing and flexibility called for in designing of nonclinical studies of anticancer pharmaceuticals can have a different pattern from those for other pharmaceuticals (*1*, *2*).

1.1 Starting dose for clinical studies

Nonclinical evaluations should identify a pharmacologically active and safe dose. For selection of the starting dose for first-in-human clinical trials, a MABEL approach should be considered (*3*). Toxicology studies to determine a NOAEL/NOEL (no observed effect level) are not considered essential to support clinical use of an anticancer medicinal product.

1.2 Study duration

For medicinal products intended for the treatment of patients with advanced cancer, nonclinical studies of 3 months' duration are usually considered sufficient to support phase III clinical studies and, in most cases, licensing.

1.3 Reproductive toxicity

With regard to reproduction toxicology, an embryo-fetal toxicity study should be available for licensing but is not considered essential to support clinical trials in patients with advanced cancer. Fertility and prenatal and postnatal toxicological studies are in general not warranted to support clinical trials or licensing for rDNA-derived biotherapeutics intended for the treatment of patients with advanced cancer (*2*).

2. Monoclonal antibodies

For monoclonal antibodies, the immunological properties of the antibody should be described in detail, including its antigenic specificity, complement-binding, and any unintentional reactivity and/or cytotoxicity towards human tissues distinct from the intended target. For monoclonal antibodies and other related antibody products directed at foreign targets (i.e. bacterial, viral targets, etc.), a short-term (i.e. 2 weeks' duration) safety study in one species (with the choice of species justified by the sponsor) can be considered; no additional toxicity studies, including reproductive toxicity studies, are needed. When animal models of disease are used to obtain proof of principle, a safety assessment can be included to provide information on potential target-associated safety aspects. Where this is not feasible, appropriate risk mitigation strategies should be adopted for clinical trials.

2.1 Antibody-drug/toxin conjugates

Species selection for an antibody-drug/toxin conjugate (ADC) incorporating a novel toxin/toxicant should follow the same general principles as an unconjugated antibody. If two species have been used to assess the safety of the ADC, an additional short-term study or an arm in a short-term study should be conducted in at least one species with the unconjugated toxin. In these cases a rodent is preferred unless the toxin is not active in the rodent. If only one pharmacologically relevant species is available, then the ADC should be tested in this species. A novel toxicant calls for an approach to species selection similar to that used for a new chemical entity on a case-by-case approach – see, for example, reference (*2*) below. For toxins or toxicants which are not novel and for which a sufficient body of scientific information is available, separate evaluation of the unconjugated toxin is not warranted. Data should be provided to compare the metabolic stability of the ADC in animals with humans.

References

1. *Preclinical safety evaluation of biotechnology-derived pharmaceuticals*. ICH Guideline S6(R1). Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2011.
2. *Nonclinical evaluation for anticancer pharmaceuticals*. ICH Guideline S9. Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2009.
3. *Guideline on strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products*. London, European Medicines Agency, 2007 (EMEA/CHMP/SWP/ 28367/07).

Appendix 5

Animal species/model selection

1. Species selection

The biological activity, together with species- and/or tissue-specificity, of many rDNA-derived biotherapeutics often precludes standard pharmacological/toxicity testing designs in commonly used species (e.g. rats and dogs). Pharmacological and safety evaluation programmes should include the use of relevant species. A relevant species is one in which the test material is pharmacologically active due to the expression of the receptor or an epitope (in the case of monoclonal antibodies). In addition to receptor expression, the cellular/tissue distribution of receptors is an important consideration in the selection of appropriate species.

A number of factors should be taken into account when determining species relevancy. Comparisons of target sequence homology between species can be an appropriate starting point, followed by in vitro assays to make qualitative and quantitative cross-species comparisons of relative target-binding affinities and receptor/ligand occupancy and kinetics. Assessments of functional activity are also recommended. Functional activity can be demonstrated in species-specific cell-based systems and/or in vivo pharmacology or toxicology studies. Modulation of a known biological response or of a PD marker can provide evidence for functional activity to support species relevance.

Consideration of species differences in target-binding and functional activity in the context of the intended dosing regimens should provide confidence that a model is capable of demonstrating potentially adverse consequences of target modulation. When the target is expressed at very low levels in typical healthy preclinical species (e.g. inflammatory cytokines or tumour antigens), binding affinity and activity in cell-based systems can be sufficient to guide species selection.

Tissue cross-reactivity in animal tissues is of limited value for species selection. However, in specific cases (i.e. where the approaches described above cannot be used to demonstrate a pharmacologically relevant species) TCR studies can be used to guide the selection of species to be used in toxicology studies by comparison of tissue-binding profiles in human and those animal tissues where target-binding is expected (see also section B.3.3). An animal species which does not express the desired epitope may still be of some relevance for assessing toxicity if comparable unintentional tissue cross-reactivity to humans is demonstrated.

When no relevant species exists, the use of relevant transgenic animals expressing the human receptor or the use of homologous proteins should be considered.

