Applied Virology

Editor

EDOUARD KURSTAK

Faculty of Medicine
University of Montreal
Montreal, Quebec, Canada

Associate Editors

WIDAD AL-NAKIB

Faculty of Medicine
Kuwait University
Safat, Kuwait

CHRISTINE KURSTAK

Hospital Hôtel-Dieu
University of Montreal
Montreal, Quebec, Canada

1984

ACADEMIC PRESS, INC.

(Harcourt Brace Jovanovich, Publishers)

Orlando San Diego New York London
Toronto Montreal Sydney Tokyo

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data

Main entry under title:

Applied virology.

Includes index.
1. Viral vaccines. 2. Virus diseases. I. Kurstak, Edouard.
QR189.5.V5A66 1984 616.9'2505 84-9272
ISBN 0-12-429601-7 (alk. paper)

PRINTED IN THE UNITED STATES OF AMERICA

84 85 86 87 9 8 7 6 5 4 3 2 1

Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

J. Abb (191, 365), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany

N. Ajjan (241), Mérieux Institute, 69223 Lyon, Cedex 1, France

C. Algan[1] (159), Regional Office for Africa, World Health Organization, Brazzaville, Congo (Popular Republic)

J. W. Almond (31), Department of Microbiology, University of Leicester, Leicester LE1 7RH, England

W. Al-Nakib (401), Department of Microbiology, Faculty of Medicine, Kuwait University, Safat, Kuwait

F. Assaad (131), Division of Communicable Diseases, World Health Organization, 1211 Geneva 27, Switzerland

Lorne A. Babiuk (349, 431), Department of Veterinary Microbiology, Western College of Veterinary Medicine, and Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada

J. E. Banatvala (221), Department of Virology, St. Thomas' Hospital and Medical School, London SE1 7EH, England

T. A. Bektimirov (131), Virus Diseases Unit, World Health Organization, 1211 Geneva 27, Switzerland

Jennifer M. Best (221), Department of Virology, St. Thomas' Hospital and Medical School, London SE1 7EH, England

R. Branche (241), Mérieux Institute, 69223 Lyon, Cedex 1, France

F. Brown (17), The Animal Virus Research Institute, Pirbright, Surrey GU24 0NQ, England

[1]Present address: WHO Program Coordinator, P.O. Box 362, Antananarivo, Madagascar.

A. J. Clayton (123), Laboratory Centre for Disease Control, Ottawa, Ontario K1A 0L2, Canada
F. Deinhardt (191, 365), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany
D. M. A. Evans (31), Division of Viral Products, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England
Morag Ferguson (31), Divison of Viral Products, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England
A. Paul Fiddian (103), Department of Clinical Immunology and Chemotherapy, Clinical and Applied Research Division, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, England
T. H. Flewett (343), Regional Virus Laboratory, East Birmingham Hospital, Birmingham B9 5ST, England
P. Fournier (241), Mérieux Institute, 69223 Lyon, Cedex 1, France
G. J. Galasso[2] (87), Development and Applications Branch, Microbiology and Infectious Diseases Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205
Henrik Garoff (51), European Molecular Biology Laboratory, 6900 Heidelberg, Federal Republic of Germany
V. Gauss-Müller (365), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany
P. D. Griffiths (401), Virus Laboratory, Royal Free Hospital and School of Medicine, Hampstead, London NW3 2QG, England
Colin R. Howard (171, 327), Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London WC1E 7HT, England
W. Jilg (191), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany
Leevi Kääriäinen (51), Recombinant DNA Laboratory, University of Helsinki, 00380 Helsinki, Finland
C. Kurstak (479), Laboratory of Virology, Hospital Hôtel-Dieu de Montreal, University of Montreal, Montreal, Quebec H2W 1T8, Canada
E. Kurstak (479), Comparative Virology Research Group, Department of

[2]Present address: Office of Extramural Research and Training, National Institutes of Health, Bethesda, Maryland 20205.

Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Quebec H3C 3J7, Canada

Kenneth Lundström (51), Recombinant DNA Laboratory, University of Helsinki, and Research Laboratory of State Alcohol Company, 00100 Helsinki, Finland

Raymond G. Marusyk (211), Neurovirology Research Unit, Department of Medical Microbiology, Faculty of Medicine, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

P. D. Minor (31), Division of Viral Products, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England

Thomas P. Monath (377), Division of Vector-Borne Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, U.S. Public Health Service, Department of Health and Human Services, Fort Collins, Colorado 80522

B. Montagnon (241), Mérieux Institute, 69223 Lyon, Cedex 1, France

F. A. Murphy (17), Division of Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Bruce L. Nicholson (445), Department of Microbiology, University of Maine at Orono, Orono, Maine 04469

Erling Norrby (3), Department of Virology, School of Medicine, Karolinska Institute, SBL, S-10521 Stockholm, Sweden

Siobhan O'Shea (221), Department of Virology, St. Thomas' Hospital and Medical School, London SE1 7EH, England

J. F. Obijeski (17), Genentech, Inc., South San Francisco, California 94080

Nils Oker-Blom[3] (51), Department of Virology, University of Helsinki, 00290 Helsinki, Finland

Ilkka Palva (51), Recombinant DNA Laboratory, University of Helsinki, 00380 Helsinki, Finland

Thomas W. F. Pay (73), Foot-and-Mouth Disease Vaccine Division, Wellcome Biotechnology Ltd., Pirbright, Surrey GU24 0NQ, England

Ralf F. Pettersson (51), Recombinant DNA Laboratory, University of Helsinki, 00380 Helsinki, Finland

Alfred M. Prince (199), Laboratory of Virology, The Lindsley F. Kimball Research Institute of the New York Blood Center, New York, New York 10021

M. Roggendorf (191, 365), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany

[3]Present address: Office of the Chancellor, University of Helsinki, 00170 Helsinki, Finland.

M. Roumiantzeff (241), Mérieux Institute, 69223 Lyon, Cedex 1, France
R. Scheid (365), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany
G. C. Schild (31), Division of Viral Products, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England
Robert E. Shope (231), Department of Epidemiology and Public Health, Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, Connecticut 06510
M. Spitz (31), Immunology Section, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England
W. Stephan (63), Biotest Pharma GmbH, D-6000 Frankfurt am Main, Federal Republic of Germany
P. Tijssen (479), Comparative Virology Research Group, Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Quebec H3C 3J7, Canada
P. Trotemann (241), Mérieux Institute, 69223 Lyon, Cedex 1, France
G. van der Groen (299), Institute of Tropical Medicine, B-2000 Antwerp, Belgium
M. H. V. Van Regenmortel (463), Institute of Molecular and Cellular Biology, 67000 Strasbourg, France
J. C. Vincent-Falquet (241), Mérieux Institute, 69223 Lyon, Cedex 1, France
K. von der Helm (365), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany
P. R. Young (327), Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London WC1E 7HT, England
R. Zachoval (191), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany
G. Zoulek (191), Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, 8000 Munich 2, Federal Republic of Germany
Arie J. Zuckerman (171), Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London WC1E 7HT, England

Preface

The chief aim of "Applied Virology" is to discuss the practical applications of recent developments in basic virology, not only to virology but to other disciplines as well, and to demonstrate the impact of virus diseases on the environment, economy, and the health of man, animals, and plants. The chapters in this volume, written by well-known experts, provide a large body of practical information on the modern strategy used to produce virus vaccines, on antiviral chemical compounds, on simple, rapid, and specific diagnostic techniques, and on epidemiology in relation to the prevention and control of virus diseases. "Applied Virology" reflects the current tendency to produce safe and efficacious vaccines by genetic engineering technologies and considers their laboratory study and field applications. Noninfectious, synthetized peptides used as safe virus vaccines are reviewed with special attention to their immunogenicity, multispecificity, and usefulness in case of epidemics. A distinct but equally essential step for the study of epidemics and the control of diseases in relation to the variation among viruses is the rapid, sensitive, and reliable diagnosis of virus diseases. The applications of advanced technologies for this purpose are also discussed.

It is our hope that "Applied Virology" will be of use to all concerned with virus diseases, e.g., hospitals, veterinary clinics, infectious disease control centers, plant protection institutes, departments of agriculture and public health, and those who endeavor to improve the quality of life and environment by using advanced diagnostic and disease control technologies. It will be an aid to all virologists, and will fill the needs of those working in developing countries, in which virus diseases continue to be an unresolved health, economic, and environmental problem.

Virus diseases remain a worldwide problem, contributing to human disability and mortality and causing severe economic losses by affecting livestock and crops in all countries. Even with the preventive measures taken in the United States, losses due to viral diseases exceed billions of dollars annually. These costs are substantially higher in Africa, Asia, the Middle East,

and South and Central America, where virus diseases hamper the development of several countries.

I wish to express my sincere gratitude to the contributors for the effort and care with which they have prepared their chapters, to the Faculty of Medicine of University of Montreal and the Faculty of Medicine of Kuwait University for the help provided, and the staff of Academic Press for their part in the production of this volume.

Edouard Kurstak

Introduction

Biblical writings, ancient scripts, and archeological findings testify to the length of time virus diseases have scourged humanity. For example, examination of the 3000-year-old mummy Pharaoh Ramses V reveals pockmarks suggesting that he may have contracted smallpox, whereas Rhases, a Persian physician who lived about 1000 years ago, described how smallpox spread from Africa, where it was endemic, to Europe and the Middle East during the first millenium AD (Becker, 1983). A commemorative carving in stone, dating from the eighteenth Egyptian dynasty (1580–1350 BC), shows a young man with a withered and shortened leg typical of poliomyelitis virus paralysis (Melnick, 1984).

Virus diseases continue to contribute significantly to human morbidity or mortality and to cause severe economic loss by affecting livestock and crops, particularly in developing countries. For example, an estimated 200 million persons are chronically infected with hepatitis B virus, one million children are estimated to die annually from measles, five million persons are killed by acute gastroenteritis (mainly rotaviruses) annually, and the list goes on and on (Kurstak and Marusyk, 1984). The economic losses can be staggering (exceeding one trillion dollars in the United States alone). Those due to animal or plant viruses are equally enormous, and the diseases caused are a major factor in chronic malnutrition in many parts of the world. Reduction of crops by 75–90% by plant viruses occurs frequently, causing disruption of the fragile nutritional balance for large populations. Plant pathologists are at a disadvantage in protecting crops from virus infections since immunization is not possible. The main traditional strategies used to protect crops are cultivation measures such as starting with virus-free material, removal of vectors or diseased material, and restriction of epidemics through, e.g., crop rotation and plant or culture spacing. Chemotherapy has hitherto been impossible or impractical in this area, but new advances in this field may be most exciting in the area of plant virology.

Virology, as a science, has passed through a relatively long descriptive

phase, a shorter but explosive expansion phase over the last two decades in which advanced techniques in biochemistry, immunology, molecular biology, genetics, etc., provided deeper insights in the molecular structure, functioning, and reproduction of viruses, to a new phase: applied virology. Applied virology is a direct result of several breakthroughs in molecular biology, immunology, and diagnostic procedures. Wildy (1984) distinguished virologists, i.e., those who want to learn more about viruses ("second-phase virologists"), from "viropractors" ("third-phase virologists") whose purpose is to exploit viruses as tools. The latter are becoming very important in disparate areas such as genetic engineering, development of vaccines and diagnostic procedures, and insect control, and this group may, as Wildy (1984) pointed out, be growing faster than that of "pur sang" virologists.

Parallel with the development of virology, attempts have been made to combat virus diseases. A milestone in this respect was the large-scale production of poliovirus in cell cultures and its use, after formaldehyde inactivation, as a vaccine in the early 1950s by J. Salk. The subsequent discovery that the virus thus produced was sometimes contaminated with the undesirable SV40 virus (latent in cells), which can transform cells *in vitro,* necessitated the application of more rigorous control measures. Though inactivated viruses are efficient vaccines in cases in which viremia are an essential part of the disease, attenuated live viruses have been preferred to provide local immunity. Live vaccines have been established primarily by adaptation of the virus in question to an unnatural host. The attenuated virus may contain a large number of mutations, e.g., 35 for poliovirus type 1 vaccine (Nomato *et al.,* 1981). The attenuated virus may revert to the virulent strain again (e.g., 1 per 3×10^6 doses of live polio vaccine; CDC, 1982). Though in developed countries this vaccine has been widely used, it is less used in developing countries since it interferes with other enteric infections.

Attenuated live-virus vaccines are widely preferred, but they also carry significant risks. They may revert back to wild type or revert to novel strains with an unpredictable host range. Moreover a live-virus vaccine may be attenuated for one host but not for another, as exemplified with several attenuated live rabies vaccines. Emergence of novel strains is occurring at a higher rate than generally assumed and could potentially be very hazardous. Too often it is assumed that viruses have a certain constant host range and that each virus exhibits a distinct and unique antigenicity. Canine parvovirus for example demonstrates the fallacy of these "laws." In the Spring of 1978 articles appeared in both the lay and scientific press concerning the rapid spread of canine parvovirus, simultaneously in different regions, which caused a fulminant enteritis of high morbidity and mortality (up to 80%) in

dogs of all ages and myocarditis in young puppies. This virus not only caused a syndrome resembling the panleukopenia–enteritis syndrome in felines and mink (Kurstak and Tijssen, 1981), but the virus proved to be very closely related to the mink parvovirus (Tratschin *et al.*, 1982). This sudden host reversion of the virus from mink to dogs raises important questions with respect to the cause of this reversion, the possible reversion to man, and measures which can be taken to decrease the chances of such reversions. It should be stressed that the mink parvovirus is serologically undistinguishable from the feline panleukopenia parvovirus and that the mink parvovirus (mink enteritis virus) had an equally sudden outbreak in 1947 and spread from Canada rapidly around the world. The possibility that the canine parvovirus derives from the mink parvovirus must, therefore, be considered seriously. Adaptation could be a result of attempts to achieve attenuation of wild-type mink parvovirus. Retrospective analysis of sera revealed that the appearance of antibodies to this adapted virus in canine sera coincided with the disease, except in Belgium where some sera collected in 1976 and 1977 contained antibody (Schwers *et al.*, 1979). It is, however, difficult to understand how the virus could be present for two years without being detected since it is very pathogenic.

New horizons for the production of safe vaccines have been opened by new genetic engineering technologies. Genetic engineering techniques may be used to produce stable deletion mutants instead of point mutation strains which lose thereby a certain biological function but are still able to replicate in certain cells (Jones and Shenk, 1979). There is, nevertheless, a tendency to shift from whole-virus vaccines to vaccines of subunits or synthetic polypeptides. Despite an early view that the feasibility of the development of synthetic vaccines would be rather limited (Arnon, 1980), this approach has gained support in recent years. Current DNA technology also provides the tools to produce hybrid vaccines (e.g., recombinants of hepatitis B and vaccinia genes). Bacterial plasmids may be used as cloning vehicles for DNA and RNA viruses. Though these methods are still primarily used for the analysis of individual genes and the determination of their nucleotide sequences, cloning will have important applications with respect to the use of a single or a few viral gene(s) for the production of vaccines.

Another exciting avenue is the production of antiidiotype antibodies using hybridoma techniques. Monoclonal antibodies with an antiidiotype structure mimicking the crucial epitope on the virus seem to have great potential for immunization and protection of the individual from a virus without vaccination with the virus, one of its products, or synthetic epitopes of the virus (Kennedy *et al.*, 1984; Koprowski *et al.*, 1984).

In addition to the development of genetic engineering systems and

hybridoma techniques to develop vaccines and the synthesis of small peptides (epitopes) on a large scale, novel delivery systems and immunoenhancers are being developed.

These new technologies will not only have a tremendous impact on the knowledge, development, and quality of new viral reagents and vaccines but will also decrease very significantly the cost of the control of virus diseases. For example, it can be expected that vaccines which now cost more than $100, and therefore cannot be applied to control epidemics or endemics satisfactorily, can be produced for, e.g., 1% of the original cost, and are more effective than the original products. The worldwide excitement about these prospects hardly needs to be stressed.

It can also be seen that the comprehensive knowledge available on virus replication allows rational approaches to the design of antiviral drugs. Developments in this area will have important consequences, e.g., for the prophylaxis of viral diseases in plants. The number of antiviral drugs which have become available is still rather limited despite extensive efforts. The main problem is to find compounds which selectively inhibit the virus without having toxic effects on cells. Hitherto, research on antiviral drugs has been concentrated on a relatively few viruses (herpes, influenza), but it is expected that this number will increase rapidly due to recent advances in the understanding of virus replication and the development of sophisticated techniques. Though different stages of virus reproduction may be blocked, the stage of viral nucleic acid synthesis is most commonly chosen since the differences between viral enzymes and cellular enzymes may be exploited.

Breakthroughs in methodologies to diagnose virus diseases have been particularly prominent in the last few years and are expected to have a major impact on applied virology. The most important advances are due to the development of the hybridoma technique and the replacement of radiomarkers on antibodies or antigens by enzymes. Traditionally, radioimmunoassays have been used on a large scale, but important disadvantages hampered their transfer to less sophisticated laboratories or to developing countries. For example, expensive counters are needed and the radioactive reagents have generally short shelf lives. Moreover, the hazards of radioactive labels and the sophistication of the procedures and equipment restrict their use to well-equipped central laboratories in which large numbers of routine assays are performed. The enzyme immunoassays which found their inception in virology in 1969 (Kurstak *et al.*, 1969) are equally sensitive, are simple, and are performed with relatively very stable reagents. The costs are low so that these techniques are applicable anywhere. The rapid development of very specific reagents by the hybridoma technique will accelerate even more the already explosive expansion of this field.

The transfer of these new biotechnologies within developed countries and

to developing countries presents an enormous challenge and requires, in addition to suitable support, adjustments in education and infrastructure in order to prevent the establishment of heavy dependence on profit-oriented large companies or countries.

The new era of applied virology allows us to benefit from decades of fundamental and molecular research in biochemistry, immunology, and genetics of viruses. Without a doubt, applied virology has enormously increased the potential for the control of virus diseases. The establishment of adequate programs and the designation of necessary resources at national and international levels are required to achieve these goals.

Edouard Kurstak

REFERENCES

Arnon, R. (1980). *Annu. Rev. Microbiol.* **34,** 593–618.

Becker, Y. (1983). "Molecular Virology." Martinus Nijhoff, The Hague.

Centers for Disease Control (1982). *Morbidity and Mortality Weekly Report*, Jan. 29.

