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Advisory Group of Independent Experts to review the smallpox research programme (AGIES)

Comments on the *Scientific Review of Variola Virus Research, 1999–2010*

December 2010

GLOBAL ALERT AND RESPONSE

World Health Organization

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Acronyms and abbreviations

ACVVR	WHO Advisory Committee on Variola Virus Research
AGIES	Advisory Group of Independent Experts to review the smallpox research programme
CDC	Centers for Disease Control and Prevention, United States of America
CPXV	cowpox virus
ECTV	Ectromelia/mouse virus
HIV	human immunodeficiency virus
MPXV	monkeypox virus
OPV	orthopoxvirus
PCR	polymerase chain reaction
RPXV	rabbitpox virus
SRC VB VECTOR	The Russian Federation State Research Centre of virology and Biotechnology
USFDA	United States Food and Drug Administration
VACV	vaccinia virus
VARV	variola virus
WHO	World Health Organization

Introduction

In May 2007, resolution WHA60.1 of the World Health Assembly requested the Director-General of the World Health Organization (WHO) to undertake a major review, in 2010, of the state of smallpox research and additional related research needed for public health purposes. This was undertaken so that the sixty-fourth World Health Assembly, in 2011, could come to a global public consensus on the timing of the destruction of existing variola virus (VARV) stocks.

In November 2008, the WHO Advisory Committee on Variola Virus Research (ACVVR) decided that a comprehensive review of the literature and of unpublished data concerning live variola virus research should be undertaken by a group of scientists endorsed by the Committee and representing all areas of research and development on orthopoxviruses (OPVs).

This resulted in the creation, under the supervision of the ACVVR, of a consolidated document entitled *Scientific review of variola virus research, 1999–2010* (hereinafter called 'the Scientific Review'). The Scientific Review comprised six chapters.

1. Smallpox vaccines
2. Laboratory diagnostics for smallpox (variola virus)
3. Variola genomics
4. The status of WHO Collaborating Centre repositories of variola virus and nucleic acid
5. Animal models and pathogenesis
6. Antiviral drug development for the treatment of smallpox – status of small-molecule therapeutics.

This Scientific Review has now been considered by a group of independent experts from outside the variola virus field (the Advisory Group of Independent Experts to review the smallpox research programme, or AGIES). The AGIES was constituted by the WHO smallpox secretariat. The comments and subsequent recommendations of AGIES are summarized in this document.

Terms of Reference and Membership of the Advisory Group of Independent Experts to review the smallpox research programme (AGIES)

Terms of Reference

The Advisory Group of Independent Experts (AGIES) was formed to act as an advisory body to WHO on matters of strategy and evaluation of the smallpox research programme, with the following functions:

1. To read the document entitled *Scientific review of variola virus research, 1999–2010*, in order to:

I. Review the results of smallpox research already undertaken, and the plans and requirements for further research essential for global public health purposes.

II. Assess whether additional research using live variola virus is necessary from a global public health perspective.

2. To deliver a report to WHO at the WHO Advisory Committee on Variola Virus Research meeting (17 November 2010) summarizing achievements to date and additional work needed to ensure high standards of security related to a potential re-emergence of smallpox from a public health perspective.

Membership of AGIES

Members of the AGIES were selected and appointed by the Director-General of WHO to serve in their personal capacities, representing the broad range of disciplines relevant to the review of the smallpox research programme, while ensuring optimal diversification and balance of personal experience, professional background, gender, and geographical origin. For a full list of these members and their short biographies, see Appendix.

Declaration of Interests

In line with WHO policy all members of the AGIES have completed and signed a Declaration of Interests. One expert has declared a potential conflict of interest in the subject matter of this meeting. No relevant conflict of interests has been declared by the other experts. The declared interest reported by Gerd Sutter was assessed to be minimal and unlikely to affect, or to be reasonably perceived to affect, his judgment. However the Secretariat has determined that his declared interests should be disclosed. Professor Gerd Sutter's declared interest is as follows: the Institute for Infectious Diseases and Zoonoses, Ludwig-Maximilians-Universität München, where he works, has accepted research support from the German Government for a project relating to "Comparative Analysis of vaccinia viruses MVA as candidate next generation smallpox vaccines"; and BIOSAFE, a research consortium funded by the EC, for a project relating to deliberate releases of biological agents. This consortium includes the company Novartis Italy as one of the partners.

Executive Summary

AGIES found the *Scientific review of variola virus research, 1999–2010* to be clearly written and comprehensive, and to provide an accurate and up-to-date review of variola virus research, including the impact of regulatory restrictions on current and future research.

This full report is presented in three parts, in line with the terms of reference provided to the AGIES. In Part 1, a summary of each chapter of the Scientific Review is provided, followed by specific comments. Part 2 contains the committee's recommendations for further research and comments on variola virus (VARV) repositories. Part 3 summarizes AGIES recommendations for ensuring high standards of security related to re-emergence of smallpox.

Recommendations for further research and for use of live variola virus

Part 1. Genomics, diagnostics and repositories

Genomics

Near complete genomic sequences are available for approximately 50 isolates of variola virus. Since variola virus genome has only limited genomic diversity and shows major homologies with genomes of other orthopoxviruses, the AGIES feels that there is no public health need for sequencing of additional variola virus isolates.

Diagnostics

Several nucleic acid-based assays have been developed. Some have used cloned or synthetic genetic elements from variola virus DNA for assay evaluation and development, others have used intact genomic variola DNA; some have used cloned variola DNA as positive controls. Their further development does not require the use of live VARV.

In the absence of clinical disease, it is not possible to determine the sensitivity, specificity, positive and negative predictive value of these tests in clinical situations.

There is a need for regulatory validation of these assays. Head-to-head comparisons and further optimization of the available assays, especially real-time polymerase chain reaction (PCR) and microarray platforms, should be undertaken. Newer assays should be developed as advances in diagnostic technology are made.

Several serological tests are available to detect antibodies to orthopoxviruses. Antigen capture assays are early in development; to date, these are generic for the orthopoxviruses, and none are variola specific. These may also benefit from head-to-head comparison. Efforts to improve their performance characteristics are warranted; newer assays may be developed as advances in diagnostic technology are made.

Live variola virus

The AGIES is of the view that live variola virus is not required for the further development of diagnostic tests nor for technical assay validation.

Repositories

See Part 3. Security issues.

Part 2. Vaccines, animal models and drugs – future research and requirement for use of live variola virus

Vaccines

The AGIES believes that attempts must continue to develop vaccines that are safer than, and at least as efficacious as, the original and/or existing licensed vaccines against smallpox.

In order to prepare for a potential outbreak of smallpox, strategies for efficacious therapeutic immunizations, such as post-exposure vaccination or delivery of anti-vaccinia immunoglobulins and/or monoclonal antibodies, need to be developed. These approaches should help to shorten the response time of public health systems in case of an outbreak. In addition, passive immunization may ameliorate the adverse effects of available vaccines in special subgroups.

Animal models

Neither variola models in animals nor natural poxvirus infection in animals can exactly model human smallpox.

Although current non-human primate models using VARV are suboptimal, research conducted into developing them further over the last decade has achieved limited success. The only reason for attempts to develop such models is to meet the current stringent regulatory requirements, in the absence of human variola virus infection. The AGIES's opinion was that a more productive approach would be for the regulatory requirements for vaccine and drug approval for variola virus infection to be reconsidered, given that human infection with the virus no longer occurs.

Therefore, the AGIES recommends that rather than develop animal models using VARV, research should concentrate on improving surrogate models that use infection with other orthopoxviruses in their natural hosts (e.g. monkeypox virus (MPXV), cowpox virus (CPXV), rabbitpox virus (RPXV), ectromelia/mousepox virus (ECTV), infections in non-human primates, rabbits and rodent models).

Such models would allow studies on pathogenesis of poxvirus infection, the analysis of drug and vaccine efficacy, and the establishment of criteria to evaluate protection.

Drugs

Two anti-variola drugs, namely, CMX001 and ST-246®, are in advanced stages of development. Resistance to each of these drugs has been described *in vitro* but the risk of treatment-induced resistance *in vivo* is not known. The AGIES recommends that if the development of resistance *in vivo* is deemed by ACVVR to be a significant possibility, then additional drugs with alternative mechanisms of antiviral action should be developed. However, in the first instance, efforts should primarily focus on cidofovir and ST-246®.

Live variola virus

At present, assuming that regulatory issues around vaccine and drug testing are resolved, the only indication for use of live VARV is to test the efficacy of drugs *in vitro*.

