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WHO Advisory Committee on Variola Virus Research

Report of the second meeting

Geneva, Switzerland
15-16 February 2001

WORLD HEALTH ORGANIZATION
Department of Communicable Disease Surveillance and Response

Contents

Summary

The committee was assembled to evaluate the progress of research on variola virus. Analysis of the various areas of research shows that considerable progress has been made. These areas are: state of strain collections and viability studies, phylogenetic analysis using DNA amplification technologies, detection and differentiation of orthopoxvirus DNA, nucleotide sequence analysis of variola virus DNA, serological procedures for the detection of variola virus, antiviral agents and animal models of smallpox.

1. Introduction

1.1 Dr David Heymann, Executive Director, Communicable Diseases welcomed participants to the meeting and indicated that the main aims were to:

- review progress on the agreed programmes of research on variola viruses
- determine if the research was progressing at the pace necessary for the work to be completed before the planned destruction date in 2002
- identify if there were any significant gaps in the present research programme
- advise, as appropriate, on other possible directions of research.

1.2 Dr Heymann asked the Committee to keep in view the proposed destruction timetable for live virus stocks and the summary conclusions of the previous meeting of the Advisory Committee during their discussions. He also thanked the members of the scientific subcommittee for their help in reviewing the submitted research protocols from those engaged in smallpox virus studies.

1.3 Dr Robert Drillien was unanimously appointed Chairman and Dr Peter Greenaway was appointed Rapporteur. Meeting participants are listed in Annex 2.

1.4 Dr Drillien thanked WHO for organizing the meeting and encouraged broad discussion of the research findings that were to be presented. Following discussion, the initial agenda was modified to accommodate several unlisted presentations. The modified agenda is in Annex 3.

2. Working paper submitted by Drs Henderson and Fenner

2.1 A working paper submitted to the Committee by Drs Henderson and Fenner, who were unable to attend the meeting was presented. The Committee noted the critical views of the authors with respect to the usefulness of conducting further research on variola virus and their concern about respecting the destruction deadline.

3. State of strain collections and viability studies

3.1 It was noted that the Centers for Disease Control and Prevention (CDC), Atlanta, held 451 virus isolates derived from a number of different national collections. The majority of these were variola virus isolates and a database has been developed linking these with available diagnostic and epidemiological data. 49 strains, selected on the basis of geographical area and year of isolation and low passage history, were taken for further analysis. Of these, 45 were subsequently shown to be viable. These covered isolates from Asia (21), Africa (16), Europe (5), South America (2) and North America (1). Many of the viable isolates showed uniform plaque morphology and grew to high titre in tissue culture. 37/45 viable isolates were from *in vitro* cultured material; the remainder were from crust (non-passaged) samples.

3.2 Collection of the samples currently held at the State Research Center of Virology and Biotechnology ("VECTOR"), Koltsovo, Novosibirsk region, first began in Moscow in the mid 1950s. This collection was augmented with isolates obtained by the WHO Collaborating Centre in Moscow during the diagnostic studies that supported the smallpox eradication programme. The collection includes primary scab material, frozen liquid cultures and lyophilised samples. Not all samples in the collection have been tested for viability. 5 primary scab isolates, 4/9 frozen cultures and 6/6 lyophilised strains have demonstrable viability. Difficulties have been experienced in obtaining support for further work but funding is now anticipated.

3.3 Collaboration between staff at CDC and "VECTOR" has been initiated to ensure that any future work on virus characterization is adequately coordinated, including the transfer of reagents.

3.4 The Committee concluded that additional work may be needed to assess the viability of the stocks held in "VECTOR". There may also be benefit in undertaking further molecular characterization of additional strains. This would help to identify strains from which further DNA sequences could be determined.

4. Phylogenetic analysis using DNA amplification technologies

4.1 A number of PCR-based amplification technologies were described to facilitate the characterization and phylogenetic analysis of variola virus isolates. These included restriction enzyme fragment length polymorphism (RFLP) of PCR amplified products using a variety of primers and multiplex PCR analysis. As a general rule, primers in the central conserved genomic region were used for the comparison of all orthopoxviruses whereas those located towards the genomic termini were used to provide species and strain specific data.

4.2 RFLP analysis of some 20 amplicons that covered the entire variola virus genome was shown to be capable of discriminating between different orthopoxvirus species and different variola virus strains. Separation of DNA fragments on agarose

gels after either HincII, BstUI or HpaII digestion followed by analysis using pattern recognition software was used to develop phylogenetic maps of the different variola viruses and other orthopoxviruses analysed. These were used to demonstrate that camelpox virus was phylogenetically closer to variola virus than first thought.