Jones, N., and Shenk, T. (1979). *Cell* **17,** 683–689.

Kennedy, R. C., Melnick, J. L., and Dreesman, G. R. (1984). *Science* **223,** 930–934.

Koprowski, H., Herlyn, D., Lubeck, M., DeFreitas, E., and Sears, H. F. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81,** 216–219.

Kurstak, E., Côté, J. R., and Belloncik, S. (1969). *C.R. Acad. Sci., Paris* **268,** 2309–2312.

Kurstak, E., and Tijssen, P. (1981). *In* "Comparative Diagnosis of Virus Diseases" (E. Kurstak and C. Kurstak, eds.), Vol. 3, pp. 3–65. Academic Press, New York.

Kurstak, E., and Marusyk, R. G. (1984). "Control of Virus Diseases." Dekker, New York.

Melnick, J. L. (1984). *In* "Karger Gazette," Issue 46–47, pp. 1–5.

Nomato, A., Kitamura, N., Lee, J. J., Rothberg, P. G., Imura, N., and Wimmer, E. (1981). *Virology* **112,** 217–227.

Schwers, A., Pastoret, P. P., Burtonboy, G., and Thiry, E. (1979). *Ann. Méd. Vét.* **123,** 561–570.

Tratschin, J. D., McMaster, G., Kronauer, G., and Siegl, G. (1982). *J. Gen. Virol.* **61,** 33–39.

Wildy, P. (1984). *In* "The Microbe 1984" (B. W. J. Mahy and J. R. Pattison, eds.), p. 1. Cambridge Univ. Press, Cambridge.

1

Strategy for Virus Vaccine Development

ERLING NORRBY

Department of Virology, School of Medicine, Karolinska Institute, Stockholm, Sweden

I. INTRODUCTION

The introduction of vaccines against different viral diseases has led to some of the major advances in modern biomedicine. Smallpox has been eradicated worldwide and poliomyelitis has become a rare disease in industrialized countries. Childhood diseases such as measles, rubella, and mumps are now less prevalent in many industrialized countries. Although these achievements are impressive, much remains to be done. There is a need to improve already existing vaccines and to develop new kinds of vaccines. The latter category should include those viral vaccines that have not been developed to date because the viruses encompass too many different serotypes or cannot replicate effectively in available *in vitro* systems.

The history of the development of viral vaccines reflects the emergence of methods for *in vitro* propagation of viruses and their biochemical characterization. Originally, animals were used for preparation of live vaccines, but later, propagation of viruses in embryonated hen's eggs allowed the production of both live and killed vaccines. The major breakthrough occurred when the technique for dispersion of cells from organs and establishment of monolayer tissue cultures was introduced. Several inactivated and live vaccines, e.g., those against poliomyelitis, were rapidly developed.

ISBN 0-12-429601-7

In the last decade further development of vaccines has relied mostly on the introduction of new genetic and biochemical techniques. Methods have been developed for isolation of structural surface proteins of viruses. Hybrid DNA technology presents possibilities for synthesis of selected virus structural components in bacteria, for manipulation of viral genomes to establish genetically more stable live vaccines, and for introduction of extraneous genes into genomes of large viruses. The development of the hybridoma technique has increased the possibility of defining the antigenic epitopes on the surface of virions, which are of primary importance for induction of immunity against disease. Monoclonal antibodies also can be used for purification of viral vaccine antigens by immunoadsorption techniques. The most recent development concerns the use of synthetic peptides representing only a small selected fraction of a virus polypeptide (6–20 amino acids) as immunogens.

II. CHOICE OF VIRAL VACCINES

The selection and use of available vaccines are determined by a wide range of different factors. Industrialized and developing countries have different priorities because of variations in the medical importance of viral diseases and in the available resources for production or importation and distribution of vaccines. The choice between live and killed vaccines has frequently led to intense debates.

In a somewhat simplified way it can be stated that inactivated vaccines are to be preferred over live vaccines because they provide better medical safety, since there is no replication of virus in the body. However, the usefulness of an inactivated vaccine obviously is dependent on whether it can provide the proper immunity. From a general point of view it appears that inactivated vaccines may provide efficient immunity in situations in which viremia is an essential part of the pathogenetic process, e.g., in poliomyelitis and hepatitis.

It has been argued that live vaccines are markedly superior to inactivated vaccines in providing local immunity. However, this is only partly true, because it has been shown, for example, that inactivated polio vaccine used under optimal conditions not only provides a protection against viremia but also restricts local replication of virus in the gut. In the case of live vaccines, the capacity of the attenuated virus to spread from the infected individual to others may pose problems. This does not occur with measles and mumps vaccines, but in the case of live polio vaccine, dissemination of virus does occur. It has been emphasized that such a spread may assist in providing

an extensive vaccine coverage but the repeated cycles of replication of the genetically unstable virus may occasionally cause harmful effects.

III. LIVE VIRUS VACCINES

Several different forms of live virus vaccines can be categorized (Table I). Most live vaccines have been established by adaptation of a particular virus to an unnatural host. For example, passaging in monkey kidney cell cultures was used to attenuate poliovirus strains. A comparison of genomes of attenuated and virulent virus strains has shown that vaccine virus accumulates a large number of mutations. Thus, type 1 poliovirus vaccine strain was found to have undergone 35 base changes compared to the wild virus strain from which it was derived (Nomato *et al.*, 1981). This observation emphasizes the problem in predicting genetic changes which are required to render a virus avirulent. We have almost no knowledge about the genetic basis for the attenuation of the currently used live vaccines. Instead this development is based on what Chanock refers to as a "genetic roulette" (Chanock, 1983).

The live polio vaccines have been used extensively, leading to a massive reduction in the occurrence of poliomyelitis particularly in industrialized countries (Nightengale, 1977). However, a certain price has to be paid in connection with the use of this vaccine because of the lack of genetic stability of the vaccine viruses. Emergence of virulent virus may cause both vaccine-induced and vaccine-associated paralytic cases. In the United States it has been found that polio immunization is associated with paralytic poliomyelitis in one case per 3.2 million doses of live vaccine distributed (Centers for Disease Control, 1982a). From 1969 to 1980, 92 cases of paralytic

TABLE I
Categories of Live Virus Vaccines

Vaccine	Category and/or example
Wild virus	Restricted pathogenesis, adenovirus vaccine in enteric coated capsules
Attenuated virus	Naturally occurring variant; e.g., vaccinia virus
	Adaptation to unnatural host cells; e.g., mumps virus
	Reassortment (recombination) between virulent and attenuated virus; e.g., influenza virus, rotavirus (virions with segmented genomes)
	General mutagenesis and selection; experimental
	Specific mutagenesis; experimental

poliomyelitis associated with vaccination were reported. Twenty-five cases occurred in otherwise healthy vaccine recipients, 55 cases occurred in healthy close contacts of recipients, and 12 cases developed in individuals (recipients or contacts) who had an immune deficiency condition. In developing countries the replication of attenuated poliovirus frequently is restricted due to interference by other enteric infections. Regrettably it is difficult to use the live vaccine effectively in these countries. Therefore attempts are being made to use the inactivated polio vaccine or a combination of inactivated and live vaccine.

The use of live measles, rubella, and mumps vaccines has increased in many parts of the world. The live measles vaccine has been extensively used in the United States and has lead to a virtual extinction during 1982 of indigenous measles (Centers for Disease Control, 1982b). The small number of cases which now occur in the United States are due to infection imported from other countries.

One problem in the use of live measles vaccines in developing countries is the thermolability of the freeze-dried product. Thus, in the absence of an effective cold chain under primitive field conditions the vaccine is readily inactivated. The availability of more thermostable vaccine (Heymann *et al.*, 1982) will improve this situation.

In addition to the problem of effective distribution of the live measles vaccine in developing countries, the age of the recipient of the vaccination poses a dilemma. Since measles virus causes severe infections during the first year of life in these countries, it has been recommended that vaccination should be performed at 6–9 months of age. Due to the presence of immunity of maternal origin in many vaccine recipients at this age, the frequency of vaccine takes is markedly reduced. As a consequence a second vaccination has to be given at the age of 1½ years. The effect of this two-dose scheme needs to be further analyzed. In one study, an altered immune response after a previous active/passive immunization has been suggested (Wilkins and Wehrle, 1979). In countries where extensive vaccination occurs, levels of maternal antibodies may become reduced in the future as circulating vaccine-induced antibodies gradually substitute for antibodies deriving from virulent measles. As this occurs the age for vaccination may have to be lowered in industrialized countries as well.

Other live virus vaccines obtained by adaptation of the virus to unnatural host systems are now at different stages of development. These include live vaccines against varicella (Arbeter *et al.*, 1982; Neff *et al.*, 1981), cytomegalovirus (CMV) infections (Starr *et al.*, 1981), and type A hepatitis (Provost *et al.*, 1982). One problem associated with the two herpesvirus vaccines is the fact that the vaccine virus can establish latent infections, which may become activated at a later date. This may be acceptable if it

can be well documented from clinical-virological experiences that the main complications with these virus infections correlate to the primary and not to the activated latent infection. In the case of varicella one would therefore have to require documentation showing that the vaccine virus does not provide a source for future development of herpes zoster as frequently as after infections with wild virus. In the case of cytomegalovirus, one would have to base the use of a live vaccine on a documented lower frequency of activated latent infections vs primary infections in such situations as pregnancy and in immune-suppressed individuals receiving organ transplants. The development of a live vaccine against hepatitis A has become feasible by the successful isolation of the virus in selected tissue culture systems. Repeated passaging of a virus isolate in such a cell system has led to an attenuation of its virulence for marmosets.

In the case of viruses which have segmented genomes, such as reoviruses and orthomyxoviruses, the genetic technique of reassortment facilitates the establishment of attenuated virus strains. A particular problem in the case of the medically important rotaviruses has long been their fastidious nature. However, recently successful propagation of the three serotypes of human rotaviruses in cell cultures was described (Urasawa *et al.,* 1981), which may lead to new approaches to vaccine production. Previously, attempts have been made to establish combined infections in human cells from temperature-sensitive mutants of bovine rotaviruses, which have a capacity to replicate under *in vitro* conditions, and from noncultivatable human rotaviruses (Chanock, 1981). Selection of the derived viral reassortments was achieved by exposing progeny from the mixed infection to a potent bovine rotavirus antiserum which neutralized this virus but not the human rotavirus. The surviving virus was then isolated in tissue culture at a restrictive temperature. This procedure has produced viral reassortments which could grow to high titer in human tissue cultures and which were specifically neutralized by antiserum to the human rotavirus serotype 1. Different procedures are now available to develop both live and inactivated rotavirus vaccines.

The technique of reassortment has been used extensively in the case of influenza A viruses (Chanock, 1981, 1982). The most recent approach was to characterize the capacity of a set of isolates of avian influenza viruses to grow in primate cells *in vivo* and to select strains which had a restricted replicating capacity (Murphy *et al.,* 1982b). Such strains might then be used in reassortment experiments together with virulent human influenza A virus strains. The aim was to isolate a vaccine virus having surface antigen properties of the human influenza A virus, but with an extensively genetically restricted capacity to replicate in human cells. Since the avian influenza parent of this hybrid is distinct in many genes from its human counterpart, one can hope that the variants established show a high degree of genetic

stability with regard to their restricted capacity to propagate in human cells. The technique of reassortment has also been used previously in combination between virulent human influenza A virus strains and genetically modified human influenza A strains. Both chemically induced temperature-sensitive and cold-adapted genetically modified strains (Chanock and Murphy, 1980) have been employed. Products of this kind are still at the experimental stage and so far no completely genetically stable influenza A virus recombinants have been defined. However, there is hope for the successful development of an effective live influenza vaccine (Wright *et al.*, 1982a). The use of such a vaccine for local application should lead to a much more efficient local immunity than the currently employed inactivated vaccines (Murphy *et al.*, 1982a).

The method of general mutagenesis and selection has also been used to identify attenuated strains of other viruses. Attempts have been made to prepare a temperature-sensitive respiratory syncytial (RS) virus vaccine. So far the virus strains isolated have shown selected characteristics in tissue culture systems and in animals. However, when the strains were administered to young children a reversion to a certain degree of virulence was encountered (Wright *et al.*, 1976, 1982b). In view of the pronounced medical importance of RS virus infections in young children it is to be hoped that future attempts to produce a vaccine that can protect against these infections will be successful.

As an alternative to general mutagenesis as a mechanism for establishing stable attenuated strains of viruses, the method of directed mutagenesis, preferably by introduction of deletions, offers interesting possibilities. This technique has become available as a consequence of the development of hybrid DNA technology and gene-sequencing methods. The application of this technique requires that the viral genome be in a DNA form, but this does not mean that it is restricted to DNA viruses. Experimental studies have been made with genomes from DNA viruses such as papova- and adenoviruses. In the adenovirus system, deletion mutants without transforming activity but with full capacity to replicate in certain cells could be established (Jones and Shenk, 1979). Deletion mutants with altered host range or replication capacity would be of considerable interest not only in developing other transforming viruses, but also in ensuring that an attenuated virus cannot regain virulence. As mentioned, directed mutagenesis can also be applied to RNA viruses, provided these viruses can be transcribed into a cDNA copy by use of reverse transcriptase. Recently, the entire genome of poliovirus in a cDNA form was established, and it is of particular interest that this DNA was shown to have infectious properties (Racaniello and Baltimore, 1981). Possibilities now exist for the generation of deletion mutants with stable properties of attenuation.

Hybrid DNA technology might also be applied in a different context. Extraneous DNA can be introduced into the genome of large DNA viruses. In one study (Smith *et al.*, 1983), the gene that directs the synthesis of hepatitis B virus surface antigen was recombined into vaccinia virus. The infected cells synthesized not only vaccinia virus but also hepatitis B virus surface antigen. In a way this represents a combination of *li*ve and *ki*lled ("like"?) vaccine, but the inactive component of this vaccine is special in that it is processed in a cell as if it were part of a replicating virus. This may have advantages with regard to the presentation of the antigen to the immune system. The potential use of this kind of approach should be further studied and animal virus vectors other than vaccinia virus might be considered.

IV. CATEGORIES OF VACCINES CONTAINING NONREPLICATING ANTIGENS

Three different categories of inactivated vaccines can be determined: whole virus products, subunit vaccines, and synthetic polypeptides (Table II).

The *whole virus products* include vaccines against influenza and poliomyelitis as well as more regionally used vaccines against rabies, tick-borne encephalitis, Japanese B encephalitis, and Rift Valley fever.

The whole virus inactivated influenza vaccine is prepared from egg-grown material and has been available for several decades. There are three major problems connected with the use of this vaccine. First, the formalin-inactivated product is given parenterally, thereby inducing circulating, humoral immunity. As a consequence the efficacy in terms of local immunity is less pronounced. Second, the continued changes in the virus in terms of antigenic drift and occasional antigenic shifts demand that strains used in the

TABLE II
Categories of Inactivated Vaccines

Vaccine	Examples
Whole virus products	Rabies, influenza, polio
Subunit vaccines	Naturally occurring components; hepatitis B virus
	Split virus; e.g., influenza
	Hybrid DNA synthesis; e.g., hepatitis B virus (experimental)
Synthetic polypeptides	Foot-and-mouth disease virus (experimental)

vaccine be selected as close to the currently circulating strains as possible. Third, repeated use of the vaccine gradually gives less effective immunity. This is due to the "original antigenic sin" which infers that the antibody response upon repeated immunization becomes more and more directed against the antigen which was originally presented to the individual than that which is present in the most recently administered vaccine. A disadvantageous effect of yearly revaccinations has been documented (Hoskins *et al.*, 1979).

The inactivated whole virus polio vaccine has been shown to provide excellent protection against disease and to restrict the circulation of poliovirus in the community by induction of a local mucosal immunity (Böttiger *et al.*, 1979). However, only a few countries have retained an immunization program based exclusively on this vaccine. Since the 1960s most countries have shifted instead to the live vaccine. Sweden is one of the countries that has retained an immunization program based on inactivated vaccine. By use of four properly spaced injections of a product of good potency a lifelong immunity appears to be established (Böttiger and Fagraeus, 1980). One problem with the inactivated polio vaccine concerns the cells to be used as a substrate for preparation of antigen. Primary monkey kidney cells do not represent a satisfactory source of cells, and in addition monkeys have become increasingly more difficult to obtain. Certain countries such as the Netherlands have established local breeding colonies of monkeys in order to provide materials sufficient for the national production of vaccines. Attempts have been made to exchange the primary monkey kidney cells for human diploid cells and also human lymphoblastoid cells lines, but the yield of virus in these systems is relatively poor. The solution to this problem appears to be the use of established nontumorigenic epithelioid cell lines. Previously negative attitudes toward the use of such cell lines have prevailed (cf. Hilleman, 1973) and there has been a fear that possibly tumorigenic genetic material could be transferred together with the vaccine antigen. However, recently accumulated knowledge concerning mechanisms of tumorigenesis and also the development of chemical techniques which allow the preparation of a product devoid of contaminating genetically active nucleic acids have changed this view. The World Health Organization now accepts the use of nontumorigenic cell lines for vaccine production with certain provisions. The cells used, e.g., Vero cells, must not be tumorigenic in nude mice and must not show any chromosomal abberations. After purification the final vaccine product should not contain nucleic acids exceeding a defined size. An inactivated poliovaccine prepared in Vero cells was recently licensed for use in France.

Most of the currently used inactivated rabies vaccine is prepared in a human diploid cell line. This vaccine provides good immunity but regret-

tably the virus yield from cells is poor, making the vaccine expensive. Attempts are now being made to produce the rabies vaccine in a nontumorigenic established cell line (Vero cells).

The preparation of *subunit vaccines* has attracted interest, particularly in cases of different enveloped viruses. However, capsid components from naked viruses such as foot-and-mouth disease virus (FMDV) and adenoviruses might also be used as subunit vaccines. Two different kinds of subunit vaccines from lipid-containing viruses have been prepared. One is the recently introduced inactivated hepatitis B vaccine (Hilleman, 1982). This is a unique vaccine in that the antigen material comes from individuals carrying hepatitis B virus infections. Circulating subvirion hepatitis B virus surface antigen is isolated from serum material and is subsequently chemically inactivated. This kind of antigen preparation has been shown to induce humoral immunity and to protect against subsequent exposure to hepatitis B virus. The duration of the immunity has not yet been established, although it appears to last for several years. Current discussions concern the selection of the target groups on which to use this vaccine. Usage has to be selective, first, because the vaccine is expensive, and second, because the product should be applied only in cases in which increased risk for infection has been clearly established. The latter situation prevails only under conditions of repeated, more than minimal exposure to possibly contaminated blood.