Part 3. Security issues

Monitoring containment policies

Stringent regular review of the quality assurance and containment practices at the Russian Federation's State Research Center of Virology and Biotechnology (SRC VB VECTOR) and the United States Centers for Disease Control and Prevention (CDC) must be continued.

Genetically engineered VARV, mutants of VARV or poxviruses containing parts of the VARV genome

The AGIES makes the following recommendations.

New strategies should be designed to address the potential for *de novo* synthesis of live VARV, including adoption of national policies on the issue by WHO Member States. It is recommended that recent biosecurity proposals (Bügl et al. 2007)[1] be considered at national policy level.

The World Health Organization should seek an updated validation from all countries regarding their stocks of variola virus DNA (in various forms, such as fragments, amplicons and/or plasmids).

In light of the existing restrictions on laboratories, other than the two WHO collaborating laboratories that retain more than 20% of the VARV genome, it is recommended that both CDC and VECTOR provide documentation to WHO cataloguing which segments of DNA have been distributed to which laboratories. Advice should also be sought on whether the complete genome has been distributed (or should be distributed), albeit as different gene segments comprising <20% of the genome, to a range of laboratories.

[1] Bügl H et al. (2007). DNA synthesis and biological security. *Nature Biotechnology*, 25:627–629.

PART 1. Review of results on smallpox research

General comments

Overall, the AGIES found the *Scientific review of variola virus research, 1999–2010* to be clearly written, comprehensive and to provide an accurate and up-to-date review of variola virus research, including the impact of regulatory restrictions on current and future research.

It was noted that specific recommendations were not given in the body of the Scientific Review. AGIES has been informed however that this was not the remit of the authors.

In this part, a summary of each chapter followed by specific comments is provided.

As would be expected, there is some overlap in the material presented, but the AGIES members note that in Chapters 2, 3 and 4 of the Scientific Review, there are discrepancies in the number of variola isolates that have been sequenced: varied numbers are given, including 45, 46, 49 and 50 isolates. These discrepant numbers may be related to the use of data from different time points, or of data from selected research groups. There is a need to update and clarify the correct number.

Chapter 1. Smallpox vaccines

Summary

This chapter recognizes the eradication of smallpox, accomplished in 1980 through a worldwide vaccination campaign using live vaccinia vaccines, as the greatest public health achievement in history. Since the eradication, however, world stability has been challenged by terrorism, and the threat of the use of smallpox as a bio-weapon has become a possibility. This threat has been exacerbated since the cessation of vaccination against smallpox and because vaccination with live vaccine is contraindicated in a significant proportion of the world's susceptible population.

This chapter summarizes progress in vaccine development, starting with the vaccines used during eradication campaigns, such as those based on vaccinia virus (VACV) strains, such as the NYCBH Dryvax®, Lister-Elstree or Tian-Tan, which, though efficacious, were mostly produced on the skin of live animals, the use of which was associated with varying degrees of adverse events. In the interest of public health, the development of new, safe and efficacious vaccines is under way, with several potential vaccines at different levels of development. The chapter critically reviews the progress made in the research into smallpox vaccine development and advances approved by WHO between 1999 and 2010. Overall, it recognizes that vaccines based on the original vaccinia viruses (initially animal-derived, then replication competent cell culture-derived) provide adequate protection, but have inherent safety problems that must be overcome through development of new vaccines. Highly attenuated or genetically modified vaccines and subunit vaccines have the potential to reduce adverse effects, but their efficacy must be improved.

The development, clinical trials and plans for stockpiling of ACAM2000, a plaque-purified, live vaccine produced in tissue culture from vaccinia virus strain NYCBH, represents an advance for public health and emergency preparedness. However, concerns about severe adverse events – mainly cardiac complications – remain, and its suitability for use in children is untried and unknown. For those susceptible to these adverse events, immunoglobulin therapy may be useful. However, no trials on its use have been carried out. Among the cell culture-derived vaccines with potential for improved safety are the highly attenuated vaccine candidates based on the vaccinia viruses MVA and LC16m8, which were generated upon serial passage in tissue culture.

Genetically engineered live vaccinia virus vaccines, and subunit vaccines that use selected vaccinia virus antigens, such as A33 and B5, are also mentioned as having improved safety. Data from animal models and limited studies in humans suggest that these are less immunogenic or protective than the live virus vaccines based on conventional first-generation, cell culture-produced or tissue culture-attenuated vaccinia viruses.

The Scientific Review alludes to the potential for post-exposure vaccination for therapeutic intervention, but data on this promising approach are still limited.

Comments

The literature reviewed covers vaccine development, describing the initial vaccinia vaccines; improvements in production and purification techniques to develop cell culture-derived vaccines; and the ongoing improvements in attenuated vaccines. The early vaccines were used largely to eradicate smallpox in the pre-1980 period, and thereafter only in at-risk groups in selected countries. Currently, the vaccines most likely to be available in an emergency response to a smallpox outbreak are live vaccines based on cell culture-produced or cell culture-produced and plaque-purified, but fully replication competent, vaccinia viruses.

Information on the severe adverse complications of the early animal- and cell culture-derived vaccinia virus vaccines, and the evoked protective immune response (both humoral and cellular), is reviewed, including adverse effects arising from the method of production of "first generation" vaccines. Cell culture-derived vaccines have a better safety profile than the first generation of vaccines, but they remain fully replication competent and present challenges with regard to vaccine safety. The use of replication-competent virus vaccine poses a particular concern because of the existence of large population groups with conditions that contraindicate the use of replicating vaccinia virus (such as human immunodeficiency virus (HIV) infection). Modern production methods (plaque purification and cell culture), improved sterility and purity, and reduced risk of contamination by adventitious agents have resulted in the development of genetically homogenous products, and large-scale production is now possible. ACAM2000 is listed as one licensed vaccine in this category that is already approved.

Post-exposure vaccination will be required for use in a smallpox outbreak, but additional work is necessary to validate this strategy. This subject was alluded to only briefly in this chapter, possibly due to the scarcity of information.

Additional references

Two very recent publications have been found on e-pub ahead of print; these were published after the report was submitted, and may be added to the references.

One of these is a useful review of the development of subunit vaccines for human use in orthopox virus infections:

> Chapman JL et al. Animal models of *Orthopoxvirus* infection. *Veterinary Pathology,* 2010, 47:852–870.

The other paper relates to monkeypox virus (MPXV) challenge:

> Buchman GW et al. A protein-based smallpox vaccine protects non-human primates from a lethal monkeypox virus challenge. *Vaccine,* 2010, 28:6627–6636.

Chapter 2. Laboratory diagnostics for smallpox (variola virus)

Summary

This chapter reviews the available methods for diagnosis of infection with variola virus, the causative agent of smallpox. Despite the eradication of smallpox, methods to diagnose this infection are needed in view of the potential bio-warfare or bio-terrorism uses of this agent, and the serious consequences of this infection.

Clinical diagnosis of smallpox was once considered easy, though distinction from other exanthematous illnesses can sometimes pose a problem. With disease eradication, such distinction is likely to be difficult, since most contemporary physicians have no prior experience with such diagnosis.

Specimens used for diagnosis of VARV include scabs, fluid and overlying skin from skin vesicles, touch preparations from opened lesions (on glass slides, electron microscope grids or filter paper), and skin biopsy. Blood and throat swabs may be useful during the early phase of infection. Serum (preferably paired early and convalescent specimens) is used for antibody-based diagnostics. Specimen collection and transport procedures are well described.

The tests for detection of VARV infection fall into four categories:

1. detection of virus;
2. detection of viral nucleic acid;
3. detection of anti-VARV antibodies (serologic assays);
4. detection of viral protein.

Of these, nucleic acid detection strategies have made the most progress in recent years. In comparison, progress in diagnostics based on detection of the virus itself, specific antibodies, and viral proteins has been relatively limited.

Virus isolation and culture techniques

These techniques include electron microscopy (EM) and virus isolation. Both are limited by the fact that they cannot be undertaken outside the only two laboratories in the world that are equipped to handle live VARV specimens.

Electron microscopy can distinguish poxviruses (including VARV, related orthopoxviruses (OPVs) and other non-parapox viruses) from herpesviruses (which cause exanthematous diseases from which smallpox needs to be distinguished), based on their typical morphology. In addition, results can be obtained rapidly. However, VARV cannot be distinguished from other orthopoxviruses by electron microscopy and the method is insensitive, requiring high concentrations of VARV for detection.

Virus isolation can be done using either the chorioallantoic membrane of chicken eggs, or one of several cell lines, with the appearance of typical cytopathic effect. Limited availability of laboratories where this can be safely done limits the application of this procedure, despite its advantages of being a gold standard method and providing live virus for further studies.