2. Number of species

Safety evaluation programmes should normally include two relevant species. However, in certain justified cases one relevant species may suffice (e.g. when only one relevant species can be identified or when the biological activity of the biotherapeutic is well understood).

In addition, even where two species may be necessary to characterize toxicity in short-term studies, it may be possible to justify the use of only one species for subsequent long-term toxicity studies. If there are two pharmacologically relevant species for the clinical candidate (one rodent and one non-rodent), both species should be used for short-term (up to 1 month's duration) general toxicology studies. If the toxicological findings of these studies are similar, or the findings are understood from the mechanism of action of the product, then longer-term general toxicity studies in one species are usually considered sufficient. The rodent species should be considered unless there is a scientific rationale for using non-rodents. Studies in two non-rodent species are not appropriate.

The use of one species for all general toxicity studies is justified when the clinical candidate is pharmacologically active in only one species. Studies in a second species with a homologous product (see below) are not considered to add further value for risk assessment and are not recommended.

2.1 Transgenic animals

When no relevant animal species exists for testing the clinical candidate, the use of a transgenic animal expressing the human target can be considered, assuming that data exist on comparable expression and distribution of the target orthologue, and on the biology of the target in the model, and that sufficient background knowledge on the strain/model (e.g. historical background data) exist.

2.2 Homologous proteins

While useful information may also be gained from the use of homologous proteins, it should be noted that the production process, range of impurities/contaminants, PK and exact pharmacological mechanism(s) may differ between the homologous form and the product intended for clinical use. Studies with homologous proteins can be used for hazard detection and for understanding the potential for adverse effects due to exaggerated pharmacology, but are generally not useful for quantitative risk assessment. Therefore, for the purposes of hazard identification it can be possible to conduct safety evaluation studies using a control group and one treatment group, provided there is a scientific justification for the study design and the dose(s) selected (e.g. maximum pharmacological dose).

2.3 Nonclinical testing in a nonrelevant species

Pharmacological/toxicity studies in nonrelevant species may be misleading and are generally discouraged. However, where it is not possible to identify a relevant species or to use transgenic animal models, or if it is not possible to use a homologous protein for testing purposes, it may still be prudent to assess some aspects of potential toxicity in a limited toxicity evaluation in a single species (e.g. a repeated dose toxicity study of < 14 days' duration that includes an evaluation of important functional end-points such as cardiovascular and respiratory end-points.

3. Animal models of disease

In recent years there has been much progress in the development of animal models that are thought to be similar to the human disease. These animal models include induced and spontaneous models of disease, gene knock-out(s) or knock-in(s), and transgenic animals. These models may provide further insight in determining the pharmacological action of the product, PK and dosimetry, and may also be useful in the determination of safety (e.g. evaluation of undesirable promotion of disease progression). In certain cases, studies performed in animal models of disease may be used as an acceptable alternative to toxicity studies in normal animals.

Animal models of disease may be useful in the definition of toxicity end-points; selection of clinical indications; and determination of appropriate formulations, route of administration and treatment regimen. It should be noted that with these models of disease there is often a paucity of historical data for use as a reference when evaluating study results. Therefore, the collection of concurrent control and baseline data is critical for optimizing study design.

The scientific justification should be provided for the use of these animal models of disease to support safety.

Appendix 6

Explanatory notes

Note 1: The species-specific profile of embryo-fetal exposure during gestation should be considered in interpreting studies. High molecular weight proteins (> 5000 D) do not cross the placenta by simple diffusion. For monoclonal antibodies with molecular weight as high as 150 000 D, there exists a specific transport mechanism – the neonatal Fc receptor – which determines fetal exposure and varies across species.

In the NHPs and humans, IgG placental transfer is low in the period of organogenesis and begins to increase in the early second trimester, reaching the highest levels late in the third trimester. Therefore, standard embryo-fetal studies in NHPs, which are dosed from early pregnancy up to gestation day 50, may not be of value in assessing direct embryo-fetal effects in the period of organogenesis, although effects on embryo-fetal development as an indirect result of maternal effects can be evaluated. Furthermore, maternal dosing in NHPs after delivery is generally without relevance since IgG is excreted in the milk only initially (i.e. in the colostrum), and not later during the lactation and nursing phase.

Rodents differ from the NHPs and humans, as IgG crosses the yolk sac in rodents by neonatal Fc receptor transport mechanisms and exposure can occur relatively earlier in gestation than with NHPs and humans. In addition, delivery of rodents occurs at a stage of development when the pups are not as mature as those of the NHP or the human neonate. Therefore, rat/mouse dams should be dosed during lactation in order to expose pups via the milk up to at least day 9 of lactation when the offspring are at an equivalent stage of development as human neonates.

Note 2: The minimum duration of postnatal follow-up should be 1 month to cover early functional testing (e.g. growth and behaviour). In general, if there is evidence for adverse effects on the immune system (or immune function) in the general toxicology studies, immune function testing in the offspring during the postpartum phase of the ePPND study is warranted. When appropriate, immunophenotyping can be obtained as early as postnatal day 28. The duration of postnatal follow-up for the assessment of immune function can be 3–6 months depending on the functional tests used.