The second kind of subunit vaccine is the influenza vaccine prepared by dissociation of the virus envelope and isolation of its two peplomers, hemagglutinin and the neuraminidase. This kind of split vaccine has been shown to give less local reactions than the whole virus products and as a consequence larger amounts of antigen can be administered. The product therefore can be used in younger individuals although it is questionable whether this is an important target group since influenza usually is rather mild in younger children. The efficacy of immunization with a split virus product does not exceed that of the whole virus vaccine, and the previously discussed problem of decreasing efficacy of immunization upon repeated vaccinations remains.

Attempts are being made to develop split vaccines of other viruses. In the case of herpesviruses the use of envelope preparations is attractive, since such products would allow the exclusion of genetic material with a potential oncogenic capacity. Some experimental batches of vaccines have been prepared and tested in limited field trials (Cappel *et al.*, 1982; Klein *et al.*, 1981).

Data have been obtained on the production and use of inactivated whole virus measles vaccines and also to some extent on the use of subunit measles vaccines. The previously used vaccines did not provide satisfactory im-

munity but instead set the stage for development of immune pathological reactions leading to the occurrence of atypical measles. This was due to the fact that the inactivation procedures employed destroyed the antigenicity of one of the surface antigen components, the fusion component (Norrby *et al.*, 1975). It should be noted that the antihemagglutinin response induced by the vaccine could effectively neutralize the virus in laboratory tests. However, *in vivo,* there was an additional requirement for antibodies against the fusion component, presumably to prevent cell-to-cell spread of the virus. Formalin-inactivated mumps virus vaccine was also shown to lack immunogenic hemolysin (Norrby and Penttinen, 1978), but no cases of atypical mumps have been identified. Phenomena of a similar nature may also be relevant to the accentuated disease seen after immunization with formalin-inactivated RS virus (Kim *et al.*, 1969). It is therefore important that in current attempts to produce subunit vaccines of paramyxoviruses the antigenicity of both the fusion factor and the hemagglutinin are retained. However, it is also essential to give the fusion component a certain advantage in the immunization process, since it has been shown that immunization with inactivated whole virus retaining immunogenic F components induces the production of an antibody response primarily against hemagglutinin (E. Norrby, unpublished results). The efficient antibody response against the fusion component in connection with wild virus or live vaccine virus infection may be a result of the exposure of this component on the surface of infected cells. In order to achieve a matching immunity when an inactivated vaccine is used, one has to prepare peplomer aggregates containing a relatively increased proportion of fusion-to-hemagglutinin components compared to that of the envelope of intact virus. Recent findings indicate that such a product can induce both an efficient antihemagglutinin and anti-fusion component of humoral immunity (P. Osterhaus, personal communication).

Another avenue to production of subunit vaccines is the use of hybrid DNA technology. Isolated viral genes responsible for the production of surface antigen components have been cloned in *Escherichia coli.* These include the VP1 (previously VP3) protein of FMDV (Kleid *et al.*, 1981), the hepatitis B virus surface antigen (Edman *et al.*, 1981), the rabies virus G protein (Yelverton *et al.*, 1983), and the hemagglutinin of influenza virus (Lai *et al.*, 1980). The production of nonglycosylated virus-specific proteins has been demonstrated and these proteins have been shown to induce humoral immunity in experimental animals. These kinds of preparations obviously offer interesting possibilities for future vaccine production.

The feasibility of production of synthetic vaccines has been debated for years (cf. Arnon, 1980), with the general consensus that the possibility for development of this kind of product would be rather limited. This view was

based on the observations that denaturation of a protein by physical means and cleavage of a protein by proteases usually lead to a loss of antigenic properties and immunogenic activity. There were some experiments, however, showing that a C-terminal hexapeptide of tobacco mosaic virus protein coupled to bovine serum albumin induced the production of antibodies which could both precipitate and neutralize the virus. Furthermore, in work with phage MS2, two synthetic peptides representing parts of the coat protein of the virus were shown to stimulate the production of antibodies capable of binding to the virus (Langbeheim *et al.*, 1976). However, the full potential of immunization with *synthetic polypeptides* could not be realized until the genetic information provided by the combined use of hybrid DNA technology and nucleic acid sequencing methods allowing prediction of the amino acid sequence of viral proteins became available (cf. Lerner, 1982; Sutcliffe *et al.*, 1983). During the past few years peptides corresponding to parts of the influenza HA1 protein (Green *et al.*, 1982), the HB_s antigen (Lerner *et al.*, 1981), and the FMDV VP1 protein (Bittle *et al.*, 1982) have been studied. In both the influenza and FMDV system, short peptides representing the whole polypeptide were used for immunization. Only a few of the peptides induced production of neutralizing antibodies. The synthetic polypeptides of FMDV which induced neutralizing antibodies were shown to protect against experimental disease. It was found that the synthetic polypeptides should be larger than 6 amino acids and that a suitable size was 12–20 amino acids to allow an effective induction of production of antibodies. Preparation of longer polypeptides did not improve their antigenicity; instead, it has become apparent that the immunizing potency of short polypeptides is based on the fact that they are less folding dependent than the intact protein or larger fragments of the protein.

The use of synthetic peptides as immunogens offers a number of advantages. The antibodies produced have a predetermined specificity, and in the case of viruses, they can be selected to represent structures on the surface of virions. However, the relative importance of different surface virion structures in inducing immunity has to be evaluated separately. The synthetic polypeptides also are interesting in that they can induce antibody responses not seen under normal conditions. For example, it has been found that in the influenza hemagglutinin there are five major antigenic regions and that under conditions of natural infection the immune response includes antibodies only to these regions. With synthetic polypeptides, an immune response against other regions of the hemagglutinin polypeptide can be generated, and these antibodies have been found to be capable of neutralizing the virus. There are also indications that this immune response may have a broader reactivity than antibodies induced by the whole hemagglutinin.

Because the synthetic peptides themselves have poor immunogenicity, they have to be combined with substances that provide an adjuvant effect. Coupling of the peptides to carrier proteins such as albumin or keyhole limpet hemocyanin and use of an adjuvant have been tried.

V. CONCLUSIONS

Many viral diseases have been effectively prevented by use of either live or inactivated vaccines, and in the case of smallpox global eradication of disease has been achieved. Similar eradication might be possible for other mono- (or oligo-)typic viruses which are transmitted as a chain of acute infections and for which there is no nonhuman reservoir of practical importance (Report on the international conference, 1982). This is the situation for viruses such as poliomyelitis and measles. Some of the procedures used for vaccine production need to be modified. In selected cases it may be advantageous to exchange primary cells for nontumorigenic heteroploid cells in virus propagation, e.g., in the case of inactivated polio vaccine.

Methodology introduced during the last decade, i.e., hybrid DNA technology, establishment of hybridomas producing virus-specific antibodies, and immunization with synthetic peptides, offers exciting possibilities for production of vaccines under completely new conditions. Genetically unstable live vaccine virus may be rendered more stable by directed deletion mutagenesis. Inactivated vaccines may be produced without any propagation of the infectious agent. Attempts should also be made to prepare vaccines against polytypic viruses. The use of isolated gene products or short synthetic polypeptides should markedly facilitate the whole process of controlling vaccine products and, in particular, allow the induction of an efficient immunity under conditions of maximal safety. Also, the costs for vaccine production may be reduced by use of these procedures, so that vaccines could be made available to countries with more limited financial resources.

REFERENCES

Arbeter, A. M., Starr, S. E., Weibel, R. E., and Plotkin, S. A. (1982). *J. Pediatr. (St. Louis)* **100,** 886–893.

Arnon, R. (1980). *Annu. Rev. Microbiol.* **34,** 593–618.

Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G., Lerner, R. A., Rowlands, D. J., and Brown, F. (1982). *Nature (London)* **298,** 30–33.

Böttiger, M., and Fagraeus, A. (1980). *Rev. Infect. Dis.* **2,** 274–279.

Böttiger, M., Mellin, P., Romanus, V., Söderström, H., Wesslén, T., and von Zeipel, G. (1979). *Bull. W. H. O.* **57**, 99–103.

Cappel, R., Sprecher, S., Richaert, F., and de Cuyper, F. (1982). *Arch. Virol.* **73**, 61–67.

Centers for Disease Control (1982a). *Morbidity and Mortality Weekly Report* Jan. 29.

Centers for Disease Control (1982b). *Measles Surveillance Report,* **No. 11**, CDC, Atlanta, *Georgia.*

Chanock, R. M. (1981). *J. Infect. Dis.* **143**, 364–374.

Chanock, R. M. (1983). *In* "Viral Diseases in South-East Asia and the Western Pacific" (J. S. MacKenzie ed.). Academic Press, New York.

Chanock, R. M., and Murphy, B. R. (1980). *Rev. Infect. Dis.* **2**, 421–432.

Edman, C. J., Hallewell, R. A., Valenzuela, P., Goodman, H. M., and Rutter, W. J. (1981). *Nature (London)* **291**, 503–506.

Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T. M., Sutcliffe, J. G., and Lerner, R. A. (1982). *Cell* **28**, 477–487.

Heymann, D. L., Smith, E. L., Nakano, J. H., Jato, J. G., Martin, G. E., and Maben, G. K. (1982). *Br. Med. J.* **285**, 531–534.

Hilleman, M. R. (1973). *In* "Biohazards in Biological Research" (A. Hillman, M.-N. Oxman, and B. Pollack, eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hilleman, M. R. (1982). *S. Afr. Med. J.* **61**, 691–698.

Hoskins, T. W., Davies, J. R., Smith, A. J., Miller, C. L., and Allchin, A. (1979). *Lancet* **1**, 33–35.

Jones, N., and Shenk, T. (1979). *Cell* **17**, 683–689.

Kim, H. W., Canchola, J. G., Brandt, C. D., Pyles, G., Chanock, R. M., Jensen, K., and Parrott, R. H. (1969). *Am. J. Epidemiol.* **89**, 422–434.

Kleid, D. G., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., Grubman, M. J., McKercher, P. D., Morgan, D. O., Robertson, B. H., and Bachrach, H. L. (1981). *Science (Washington, D.C.)* **214**, 1125–1129.

Klein, R., Ruivovici-Klein, E., Moser, H., Monoka, R., and Hilfenhaus, J. (1981). *Arch. Virol.* **68**, 73–80.

Lai, C., Markoff, L., Zimmerman, S., Cohen, B., Berndt, J., and Chanock, R. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 210–214.

Langbeheim, H., Arnon, R., and Sela, M. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4636–4640.

Lerner, R. A. (1982). *Nature (London)* **299**, 592–596.

Lerner, R. A., Cores, N., Alexander, H., Lin, F. T., Sutcliffe, J. G., and Shinnick, T. M. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3403–3408.

Murphy, B. R., Nelson, D. L., Wright, P. F., Tierney, E. L., Phelan, M. A., and Chanock, R. M. (1982a). *Infect. Immun.* **36**, 1102–1108.

Murphy, B. R., Hinshaw, V. S., Sly, D. L., London, W. T., Hosier, N. T., Wood, F. T., Webster, R. G., and Chanock, R. M. (1982b). *Infect. Immun.* **37**, 1119–1126.

Neff, B. J., Weibel, R. E., Villarejos, V. M., Buynak, A. A. B., McLean, H. D., Morton, S. B., and Hilleman, W. M. R. (1981). *Proc. Soc. Exp. Biol. Med.* **166**, 339–347.

Nightengale, E. O. (1977). *N. Engl. J. Med.* **297**, 249–253.

Nomato, A., Kitamura, N., Lee, J. J., Rothberg, P. G., Imura, N., and Wimmer, E. (1981). *Virology* **112**, 217–227.

Norrby, E., and Penttinen, K. (1978). *J. Infect. Dis.* **139**, 672–676.

Norrby, E., Enders-Ruckle, G., and ter Meulen, V. (1975). *J. Infect. Dis.* **132**, 262–269.

Provost, P. J., Banker, F. S., Giesa, P. A., McAleer, W. J., Buynak, E. B., and Hilleman M. R. (1982). *Proc. Soc. Exp. Biol. Med.* **170**, 8–14.

Racaniello, W. R., and Baltimore, D. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4887–4891.

Report on the international conference on the eradication of infectious diseases. (1982). *Rev. Infect. Dis.* **4**, 912–984.

Smith, G. L., Mackett, M., and Moss, B. (1983). *Nature (London)* **302**, 490–495.

Starr, S. E., Glazer, J. P., Friedman, H. M., Farquhar, J. D., and Plotkin, S. A. (1981). *J. Infect. Dis.* **143**, 585–589.

Sutcliffe, J. G., Shinnick, T. M., Green, N., and Lerner, R. A. (1983). *Science* **219**, 660–666.

Urasawa, T., Urasawa, S., and Taniguchi, K. (1981). *Microbiol. Immunol.* **25**, 1025–1035.

Wilkins, J., and Wehrle, P. F. (1979). *J. Pediatr. (St. Louis)* **94**, 865–869.

Wright, P. F., Shinozaki, T., Fleet, W., Sell, S. H., Thompson, J., and Karzon, D. T. (1976). *J. Petiatr. (St. Louis)* **88**, 931–936.

Wright, P. F., Okabe, N., McKee, K. J., Maasab, H. F., and Karzon, D. F. (1982a). *J. Infect. Dis.* **146**, 71–79.

Wright, P. F., Belshe, R. B., Kim, H. W., Van-Voris, L. P., and Chanock, R. M. (1982b). *Infect. Immun.* **37**, 397–400.

Yelverton, E., Norton, S., Obijeski, J. F., and Goeddel, D. V. (1983). *Science* **219**, 614–620.

2

Genetic Engineering Technology in Vaccine Production and Control of Animal Virus Diseases

F. A. MURPHY,* F. BROWN,† AND J. F. OBIJESKI‡

*Division of Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Altanta, Georgia

†The Animal Virus Research Institute, Pirbright, Surrey, England

‡Genentech, Inc., South San Francisco, California

I. INTRODUCTION

Of all the promises of the biotechnological revolution none offers more immediate application for improving the welfare of mankind than the genetic engineering and chemical synthesis of vaccines for controlling the diseases of food- and fiber-producing animals. Microbial and viral diseases are still responsible for the largest proportion of livestock production loss in developed and developing countries. International prohibitions to the shipping of animals and animal products (established because of the risk of exotic disease importation) lower the value of agricultural production, including production of animal feed crops. Livestock loss from infectious diseases is also a major influence on human nutritional status. In addition, zoonotic animal diseases represent an important direct threat to human health (BOSTID, 1982).

Much has been done to control many animal diseases. Chemotherapy,

ISBN 0-12-429601-7

antibiotics, and anthelmintics, vector control, quarantine, test-and-slaughter programs, and vaccines have reduced disease incidence and economic loss. All of these approaches are expensive, and all place a burden on limited scientific expertise and disease control resources. In some circumstances cost-to-benefit considerations result in dilemma. For example, the cost of a pesticide for vector control may be so high that the livestock producer may face ruin either from its purchase or from the loss caused by unchecked vector-borne disease. This kind of economic dilemma may be associated with other conventional approaches to disease control, such as incomplete indemnification in test-and-slaughter programs. Genetic engineering now offers possibilities for increasing vaccine potency, safety, and stability and especially for decreasing vaccine costs. It seems clear that the cost-to-benefit equations for control programs for many infectious diseases will change for the good.

Virus disease vaccines deserve special emphasis when the potentials of genetic engineering are considered. There are several reasons.

1. Many animal viruses have explosive epizootic patterns of spread, representing great potential for economic loss over large areas. The spread of African horsesickness virus into the Middle East and Indian subcontinent from its enzootic niche in East Africa in 1959–1960 is exemplary—the economic loss and associated human suffering caused by this single epizootic was enormous. In this case, appropriate use of vaccine, i.e., immediate delivery from stored reserves of large numbers of doses of stable vaccine, might have stopped this epizootic.

2. Many viruses have physical properties which allow them to survive the same sanitary measures used to control most bacterial pathogens. Virion stability can result in reemergence, reintroduction, and transport of viruses irrespective of quarantine regulations. The spread of African swine fever into the Iberian peninsula, Haiti, the Dominican Republic, and Brazil from its enzootic niche in Central Africa is exemplary—the economic loss and associated human suffering caused by this movement of the virus has also been enormous, and the entrenchment of the virus in its new habitats promises continuing losses. Vaccine development for this disease promises to be especially difficult, but if successful could reverse this entrenchment.

3. Many animal viruses are capable of entering a persistent infection cycle with long-term continuous or intermittent shedding. In most instances such infection cycles, once established, cannot be interrupted by vaccination or other means. The consequence is that such viruses are present continuously in the environment of each new generation of animals. An example is the great economic loss caused by each of the important herpesviruses of animals. Infectious bovine rhinotracheitis, equine rhinopneumonitis, and many more herpesvirus diseases represent difficult control problems which might

be solved by development of vaccines suitable for use in young animals, before primary infection initiates persistence.

From these few examples, it seems clear that control of virus disease is a particularly worthy focus for application of genetically engineered vaccines. This conclusion is reinforced by some general characteristics of viruses themselves: most important in this regard is the relative simplicity of the antigenic determinants of viruses and their similarity in related viruses. This accounts for the potency of very small dosages of virus vaccine and for the practicality of combining antigens into polyvalent vaccines. For example, the extraordinary efficacy of the attenuated live-virus rinderpest vaccine in cattle depends on the invariable, stable antigenicity of the virus, and the ability to customize polyvalent bluetongue vaccines to match the prevalence of particular serotypes in a given area is aided by the methodologic repetitiveness of vaccine production.