Nucleic acid based diagnostics

Several techniques have been tried for detection of VARV in clinical specimens, including restriction-fragment length polymorphism (RFLP), polymerase chain reaction (PCR), PCR-RFLP, real-time PCR, sequencing of nucleic acids, and microarray platforms. With advances in technology, newer methods that are better suited to automation, like real-time PCR, sequencing and microarray chips, are increasingly being tested. These reflect the techniques most likely to be used for diagnostic purposes. Nucleic acid techniques, however, cannot distinguish between viable and non-viable virus.

Simple RFLP techniques, though useful in differentiating VARV from other orthopoxviruses, and in distinguishing various isolates of VARV, require large quantities of viral DNA and hence prior viral culture. This, combined with the increasing ease in use of newer techniques like sequencing, suggests that RFLP will soon become redundant.

The available PCR and PCR-RFLP techniques, including multiplex PCR, can differentiate between VARV and other orthopoxviruses to some degree. However, these techniques are somewhat limited by genetic heterogeneity in orthopoxviruses, and carry the risk of false-positive results because of laboratory contamination.

Several real-time PCR techniques for detection of VARV have been developed. These techniques are rapid, highly sensitive and specific, and easy to automate. These techniques use cloned or synthetic VARV DNA fragments as positive controls, and appear to distinguish VARV from other poxviruses with ease. Furthermore, these are less prone to false-positive results when compared to the usual PCR techniques. Recent developments, like portable real-time PCR machines and lyophilized reagents, make these techniques particularly attractive for use in VARV diagnostics in field situations. Multiple tests based on different parts of the viral genome can be used to increase the specificity further.

Oligonucleotide microarrays for the detection of VARV contain several DNA probes specific for VARV and other orthopoxvirus species (and possibly for other agents, like herpesviruses, that may mimic smallpox clinically), immobilized on either three-dimensional polyacrylamide-gel chips or glass slides. These methods are robust, quick, and suitable for high throughput. They have been shown to be highly reliable.

Sequencing of amplified PCR products has been used to distinguish between VARV and other related viral species. This technique can also allow tracing of the origin of various virus strains.

These assays for viral nucleic acids generally use cloned or synthetic variola virus DNA fragments of up to 500 bases in length as primers/probes and positive controls, which is

within the upper limit on the length of variola virus genomic fragments that can be used in laboratories without special facilities, and do not need live variola virus.

All the available tests are limited by the fact that they are research-based. One of the assays is available commercially, but only for research, and not for diagnostic purposes. However, none of the assays has yet attained regulatory approval, and this limits the use of these tests for any future widespread diagnostic use.

Protein-based assays

One antigen capture assay is available for detection of orthopoxviruses. It uses polyclonal antibodies to detect virion structures. The assay has not been characterized well, but may be of interest for field use. A haemagglutination and haemadsorption inhibition assay used in the pre-eradication era needs further evaluation. The specificity of these assays in distinguishing between infection with VARV and with other orthopoxviruses may be limited.

Serological assays for anti-VARV antibody

These tests may be useful when clinical specimens suitable for detection of virus or viral components are not available, to determine whether a person is positive for IgM anti-VARV antibodies and hence has had recent exposure to an orthopoxvirus. Though several serological assays for orthopoxvirus infection are available, these are not specific for particular virus species. Cell-mediated immune responses currently appear to be of little use for diagnosis of VARV infection.

Comments

The information provided in the chapter is complete and provides an accurate summary of the knowledge in the area of VARV diagnostics. None of the laboratory diagnostic tests for variola virus infection needs the use of live variola virus.

Chapter 3. Variola genomics

Summary

The variola virus genome

Technological advances in DNA sequencing and bioinformatics have had a direct impact on the ability to obtain genomic sequencing, and the speed and accuracy with which this can be done, leading to almost complete genetic sequence information on 49 VARV isolates. According to the authors, this sequence information is available in public databases.[1] Since none of the currently available sequenced VARV genomes include the terminal native hairpin sequences, a truly complete sequence is not (yet) available.

The United States Centers for Disease Control and Prevention (CDC) and the Russian Federation State Research Centre of Virology and Biotechnology (SRC VB VECTOR), currently hold about 550 VARV isolates that have not been sequenced. The value of sequencing additional isolates is, however, uncertain.

Like all poxviruses, the VARV genome consists of double-stranded DNA (~185 kb), with hairpin termini and terminal inverted repeat (TIR) sequences. All the deduced VARV genomes contain approximately 200 open reading frames (ORFs). In contrast to most other poxviruses, the TIR sequences of VARV do not encode any viral proteins. The central region of the VARV genome contains about 90 highly conserved poxvirus genes, which are essential for genomic replication, gene expression and virus assembly. Genes responsible for biological properties of VARV, such as virulence, immune response determinants and disease pathogenesis, appear to be located near the genomic termini. Based on the high rate of similarity between gene sequences of VARV and other OPVs, the VARV strains can be expected to show sensitivity to drugs that are active against other OPVs, such as ST-246® and a cidofovir derivative, i.e. CMX001. Several proteins of VARV have been studied, and some of them were found to interact with specific elements of the human immune system, and various signalling pathways.

Variola evolution

Based on genome sequencing, several distinctive groups of VARV have been identified. One group includes variola major, from Asia, with isolates from Africa; other groups consist of variola minor, from South America and western Africa. It has been estimated that VARV diverged from an ancestral poxvirus, probably from rodents in Africa, between 16 000 and 68 000 years ago. It was calculated that the average rate of mutation of VARV virus is about 10^{-6} nucleotide substitutions per site per year. By this measurement, VARV started its independent evolution about 3400 (± 800) years ago.

[1] at http://www.poxvirus.org and http://www.ncbi.nlm.nih.gov/GenBank, last accessed November 2010

The original ancestor of OPVs remains unknown, but was possibly related to current strains of cowpox virus (CPXV). It remains unknown whether it was variola major or variola minor that appeared first in human populations, but it is clear that these strains were diverging from each other by the time smallpox was eradicated.

Poxvirus genome technologies

Recent expansion in technologies for DNA sequencing, cloning and gene synthesis now allow *de novo* synthesis of the entire VARV genome and creation of a live virus, using only publicly available sequence information. The estimated cost of synthesizing all the clones necessary to make up the complete VARV may be less than US$ 200 000, and this is likely to drop in the future.

Alternative methods could also be used to generate intact VARV or VARV-like viruses: for example, recombinant OPVs. Such recombinant viruses represent a potential hazard as they would be much easier to assemble or generate than wild-type VARV.

Guidelines for variola virus genomes

Currently, VARV containment strategies include the stipulation that no laboratory other than the two relevant WHO collaborating centres be permitted to retain more than 20% of the VARV genome. In addition, any manipulation of VARV DNA must be geographically isolated from work involving the propagation of other poxviruses. These strategies remain relevant. However, new additional strategies, including the adoption of national policies, need to be designed to address the potential for *de novo* synthesis of live VARV.

Comments

It would be useful, for ease of consideration by the WHO Advisory Committee on Variola Virus Research for the original authors to table the principal uses of genome sequence information, and comment whether additional genomic sequencing is desirable for each of these uses (e.g. for studies of relationships of VARV to other poxviruses, for diagnostic purposes, for study of virus-host interactions, for identification of new drug targets, and for vaccine development). At this point, the authors only question the potential additional benefit from the perspective of a study of viral evolution.

The authors may want to comment on whether further sequencing of the terminal ends of variola virus genome is desirable and/or feasible.

The risk that a recombinant VARV might be synthesized with resistance to drugs such as ST-246® and CMX001 should be discussed.

The authors present opposing arguments regarding further sequencing of additional VARV isolates currently held at CDC and SRC VB VECTOR. On the one hand, they indicate that additional sequencing would enable recognition of more genetic variation between isolates and might lead to the discovery of new and additional interactions with the host. On the other hand, they question the value of further sequencing in light of the development of vaccines and drugs. After considering all the comments, AGIES is of the opinion that further sequencing is not indicated from a public health perspective.

It is suggested that the authors provide a listing or table of the 49 variola isolates from which near complete genome information is available (noting however that this number may actually be 50, as indicated elsewhere in the Scientific Review document), and link these to the information provided in Chapter 4. In addition, it would be interesting to depict graphically the relationship of several distinctive groups of VARV – either as three distinct clades, or as two major clades with one containing two subgroups – using phylogenetic trees. The reference for this obvious difference, based on the method of analysis, should be indicated clearly in the document (Esposito et al. 2006)[1].

In the *Variola genomics* chapter, 'Variola evolution' section, first paragraph of the section, the citation (Gubser et al.,2004)[2] should be mentioned in the text for the conserved poxvirus genes that are believed to encode for the essential elements in the viral genome.