Neurobehavioural assessment can be limited to clinical behavioural observations. Instrumental learning calls for a training period, which would result in a postnatal duration of at least 9 months and is not recommended.

Note 3: A detailed discussion of the approach to determining group sizes in cynomolgus monkey ePPND studies is available (*1*). Group sizes in ePPND studies should yield a sufficient number of infants (6–8 per group at postnatal day 7) in order to assess postnatal development and provide the opportunity for specialist evaluation if necessary (e.g. immune system).

Most ePPND studies accrue pregnant animals over weeks and months. Consideration should be given to terminating further accrual of pregnant animals into the study and adapting the study design (e.g. by caesarean section) when prenatal losses in a test item group indicate a treatment-related effect. Reuse of vehicle-control treated maternal animals is encouraged. If there is some cause for concern that the mechanism of action may lead to an effect on EFD or pregnancy loss, studies can be conducted in a limited number of animals in order to confirm the hazard.

Note 4: An example of an appropriate scientific justification would be a monoclonal antibody which binds a soluble target with a clinical dosing regimen intended to saturate target-binding. If such a saturation of target-binding can be demonstrated in the animal species selected and there is an exposure multiple of up to 10-fold the therapeutic drug levels, a single-dose level and control group would provide adequate evidence of hazard to embryo-fetal development.

Note 5: End-points to be included in an interim report of an ePPND study in NHPs are:

- Dam data – survival, clinical observations, bodyweight, gestational exposure data (if available), any specific PD end-points.
- Pregnancy data – number of pregnant animals started on study, pregnancy status at both the end of organogenesis (gestation day 50) and at gestation day 100, occurrence of abortions and timing of abortions. There is no need for ultrasound determinations of fetal size in the interim report; these are not considered essential since actual birth weight will be available.
- Pregnancy outcome data – number of live births/still births, infant birth weight, infant survival and bodyweight at day 7 postpartum, qualitative external morphological assessment (i.e. confirming appearance is within normal limits), infant exposure data (if available), any specific PD end-points in the infant if appropriate.

Reference

1. Jarvis P et al. The cynomolgous monkey as a model for developmental toxicity studies: variability of pregnancy losses, statistical power estimates, and group size considerations. *Birth Defects Research (Part B)*, 2010, 89:175–187.

Annex 5

Biological substances: WHO International Standards and Reference Panels

A list of WHO International Standards and Reference Panels for biological substances is available at: http://www.who.int/biologicals.

At its meeting in October 2013, the WHO Expert Committee on Biological Standardization made the changes shown below to the previous list.

Additions[1]

Preparation	Activity	Status
Cytokines, growth factors and biotherapeutics other than blood products		
PEGylated granulocyte colony stimulating factor	10 000 IU per ampoule	First WHO International Standard
Tumour necrosis factor alpha (human, recombinant) for use in bioassay	43 000 IU per ampoule	Third WHO International Standard
In vitro diagnostic device reagents		
Anti-hepatitis B virus e antibodies (anti-HBe) *	120 IU/ml	First WHO International Standard
Hepatitis A virus RNA for NAT-based assays	54 000 IU/ml	Second WHO International Standard
Hepatitis B virus e antigen (HBeAg) *	100 IU/ml	First WHO International Standard
Hepatitis D virus RNA for NAT-based assays *	575 000 IU/ml	First WHO International Standard
HIV-1 circulating recombinant forms RNA for NAT-based assays	Ten panel members consisting of CRFs and other variants. No unitage assigned	First WHO International Reference Panel

[1] Unless otherwise indicated, all materials are held and distributed by the National Institute for Biological Standards and Control, Potters Bar, Herts, EN6 3QG, England. Materials identified by an * in the above list are held and distributed by the Paul-Ehrlich-Institut, 63225 Langen, Germany.

Preparation	Activity	Status
Human serum immunoglobulin E	13 500 IU/ml	Third WHO International Standard
Mycoplasma DNA for NAT-based assays (for generic mycoplasma detection) *	200 000 IU/ml	First WHO International Standard
Parvovirus B19 DNA for NAT-based assays	1 410 000 IU/ml	Third WHO International Standard
Vaccines and related substances		
Inactivated poliomyelitis vaccine for D antigen assay	277 DU/ml for poliovirus type 1 65 DU/ml for poliovirus type 2 248 DU/ml for poliovirus type 3	Third WHO International Standard

Discontinuations

Anti-echinococcus serum (human); Lyophilized, 87.36 mg human serum/ampoule. First International Reference Reagent, 1975 (ECHS; 75.1106).

Anti-C complete blood-typing serum (human); Lyophilized, 100 IU/ampoule. First International Standard, 1984 (W1004; 84.1424).

Anti-E complete blood-typing serum (human); Lyophilized, 100 IU/ampoule. First International Standard, 1983 (W1005; 83.1424).