Conventional vaccine development has become rather static in the past few years; many of the very good vaccines have been in use for more than 10 years, and the poorer vaccines have not been improved. This is the case even though technological breakthroughs are applied quickly and with good effect in the vaccine industry. Four research approaches lie at the heart of the needed biotechnological revolution in vaccine development (BOSTID, 1982; Bachrach, 1981). (1) Recombinant DNA technology must be developed and adapted to large-scale systems. (2) Peptide synthesis must be developed and adapted to large-scale systems. (3) Improved adjuvants, immunoenhancers, and novel delivery systems (e.g., liposomes, slow-release carrier compounds, mechanical slow-release devices) must be developed and brought into general use. (4) Attenuated live-virus vaccines with antigenicity and safety characteristics assured by controlled deletion mutations must be designed. This is an exciting new research area which should not be overshadowed by recombinant DNA technology. Cell-mediated immunity is the principal host defense against many virus infections, and small virus proteins such as those produced by recombinant DNA technology or chemical synthesis methods do not (even with adjuvants) evoke very good cell-mediated immune responses. It is hoped that this problem will be overcome, but in case it is not, research emphasis must be directed now to this new field of mutation-controlled animal vaccine engineering. The same argument for a comprehensive, balanced research effort for the genetic engineering of human disease vaccines is being voiced—it will take very good synthetic vaccines to improve upon the present measles, rubella, or mumps attenuated live-virus vaccines.

Recent breakthroughs made with two important viral vaccines—rabies vaccine and foot-and-mouth disease vaccine—are used in this chapter to illustrate the present fast-moving state of development of genetic engineer-

ing technology. Parallel research on adjuvants and vaccine delivery systems does not seem to be keeping up. Perhaps the latter is dampened by the conservative position which must be taken with regard to the use of adjuvants with human vaccines. However, questions appropriate in human medicine, such as adjuvant exacerbation of immunopathologic diseases, are not as important in short-lived livestock species. A review of the acceptable characteristics of animal vaccine adjuvants by an international body, such as the FAO, would be of value. Such a review would clarify the limits to acceptable reactogenicity and would lead to common international standards.

II. GENETIC ENGINEERING OF RABIES VACCINE

Rabies is an ancient scourge which must still be regarded as a very important disease of man and animals (Murphy, 1983a; Shope, this volume). The human disease can be measured in terms of mortality statistics in many parts of the world, but it must be measured also in terms of individual suffering and public hysteria. The animal disease can be measured in terms of costs of control, loss of livestock production, and impact on fragile wildlife populations. In some parts of the world the incidence of human rabies can be taken as an index of a massive underlying animal (usually dog) transmission cycle. In other areas the incidence of livestock rabies can be taken as an index of a massive underlying wild animal (including bat and free-roaming dog) transmission cycle. In many of these areas the virus is entrenched and is unreachable by conventional means. Immunization of man and domestic animals plays a major role in limiting the consequences of human exposure and in limiting livestock losses. Immunization is used in control programs along with control of animal movement (stray animal control), surveillance, laboratory diagnosis, public education, and wildlife control (Baer, 1983).

Immunization of man, primarily done in postexposure settings, has little effect on the transmission cycle of the virus, but given the usual circumstances of exposure, the provision of safe and efficacious vaccine should be considered a public responsibility. For many years human rabies vaccines changed little from those developed by Pasteur; they were not potent enough and caused neurological damage. Later, vaccines such as duck embryo vaccine, developed to avoid neurological damage, suffered from lack of potency. The first great breakthrough in increasing potency safely came with the Wistar Institute human diploid cell (HDC) vaccine; this vaccine is now produced primarily by the Institut Mérieux (95% of world production). Because of the time, labor, and materials needed for its production, this vac-

cine is extremely expensive; the current five-dose postexposure treatment regimen costs more than 250 U.S. dollars (despite this, the Institut Mérieux sells a million doses per year and there is an apparent demand for another half-million doses). This cost, which is more than the mean per captia expenditure for the entire health sector in many countries, represents a cruel dilemma. How is the public health officer to advise people exposed to rabies? Is he to say, "There is a very good vaccine, but we cannot afford it"?

The resolution of this dilemma lies in the production of a human rabies vaccine that is potent, safe, and also inexpensive. Improvement in each of these three vaccine qualities represents an important challenge. Potency has been a problem compounded by very conservative requirements for certified cell culture substrates for human vaccine development and production. It is not likely that cells such as BHK-21 (or other hamster cells) will ever be allowed as substrate for human vaccines, even inactivated vaccines, in the United States, but certifiable cells must continue to be developed and tested for rabies virus yield characteristics if whole virus immunogens are ever to be improved. Safety has been a problem compounded by "live virus in inactivated products and inactive virus in attenuated live-virus products." Safety assurances require infrastructure and professional oversight. Cost control overrides all other considerations. Low cost must be designed into the research approach to vaccine development, and this must be complemented by manufacturing and distribution economies.

Immunization of animals, always done prophylactically, can be carried out with the aim of preventing economic loss (e.g., cattle immunization), reducing risk of human exposure (e.g., urban dog immunization), or interrupting the transmission cycle (e.g., urban dog immunization, wildlife immunization). Where the aim is to prevent economic loss, immunization must be safe, efficacious, and inexpensive. Some animal vaccines grown in cell cultures, e.g., inactivated and attentuated live-virus vaccines produced in BHK-21 cells, nearly meet these demands, but again, cost remains a problem. A vaccine meeting all demands would fill a great need, especially in Central and South America, where cattle rabies is a major problem (Baer, 1983; Murphy, 1983b).

Where the aim is to reduce human exposure or interrupt wildlife transmission cycles, there are common principles and approaches. In each case, immunization of the reservoir host population can better stop virus shedding and transmission than can animal removal. Animal removal, whether brought about by the usual slow epizootic of lethal rabies infections or by animal-killing programs, leaves the econiche, whether urban or wildlife, to be filled by a new generation of susceptibles (Baer *et al.*, 1971; Steck *et al.*, 1978). The approach of distributing vaccine-laden bait, as used to immunize foxes in Switzerland, can be adapted to reach other wildlife species in dif-

ferent ecosystems (e.g., skunks in Ontario, Canada) and can even be adapted to reach free-roaming urban dog populations (e.g., dogs in cities on the United States–Mexico border) (Baer, 1983). The same approach could simplify the immunization of pet dogs and cats (in a similar way to the Sabin polio vaccine delivery system).

All these wildlife immunization schemes presume the availability of a safe vaccine which is potent when administered orally (Murphy, 1983b). Conventional inactivated vaccines, even when concentrated, have proved to be worthless when administered orally (Baer *et al.,* 1971). Attenuated live-virus vaccines represent, in the authors' view, an untoward risk—risk of reversion to wild type or risk of emergence of novel, unpredictable infection cycles in species which consume the bait or in species coming in contact with the virus by indirect means. There is no guarantee that a vaccine virus developed for use in a given species will be attenuated for all species which might be exposed; in fact, there are many examples in the rabies vaccine literature of species-specific attenuation. The circumstances of wildlife or free-roaming dog immunization programs offer little possibility for recalling a faulted vaccine. If, as seems to be the case, the lack of immunogenicity of noninfectious rabies vaccines when delivered orally has been partly due to insufficient antigen content, then the high concentrations of antigen which can be produced biotechnologically might solve the problem. Certainly, such concentrated antigens must be tested as oral vaccines as soon as they become available in necessary volumes. If successful, and if usable in wildlife and free-roaming dog immunization programs, it is conceivable that rabies might be eradicated from some areas of the world.

At the time of this writing the gene encoding the rabies virus glycoprotein has been cloned by two research groups (a group from the Wistar Institute, Philadelphia, Pennsylvania, and Transgene Ltd., Strasbourg, France; and a group from Genentech Inc., San Francisco, and the Centers for Disease Control, Atlanta, Georgia) (Anilionis *et al.,* 1981; *Genetic Engineering Letter,* 1982; Yelverton *et al.,* 1983a,b).

The strategy of the work done at Genentech Inc./Centers for Disease Control has been as follows (Yelverton *et al.,* 1983a,b): (1) From polyadenylated mRNAs obtained from rabies [challenge virus standard (CVS) strain] infected cells, double-stranded cDNA species were synthesized using reverse transcriptase and DNA polymerase. (2) The cDNAs were annealed into the bacterial cloning vector ("plasmid") pBR322, and the hybrid plasmids were then used to transform *Escherichia coli* cultures. (3) A library of transformed *E. coli* colonies was screened by hybridization of plasmid DNAs with native rabies G mRNA. Candidate plasmids potentially bearing cloned rabies cDNA were confirmed by the technique of "hybrid arrest of translation," and one, pRab91, was chosen for expression. This clone con-

sisted of >2000 base pairs encoding all of the mature rabies glycoprotein; it was sequenced, and the amino acid sequence of its product was deduced from the cDNA nucleotide sequence. (4) For expression of the pRab91 clone in *E. coli,* it was annealed into an "expression vector" containing (a) an RNA polymerase binding site—the promoter, (b) a control element for the amount of mRNA produced—the operator, (c) a sequence for directing protein synthesis—the ribosome binding sequence, and (d) a codon for the beginning of translation of mRNA into protein—the initiation codon (all derived from the *E. coli* tryptophan operon). The resulting plasmid, pRabGtrp1, was used to transform *E. coli,* and large cultures were produced. (5)Whole-cell extracts of the transformed *E. coli* cultures were examined for the presence of viral protein; a protein of molecular weight (MW) 62,000 was identified as an analog of the viral glycoprotein. (6) Because the MW 62,000 protein was extremely insoluble (typical of highly hydrophobic viral glycoproteins), a plasmid was constructed containing a truncated glycoprotein cDNA sequence. This plasmid encoded a rabies glycoprotein analog lacking the most hydrophobic transmembrane end of the native protein (MW 49,000).

The two bioengineered proteins (MW 62,000 and 49,000) were shown to be reactive with antiserum raised against the native rabies glycoprotein. Most of the antigenic determinants of the native protein seemed to be conserved in the *E. coli*-produced analogs. The worry that posttranslational processing (such as carbohydrate side-chain attachment) would be needed for expression of antigenic determinants does not seem to have been justified. Protection of animals from rabies challenge with a vaccine made from these analog proteins has not been tried yet, but experiments are in progress to determine whether the bacterially produced protein will be as effective in eliciting neutralizing antibody as the native glycoprotein (Dietzschold *et al.,* 1978, 1979).

The matter of virus glycoprotein insolubility poses problems in vaccine formulation. The potency of a vaccine depends on maximum availability to the immune triggering system of the antigenic determinants, but insoluble glycoprotein complexes bury many of the determinants. The means used to solubilize the bioengineered rabies glycoprotein analogs for preliminary testing are not suitable for use in vaccines. This problem, and others, has led to consideration of an alternative immunogen, that is, a chemically synthesized small polypeptide with the same amino acid sequence as the key antigenic determinant site on the whole viral protein. Such a peptide would be designed by deduction, using the nucleotide sequence of the glycoprotein cDNA. Such a peptide could be produced on a large scale inexpensively; the peptide would be soluble, and when conjugated to a carrier it could be a potent immunogen.

The first experiments toward this goal were reported by Sutcliffe and colleagues (1983); 18 peptides, representing over half of the rabies glycoprotein amino acid sequence, were synthesized, and antibodies were raised against each of these. Although each antiserum bound native rabies glycoprotein, none was capable of neutralizing virus (cell culture neutralization test) or protecting mice or dogs against challenge. It was concluded that the key epitopic sites on the rabies glycoprotein were not represented in the group of 18 peptides, and so more peptides, representing likely regions of the glycoprotein, are now being synthesized. This kind of progress is being made even though key antigenic determinant sites on the rabies glycoprotein have not yet been identified and characterized chemically. The great success of this approach obtained with foot-and-mouth disease virus, as described in Section III, suggests that this may be the ultimate solution to human and animal rabies vaccine problems (Sutcliffe *et al.*, 1983; Dietzschold *et al.*, 1983).

III. GENETIC ENGINEERING OF FOOT-AND-MOUTH DISEASE VACCINE

Foot-and-mouth disease (FMD) is certainly the most important virus disease of the world's livestock industries. The seven immunologically distinct serotypes and more than 60 subtypes of the virus cause economic loss directly as a result of morbidity and mortality in affected animal populations, and indirectly as a result of constraint of international trade in animals and animal products (Brooksby, 1982). In FMD-free countries, such as the United States, costs relate to large disease control agencies that are maintained ready to implement an eradication scheme should the virus be introduced. In countries which are usually FMD-free, such as the United Kingdom, infrequent introductions have led to costly large-scale slaughter of livestock as part of eradication protocols. In these FMD-free countries, vaccine stockpiling is part of the control strategy—expensive insurance for use if eradication fails. In countries where the virus is enzootic or is frequently introduced there are immunization programs in place and vaccine is produced on a scale unmatched for any other animal vaccine. Over three billion monovalent dose units of vaccine are used annually in South America, Africa, and Asia (Henderson, 1981; Della-Porta, 1983).

The use of vaccine in well-organized control programs in Western Europe has reduced an annual incidence of some thousands of outbreaks per year in the 1950s and 1960s to the virtual elimination of disease in the past 10 years. However, extension of this success to other problem areas of the world has been difficult. In South America it is necessary to distribute a

trivalent vaccine two or three times a year so as to reach a sufficient proportion of the cattle population at risk. Anything less fails to reduce transmission and fails to decrease the incidence of disease. The same vaccine usage pattern is required in the Middle East and in Africa. The delivery of so much vaccine frequently represents a great financial and social burden—it is no wonder that FMD is not being controlled in many parts of the world. The underlying problem is not only the cost of present vaccines, but also the social impasse in maintaining needed enthusiasm. In the present situation in FMD enzootic areas, this impasse might be broken by the conviction that a new vaccine of great potency might lead to true control or elimination of the virus. This conviction would have to be based on vaccinating a higher proportion of cattle at risk than is usual. In such a situation, the cost-to-benefit equation for control would include the extra cost of reaching all livestock in the given area, but it would also include the extra benefit of greatly reduced virus transmission.

This worldwide situation, marked by the need for very large numbers of vaccine doses, for vaccine of increased potency (so as to reduce immunization frequency), for vaccine which is absolutely incapable of causing disease (inadequate inactivation has been a common problem in vaccine manufacture), and for vaccine which is inexpensive, has been addressed by bioengineering technology. Capacity for control, and even area eradication, of FMD will be greatly expanded by vaccines produced by transformed *E. coli* or by peptide synthesis.

The gene encoding the VP1 protein of several FMD virus serotypes has been cloned by three research groups (a group from the Plum Island Animal Disease Center, Greenport, New York, and Genentech Inc., San Francisco, in the United States; a group from the Animal Virus Research Institute, Pirbright, and the Wellcome Research Laboratories, Beckenham, in the United Kingdom; and a group from the University of Heidelberg, the Federal Research Institute for Animal Virus Diseases, Tubingen, the Max Planck Institute, Munich, Federal Republic of Germany, and Biogen S.A., Geneva, Switzerland) (Boothroyd *et al.*, 1981; Brown, 1983a,b; Kleid *et al.*, 1981; Kleid, 1982; Kupper *et al.*, 1981).

The strategy of FMD VP1 cloning has been as follows: (1) From viral RNA obtained from FMD virus particles or from virus-infected cells, double-stranded cDNA species were synthesized using reverse transcriptase and DNA polymerase. (2) Selected cDNA species were annealed into cloning vector plasmids, and these were used to transform *E. coli*. (3) A library of resulting plasmids was screened, and individual clones encoding the VP1 protein were identified, sequenced, and cleaved with restriction endonucleases to fragments encoding only desired parts of protein. (4) These cDNA fragments were annealed into expression vector plasmids containing the *E.*

coli tryptophan promoter-operator system. The plasmids were used to transform *E. coli* organisms, which were then grown in large cultures. (5) Lysates of these cultures were examined for the presence of FMD VP1 protein analogs, and the presence of biologically active proteins was demonstrated.

In the first experiments done by the group from Plum Island/Genentech, Inc. on FMD virus type A12, 17–20% of the total protein of the bacterial cultures was the viral fusion product (Kleid *et al.*, 1981). This represented 2×10^6 molecules of protein per bacterium, or about 1 mg/ml of bacterial culture fluid. This protein, when partially purified, specifically bound homologous FMD antibody, and when two doses of the analog protein were inoculated with adjuvant into cattle and swine, the double dose elicited neutralizing antibody and protective immunity against homologous virus challenge. These research programs have now been broadened to include more FMD virus serotypes, and the process is being scaled up to provide enough virus protein for formulation into vaccines for additional challenge trials and for open field trials in enzootic areas.

Despite these early successes with bacterially produced FMD VP1 protein, such theoretical and practical problems remain that there has been a parallel development of chemically synthesized peptide vaccines for FMD. A research group from the Research Institute of the Scripps Clinic, La Jolla, California, and the Animal Virus Research Institute, Pirbright, United Kingdom, has synthesized peptides corresponding to important antigenic determinant sites on the VP1 protein (Bittle *et al.*, 1982; Brown, 1983b; Lerner, 1983). The strategy of this synthesis has been as follows: (1) From VP1 cDNA nucleotide sequence data, the entire amino acid sequence of the VP1 protein of several different FMD virus serotypes had been deduced. Analysis of these amino acid sequences, especially for hydrophilic regions and for regions exhibiting variability among subtypes (variability presumed to be due to host immune selective pressure), led to selection of candidate sequences for synthesis. (2) Peptides corresponding to these (and other) amino acid sequences were chemically synthesized by the Merrifield solid-phase method. (3) The synthesized peptides were covalently coupled to a protein carrier (keyhold limpet hemocyanin) and tested for immunogenicity.

The first results of this approach have been exciting. A peptide corresponding to amino acids 141–160 elicited antibodies which neutralized virus (mouse neutralization test) and protected against challenge (guinea pig challenge protection test) after a single dose. Neutralizing antibody levels which would be expected to protect were also obtained when the peptide was inoculated into cattle. A challenge protection test in cattle is now underway.

The potential of synthetic peptide FMD vaccines is enormous: (1) Pep-

tides of different serotype specificity might be coupled to provide a balanced polyvalent vaccine. (2) Polymers of the peptides might be constructed to increase the number of antigenic determinants per molecule as a means of increasing potency (although folding of longer peptides limits epitope exposure). (3) Large-scale peptide synthesis technology, either chemical synthesis or biosynthesis technology, might reduce the cost per dose of vaccine significantly. The key FMD peptide has now been biosynthesized in *E. coli* by insertion of the appropriate synthetically constructed cDNA into an expression plasmid. The protein produced by this means has been shown to protect cattle when incorporated into a two-dose vaccination regimen. The relative advantages and disadvantages of producing peptide immunogens by biosynthesis versus chemical synthesis can now be studied. Perhaps biosynthesis will lead to more inexpensive vaccines than can be produced via chemical synthesis, but this is by no means certain. (4) Parallel development of better carriers for the haptenic viral peptides and development of better adjuvants might lead to long-term immunity in vaccine recipients. Success in this area is a major requirement for disease control and eradication (Beale, 1982; Brown, 1983b; Lerner, 1983).