In the *Variola genomics* chapter, 'Variola evolution' section, third paragraph of the section, last sentence, a reference should be provided clearly for the statement that the VARV strains were in the process of divergent evolution at the time of eradication of smallpox.

A search of the public databases in GenBank[3] showed that sequence information on VARV is indeed available in the public domain. To what extent these sequences are complete, could not be verified.

[1] Esposito JJ et al. (2006). Genome sequence diversity and clues to the evolution of variola (smallpox) virus. *Science*, 313:807–812.

[2] Gubser C et al. (2004). Poxvirus genomes: a phylogenetic analysis. *The Journal of General Virology*, 85:105–117.

[3] at http://www.ncbi.nlm.nih.gov/GenBank, last accessed November 2010

Chapter 4. Status of WHO collaborating centre repositories of variola virus and nucleic acid

Summary

This chapter outlines the history to date of self-reported holdings of stocks of variola virus and variola DNA in the post-smallpox eradication era, and documents the material held in the two designated repositories, both of which are WHO collaborating centres.

These repositories are located at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, United States of America; and the Russian Federation State Research Centre of Virology and Biotechnology (SRC VB VECTOR) in Koltsovo, Novosibirsk Region, Russian Federation.

The recommendation of 1986 of the WHO Committee on Orthopoxviral Infections is that all collections of VARV strains and their genomic DNAs ultimately be destroyed. However, it is noted that genetic material must be conserved in a reliable and biologically safe form. Currently this is as genomic DNA, amplicon collections, and in recombinant plasmids. Storage as recombinant plasmids overcomes two issues with amplicon collections, namely: (1) the potential risk of DNA degradation during long-term storage; and (2) the need to retain genomic DNA in order to maintain a full-size collection of amplicons (in the Scientific Review, Chapter 4, Section 4.2. 2.

In line with the most recent update of the WHO Recommendations[1] concerning the distribution, handling and synthesis of variola virus DNA, the two laboratories that house these repositories may distribute variola virus DNA fragments (not exceeding 20% of the total viral genome) to requesting scientists, provided both parties adhere to the WHO Recommendations obtainable from the WHO web site. The two laboratories that house repositories are required to provide WHO with annual written and verbal reports regarding the use of live variola virus and the status of the repositories.

In 2010, the SRC VB VECTOR laboratory collection contains 120 strains of VARV, 32 of which contain viable virus (in the Scientific Review, Chapter 4, Section 4.2.1). Genetic material is stored as genomic DNA (199 vials; material from 39 different VARV strains); collections of amplicons each corresponding to one VARV strain (1446 vials; 17 collections each in three repeating sets); and collections of recombinant plasmids (3795 vials; 16 collections; stored as three repeating sets).

In 2010, the CDC laboratory collection contains 451 isolates or clinical specimens (scabs). The provenance of 213 of these is unknown. Forty-six epidemiologically similar strains,

[1] WHO Recommendations concerning the distribution, handling and synthesis of Variola virus DNA, May 2008 available at http://www.who.int/csr/disease/smallpox/SummaryrecommendationsMay08.pdf , last accessed, November 2010

differing in terms of time and place, and representing the range of virus diversity, underwent full genome sequencing and are stored in this repository; three of these strains were used to create amplicon (309 vials) and plasmid (875 vials) collections.

This chapter also provides a table of external investigators provided with specified virus genome fragments (2003–2008) from the CDC laboratory.

Comments

The respective formats of the information presented by CDC and SRC VB VECTOR differs principally in the provision of information by CDC about external investigators to whom DNA has been released for experimental purposes. This information is useful in the overall context of the report. It is recommended that SRC VB VECTOR provide similar information as part of the Scientific Review, i.e. that SRC VB VECTOR document the provision of VARV material to other research institutions.

SRC VB VECTOR

It is noted that 32 of the 120 strains of variola virus at SRC VB VECTOR are viable (the Scientific Review, Chapter 4, Part 1, Section 4.2.1). Of the 59 strains summarized in Table 4.2, it appears that freeze-dried stocks (n=25) remained viable, but frozen chorioallantoic membrane stocks (n=19) and one CAM homogenate (#57, no indication of whether it was stored frozen) did not, and that seven out of 14 clinical samples (scabs) contained viable virus (the method of storage was not stated). It is recommended that the need for continuing storage of non-viable samples and material be assessed, and that, if necessary, the overall storage and preservation conditions be optimized and/or standardized to prevent further loss of material.

Full-genome sequences of 39 variola strains (32 viable and 6 non-viable isolates listed in Table 4.2, and an additional strain, Ind-70, not listed in Table 4.2) are stored. Seventeen viable variola strains are stored in the amplicon repository; 14 (plus two additional to strains in Table 4.2) are stored in the recombinant plasmid repository. DNA from 9 strains is present in both the amplicon and recombinant plasmid repositories. These belong to two epidemiological types isolated from different geographical regions (the Scientific Review, Chapter 4, Section 4.2.2,)

Centers for Disease Control and Prevention

The CDC amplicon repository contains DNA from one strain in the full genome repository, and from two not in the repository; the plasmid repository contains DNA from five strains in the full genome repository, and from one not in the repository.

Questions raised by this information

Does either laboratory have evidence of the state of the DNA (i.e. whether degradation is occurring) in their amplicon repositories?

How many strains of variola should be represented in the amplicon and plasmid repositories ?

Stocks of non-infective variola DNA fragments have been retained in South Africa. In the version of November 2010 of the Scientific Review, Chapter 4, *Executive summary*, the following phrase was used:

> "…although South Africa may still retain cloned non-infectious variola virus fragments"

This should be amended to indicate that:

> a duplicate set of DNA clones originally prepared in the United Kingdom and sent to CDC, are also held in South Africa. This duplicate set was transferred to South Africa following an agreement between WHO and the Government of South Africa to allow the Department of Health in South Africa to retain a set of the clones in exchange for destroying their variola virus stocks.

Chapter 5. Animal models and pathogenesis

Summary

The chapter focuses on pathogenesis, and the animal models available for the study of OPV diseases. It describes not only the data pertaining to variola virus, but also (and mainly) data pertaining to other OPV that infect animals, such as ectromelia virus (ECTV), cowpox virus, rabbitpox virus (RPXV), and vaccinia virus. It indicates that very little is known about the underlying pathophysiology or the virulence mechanisms of variola virus, and that the establishment of a feasible animal model is essential to understanding the pathogenesis of the virus, and for the development of new drugs and vaccines against smallpox.

Although smallpox has been eradicated, intentional re-introduction of variola virus into the human population is of great concern, due to ease of transmission of the virus from person to person, the high mortality rate (reaching approximately 30%), and low or non-existent immunity in the majority of the human population. In addition, recent years have seen an increasing number of humans infected with other OPV, such as cowpox and monkeypox virus, indicating the zoonotic potential of OPV species. Moreover, smallpox vaccines that are presently available can have severe adverse effects. The development and licensure of counter-measures, including drug treatment and vaccines, will depend on animal models for the demonstration of protective efficacy.

This chapter remarks on the importance of the development of an animal model in which VARV produces a disease similar to human smallpox, in order to provide the most convincing demonstration of protective efficacy of vaccines and antiviral drugs for smallpox. Regulatory authorities (e.g. the United States Food and Drug Administration, or USFDA) may request the use of the authentic agent (in this case VARV) for efficacy testing following the "Animal Rule", and that disease process in the animal model be faithful to the human disease. The development of an appropriate animal model would also be essential for pharmacokinetic studies for determination of correct dose of newly developed anti-variola drugs for use by humans, and the appropriate course of therapy.

A major limitation for the development of a ‘perfect’ model is the species-specificity of VARV. Attempts to infect and produce disease using VARV in rodents, rabbits and primates have been either unsuccessful, or do not reproduce the characteristics of the human disease. Hence, most studies have been performed with other OPVs in their natural animal hosts. These models recapitulate some, but not all, features of human smallpox. Individual analysis of each animal model indicates that, in some cases, virus-host interactions involve features that are species-specific, and may not be reliably generalized.

Despite these limitations, the available animal models have been adequate to demonstrate the efficacy of several candidate antiviral drugs, including cidofovir and ST-246®. However, these drugs need further refinement, and no existing model simultaneously satisfies all of the criteria required under the USFDA "Animal Rule" (US 21CRF310.610).

Two important points were made in this chapter.

1. Smallpox was eradicated before the development of modern technologies in immunology and molecular biology, making our present knowledge of this infection very limited. Therefore, studies to improve the analysis of virus pathogenesis, treatment and prevention must continue.