4.3 Parameters such as position tolerance and optimization of the electrophoretically separated fragments were investigated to determine the reliability of this analytical procedure. Dendograms generated following bootstrap analyses of the data demonstrated that the variola virus minor strains could be separated from the major strains and that there was some sub-clustering of individual African and Asian isolates. It was felt that the technology had potential for gaining further insight into strain divergence and for grouping different isolates in a way that avoided direct DNA sequencing. It was also a procedure that could be applied using relatively small amounts of material and one that could be easily automated.

4.4 It was noted that this technology could potentially be used to trace any future outbreaks back to a particular source. This provided a further incentive to use this approach to characterize all existing isolates. However, members of the Committee drew attention to the fact that there would always be a necessity to correlate molecular findings with any clinical data obtained at initial disease presentation.

4.5 It was also noted that this technology could be used to analyse molecular differences that may occur as a result of initial adaptation to tissue culture or on subsequent passage. This had not been done in a rigorous manner but initial data indicated that there was a high degree of sequence conservation when moving from a primary isolate to a tissue culture adapted strain. Available sequence analyses have already identified some microheterogeneity but the significance of this has yet to be determined.

4.6 A related procedure involving multiplex PCR analysis was described for the species-specific differentiation of orthopoxviruses. This used the variable sequences present at the termini of orthopoxvirus genomes to facilitate species differentiation. The methodology had general applicability for diagnostic testing and could be configured to provide results within approximately 4 hours.

4.7 The Committee concluded that significant progress had been made in using PCR technology to investigate phylogenetic relationships between the orthopoxviruses, particularly variola viruses.

5. Detection and differentiation of orthopoxvirus DNA

5.1 A number of presentations described methodologies for the detection and subsequent diagnosis of orthopoxvirus infections using DNA amplification technologies. A major objective for this work is the real time identification of smallpox viruses. The basic procedures used are similar to those already described for the phylogenetic analyses of different variola virus isolates. The detection and

differentiation of orthopoxvirus strains and individual strains of variola virus generally involve the generation of PCR amplified products from both conserved and variable regions of the genome. Different groups have developed different platforms for the detection of amplified DNA products.

5.2 One of the detection procedures described involved the manufacture of 'Biochips' composed of polyacrylamide pads on a glass slide support. These 'Biochips' have a relatively long shelf life and can be manufactured by automated procedures. Specific polynucleotides, capable of differentiating orthopoxvirus species after hybridization, were incorporated into different polyacrylamide pads. Fluorescent-labelled PCR product was hybridized to the 'Biochips' and the intensity of fluorescence measured. The method was capable of differentiating different orthopoxvirus strains following analysis of the fluorescent patterns obtained. The procedure was capable of delivering results within several hours. The procedures have been validated using laboratory-based analysers and further work is being done to produce a portable analyser that could be used in field conditions. The Amplification Refractory Mutation System (ARMS) was described as another diagnostic procedure. This multiplex PCR uses a primer pair that amplifies a large conserved region of the orthopoxvirus genome and variola virus-specific primers that bind within this region to initiate amplification of a smaller product. The procedure is capable of differentiating orthopoxvirus species and is suitable for deployment using several different platform technologies.

5.3 Similar work was described using TaqMan PCR technology in which the evolution of a fluorescent signal was used for detection. The procedure is reasonably rapid and could be used in a high throughput format. The specificity of the test was 100% with the sensitivity being dependent on the concentration of DNA in the starting material. It was noted that the procedure had been validated against a panel of orthopoxviruses. The equipment used was presently laboratory-based but work to develop a portable analyser was in progress.

5.4 PCR methodologies, targetting individual orthopoxvirus genes, for the laboratory identification of orthopoxviruses (including variola virus) were presented. These methodologies have been published, and use restriction fragment length polymorphism as a specificity control.

5.5 The Committee noted that enormous progress had been made in this area. However, a major limitation of these procedures was the methodology for obtaining the initial DNA samples; some reliable and rapid procedures using commercially available reagents are becoming available. It was also noted that the specificity of the procedures was totally dependent on the sequences of the primers used for amplification. The detection of nucleotide sequences in cowpox virus that were previously considered to be variola virus specific, emphasized the point that the use of a single locus for PCR amplification would be insufficient to provide an unambiguous