IV. PRIORITIES FOR GENETIC ENGINEERING OF OTHER ANIMAL VACCINES

At a conference held in 1980 and sponsored by the American Association for the Advancement of Science and other organizations, a group of scientists attempted to set research priorities for maximum impact upon the world's livestock industries (Pond *et al.*, 1980). Unlike most such exercises, this one cut through the question of how to deliver present technology worldwide and concentrated on new "research imperatives"—new scientific approaches to major problems.

The report of this conference has become quite influential. Under the heading "Animal Health," four subject areas were identified as having the highest priority for research support: (1) integrated food animal health systems—the interdisciplinary approach to identifying and quantifying factors responsible for losses in the intensive production environment; (2) food animal toxicology—the interdisciplinary approach to identifying and controlling the spectrum of chemicals (pesticides, herbicides, pollutants, natural toxins) which decrease yields in the intensive production environment; (3) human health hazards—the interdisciplinary approach to identifying and removing from animals and animal products pathogens, toxic chemicals, therapeutic drugs, growth promotants, growth permittants, hormones, etc. (substances derived from intensive production practices); and (4) genetic

engineering for disease prevention and resistance. The first three of these "research imperatives" reflect the great change occurring in agriculture in many parts of the world—the change to large-scale corporate or collective production units. Genetic engineering is listed also for the purposes of intensive production agriculture, but its application will help all livestock producers, whether they be large corporations or collectives, family farm units, or people surviving by subsistence farming.

If the promise of genetic engineering for livestock disease control is to be organized rationally, one need is to establish priorities for research support. Priorities should be set in accord with the relative importance of particular diseases—in the intensive production environment and in the production environment of developing countries. One attempt at this was made at a workshop held in 1982 and sponsored by the National Research Council and the Agency for International Development (United States), the World Bank, and the Rockefeller Foundation (BOSTID, 1982). In the report of this workshop, high priority is given to research support for bioengineered vaccines in general and for vaccines for the following diseases in particular: (1) neonatal diarrheas—including viral diarrheas; (2) bovine respiratory disease complex—including viral pneumonias; (3) African swine fever; (4) hemotropic protozoa—trypanosomiasis, theileriosis, babesiosis, anaplasmosis; and (5) animal tuberculosis. The next priority category included vaccine research for Rift Valley fever and Newcastle disease, and the third priority category included vaccine research for rinderpest, bluetongue, hog cholera, African horsesickness, the equine encephalitides, pulmonary adenomatosis and related retrovirus diseases of sheep and goats, pseudorabies, and vesicular stomatitis. Rabies and FMD vaccines were recognized as being very important, but it was considered that research on them is well funded presently. This kind of prioritization is always subject to revision according to area needs, but the overall hope is that this list, or similar lists developed by others, will be used by international funding agencies as they consider means to accelerate and broaden the biotechnological revolution in animal vaccines.

V. TECHNOLOGY TRANSFER TO DEVELOPING COUNTRIES

The promise of genetic engineering of virus disease vaccines will not be exploited fully until the technology is transferred from laboratories in developed countries to those in developing countries. At the workshop on priorities in biotechnology research cited above, Baltimore (1982) asked, "What would it take for less-developed countries to have the necessary capability to utilize the new technologies, and thereby set their own priorities

and to solve their own problems?" He answered this question with three categories of needs: education, support, and infrastructure. Education needs include graduate and postgraduate programs, technical training, personnel exchanges between laboratories in developed and developing countries, and development of effective lines of communication. Support needs include funds for laboratory facilities, equipment, and supplies. Infrastructure needs are most difficult and most crucial—they include reliable systems for supplying specialized chemicals and enzymes, specialized laboratory devices and gadgets, electricity, refrigeration and freezer space, etc. The overall commitment would be very large. Baltimore (1982) concluded: "It is not prudent for developing countries to maintain a continual dependence on the developed world where much of the current work is profit-oriented. This is not a solid base on which to build solutions to local problems."

One exercise aimed at breaking the dependence on biotechnological products from the developed countries was sponsored by the United Nations Industrial Development Organization (UNIDO); this exercise involved planning for "The Establishment of International Centres for Genetic Engineering and Biotechnology (ICGEB)" (UNIDO Mission, 1981). This exercise, completed in 1981, was based on expert committee visits to many parts of the world (including a visit to Kuwait University and the Kuwait Foundation for the Advancement of Science) and included analyses of specific regional needs. The UNIDO mission also developed model plans and cost estimates for building self-contained laboratory centers. The centers would cost (in 1980 U.S. dollars) $9,530,000 to build and about $5,000,000 per year to operate and would have a professional staff of about 30. This freestanding laboratory approach is controversial—it might be argued that such centers would function better and be cheaper if integrated into university or national laboratories. In any case, the need is clear, even if the organizational structure is not. The need is to broaden genetic engineering capabilities in response to local and regional problems and to transfer technological breakthroughs quickly. New breakthroughs will come at an increasing pace and with unpredictable impact upon animal agriculture.

REFERENCES

Anilionis, A., Wunner, W. H., and Curtis, P. J. (1981). *Nature (London)* **284**, 275–278.

Bachrach, H. L. (1981). Genetic Engineering in Plants and Animals. U.S. Department of Agriculture Workshop on Emerging Technologies, Chicago, Illinois.

Baer, G. M. (1983). *In* "Control of Virus Diseases" (E. Kurstak and R. G. Marusyk, eds.), pp. 79–92. Dekker, New York.

Baer, G. M., Abelseth, M. K., and Debbie, J. G. (1971). *Am. J. Epidemiol.* **93**, 487–490.

Baltimore, D. (1982). *In* "Priorities in Biotechnology Research for International Development" (BOSTID). National Academy Press, Washington, D. C.

Beale, J. (1982). *Nature (London)* **198,** 14–15.

Bittle, J. L., Houghton, R. A., Alexander, H., Shinnock, T. M., Sutcliffe, J. G., Lerner, R. A., Rowlands, D. J., and Brown, F. (1982). *Nature (London)* **298,** 30–33.

Boothroyd, J. C., Highfield, P. E., Cross, G. A. M., Rowlands, D. J., Lowe, P. A., Brown, F., and Harris, T. J. R. (1981). *Nature (London)* **290,** 800–802.

BOSTID (1982). "Priorities in Biotechnology Research for International Development" (Board on Science and Technology for International Development, National Research Council). National Academy Press, Washington, D.C.

Brooksby, J. B. (1982). *Intervirology* **18,** 1–23.

Brown, F. (1983a). *In* "Viral Disease in South-East Asia and the Western Pacific" (J. S. MacKenzie, ed.). Academic Press, New York.

Brown, F. (1983b). *In* "Control of Virus Diseases" (E. Kurstak and R. G. Marusyk, eds.). Dekker, New York.

Della-Porta, A. J. (1983). *Aust. Vet. J.* (in press).

Dietzschold, B., Cox, J. H., and Schneider, L. G. (1978). *Dev. Biol. Stand.* **40,** 45–56.

Dietzschold, B., Cox, J. H., and Schneider, L. G. (1979). *Virology* **98,** 63–75.

Dietzschold, B., Wiktor, T. J., MacFarlan, A., and Varrichio, A. (1983). *J. Virol.* (in press).

Genetic Engineering Letter (1982). **2, 3,** and also *Business Week* (November, 1982).

Henderson, W. (1981). United Nations Industrial Development Organization, UNIDO/IS.273. Vienna, Austria.

Kleid, D. G. (1982). Engineering Foundation Conferences; Biochemical Engineering III. Santa Barbara, California.

Kleid, D. G., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., Grubman, M. J., McKercher, P. D., Morgan, D. O., Robertson, B. H., and Bachrach, H. L. (1981). *Science (Washington, D.C.)* **214,** 1125–1129.

Kupper, H., Keller, W., Kurz, C., Forss, S., Schaller, H., Franze, R., Strohmaier, K., Marquardt, O., Zaslavsky, V. G., and Hofschneider, P. H. (1981). *Nature (London)* **289,** 555–559.

Lerner, R. A. (1983). *Sci. Am.* **248,** 66–74.

Murphy, F. A. (1983a). *In* "Viral Diseases in South-East Asia and the Western Pacific" (J. S. MacKenzie, ed.). Academic Press, New York.

Murphy, F. A. (1983b). *In* "Control of Virus Diseases" (E. Kurstak and R. G. Marusyk, eds.). Dekker, New York.

Pond, W. G., Merkel, R. A., McGilliard, L. D., and Rhodes, V. J. (1980). "Animal Agriculture—Research to Meet Human Needs in the 21st Century." Westview Press, Boulder, Colorado.

Steck, F., Haflinger, U., Stocker, C., and Wandeler, A. (1978). *Experientia* **34** (12), 1662–1675.

Sutcliffe, J. G., Shinnick, T. M., Green, N., and Lerner, R. A. (1983). *Science (Washington, D.C.)* **219,** 660–666.

UNIDO Mission (Heden, C.-G., Boyer, H. W., Bukhari, A., Chakrabarty, A., Narang, S., Riazuddin, S., and Wu, R. (1981). UNIDO/IS.254, Vienna, Austria.

Yelverton, E., Obijeski, J. F., Holloway, B. P., and Goeddel, D. V. (1983a). *Virology* (in press).

Yelverton, E., Norton, S., Obijeski, J. F., and Goeddel, D. V. (1983b). *Science (Washington, D.C.)* **219,** 614–620.

3

Location and Sequence of a Critical Antigenic Site on VP1 of Poliovirus Type 3 and Its Relevance to the Development of Novel Vaccines against Poliomyelitis

P. D. MINOR,* D. M. A. EVANS,* MORAG FERGUSON,* M. SPITZ,† J. W. ALMOND,‡ AND G. C. SCHILD*

**Division of Viral Products and †Immunology Section, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, England*

‡Department of Microbiology, University of Leicester, Leicester, England

I. INTRODUCTION

The causative agents of poliomyelitis (Minor *et al.*, 1982), classified into poliovirus types 1, 2, and 3 on the basis of their serological characteristics, are members of the family Picornaviridae, a large group of viruses of considerable importance in human and veterinary disease. They are small viruses possessing single-stranded, messenger-sense RNA genomes of about 2.6×10^6 molecular weight (~ 7.5 kilobases) enclosed in a protein capsid composed of 60 copies each of four virus-encoded polypeptides (VP1, VP2, VP3, and VP4) ranging in molecular weight from 4000 to 30,000. The virus

ISBN 0-12-429601-7

genome encodes a single large polyprotein which is specifically cleaved to produce the viral capsid proteins (VP1, VP2, VP3, VP4) and several non-capsid proteins, including an RNA polymerase, a viral protease, and other proteins of yet unknown function. Complete processing of the polyprotein seems to occur by the combined action of cellular and virus-encoded proteases.

Poliomyelitis, although well controlled by the use of vaccines in developed countries, remains a major problem in the developing world (Paccaud, 1979; WHO, 1980, 1981). Epidemics of poliomyelitis in unvaccinated populations are chiefly caused by type 1 poliovirus, but in countries with well-established vaccination programs where paralytic cases have been reduced to a very small number, some of the few cases reported are associated with the use of live attenuated virus vaccines, particularly in cases involving poliovirus types 2 and 3.

Current research on the characterization of polioviruses emphasizes the biochemical analysis of poliovirus genomes, and the comparison of the antigenic characters of different strains using monoclonal antibodies. Detailed knowledge of poliovirus genome sequences may also contribute to the development of improved live-virus vaccines, with genetic determinants optimized for antigenicity and stabilized for attenuation. Precise definition of the structure of the antigenic sites involved in virus neutralization will be of importance in the development of novel inactivated vaccines. Two approaches are feasible: the first involves expression in bacteria of a cloned VP1 gene; the second involves the chemical synthesis of peptides representing sequences from VP1. Considerable progress has already been made in the development of novel vaccines against another picornavirus, foot-and-mouth disease virus, using each of these approaches (Kleid *et al.*, 1981; Bittle *et al.*, 1982).

This chapter reviews progress in our laboratory on the antigenic analysis of poliovirus type 3 using monoclonal antibodies, with particular emphasis on the epitopes involved in virus neutralization and on the location and amino acid sequence of an antigenic site on VP1 which appears to have a critical role in neutralization.

II. ANTIGENIC ANALYSIS OF POLIOVIRUS USING MONOCLONAL ANTIBODIES

Examination of poliovirus particles present in virus harvests has revealed two major populations: infectious virus which sediments at 155 S and empty 80 S capsids (Mayer *et al.*, 1957; Minor *et al.*, 1980). These particles exist in distinct antigenic forms which have been termed D and C antigen, re-

spectively (Mayer *et al.*, 1957). However, there is little information on the detailed antigenic structure of these particles. To characterize the antigenic determinants on infectious and empty particles, we have developed hybridoma cell lines secreting monoclonal antibodies to poliovirus. The antibodies were tested for virus neutralizing activity and for their ability to bind to D and C antigen in antigen-blocking tests. In addition, their reactivity with isolated poliovirus capsid proteins in immunoblot tests was examined.

To develop monoclonal antibodies, mice or rats were immunized with poliovirus type 3 (Table I) and hybridoma cell lines were prepared by fusion of mouse spleen cells with P3 × 63 Ag8 cells and rat spleen cells with rat Y_3 Ag 1.2.3 cells (Minor *et al.*, 1982). Cell hybrids were screened for secretion of antibodies to poliovirus type 3 in radioimmunoassay or single radial diffusion (SRD) antigen-blocking tests (Ferguson *et al.*, 1982), and secretor cell lines were cloned. Fifty-one stable, cloned cell lines secreting antibody to poliovirus type 3 were obtained, and the antibody was characterized for immunoglobulin class by sedimentation in sucrose gradients and the migration rates of the immunoglobulin chains in polyacrylamide gel electrophoresis. Thirty-one of the antibodies were of the IgG class and 20 were IgM.

The antibodies were tested by SRD antigen-blocking tests (Fig. 1) to determine their reactivities with D and C antigens of poliovirus 3. The method used was a modification of the autoradiographic SRD method of Schild *et al.* (1980). Briefly, [^{35}S]methionine-labeled 155 S or D or 80 S C peaks of poliovirus antigen from sucrose gradients were mixed with the test monoclonal antibody before adding to wells in agarose gels containing low concentrations of hyperimmune anti-poliovirus type 3 serum. Diffusion of radiolabeled antigen in the gel after 24–48 hr was detected by autoradiography. Test antibody which reacts with D and/or C antigen inhibits its diffusion into the gel compared with control antigen treated with phosphate-buffered saline (PBS).

Antigen-blocking titers of ascitic fluids prepared from representative hybridomas are shown in Table I. Three categories of reactivity of the antibodies were seen: those with SRD blocking activity exclusively for D antigen (e.g., NIBp 134, NIBp 194), those blocking exclusively C antigen (e.g., NIBp 131, NIBp 198), and those blocking both D and C antigen (e.g., NIBp 175, NIBy 25-4-12). Of the 51 monoclonal antibodies, 8 blocked D and C antigens, indicating that they reacted with determinant(s) common to both infectious and empty particles. Of the remaining 43 antibodies, 19 blocked exclusively D antigen and 24 blocked exclusively C antigen.

The monoclonal antibodies were tested for neutralizing activity against a range of type 3 virus strains in microtiter assays in Hep 2c cells using 100 $TCID_{50}$ as the challenge dose of virus. The neutralization titers of the rep-

TABLE I

Characteristics of Representative Monoclonal Antibodies to Poliovirus Type 3[a]

Immunizing virus[b]	Hybridoma designation	Particle specificity[c]	SRD antigen blocking titer[d]		Virus neutralization titer[e]	Capsid protein specificity	Immunoglobulin class[f]
			D antigen	C antigen			
119	NIBp 134	D	100,000	<2	4,000	VP1[g]	IgM
119	NIBp 135	D	1,000	<2	<10	?	IgM
119	NIBp 139	D	100,000	<2	<10	?	IgM
119	NIBp 165	D	300	<2	256	VP1[g]	IgM
Leon/USA/39	NIBp 194	D	1,000	<2	150	VP1[g]	IgG
10/USA/57	NIBy 25-4-12	D + C	100,000	10,000	70,000	VP1[g]	IgM
Sabin vaccine	NIBy 27-4-4	D + C	500	20	10,000	VP1[g]	IgM
Leon/USA/39	NIBp 175	D + C	100,000	100,000	14,000	VP1[g]	IgG
10/USA/57	NIBy 25-1-14	D + C	3,000	1,000	20,000	VP1[g]	IgG
Sabin vaccine	NIBy 25-2-11	C	<2	50	<10	VP1[h]	IgG
119	NIBp 131	C	<2	10,000	<10	?	IgM
Leon/USA/39	NIBp 176	C	<2	300	<10	VP3[h]	IgM
Sabin vaccine	NIBp 196	C	<2	300	<10	VP1[h]	IgG
Sabin vaccine	NIBp 198	C	<2	100,000	12,000	VP1[i]	IgM

[a] Data from Ferguson *et al.* (1983).

[b] Virus 119 is a virulent revertant of Sabin type 3 vaccine virus isolated from a fatal case of poliomyelitis. For origins of viruses, see Ferguson *et al.* (1982).

[c] Based on autoradiographic SRD antigen blocking tests.

[d] Dilution of antibody, which reduces by 75% the zone size obtained when D or C antigen is mixed with PBS.

[e] Dilution of antibody just neutralizing 100 $TCID_{50}$ virus in microtiter assays.

[f] Determined by migration of ^{35}S-labeled immunoglobulin chains in polyacrylamide gel electrophoresis.

[g] Determined by sequencing RNA from resistant mutants (Minor *et al.*, 1983; Evans *et al.*, 1983).

[h] Immunoblot reactivity determined by the method of Thorpe *et al.* (1982).

[i] P. D. Minor, unpublished observations.