2. Until now, disease severity has usually been evaluated by time and rate of death, viraemia, and lesion count, which are crude measures. Since the death rate in animal models differs from that observed in human infection, it is still necessary to develop a panel of surrogate endpoints that better reflect the disease.

It was also pointed out that it is possible that no single combination of conditions will result in a model that simultaneously satisfies all of the criteria for the ideal model of smallpox infection: different models may be required to assess different indications.

The point is made that primates seem to be the most suitable known models for the study of OPV infection. While the interaction of VARV with the human immune system can only be approximated in the monkey models, the extrapolation from primates to humans is less tenuous than that from rodents to humans. Whether MPXV in monkeys is more faithful to human smallpox than VARV in monkeys, is the focus of intense investigation. Both primate models may provide insight into the development of diagnostic, prophylactic, and therapeutic strategies. However, it should be kept in mind that the *in vivo* use of VARV seems impractical.

Comparison of animal models

The advantages and drawbacks of various animal models are detailed in the text of the chapter. These include ECTV in different strains of mice (in which it is a natural pathogen), use of CPXV (e.g. strain Brighton Red) in mice, vaccinia virus infection in mice, and RPXV (strains of vaccinia virus that caused outbreaks of rabbitpox in rabbit breeding facilities).

A major advantage of the mouse models is the availability of inbred strains of mouse and of laboratory reagents to investigate immune response, allowing the analysis of specific factors that may be related to susceptibility/resistance and response to treatment or vaccine. However, it must be considered that both the virus and the mouse strain influence the disease pattern, lethality and immune response observed.

Although rabbits exposed to RPXV via the aerosol route or by intradermal inoculation develop a systemic syndrome similar to human smallpox, the main issue with using rabbits as an animal model is that a detailed analysis of host immune response is limited, due to the lack of reagents and inbred rabbit strains.

Monkeypox and non-human primate models

Monkeypox is maintained in nature by association with rodent reservoirs. It is also a human pathogen that produces many of the signs and symptoms of smallpox, although with a lower potential for person-to-person transmission. Therefore, it is relevant to study this model not only as a surrogate for smallpox, but also as a disease entity in its own right.

Ground squirrels and prairie dogs (Hutson et al. 2009, 2010) have been investigated as MPXV experimental models. Since this chapter was written, mouse models have been reported. These demonstrated that low infectious doses can result in lethal, systemic infection (Americo et al. 2010; Stabenow et al. 2010).

Monkeypox virus infection of primates, mostly cynomolgus macaques (either *Macaca iris* or *Macaca fascicularis,* but also rhesus monkeys – *Macaca mulatta* – has been accomplished via the aerosol, intramuscular, intratracheal, intrabronchial and intravenous routes of exposure. Natural transmission of MPXV (and VARV) probably occurs by a combination of aerosol, fomites, and mucosal exposures. Therefore, aerosol exposures are most appropriate for modelling primary exposures following a biological warfare attack. However, aerosol exposures require BSL-4 bio-containment in a Class III cabinet, and are less readily controlled in comparison with intravenous exposure. Here, the intratracheal model (Stittelaar et al. 2005) might be more practical.

Changes in various clinical parameters measured in monkeys exposed to aerosolized MPXV occur in a sequence similar to that in humans, but are more accelerated.

Intravenous exposure of cynomolgus macaques to MPXV also resulted in uniform systemic infection, which was dose dependent. This model was used to test the efficacy of a candidate vaccine, the highly attenuated modified vaccinia Ankara (MVA). Additionally, MPXV intratracheal challenge models have been used to monitor the efficacy of MVA vaccination in comparison, and in combination, with the licensed Dryvax® vaccine (Stittelaar et al. 2005). The intravenous (IV) MPXV model was also used to demonstrate the efficacy of a number of candidate antiviral drugs, including cidofovir and ST-246®.

There has been a reluctance to accept the intravenous challenge model, since it is not the 'natural route' of VARV infection. However, protection against an overwhelming intravenous dose is a very stringent criterion, and protection against intravenouschallenge may predict efficacy against peripheral challenge routes. Alternative exposure models, including the intratracheal route, are being explored.

Comments

A number of recent papers not included in the chapter exist that may provide an important update to data regarding OPV animal models.

One of these proposes another model, by infecting the common marmoset *Callithrix jacchus* with calpox virus, a new isolate of CPXV. The authors of this paper mentioned that existing non-human primate models, using VARV and MPXV, need very high viral doses that have to be applied intravenously or intratracheally to induce a lethal infection in macaques. They demonstrated that marmosets infected with calpox virus, not only via the intravenous but also via the intranasal route, reproducibly develop symptoms resembling human smallpox after infection with much less virus than is used in monkeypox virus infection. *In vivo* titration resulted in an MID50 (minimal monkey infectious dose 50%) approximately 10 000-fold lower than MPXV and VARV doses applied in the macaque models (Kramski et al. 2010). Although interesting, this model still has limitations, such as high death rates caused by CPXV in this particular host (but not in other hosts), the scarcity of basic information regarding the components of the immune system of marmosets, and limitations of reagents to study immune responses in this animal model.

Another paper demonstrates ECTV infection of C57/Bl6 mice via the intranasal route. The authors showed that this infection induces a slower disease progression and lower mortality rate than infection in A/Ncr mice, and that this model would be closer to VARV infection of humans. The model was also demonstrated to be suitable for analysis of antiviral drugs (Parker et al. 2009).

Additional references

1. Americo JL, Moss B, Earl PL. Identification of wild-derived inbred mouse strains highly susceptible to monkeypox virus infection for use as small animal models. *Journal of Virology*, 2010, 84:8172–8180.

2. Kramski M et al. A novel highly reproducible and lethal nonhuman primate model for orthopox virus infection. *PLoS One*, 2010, 5:e10412.

3. Parker S et al. Mousepox in the C57BL/6 strain provides an improved model for evaluating anti-poxvirus therapies. *Virology*, 2009, 385:11–21.

4. Stabenow J et al. A mouse model of lethal infection for evaluating prophylactics and therapeutics against monkeypox virus. *Journal of Virology*, 2010, 84:3909–3920.

5. Stittelaar KJ et al. Modified vaccinia virus Ankara protects macaques against respiratory challenge with monkeypox virus. *Journal of Virology*, 2005, 79:7845–7851.

6. Hutson CL et al. A prairie dog animal model of systemic orthopoxvirus disease using West African and Congo Basin strains of monkeypox virus. *Journal of General Virology*, 2009, 90:323–333.

7. Hutson CL et al. Dosage comparison of Congo Basin and West African strains of monkeypox virus using a prairie dog animal model of systemic orthopoxvirus disease. *Virology*, 2010, 402:72–82.

Chapter 6. Antiviral drug development for the treatment of smallpox – status of small-molecule therapeutics

Summary

This chapter reviews the progress made in obtaining efficient antiviral drugs for the treatment of smallpox. In the past decade, extensive research and development efforts have been undertaken in this field. These were necessary because eradication of naturally occurring smallpox was achieved before modern approaches of molecular biology and biochemistry for rational antiviral drug development had become available. The particular public health importance of therapeutic means against potential re-emergence of variola virus or other highly virulent orthopoxviruses further supported the urgency of this research programme. The overall objective of the research programme was to generate at least two approved drug substances, acting by different mechanisms, and suitable for oral application.

The authors provide a detailed description of the process of antiviral drug development, including the screening for potentially useful compounds – both *in vitro* (tissue culture assays) and in preclinical *in vivo* testing (animal models) – and the possible pathways through clinical evaluation to licensure/marketing authorization of an antiviral drug substance. Emphasis is given to the exemplary Investigational New Drug (IND) procedure implemented by the USFDA. This is appropriate, as the USFDA is likely to be the first regulatory authority to license an antiviral agent against smallpox.

The main text of the chapter provides an overview of the many different compounds being looked at, representing various classes of drugs, and grouped according to the proposed mechanisms of action. Due to the necessary restrictions applied to work with VARV, most research has been carried out with surrogate viruses such as vaccinia virus, cowpox virus, ectromelia/mousepox virus, and monkeypox virus. The status of the development and use of primate animal models for drug testing is reported: the cynomolgous macaque/MPXV infection model is indicated to be the most practical and valuable primate animal model for efficacy testing.

Finally, the authors provide a detailed description of work conducted with the first drug candidates entering clinical development. Importantly, it is reported that three drug substances have been granted Investigational New Drug status by USFDA, and have proceeded to testing in humans. Two of these compounds are based on cidofovir, a small molecule nucleoside analogue that selectively inhibits the viral DNA polymerase. Intravenous cidofovir (Vistide®), a substance approved by USFDA for treatment of retinitis caused by human cytomegalovirus, has been efficacious for treatment of experimental infections with OPV in animal models including MPXV and VARV. Clinical development of Vistide as smallpox drug is hampered by the need for intravenous infusion, the likely requirement of higher dosage than that used for retinitis treatment in man, and this drug's potential for nephrotoxicity.

The newly developed derivative CMX001 (HDP-cidofovir) is a lipid analogue of cidofovir that is suitable for oral application because it uses lipid uptake pathways *in vivo*. Efficacious for the treatment of OPV infections in animal model testing, CMX001 has been demonstrated to be orally bioavailable in humans. There are no data from toxicity studies or trials in humans suggesting nephrotoxicity. Phase II clinical trials with CMX001 are ongoing.

The third drug substance in clinical development is the small molecule compound ST-246® (Tecovirimat, SIGA Technologies), which specifically targets the function of a major OPV envelope protein encoded by gene homologues of the VACV open reading frame *F13L*. This envelope protein is important for the formation of extracellular virus particles and for the efficient *in vitro* and *in vivo* spread of infectious virus. Thus, in the presence of ST-246®, the production of fully functional infectious progeny is severely affected, and *in vivo* infection is efficiently blocked. ST- 246 proved highly efficient for treatment of OPV infections in all available animal models, and because of the extremely promising preclinical safety profile of the drug, the USFDA has granted Fast Track status to the clinical development of Tecovirimat. Fast Track is a regulatory process designed to expedite the review of drugs to treat serious diseases and fill an unmet medical need, with the purpose of getting important new drugs, e.g. those showing superior performance or significantly decreased toxicity, more quickly to the patients.

Comments

The information in the chapter represents an adequate overview of the efforts and achievements made in anti-smallpox drug development. The AGIES acknowledges the fact that two promising drug candidates (CMX001, ST-246®) have proceeded to advanced stages in development. These two drug candidates interfere with different steps in the virus life cycle to inhibit OPV replication, and both fulfil the requirement of suitability for oral administration. ST-246® targets a single OPV protein with very high specificity, which explains the excellent preclinical data with regard to efficacy, and, importantly, its profile of minimal toxicity. In comparison to ST-246®, the drug CMX001 might be somewhat less efficient to treat OPV, but holds significant promise also for the treatment of other viral infections. Thus, the research programme on drug development may be close to reaching its overall objectives – probably even closer than suggested by the rather unassuming report of the expert authors.

PART 2 . Recommendations for further research and comments on repositories

Section 1. Genomics, diagnostics and repositories

Genomics

Near-complete genomic sequences are available for approximately 50 variola virus isolates from around the world. These isolates show only very limited genomic diversity and share many homologies to genomes from other orthopoxviruses. In view of this, and the fact that variola virus has a DNA genome, with lower likelihood of major genomic variation, the AGIES felt that there is no public health need for sequencing of additional variola virus isolates, though this may appear interesting from a scientific perspective.

The AGIES believes that current containment strategies for variola virus are more or less historically based, and are inadequate in the current era, where modern molecular techniques allow for *de novo* synthesis of intact VARV in any lab with the appropriate equipment and/or financial resources. In addition, the technology to genetically modify poxviruses is generally easily available, and can be used to circumvent the WHO recommendations that prohibit genetic engineering of VARV. The AGIES therefore recommends that new strategies be designed to address the potential for *de novo* synthesis of live VARV, including adoption of national policies by WHO Member States. It is recommended that recent biosecurity proposals (Bügl et al. 2007)[1] be considered at national policy level.

Laboratory diagnostics

Diagnosis of poxvirus infection can be made using one or more of several techniques, including electron microscopy, virus isolation, detection of viral nucleic acids, detection of viral proteins within an intact virion, and serological response of the host.

Electronic microscopy, though an easy and quick technique, is limited by the fact that, for variola virus directed diagnostics, it can only be undertaken in the two laboratories in the world equipped to handle live VARV specimens.

Several assays for specific detection of variola virus nucleic acid, using various technological platforms, including PCR assays, quantitative (real-time) PCR assays and microarray chip-based methods, have been developed. These assays generally use cloned or synthetic variola virus DNA fragments. Real-time PCR assays can utilize fragments of up to 500 bases in length

[1] Bügl H et al. (2007). DNA synthesis and biological security. *Nature Biotechnology*, 25:627–629.

as primers/probes and positive controls, which is within the upper limit on the length of variola virus genomic fragments that can be used in laboratories without special facilities, and do not need live variola virus. There is a need for regulatory validation of these current assays. Head-to-head comparisons of available techniques, and efforts to optimize further the available nucleic acid based assays, in particular real-time PCR techniques and microarray platforms, should be undertaken.

Several serological tests are available to detect antibodies to orthopoxviruses. Antigen capture assays are early in development; to date, these assays are generic for OPVs, but not variola specific. These may also benefit from head-to-head comparison, and efforts to improve their performance characteristics and to make them more rapid, standardized and automated.

The current molecular and serological assays may also need adaptation with time, to keep up with future advances and developments in techniques and equipment in their respective fields.

The AGIES believes that there is no need to use live variola virus for work related to development and improvement of diagnostic tests for this infection.

Repositories

The information in the Scientific Review raises the issue of the purpose of retaining non-viable strains of variola in either repository (independent of consideration by the World Health Assembly of a time line for destruction of all viable variola stocks, following this review).

A decision will be needed, in time for future work, regarding the characteristics of strains, and the optimal number required in the amplicon and plasmid DNA repositories in order to ensure adequate epidemiological representation.

The AGIES experts feel it would be of value to know which segments of DNA have been distributed to which laboratories, and whether the rule of 20% is still applicable.

Section 2. Vaccines, animal models and drugs

Vaccines

The AGIES believes that attempts must continue to develop vaccines that are safer than, and at least as efficacious as, the original and/or the existing licensed vaccines against variola virus infection.

The group noted that two highly attenuated cell culture vaccines, namely MVA and LC16m8 vaccines, are already moving towards licensure. It was felt that, in future, research should be undertaken towards the creation of genetically engineered viral strains, through the removal of genes responsible for adverse effects from the currently available vaccine strains. These new strains can then be tested for protection against infection with orthopoxviruses in animal models of such infection, and for safety and immunogenicity in human clinical trials. Similarly, further research work should also be undertaken on promising subunit vaccine candidates, since these can be expected to be safer than existing vaccines, though their efficacy remains unproven.

A major stumbling block to the development of new vaccines is the inability to demonstrate the development of protective immunity against smallpox in humans, because the disease no longer exists. The ACVVR has pointed out that, under the circumstances, current regulatory requirements require the use of live variola virus in a non-human primate model for demonstration of efficacy. The AGIES recognizes that excellent surrogate animal models of infection with various OPVs are available (such as cynomolgous macaques, rabbits and rodents), and that these might obviate the need to use live VARV for drug and vaccine efficacy testing. From a public health perspective, the risks associated with the use of live VARV for *in vivo* animal studies on vaccines outweigh the benefits of these animal models over the models that rely on other orthopoxviruses in hosts mostly identified by naturally occurring infections. Thus, AGIES feels that the latter surrogate models may be useful for testing the efficacy of newer vaccines. Further, the AGIES recommends that, to assist the development of newer vaccines, researchers in the field and regulatory authorities should urgently discuss, and jointly arrive at a consensus on, acceptable surrogate markers of protection and the most appropriate surrogate model(s) for testing vaccines against variola virus infection.

In addition to known contraindications, the impact of immunodeficiency states (e.g. HIV infection, malnutrition and others) on the use of immunization with currently available smallpox vaccines remains unknown. From a public health perspective, prophylactic immunization of large populations is, therefore, unwarranted at present. In preparedness for a potential outbreak of smallpox, development of strategies for efficacious therapeutic immunizations, such as post-exposure vaccination or delivery of anti-vaccinia immunoglobulins and/or monoclonal antibodies, is also highly recommended. These approaches should help to shorten the response time of public health systems in case of an outbreak. In addition, passive immunization may ameliorate the adverse effects of the available vaccines.

Animal models and pathogenesis

It may be important and highly desirable to have an animal model in which VARV produces a disease similar to human smallpox. In this context, the AGIES noted the opinion expressed in the Scientific Review document that live variola virus is needed for cutting edge studies on pathogenesis of variola virus infection.