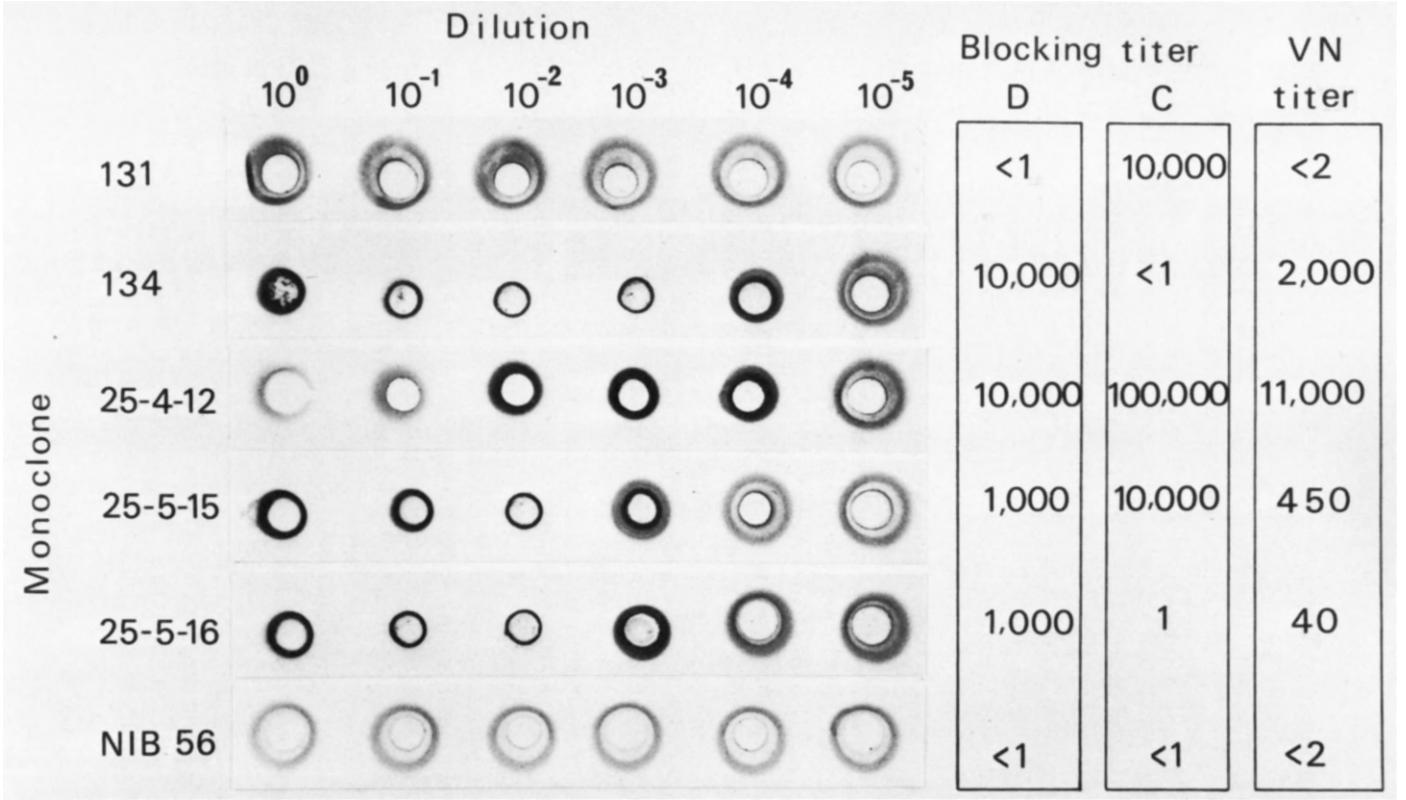


Fig. 1. Autoradiographic exposure of a simple-radial-immunodiffusion blocking assay (Schild *et al.*, 1980) of monoclonal antibodies to poliovirus type 3. The gel contains a low concentration of polyclonal antibody to poliovirus type 3. Mixtures of monoclonal antibody dilution (10^0 to 10^{-5}, and ^{35}S-labeled poliovirus type 3 D antigen were added to wells in the immunoplate. Antibody reacting with D antigen blocked the diffusion of the antigen in the gel as seen by comparisons with a control antibody NIB 56. Antibodies 134, 24–4–12, 25–5–15, and 25–5–16 react with D antigen. The blocking titers of the monoclonal antibodies to C antigen and their virus-neutralizing (VN) titers are also shown. Antibody 131 is specific for C antigen, and 134 and 25–5–16 are specific for D antigen. The remaining antibodies, 25–4–12 and 25–5–15, react with both D and C antigens.

resentative group of viruses is shown in Table I. Efficient neutralization was exhibited by antibodies of both IgG and IgM classes. In addition, antibodies of each immunoglobulin class exhibited D, C, or D + C antigen-blocking activity. The neutralization activities against poliovirus 3 (Sabin vaccine strain Leon $12a_1b$) for all 51 antibodies is summarized in Table II. All antibodies which reacted with the determinant(s) common to D and C particles possessed neutralizing activity at titers $\geq$ 1:100, whereas only 12 of the 19 D-specific antibodies had detectable neutralizing activity. Where neutralizing activity was detected it was often at low titer. It is of interest that not all antibodies which bind to infectious virus, as indicated by their blocking titers with D antigen, possessed neutralizing activity. This suggests that virus neutralization involves critical antigenic sites on the infectious particle and is not a necessary consequence of binding of antibodies to any antigenic site on the virus surface. All neutralization was poliovirus type 3 specific, suggesting that antigenic sites common to the other poliovirus serotypes are not involved in neutralization. Surprisingly, one antibody which reacted exclusively with C antigen in SRD antigen blocking tests appeared to neutralize virus infectivity in the standard neutralization test. The mechanism of neutralization of this antibody is unknown. However, antibodies with virus neutralization activity which do not react with free infectious virus may nevertheless react with particles after attachment to cells or at a late stage in replication, during maturation of release. It is of interest that antibodies reacting with poliovirus C, but not with D, are the most abundant antibodies to poliovirus detected in some collections of human sera (G. C. Schild, J. M. Wood, and D. I. Magrath, unpublished data), and their relevance to immunity requires further study.

Antibodies which bound to the determinants common to D and C particles possessed neutralizing activity against a wide range of poliovirus type 3 strains—in some cases, against all of the 30 strains tested. In contrast D-specific antibodies often reacted with a narrow range of strains in neutralization and antigen-blocking tests. Thus some antigenic determinants involved in neutralization appear to be highly conserved within a serotype whereas others are unique for individual strains. Several antibodies reacted only with Sabin vaccine virus or with strains the RNA of which gave T1 oligonucleotide maps identical or similar to that of Sabin virus, showing that they were genetically derived from vaccine (Ferguson *et al.*, 1982) (see Table III). Such monoclonal antibodies will be of use as diagnostic reagents for the identification of vaccine-derived viruses.

The reactivity of the 51 monoclonal antibodies with separated poliovirus capsid proteins was investigated using the method described by Thorpe *et al.* (1982). The denatured poliovirus proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and then transferred elec-

TABLE II

Neutralization Titers of Monoclonal Antibodies against Poliovirus 3[a]

D + C[b] antigen specificity of antibody	No. of antibodies tested[c]	No. of antibodies with virus neutralizing activity at stated titers[d]					Immunoblot reactivity
		<10	10^1 to $<10^2$	10^2 to $<10^3$	10^3 to $<10^4$	$\geq 10^4$	
D + C reactive	8	0	0	3	0	5	0
D specific	19	7	8	3	0	1	0
C specific	24	23	0	0	1	0	5 VP1 1 VP3
Total	51	30	8	6	1	6	6

[a] Data from Ferguson *et al.* (1983).

[b] Based on autoradiographic SRD antigen blocking assays.

[c] Each antibody was obtained from an independent hybridoma clone in fusions involving the lymphocytes of mice or rats immunized with poliovirus type 3 strains (Minor *et al.*, 1982).

[d] Dilution of antibody just neutralizing 100 $TCID_{50}$ of virus in microtiter assays.

TABLE III

Virus Neutralization Activities of Monoclonal Antibodies against Strains of Poliovirus Type 3[a]

Type	Neutralization titer of monoclonal antibody		
	NIBp 134	NIBp 138	NIBp 165
Type 3 strains with Sabin-like maps[b]			
Sabin vaccine strain	4,100	64	256
106	10,000	90	256
116	>30,000	20	45
119	3,600	32	181
122	>60,000	45	90
131	41,000	8	256
285/UK/74	10,000	11	32
5229/UK/74	10,000	45	128
11340/UK/74	>30,000	128	720
Type 3 strains with non-Sabin-like maps			
Saukett/USA/50 (COP)	900	<4	<4
30/USA/52	<4	<4	<4
190/USA/52	10,000	<4	<4
714/India/58	10,000	<4	<4
6/UK/62	<4	<4	<4
476/UK/62	<4	<4	<4
10/USA/52	640	<4	<4
77689/USA/53	90	<4	<4
Type 1 Mahoney/USA/41	<4	<4	<4
Type 2 MEF/USA/42	<4	<4	<4

[a] Data from Ferguson *et al.* (1983).

[b] T1 oligonucleotide maps of poliovirus RNA were performed as described by Minor (1982). Isolates with maps resembling that of the Sabin type 3 vaccine are likely to have been derived from vaccines (see Minor *et al.*, 1982).

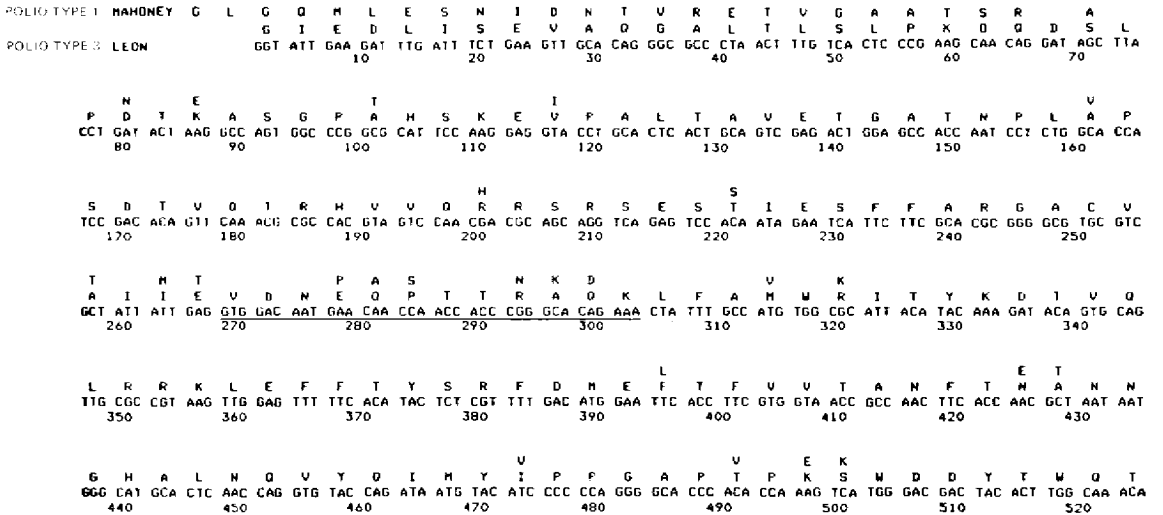
trophoretically onto nitrocellulose paper. Nitrocellulose strips were treated with monoclonal antibody, and antibodies binding specifically to the virus polypeptides were detected by application of ^{125}I-labeled anti-mouse Fab fragments followed by autoradiography. The results are shown in Table II. None of the antibodies specific for D antigen, nor those that reacted with antigenic determinants common to D and C antigen, were found to bind to separated polypeptides. This suggests that the antigenic sites present on infectious particles, including those responsible for virus neutralization, are

complex and dependent on the three-dimensional conformation of viral polypeptide (VP1) at the virus surface. In contrast, six of the C-specific antibodies reacted with denatured proteins, five with VP1, and one with VP3. These determinants are therefore likely to be specified simply by amino acid sequence rather than by tertiary confirmation of the protein.

The six antibodies found positive in immunoblot tests with poliovirus 3 were also examined in tests with poliovirus 1 and 2 capsid proteins; the results are shown in Table IV. Monoclonal antibodies NIBp 196 and 203 reacted with VP1 of poliovirus types 2 and 3 whereas NIBp 118, NIBy 25-2-11, and NIBy 25-4-4 reacted only with VP1 of the homotypic virus. Similar intertypic reactions were found in antigen-blocking tests with these monoclonal antibodies. Although antibody NIBp 176 reacted with VP3 of all three poliovirus types in immunoblot experiments, it reacted only with type 3 C antigen in antigen blocking tests; this finding suggested that the antigenic determinant against which it is directed is in a different conformation in poliovirus types 1 and 2. Polyclonal antisera to poliovirus which cross-react with all three poliovirus types in immunoblot (Thorpe *et al.*, 1982) and immunoprecipitation (Blondel *et al.*, 1982) studies have been described. This, however, is the first report of monoclonal antibodies recognizing common determinants in different poliovirus serotypes. The finding of shared, sequence specified antigenic determinants between poliovirus types is consistent with the observation of high levels ($\simeq 90\%$) of predicted amino acid sequence homology between polioviruses of types 1 and 3 (Stanway *et al.*, 1983a).

III. LOCATION AND PRIMARY STRUCTURE OF THE ANTIGENIC SITE FOR NEUTRALIZATION OF POLIOVIRUS TYPE 3

Monoclonal antibodies are potent reagents for use in studies to locate and identify the antigenic sites on virus capsid proteins which are involved in virus neutralization (Minor *et al.*, 1983; Evans *et al.*, 1983). We have previously used antigenic mutants resistant to neutralizing monoclonal antibodies to identify a single antigenic site for the neutralization of poliovirus type 3. Evidence based on oligonucleotide mapping (Minor *et al.*, 1983) suggested that this operationally defined site corresponded largely to one physicochemical site on the capsid protein VP1 (Fig. 2). Evans *et al.* (1983) have presented conclusive evidence summarized below that the great majority of the mutations conferring resistance to neutralization are confined to an eight-amino acid region of VP1, specified by a sequence of viral RNA 277–300 bases from the start of the region coding for VP1.


POLIO TYPE 1 MAHONEY G L G Q M L E S N I D N T V R E T V G A A T S R A
G I E D L I S E V A Q G A L T L S L P K Q Q D S L
POLIO TYPE 3 LEON GGT ATT GAA GAT TTG ATT TCT GAA GTT GCA CAG GGC GCC CTA ACT TTG TCA CTC CCG AAG CAA CAG GAT AGC TTA
10 20 30 40 50 60 70
N E T I V
P D T K A S G P A H S K E V P A L T A V E T G A T N P L A P
CCT GAT ACT AAG GCC AGT GGC CCG GCG CAT TCC AAG GAG GTA CCT GCA CTC ACT GCA GTC GAG ACT GGA GCC ACC AAT CCT CTG GCA CCA
80 90 100 110 120 130 140 150 160
H S
S D T V Q I R H V V Q R R S R S E S T I E S F F A R G A C V
TCC GAC ACA GTT CAA ACG CGC CAC GTA GTC CAA CGA CGC AGC AGG TCA GAG TCC ACA ATA GAA TCA TTC TTC GCA CGC GGG GCG TGC GTC
170 180 190 200 210 220 230 240 250
M T P A S N K D S V K
T I I E V D N E Q P T T R A Q K L F A M W R I T Y K D T V Q
GCT ATT ATT GAG GTG GAC AAT GAA CAA CCA ACC ACC CGG GCA CAG AAA CTA TTT GCC ATG TGG CGC ATT ACA TAC AAA GAT ACA GTG CAG
260 270 280 290 300 310 320 330 340
L E T
L R R K L E F F T Y S R F D M E F T F V V T A N F T N A N N
TTG CGC CGT AAG TTG GAG TTT TTC ACA TAC TCT CGT TTT GAC ATG GAA TTC ACC TTC GTG GTA ACC GCC AAC TTC ACC AAC GCT AAT AAT
350 360 370 380 390 400 410 420 430
V E K
G H A L N Q V Y Q I M Y I P P G A P T P K S W D D Y T W Q T
GGG CAT GCA CTC AAC CAG GTG TAC CAG ATA ATG TAC ATC CCC CCA GGG GCA CCC ACA CCA AAG TCA TGG GAC GAC TAC ACT TGG CAA ACA
440 450 460 470 480 490 500 510 520

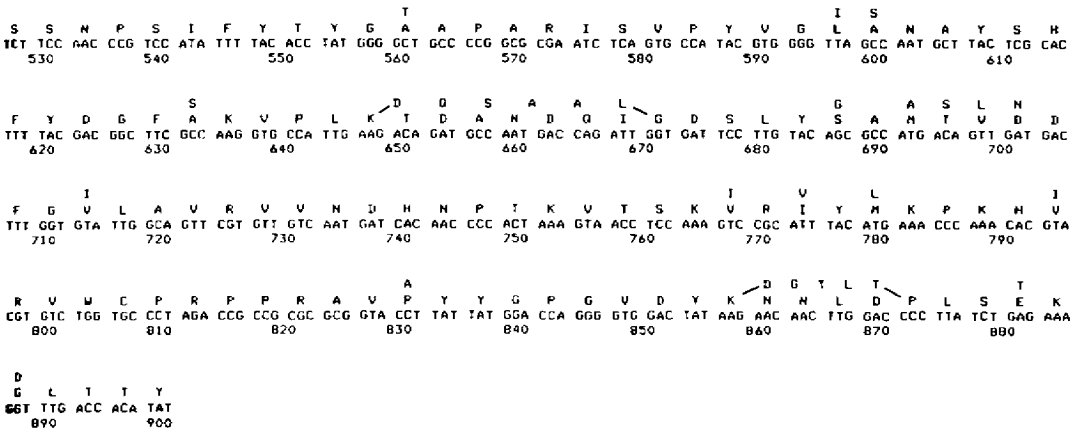


Fig. 2. Nucleic acid sequence of the portion of the type 3 poliovirus (Leon) genome coding for VP1. The amino acid sequence of the type 1 poliovirus (Mahoney) is shown where it differs from that of poliovirus type 3 (Leon). The underlined segment indicates the region containing a critical antigenic site of VP1 for poliovirus 3 (Leon) as shown in Table VI. Animo acids are indicated by the single-letter code. The nucleic acid sequence of poliovirus type 3 was established from cloned cDNA as described by Stanway *et al.* (1983b).