However, attempts at inoculation of VARV into various non-human primates have led to highly artificial models and outcomes that do not resemble human disease. Further, it appeared that the use of animal models of VARV was impractical, given the current stringent regulations for *in vivo* work using this virus. Therefore, AGIES felt that it may be more appropriate to focus on improving the animal models that use infection with other orthopoxviruses in their more natural hosts (e.g. monkeypox virus, cowpox virus, rabbitpox, mousepox virus infections in non-human primates, rabbits, and various rodent models), since these appear to be appropriate and adequate surrogates of human variola virus infection. These models not only allow studies on pathogenesis of poxvirus infection, but also allow for testing the effectiveness of vaccines and drugs against such infection, including determination of biomarkers associated with disease progression and/or protection, analysis of drug and vaccine efficacy, studies on drug pharmacokinetics and pharmacodynamics, and establishment of criteria to evaluate protection.

Although current non-human primate models using VARV are suboptimal, the amount of research conducted into developing them further, and success in achieving this goal over the last decade, has been rather limited. The only reason for attempting to develop such a model is to meet the current stringent regulatory requirements, in the absence of human variola virus infection. The AGIES's opinion was that a more productive approach would be for the regulatory requirements for vaccine and drug approval for variola virus infection to be reconsidered, given that human infection with the virus no longer occurs.

Drug development

The Scientific Review document provided to the AGIES argues that work with live VARV may be indispensable for the development and approval of antiviral drugs against smallpox. This argument appears reasonable, at least for *in vitro* testing. Until the use of appropriate animal models that utilize infection with alternative orthopoxviruses, as with surrogates, is generally agreed on and successfully implemented, the AGIES supports the development of macaque infection model using monkeypox virus (or other more recently published primate and possibly C57Bl mouse models) for *in vivo* efficacy testing of future candidate drugs.

The AGIES noted with concern that the only compelling scientific and public health reason to keep live variola stocks is to meet current restrictive regulatory requirements for vaccine and drug development. It, therefore, strongly recommends that researchers and regulatory authorities meet and jointly define future alternative models for testing vaccines and drugs against variola virus, in preparation for destruction of variola stocks. The group further feels

that, in the interim period, it may be possible to identify a limited number of strains of variola virus for such testing, and to retain only these for this limited use.

The AGIES notes that two promising drug candidates, acting by different mechanisms, are in an advanced stage of development, but no rational estimates are available on timelines for final regulatory drug approval for newly developed agents with anti-poxvirus activity (e.g. CMX001 or ST-246®). Efficacy testing in humans for drug approval against smallpox is not possible. Regulatory authorities have established new regulatory pathways for such indications in the form of the USFDA 'Animal Rule,'. However, to date, there is no experience with drug approval using the 'Animal Rule,' and there is uncertainty around how to implement it for the development of anti-smallpox drugs. Moreover, all currently available infection models with VARV in animals (non-human primates) must be considered suboptimal, since these do not replicate human disease, and are impractical to work with. Hence, investigators in the field of poxvirus research and regulatory authorities need to work together to arrive at an acceptable pathway or criteria to be followed for approval of such drugs. With the ultimate goal of eliminating variola stocks in mind, the AGIES would also like the experts to consider whether development of an *in vitro* model in which relevant variola genes are expressed in a heterologous system would provide an alternative to the use of variola virus.

The development of resistance has been described during *in vitro* passage experiments in the presence of each of these drugs; such passage experiments have led to the identification of molecular mechanisms of resistance to these drugs in orthopoxviruses. Although the likelihood of the development of resistance to these drugs following *in vivo* use is not known, the AGIES felt that it may be desirable to continue research into the development of additional drugs with activity against poxviruses that act by different mechanism(s) to those of the drugs in advanced stages of development.

The complete status of drug development is difficult to estimate, because some potentially promising compounds have not yet been sufficiently tested, and/or data on compound development have not been publicly released because of patent protection issues.

PART 3 . Ensuring high standards of security related to the re-emergence of smallpox

The re-emergence of smallpox may result from intentional or unintended release of natural or genetically engineered variola virus. As far as is known, live variola virus has not yet been created by genetic engineering. However, the tools to do so are available, another more complex micro-organism *(Mycoplasma mycoides)* has been created, and with continual rapid advances in technology the time required to engineer living organisms will decrease.

Currently, variola virus containment strategies include the fact that no laboratory in the world other than the two designated WHO collaborating centres is permitted to maintain stocks of live variola virus or more than 20% of the VARV genome, and genetic engineering of VARV is prohibited. In addition, any manipulation of VARV DNA must be geographically isolated from work involving the propagation of other poxviruses. However, the technology to genetically modify poxviruses is generally easily available, and could be used to circumvent the WHO recommendations that prohibit genetic engineering of VARV.

Regulatory requirements for new drugs and vaccines currently state that testing must include live variola virus. Furthermore, it is the opinion of AGIES that variola is currently the best target for *in vitro* testing of new drug candidates.

Monitoring containment policies

Stringent regular review of the quality assurance and containment practices at SRC VB VECTOR and CDC must be continued.

Genetically engineered VARV, mutants of VARV or poxviruses containing parts of the VARV genome

The WHO prohibition on genetic engineering of VARV is essential, but relies on individuals to comply. The AGIES, therefore, recommends that new strategies be designed to address the potential for *de novo* synthesis of live VARV, including adoption of national policies by WHO Member States. It is recommended that recent biosecurity proposals (Bügl et al. 2007)[1] be considered at national policy level.

The AGIES experts feel that WHO should seek an updated validation from all countries regarding their stocks of variola virus DNA (in various forms, such as fragments, amplicons and/or plasmids).

With respect to the current prohibition on laboratories other than the two WHO collaborating laboratories retaining more than 20% of the VARV genome, it is recommended

[1] Bügl H et al. (2007). DNA synthesis and biological security. *Nature Biotechnology*, 25:627–629.

that both CDC and SRC VB VECTOR provide documentation to WHO detailing which segments of DNA have been distributed to which laboratories. Advice should also be sought on the proportion of the variola virus genome covered by separate segments of <20% each already distributed to a range of laboratories, and whether the complete variola virus genome has already been distributed (or should be distributed) in this manner.

Appendix . Profiles of AGIES members

Professor Dr Rakesh Aggarwal, Lucknow, India

Professor Aggarwal obtained his M.D. at the All India Institute of Medical Sciences, New Delhi in 1986. He trained as a gastroenterologist-hepatologist, and also holds a Postgraduate degree in Epidemiology from the London School of Hygiene and Tropical Medicine. He is working as a Professor of Gastroenterology at the Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.

Professor Aggarwal has conducted extensive research on various aspects of hepatitis viruses, in particular the hepatitis E virus. This has included studies on epidemiological, clinical, laboratory and immunological aspects of this infection. He has also undertaken health economic analyses on the role of hepatitis B virus immunization.

His current duties and responsibilities include taking care of inpatients and outpatients with liver disease, teaching and supervision of gastroenterology-hepatology fellows, and running an active hepatitis research laboratory. He is Principal Investigator of several clinical and laboratory studies, primarily in the field of viral hepatitis and other liver diseases.

Dr Suleyman Al-Busaidy, Muscat, Oman

Dr Suleiman Al-Busaidy is an Omani microbiologist, currently holding the Directorship position of the Central Public Health Laboratory (CPHL) in the Ministry of Health, Muscat, Oman. As the Director of the Central Public Health Laboratories, his responsibilities include the provision of scientific and managerial leadership in developing, promoting and integrating public health laboratory services into practice, towards prevention and control of diseases. Under his leadership, the CPHL has attained WHO recognition as a Regional Reference Laboratory for Polio, Measles & Rubella, and Tuberculosis, as well as being the provider for the Eastern Mediterranean External Quality Assurance Scheme in Microbiology. Dr. Suleiman established the Virology Laboratory in Oman.

Dr Luciana Barros de Arruda, Rio de Janeiro, Brazil

Dr Luciana Barros de Arruda has undergraduate Bachelor's degree in Microbiology and Immunology from the Federal University of Rio de Janeiro (1997), Master's degree in Biological Sciences (Biophysics) from the Federal University of Rio de

Janeiro (1999), and a PhD in Life Sciences (Biophysics) from the Federal University of Rio de Janeiro (2003). She is an associate professor at the Department of Virology, Institute of Microbiology, at the Federal University of Rio de Janeiro (UFRJ). Her MsC was performed at UFRJ, in the Department of Immunology, where she studied the modulation of B lymphocytes response against the parasite *Trypanosoma cruzi*. Her PhD was performed at UFRJ and Johns Hopkins University (Baltimore, MD, USA), where she started to work on the development of genetic vaccine against HIV.

Currently, Luciana Barros de Arruda coordinates the laboratory of Genetic and Immunology of Viral Infections, at the Department of Virology, UFRJ. Her research group develops projects focusing on the immune response against arbovirus (Dengue and Sindbis virus) and HIV, including the study of humoral and cellular immune response in human and animal experimental models, and the development of antiviral vaccines.

Professor Zhihong Hu, Wuhan, People's Republic of China

Professor Zhihong Hu received her BSc degree (Virology and Molecular Biology) in 1986 from Wuhan University, China, and her MSc degree (Virology) in 1989 from Wuhan Institute of Virology, Chinese Academy of Sciences (CAS), afterwards becoming a staff member of the institute. She obtained her PhD degree (Virology) from Wageningen Agricultural University in 1998.

Since 1997, she has been Professor at Wuhan Institute of Virology (CAS), and was the Director General of the Institute from 2000 to 2008. Professor Zhihong Hu's research interest focuses on virology, especially on molecular biology and application of baculovirus. Since 2003, her research has extended to emerging infectious viral diseases, such as Severe Acute Respiratory Syndrome (SARS) and Crimean-Congo haemorrhagic fever (CCHF).

Professor Siripen Kalayanarooj, Bangkok, Thailand

Professor Siripen Kalayanarooj has an undergraduate Bachelor's degree in the field of Medical Science from Mahidol University, Bangkok (1975), and a Medical Doctorate with honors (1977) and Sub-Board in Paediatric Infectious Diseases (1983) from the University of California, Los Angeles, USA.

Since 1997, she has been the Director of the WHO Collaborating Centre for Case Management of Dengue, Dengue Hemorrhagic Fever (DHF) and Dengue Shock syndrome (DSS) at the Queen Sirikit National Institute of Child Health (QSNICH), previously known as the Children's Hospital in Bangkok, Thailand. She is also the QSNICH Chief of Infectious Diseases Unit (Since 2005) and Chairman of the Hospital Infectious Control Committee. QSNICH is a tertiary care/referral hospital under the Department of Medical Services at the Thai Ministry of Public Health, and is affiliated with the Faculty of Medicine of Rangsit University.

Professor Siripen Kalayanarooj's duties and responsibilities are the following:

- policy advocacy: acting as the team leader of the country responsible for the clinical management of dengue, DHF and DSS, in order to minimize the dengue case fatality rate;
- service: taking care of paediatric patients, both in and out patients, especially patients with infectious diseases and dengue; giving advice to all physicians, paramedical personnel, and the general population concerning vaccines and paediatric infectious diseases;
- teaching: supervising paediatric fellows in infectious diseases, residents and medical students, in general paediatrics and paediatric infectious diseases; giving lectures, providing consultations, and organizing the workshops for all physicians and paramedical personnel for national and international participants concerning dengue, DHF and DSS;
- research: acting as the Principle Investigator of the collaborative studies to conduct clinical and biomedical research regarding dengue.

Professor Dr Michael C Kew, Cape Town, South Africa

Professor Kew was Professor of Medicine at the University of Witwatersrand, Johannesburg from 1978 to 2009. He had been Director of the Medical Research Council – Molecular Hepatology Research Unit at the university. This unit documented the various causes of liver disease but particularly Hepatitis B virus infection and its role in causing liver cancer, which prompted the South Africa Department of Health to require all babies born in South Africa to be vaccinated against the Hepatitis B virus.

Currently, Professor Kew is Honorary Research Associate in the Department of Medicine at the University of Cape Town and at the Groote Schuur Hospital, Cape Town. He is Emeritus Professor and Honorary Research Professor at the University of Witwatersrand, Johannesburg.

Professor Rosemary Sang, Nairobi, Kenya

Dr Rosemary Sang is the Head of the Arbovirology & Hemorrhagic fevers unit of the Centre for Virus Research – a centre of the Kenya Medical Research Institute (KEMRI). She is an expert in Arbovirology with interests in arbovirus epidemiology, surveillance and response, and pathogen discovery. KEMRI is a State corporation established as the national body responsible for carrying out health research in Kenya. Dr Sang also serves as honorary lecturer in virology at the Institute of Tropical Medicine and Infectious Diseases (ITROMID) Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya.

Dr Rosemary Sang is responsible for KEMRI's Health Safety and Environment issues and she is the Chair to the Health, Safety and Environment Advisory Committee

(HSEAC). The HSEAC is charged with ensuring that safe work practices and adherence to established policies and guidelines are followed. Dr Sang is a member of the KEMRI Institutional Biosafety Committee (KEMRI-IBC). The KEMRI-IBC was established under the Biosafety Act, 2009, following NIH Guidelines for Research Involving Recombinant DNA Molecules and other applicable International Guidelines to provide local review and oversight of all forms of research utilizing recombinant DNA and safe handling of infectious biological materials.

Professor Tania Sorrell, Sydney, Australia

Tania Sorrell is the Director of the Sydney Emerging infections and Biosecurity Institute (SEIB), Director of the Centre for Infectious Diseases and Microbiology and Professor of Clinical Infectious Diseases at the University of Sydney, and a Senior Physician (previously Department Director) in Infectious Diseases at Westmead Hospital, Sydney.

She has longstanding interests in prevention, diagnosis and treatment of infectious diseases, especially in immunocompromised hosts, and in the emergence of resistant micro-organisms. Her research into the serious fungal infection, cryptococcosis, has provided new insights into host-microbial interactions and new drug development. She has developed new diagnostics for fungal diseases and is on international committees developing guidelines for antifungal therapy. She has authored more than 190 publications in refereed journals, 20 invited reviews, and 30 book chapters, and is on the Editorial Board of Clinical Infectious Diseases.

Professor Sorrell established clinical infectious diseases as a specialty within internal medicine in Australia, training or fostering the careers of many of the current national leaders in clinical infectious diseases and translational research in infectious diseases/microbiology.

She has served/serves on State and national advisory committees in infectious diseases, pandemic planning for influenza, approval of therapeutic agents, and both the Research and Human Ethics Committees of the National Health and Medical Research Council of Australia. She is a past president of the Australasian Society for Infectious Diseases.

Professor Dr Gerd Sutter, Munich, Germany

Professor Gerd Sutter is Full Professor and Chair for Virology at the Ludwig-Maximilians-Universität München. From 2003 to 2009, Professor Sutter headed the Division of Virology, Paul-Ehrlich-Institut, an institution of the Federal Republic of Germany reporting to the Federal Ministry of Health. From 1994 to 2003, he served as Research Group Leader at the National Research Center Helmholtz Zentrum München. From 1990 to 1993, Professor Sutter was a Post-doctoral Fellow at the Laboratory of Viral Diseases, National Institutes of Health, Bethesda, USA.

Professor Sutter is an awardee of the Bundesministerium für Bildung und Forschung (BMBF) on Infectious Disease Research (1990–1996). He has served as an expert to various international institutions, such as the National Institute of Allergy and Infectious Diseases (NIAID, NIH, USA), and WHO advisory committees, such as the WHO Initiative for Vaccine Research – Informal Consultation on Characteristics and Quality Aspects of Vaccines.

Professor Sutter's research interests include vaccine development with emphasis on the use of poxvirus vaccines and vectors and the prevention of zoonotic and emerging virus infections, and the study of (pox)viral modulation of the host immune system including evasion of innate and adaptive responses to infection.

Dr Stefan Wagener, Winnipeg, Canada

Dr Stefan Wagener is the Scientific Director for Biorisk Management at the National Microbiology Laboratory (Public Health Agency of Canada), in Winnipeg. He also serves as an advisor to the WHO and the Canadian Global Partnership Programme (Department of Foreign Affairs and International Trade Canada), programming and working in the Former Soviet Union countries, Asia and Africa.

After obtaining his MS and PhD in Germany, he joined Michigan State University working as a research scientist, before switching to the field of Occupational Health and Safety. At Michigan State University, he served as a biological hazard specialist to local and State emergency agencies for bioterrorism, and as a containment facility and biological safety specialist. From 2001 to 2006, he managed all operational and safety aspects of Canada's Level 4 facility, the Canadian Science Centre for Human and Animal Health in Winnipeg, Manitoba, as the Chief Administrative Officer for the Canadian Food Inspection Agency and Health Canada.

Currently, as the Scientific Director, he manages a comprehensive international scientific programme involving biorisk management, training and research, as well as developing new and advanced tools for biosafety and biosecurity based on the Assessment, Mitigation and Performance (AMP) model. Stefan Wagener is a Past-President of the American Biological Safety Association (ABSA) and was the Chair of the 2007 European Committee for Standardization (CEN) workshop developing the first international laboratory biorisk management standard: CWA 15793:2008. He currently chairs the CEN workshop 55, developing a Guidance Document for the CWA 15793:2008.