TABLE IV

Polypeptide Specificity of Nonneutralizing, C Antigen-Specific Monoclonal Antibodies[a]

Monoclonal antibody	C antigen blocking titer	Type 3 Immunoblot reactivity			C antigen blocking titer	Type 2 Immunoblot reactivity			C antigen blocking titer	Type 1 Immunoblot reactivity		
		VP1	VP2	VP3		VP1	VP2	VP3		VP1	VP2	VP3
NIBy 25-2-11	50	+	–	–	<10	–	–	–	<10	–	–	–
NIBy 25-4-4	50	+	–	–	<10	–	–	–	<10	–	–	–
NIBp 118	30	+	–	–	<10	–	–	–	<10	–	–	–
NIBp 196	300	+	–	–	100	+	–	–	<10	–	–	–
NIBp 203	300	+	–	–	100	+	–	–	<10	–	–	–
NIBp 176	300	–	–	+	<10	–	–	+	<10	–	–	+

[a]Data from Ferguson *et al.* (1984).

Mutants of a single parental poliovirus type 3 strain (P3-Leon-USA-1937) were selected in the presence of several individual virus-neutralizing monoclonal antibodies. Sixteen mutant groups were identified by their distinct patterns of resistance against 11 monoclonal antibodies. Their reaction patterns are presented in Table V, which identifies the monoclonal antibodies used to select the various groups of mutant viruses. For the purpose of this analysis, an antigenic site was defined as an area of the virus where a mutation affecting the binding of one monoclonal antibody affects the reactions of others directed against the same site (Minor *et al.*, 1983). Such an operationally defined site need not necessarily be made up of a contiguous

TABLE V
Reaction of Neutralizing Monoclonal Antibodies with Antigenic Mutants of Type 3 Poliovirus[a,b]

Mutant	25-1-14	25-4-12	27-4-4	199	194	134	208	175	204	197	165	Selecting monoclonal antibodies
1	R	R		R						R		25-1-14, 24-4-12, 25-5-5, 27-4-4
2	R	R		R	R					R	R	25-1-14, 25-4-12, 199, 165, 25-5-5
3	R	R	R	R	R		R	R	R	R	R	25-4-12, 27-4-4, 175, 204, 25-5-5
4	R	R	R	R	R		R	R		R	R	25-4-12, 27-4-4, 204, 165, 132
5				R	R	R	R	R			R	199, 134, 132, 165, 175, 204
6				R	R	R	R				R	199, 165, 132
7				R	R	R	R	R	R		R	134, 165
8			R			R	R			R		27-4-4
9	R	R	R	R	R	R	R	R		R	R	27-4-4, 199, 175, 204
10	R		R	R	R	R	R	R	R	R	R	27-4-4, 134, 175
11				R	R		R	R		R	R	199, 175, 204, 165
12		R		R	R	R	R	R			R	175
13	R	R		R		R	R	R			R	175, 204
14	R	R						R		R	R	175
15	R	R	R	R	R			R	R	R	R	175, 204
16	R	R		R	R		R		R		R	199, 204, 165

[a] Data from Evans *et al.* (1983).

[b] Reaction was assessed by neutralization of 5×10^4 $TCID_{50}$ units of virus by a 1:10 dilution of antibody ascitic fluid. R, resistant. The wild type was neutralized by all antibodies.

sequence of amino acids (Caton *et al.*, 1982). The RNA of representative strains from each mutant group was sequenced to define precisely the positions of amino acid substitutions in VP1 which conferred resistance. This was done by the dideoxy sequencing method (Caton *et al.*, 1982) using a primer restriction fragment prepared from cloned poliovirus cDNA (Cann *et al.*, 1983). A sequence of at least 60 bases, in most cases up to 150 bases, was identified for each mutant. The sequences of all the mutants were the same except for the single-point mutations shown in Table VI. Where mutations were detected there was only one per mutant and all would result in amino acid substitutions.

Base changes were detected in viruses of 15 of the 16 mutant groups and were concentrated into a single region of only eight codons (Table VI). Only one mutant group (group 9) had an amino acid substitution at position 4 (glutamic acid to glycine). The amino acids at positions 5 and 6 were conserved in all the mutant groups. The remaining mutations were distributed between positions 7 and 11. For example, mutants of groups 1, 2, and 14 showed different point mutations in position 7, and at least two distinct mutations were located in each of positions 8 to 11. Furthermore, different mutations which occurred in the same position resulted in different patterns of resistance to neutralization. Single base changes at the six variable codons could generate 33 possible different mutants. We have therefore isolated nearly half of the total number of theoretically possible mutants.

In only one of the groups, group 6, was no mutation detected in the 150 bases sequenced by primer extension, indicating that in this instance the mutation was elsewhere in the genome. This mutation might nevertheless affect the same single antigenic site in the tertiary structure of the capsid. This would not be without precedent. The hemagglutinin molecule of influenza A virus has been found to contain five operationally defined antigenic sites all of which are coded for by noncontiguous portions of the primary gene sequence (Caton *et al.*, 1982). Other reports have suggested the existence of multiple antigenic sites for poliovirus type 1 (Emini *et al.*, 1982, 1983).

The amino acid sequence for poliovirus type 1 (Mahoney strain) in the region we propose for the antigenic site of type 3 virus (positions 4–11) is also shown in Table VI. There are only two positions within this region which are conserved between types 3 and 1, and one of these is not concerned between the wild-type 1 vaccine progenitor strain (Mahoney) and the Sabin type 1 vaccine strain, LSc. In contrast the two amino acids at positions 5 and 6 (glutamine and proline) were conserved in all mutants derived from the Leon type 3 virus, the Sabin type 3 vaccine, and a virulent type 3 vaccine-associated strain (119) (A. J. Cann, unpublished data); however, they are not conserved between polioviruses of types 3 and 1. The

same two positions are conserved between the wild type 1 vaccine progenitor strain (Mahoney) and the Sabin type 1 vaccine strain (LSc). Thus, the proposed antigenic site shows low overall sequence homology between these two types of poliovirus. The significance of these observations with respect to type specificity is not clear. Nevertheless, it is possible that for poliovirus type 3 the conserved amino acids in positions 5 and 6 may contribute to the type specificity of the neutralization reaction.

Of the 21 monoclonal antibodies we have characterized which neutralize virus infectivity, none bound to isolated VP1 which had been denatured during its preparation. However, the genetic and molecular studies of a large collection of over 120 mutants of poliovirus type 3 selected for resistance to 16 of the 21 antibodies indicate that all 16 antibodies bind to the same eight-amino acid region of VP1. In addition, resistant mutant viruses with identical point mutations within this antigenic site were isolated by selection in the presence of either D-specific or D- and C-reactive monoclonal antibodies. Thus, neutralizing monoclonal antibodies with D and C antigen or D antigen specificity appear to react with the same antigenic region of VP1. In addition, some of the viruses which have point mutations in this region are resistant to NIBp 198, the C-specific antibody with neutralizing activity, suggesting that this antibody also reacts with the same antigenic site on VP1 as do the other neutralizing antibodies. However, nonneutralizing C or C-specific antibodies may be directed against other sites, e.g., NIBp 176, which reacts with VP3.

IV. DISCUSSION AND CONCLUSION

It seems probable that there are close analogies between the antigenic structure of different poliovirus serotypes and possibly between poliovirus and other picornaviruses. Emini *et al.* (1982, 1983) have shown that neutralizing monoclonal antibodies for poliovirus type 1 react with epitopes in VP1. Blondel *et al.* (1983) have described a neutralizing monoclonal antibody to poliovirus type 1 which immunoprecipitated both D and C antigen and also isolated VP1. Nevertheless a further D-specific neutralizing antibody against poliovirus type 1 with neutralizing activity failed to react with separated homologous poliovirus 1 capsid polypeptides. Similarly, several neutralizing monoclonal antibodies against foot-and-mouth disease virus failed to react with the separated capsid polypeptides of the homologous virus (Meloen *et al.*, 1983). With the exception of the single D- and C-specific antibody of Blondel *et al.* (1983), these findings are in accordance with the studies of poliovirus type 3 reported here. The relative infrequency of reports of neutralizing monoclonal antibodies to picornaviruses which

TABLE VI

Point Mutations and Consequent Amino Acid Substitutions in Groups of Mutant Viruses Derived from Leon Type 3 Poliovirus[a,b]

Group		Point mutation amino acid substitution											
		1	2	3	4	5	6	7	8	9	10	11	12
Type 1 (Mahoney)		GUG Val	GAU Asp	AAC Asn	CCA Pro	GCU Ala	UCG Ser	ACC Thr	ACG Thr	AAU Asn	AAG Lys	GAU Asp	AAG Lys
Type 3 (Leon)		GUG Val	GAC Asp	AAU Asn	GAA Glu	CAA Gln	CCA Pro	ACC Thr	ACC Thr	CGG Arg	GCA Ala	CAG Gln	AAA Lys
Mutant group	1							AUC Ile					
	2							GCC Ala					
	3								AAC Asn				
	4									CAG Gln			
	5											CUG Leu	
	6												
	7										ACA Thr		
	8								UCC Ser				

9	GGA Gly					
10						CCG Pro
11					GUA Val	
12						CGG Arg
13						CAC His
14		AAC Asn				
15				UGG Trp		
16			AUC Ile			

[a]Data from Evans *et al.* (1983).

[b]The RNA of representative strains of each mutant group was sequenced in the region of 393 to about 240 bases downstream from the 5′ end of the VP1 coding region of the genome. The dideoxy sequencing method was used, using a restriction fragment primer prepared from cloned poliovirus cDNA. The primer was prepared by digesting plasmid DNA with *E. coli* and *Sph*I and labeled by incubation with 1 unit of Klenow fragment from DNA polymerase I and 100 μCi of [$\alpha - {}^{32}$P]dATP (3000 Ci/mmol; Amersham International). The products were denatured and loaded onto a preparative slab polyacrylamide gel. After electrophoresis, the 47-base restriction fragment was detected by autoradiography, excised, and eluted from the gel. The primer was purified by exclusion chromatography and then annealed onto the virion RNA and incubated with dideoxyribonucleotides and deoxyribonucleotides in appropriate proportions together with 5 units of avian myeloblast reverse transcriptase (Life Sciences, Inc.). After incubation at 37°C for 25 min, the reaction products were denatured and loaded onto sequencing gels. After electrophoresis the gels were fixed in 10% acetic acid, washed and dried, and subjected to autoradiography.

react with separated capsid proteins implies that the antigenic sites of these viruses involved in neutralization are determined largely by the conformational arrangement of the capsid proteins in the intact virus.

For poliovirus type 3 we have shown that an eight-amino acid region of VP1, at positions 93–100 from its carboxy terminus, represents an antigenic site on the virus which has a major role in virus neutralization. However, the possibility that other antigenic regions of VP1, or the other capsid proteins of the virus, also play some part in the neutralization of virus by antibody cannot be excluded, although our studies suggest that the site we have identified plays the predominant role. Studies in progress in our laboratory involve the examination of the antigenic properties of synthetic peptides based on this site and the expression in bacterial cells of antigenic proteins coded for by cloned poliovirus cDNA.

Although studies with monoclonal antibodies are of value in locating and identifying the antigenic sites involved in virus neutralization, critical information on the three-dimensional structure of such sites at the virus surface must await the application of more precise analytical methods, such as X-ray crystallography.

REFERENCES

Bittle, J. L., *et al.* (1982). *Nature (London)* **298,** 30–33.

Blondel, B., Crainic, R., Akacem, O., Bruneau, P., Girard, M., and Horodniceanu, F. (1982). *Virology* **123,** 461.

Blondel, B., Akacem, O., Crainic, R., Couillin P., and Horodniceanu, F. (1983). *Virology* **126,** 707.

Cann, A. J., Stanway, G., Hauptmann, R., Minor, P. D., Schild, G. C., Clarke, C. D., Mountford, R. C., and Almond, J. W. (1983). *Nucleic Acid Res.* **11,** 1267.

Caton, A., Brownlee, G. G., Yewdell, J. W., and Gerhard, W. (1982). *Cell* **31,** 417–427.

Emini, E. A., Jameson, B. A., Lewis, A. J., Larsen, G. R., and Wimmer, E. (1982). *J. Virol.* **43,** 997–1005.

Emini, E. A., Shaw-Yi, Kao, Lewis, A. J., Crainic, R., and Wimmer, E. (1983). *J. Virol.* **46,** 466.

Evans, D. M. A., Minor, P. D., Schild, G. C., and Almond, J. W. (1983). *Nature (London)* **304,** 459.

Ferguson, M., Qi, Yi-Hua, Minor, P. D., Magrath, D. I., Spitz, M., and Schild, G. C. (1982). *Lancet* **2,** 122–124.

Ferguson, M., Minor, P. D., Magrath, D. I., Qi, Yi-Hua, Spitz, M., and Schild, G. C. (1984). *J. Gen. Virol.* **65,** 197.

Kleid, D. G., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., Grubman, M. J., McKercher, P. D., Morgan, D. O., Robertson, B. H., and Bachrach, H. L. (1981). *Science* **214,** 1125.

Laemmli, U. K. (1970). *Nature (London)* **227,** 680.

Mayer, M. M., *et al.* (1957). *Immunol.* **78,** 435.

Meloen, R. H., Briaire, J., Woortmeyer, R. J., and van Zaane, D. (1983). *J. Gen. Virol.* **64,** 1193.

Minor, P. D. (1982). *J. Gen. Virol.* **59**, 307.

Minor, P. D., Schild, G. C., and Wood, J. M. (1980). *J. Gen. Virol.* **51**, 147.

Minor, P. D., Kew, O., Schild, G. C. (1982). *Nature (London)* **299**, 109.

Minor, P. D., Schild, G. C., Bootman, J., Evans, D. M. A., Ferguson, M., Reeve, P., Spitz, M., Stanway, G., Cann, A. J., Hauptmann, R., Clarke, L. D., Mountford, R. C., and Almond, J. W. (1983). *Nature (London)* **301**, 674–679.

Paccaud, M. F. (1979). *World Health Statistics Annual* **32**, 198.

Schild, G. C., Wood, J. M., Minor, P. D., and Dardewate, C. N. (1980). *J. Gen. Virol.* **51**, 157.

Stanway, G., Cann, A. J., Hauptmann, R., Hughes, P., Clarke, L. D., Mountford, R. C., Minor, P. D., Schild, G. C., and Almond, J. W. (1983a). *Nucleic Acids Res.* **11**, 5629.

Stanway, G., Cann, A. J., Hauptmann, R., Mountford, R. C., Clarke, L. D., Reeve, P., Minor, P. D., Schild, G. C., and Almond, J. W. (1983b). *Eur. J. Biochem.* **135**, 529.

Thorpe, R., Minor, P. D., Mackay, A., Schild, G. C., and Spitz, M., (1982). *J. Gen. Virol.* **63**, 487.

WHO Weekly Epidemiological Record **14**, 361, (1980); **23**, 180 (1981); **28**, 221 (1981); **42**, 329 (1981); **43**, 337 (1981); **48**, 377 (1981).

4

Synthesis of a Viral Membrane Protein in *Bacillus subtilis*

RALF F. PETTERSSON,* KENNETH LUNDSTRÖM,*,‡ HENRIK GAROFF,§ ILKKA PALVA,* LEEVI KÄÄRIÄINEN,* AND NILS OKER–BLOM†,[1]

**Recombinant DNA Laboratory and †Department of Virology, University of Helsinki, Helsinki, Finland*

‡*Research Laboratory of State Alcohol Company, Helsinki, Finland*

§*European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany*

I. INTRODUCTION

In principle, the ideal vaccine against virus infections is one that contains only the purified structural protein(s) necessary to elicit neutralizing antibodies. Such a vaccine would lack nucleic acids, which at least in theory could be harmful, and would have fewer side effects normally caused by host cell contaminants present in whole-virus preparations. For enveloped viruses, the crucial protein(s) is a membrane glycoprotein located on the virus surface. Examples of such proteins in RNA viruses are the hemag-

[1]Present address: Office of the Chancellor, University of Helsinki, Helsinki, Finland.

ISBN 0-12-429601-7

glutinin (HA) of influenza virus (Wilson *et al.*, 1981), the G protein of rabies (Cox *et al.*, 1977), the hemagglutinin-neuraminidase (HN) of paramyxoviruses (Choppin *et al.*, 1981), the glycoprotein of tick-borne encephalitis virus (Heinz *et al.*, 1983), and one of the glycoproteins in alphaviruses (Kääriäinen and Söderlund, 1978). Corresponding proteins have also been identified in DNA viruses, such as the surface antigen of hepatitis B (HBsAg) (Szmuness *et al.*, 1981), or one or several of the membrane proteins of herpes simplex virus (HSV) (Lee *et al.*, 1982; Seigneurin *et al.*, 1983). In a few nonenveloped viruses, one single capsid protein has been found to be responsible for eliciting neutralizing antibodies, e.g., VP1 of poliovirus (Chow and Baltimore, 1982; Minor *et al.*, 1983) and foot-and-mouth disease virus (FMDV) (Wild *et al.*, 1969; Bachrach *et al.*, 1975). Domains on the surface of the glycoproteins of influenza virus (Wilson *et al.*, 1981; Green *et al.*, 1982), tick-borne encephalitis (Heinz *et al.*, 1983), or short amino acid sequences within VP1 of the picornaviruses (Minor *et al.*, 1983; Evans *et al.*, 1983) have been defined as being crucial antigenic determinants for eliciting neutralizing antibodies. This has raised the possibility of using short synthetic peptides as vaccines (Beale, 1982; Lerner, 1982).

A simple general method for the preparation of water-soluble viral envelope glycoprotein complexes (rosettes) is available (Helenius and von Bonsdorff, 1976). Using this procedure, highly purified envelope glycoprotein preparations against, e.g., orthomyxoviruses (K. Matlin, D. Soldateschi, and K. Simons, personal communication), paramyxoviruses (Morein *et al.*, 1983), bunyaviruses (Kuismanen *et al.*, 1982), alphaviruses (Helenius and von Bonsdorff, 1976), flaviviruses (Heinz and Kunz, 1980), rubella virus (Oker-Blom *et al.*, 1983), and rhabdoviruses (Matlin *et al.*, 1982) have been prepared on a laboratory scale. Examples of such preparations are shown in Fig. 1. Purified glycoproteins can furthermore be introduced into the membranes of artificial liposomes—structures generally referred to as "virosomes" (Almeida *et al.*, 1975; Morein *et al.*, 1978). Both these types of vaccines have been shown to be highly effective in model animal systems (Morein *et al.*, 1978, 1983; Oxford *et al.*, 1981). Recently, Morein *et al.* (1984) have reported further enhancement of the immunogenic capacity of subunit vaccines by constructing a cagelike immunostimulating complex (ISCOM) containing membrane proteins of envelope viruses built around a micelle complex of a glycoside. In contrast to these various glycoprotein complexes, discouragingly enough, monomeric forms of the spike proteins have been shown to be rather poor in eliciting neutralizing antibodies (Morein *et al.*, 1978; Dietzschold *et al.*, 1983). Since the technical problems appear largely to have been solved, why then have the purified subunit vaccines not been made commercially available for mass vaccination programs? The

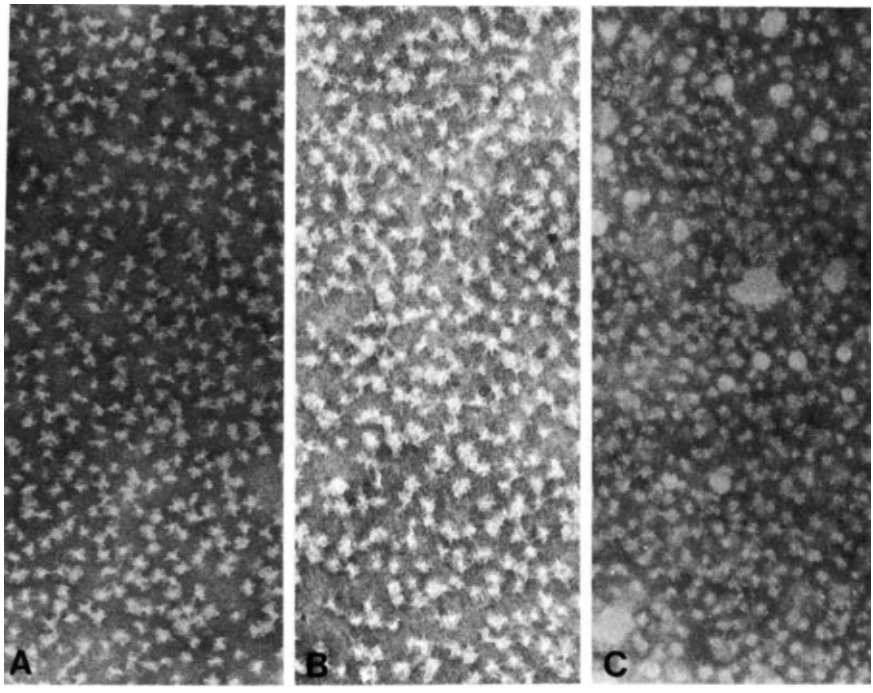


Fig. 1. Electron micrographs of negatively stained soluble membrane glycoprotein complexes of (A) Semliki Forest virus (togavirus), (B) Uukuniemi virus (bunyavirus), and (C) rubella virus (togavirus).

simple answer is that purification of these viral components from viruses grown in tissue cultures is as yet not economically feasible, especially in considering vaccines for mass production in developing countries.

Great hopes have therefore been put on recombinant DNA techniques for the production of large quantities of viral proteins in bacteria and yeast and perhaps even in tissue cultures of animal cells. The methods involve the programming of the cells with the molecularly cloned gene encoding the crucial viral protein needed to elicit neutralizing antibodies. Genetic engineering has made it possible to express cloned genes efficiently in *Escherichia coli* using bacterial or bacteriophage promoters. Since prokaryotes are not able to add glycans to the viral membrane proteins, attempts to program eukaryotic cells, including yeast, with cloned genes have also been made. Whether the glycans of glycoproteins are important for the generation of neutralizing antibodies is still a question.

Using the recombinant DNA techniques, an FMDV vaccine that gives rise to neutralizing protecting antibodies in swine, albeit rather inefficiently,

has been produced in *E. coli* (Kleid *et al.*, 1981). Expression of HBsAg in *E. coli* (Burrell *et al.*, 1979) and yeast (Valenzuela *et al.*, 1982), influenza virus HA in *E. coli* (Emtage *et al.*, 1980; Davis *et al.*, 1983) and animal cells (Gething and Sambrook, 1982), rabies G protein in *E. coli* (Yelverton *et al.*, 1983), poliovirus proteins in *E. coli* (M. Chow and D. Baltimore, personal communication; van der Werf *et al.*, 1983) has been reported. With the exception of HBsAg produced in eukaryotic cells (Dubois *et al.*, 1980; Valenzuela *et al.*, 1982), the foreign proteins remain within the cell, thus causing problems in obtaining a pure subunit vaccine. In addition, rather low levels of expression of the membrane proteins have been obtained. Special problems arise when membrane protein genes are expressed (Pettersson, 1982; Yelverton *et al.*, 1983; Davis *et al.*, 1983). Due to the presence of the NH_2-terminal hydrophobic signal peptide necessary for translocation of the protein through the rough endoplasmic reticulum in animal cells, and the COOH-terminal stretch of hydrophobic amino acids necessary to anchor the protein in the lipid bilayer, the full gene product will associate with lipids and thus remain attached to the cloning host. Purification of such membrane-bound forms is bound to be very difficult. To circumvent these problems, e.g., the influenza virus HA gene has been modified such that the segment coding for the COOH-terminal anchor has been excised. Using an SV40 promoter and its own signal sequence, it was possible to synthesize a water-soluble secreted form of HA in monkey cells (Gething and Sambrook, 1982). Similar results have been obtained with the G protein of vesicular stomatitis virus (VSV) (Rose and Bergmann, 1982).

In these cells the signal peptide is cleaved off, thus releasing the protein from the membranes. In prokaryotes, the viral signal peptide is most likely not cleaved off. The DNA sequence encoding the signal sequence must therefore be removed and replaced by a bacterial one, so that the bacterial signal peptidases will recognize the correct cleavage site and process the protein into a soluble form. Whether the secreted forms of influenza HA and VSV G protein will elicit neutralizing antibodies is still not known.

To facilitate easy purification of viral membrane proteins produced from cloned genes, we have turned to another bacterial host, *Bacillus subtilis*. This organism has several beneficial properties as compared to *E. coli*. It is a good secretor of various cellular enzymes, it is a gram-positive bacterium lacking an outer membrane, it is nonpathogenic, it is a well-known industrial organism suitable for large-scale fermentation, its genetics is rather well known, and suitable plasmids are available as cloning vectors. The production of HBcAg and FMDV VP1 in *B. subtilis* (Hardy *et al.*, 1981) has been reported. However, in these cases the products remained cell associated. In order to get the products excreted from *B. subtilis* we have constructed a "secretion vector" (Palva, 1983). This vector contains

the promoter and signal sequence regions of the α-amylase gene from *Bacillus amyloliquefaciens*. Foreign genes can be joined at various distances downstream from the junction between the signal sequence and the exoamylase at a *Hin*dIII restriction enzyme site. Using a set of these vectors, secretion of *E. coli* β-lactamase (Palva *et al.*, 1982) and human α_2-interferon (Palva *et al.*, 1983) has been achieved. Here we report the joining of a viral membrane protein gene—the E1 protein of Semliki Forest virus (SFV)—to the secretion vector. Although E1 is readily synthesized in *B. subtilis*, only cell-associated protein was detected. The reason for the apparent lack of secretion is discussed.

II. CONSTRUCTION OF A SECRETION VECTOR FOR EXPRESSION OF FOREIGN GENES IN *BACILLUS SUBTILIS*

To construct a cloning vector with the aid of which foreign genes could be expressed so that the product would be secreted out of the cell (secretion vector), we wished to isolate the promoter and signal sequence regions from a gene whose product is normally secreted efficiently from bacilli. To this end, the gene encoding the α-amylase in *B. amyloliquefaciens* was isolated by shotgun cloning of partially *Mbo*I-digested chromosomal DNA using pUB110 as vector and *B. subtilis* as host (Palva, 1982). This gene was chosen since α-amylase is the major secretory protein of *B. amyloliquefaciens* and all detectable α-amylase is found outside the cell. The gene was identified in a 2.3-kilobase (kb) DNA fragment (Palva *et al.*, 1981) and completely sequenced (Takkinen *et al.*, 1983). The regions containing the promoter and the signal sequence were located on the gene by comparing the N-terminal amino acid sequences of the *in vitro* synthesized preamylase and the *in vivo* synthesized exoamylase with the nucleotide sequence of the cloned gene (Palva *et al.*, 1981). Next the DNA sequence coding for the exoamylase was removed by sequential digestion with exonuclease III and nuclease *Bal*31. Starting from a suitable *Eco*RI restriction enzyme site close to the 5′ end of the exoamylase, about 200 nucleotides were removed, leaving the signal sequence intact. Synthetic *Hin*dIII or *Eco*RI linkers were then ligated to the new ends of the heterogeneously digested molecules. A set of plasmids (pKTH50–58) containing the promoter, the whole signal sequence, and 0–42 nucleotides from the exoamylase region was thus obtained (Palva 1983). To these plasmids foreign genes can be joined via the restriction enzyme linkers in the desired reading frame. Using these vectors, *E. coli* TEM β-lactamase (Palva *et al.*, 1982) and human α_2- interferon (Palva *et al.*, 1983) have been successfully expressed in and secreted from *B. subtilis*.

Since genes from which 3′-terminal coding sequences have been re-

moved have lost their natural translation termination codon, we have also introduced a synthetic oligonucleotide [5′d(TGATTGATTGA)3′] containing the termination codon TGA in all reading frames downstream from the *Hin*dIII or *Eco*RI linkers. Thus, translation of any gene lacking its own termination codon will stop within this oligonucleotide (Pettersson *et al.*, 1983). Figure 2 shows the schematic structure of the secretion vector.

III. MODIFICATION OF THE E1 MEMBRANE PROTEIN GENE OF SEMLIKI FOREST VIRUS

To study whether the secretion vector could be used to express a viral membrane protein into a secreted water-soluble form, we used the glycoprotein E1 of Semliki Forest virus (SFV), a member of the alphaviruses within the Togaviridae family, as a model. The molecular biology of this virus is well understood (Kääriäinen and Söderlund, 1978), and the virus is an excellent model for developing, for example, subunit vaccines (Morein *et al.*, 1978). The monomeric spike protruding from the surface of SFV consists of one copy each of the glycoproteins E1, E2, and E3 (Ziemiecki and Garoff, 1978; Kääriäinen and Söderlund, 1978). The genes coding for these membrane proteins, as well as the capsid protein, have been cloned from a cDNA prepared from the viral genome and completely sequenced (Garoff *et al.*, 1980a,b). A full-length copy of all the structural genes including the noncoding flanking regions has been constructed (Kondor-Koch and Garoff, 1982). The overall organization of the structural genes of SFV is shown in Fig. 3A. The hydrophobic signal sequence of E1 is located within the 6K

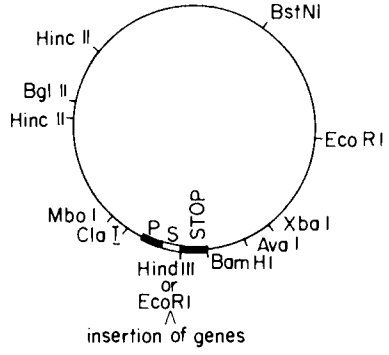


Fig. 2. General structure of the "secretion vector" constructed for the expression of foreign gene products in *B. subtilis*. P, promoter; S, signal sequence of the α-amylase gene of *B. amyloliquefaciens*; STOP, synthetic oligonucleotide encoding the translation termination signal TGA in all three reading frames. Reproduced from Pettersson *et al.* (1983) with permission of the publishers, Elsevier, Amsterdam.

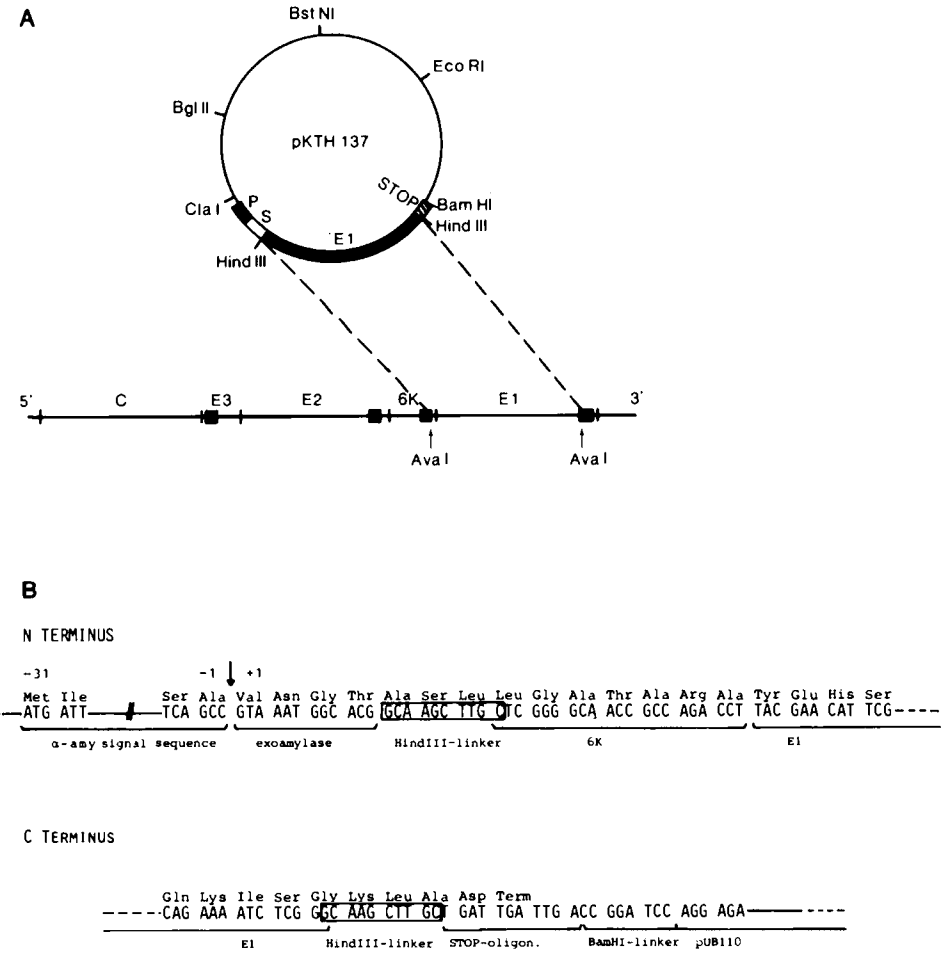


Fig. 3. Joining of the truncated E1 gene of SFV to the secretion vector. (A) The E1 gene was excised from the cloned cDNA by *AvaI*, subcloned in pBR322 and *E. coli*, and transferred to the secretion vector (Fig. 2), giving plasmid pKTH137. (B) Nucleotide sequence at the N- and C-terminal junctions of the E1 gene and the secretion vector.

polypeptide and the protein is anchored to the lipid bilayer by a 24-residue-long stretch of hydrophobic amino acids (Garoff *et al.*, 1980a). To avoid the possibility that E1 would remain attached to the bacterial membrane by these hydrophobic sequences, suitable *Ava*I sites were used to excise the E1 gene from the cloned cDNA so that the regions encoding the two hydrophobic sequences were removed (Fig. 3A). The ends were then filled in with reverse transcriptase, *Hin*dIII linkers were added, and the modified gene was joined to the *Hin*dIII site of pBR322 and cloned in *E. coli*.

IV. JOINING OF THE E1 GENE TO THE SECRETION VECTOR

The modified E1 gene was excised from pBR322 with *Hin*dIII and joined to a secretion vector (pKTH54) that gave the right reading frame. The nucleotide sequences at the 5′ and 3′ junctions were determined and are shown in Fig. 3B. At the 5′ end, E1 and 20 nucleotides (7 amino acids) derived from the 6K gene was joined via the *Hin*dIII linker (10 nucleotides encoding 3 extra amino acids) to the twelfth nucleotide (fourth amino acid) of the exoamylase. The 3′ end, lacking 71 nucleotides (23 amino acids) from the C terminus, including the hydrophobic anchor, was joined via the *Hin*dIII linker to the synthetic translation termination oligonucleotide. Translation of E1 should thus stop at the second TGA codon, which is in frame with the E1 gene. This would give only 4 extra amino acids, 3 of which would be linker encoded (Pettersson *et al.*, 1983).

V. EXPRESSION OF E1 IN *BACILLUS SUBTILIS*

The hybrid plasmid containing the modified E1 gene (pKTH137) was transformed into *B. subtilis*, and the expression of E1 was studied at the end of the exponential growth phase. Samples were taken from the cells and the cleared medium and analyzed by immunoblotting using a specific antiserum against E1 (Väänänen, 1982) and iodinated *Staphylococcus aureus* protein A. An autoradiogram of such an analysis is shown in Fig. 4. An immunoreactive protein probably representing E1 was readily detected in the cells (lane 2), whereas almost no protein was found outside the cell (lane 3). The same result was also obtained at earlier and later stages of the growth curve. The level of expression was low, being about 100 μg of E1 per liter. This is about 10^{-3} to 10^{-4} the amount of α-amylase, 10^{-2} of *E. coli* β-lactamase, and 10^{-1} of human α_2-interferon produced from their respective cloned genes (Palva, 1983). We can therefore conclude that a low level of E1 is being made in *B. subtilis* and that almost all of it is cell associated.

One possible reason for the apparent lack of secretion of E1 could be the presence of proteases that rapidly degrade the secreted product. *B. subtilis* is known to export much serine protease and metalloprotease. We therefore analyzed the production of E1 in the presence of protease inhibitors. The effect of Trasylol and benzamide, two serine protease inhibitors, is shown in Fig. 4. Under these conditions some E1 is also found in the medium (lanes 4 and 6). The mobility of the extracellular form is slightly faster than that of the cell-associated form (lanes 3 and 5). We do not, however, know whether the extracellular form is properly cleaved at the expected cleavage site. Based on these results, it seems likely that the low level of expression and the apparent lack of secretion is due to the rapid degradation of E1 by

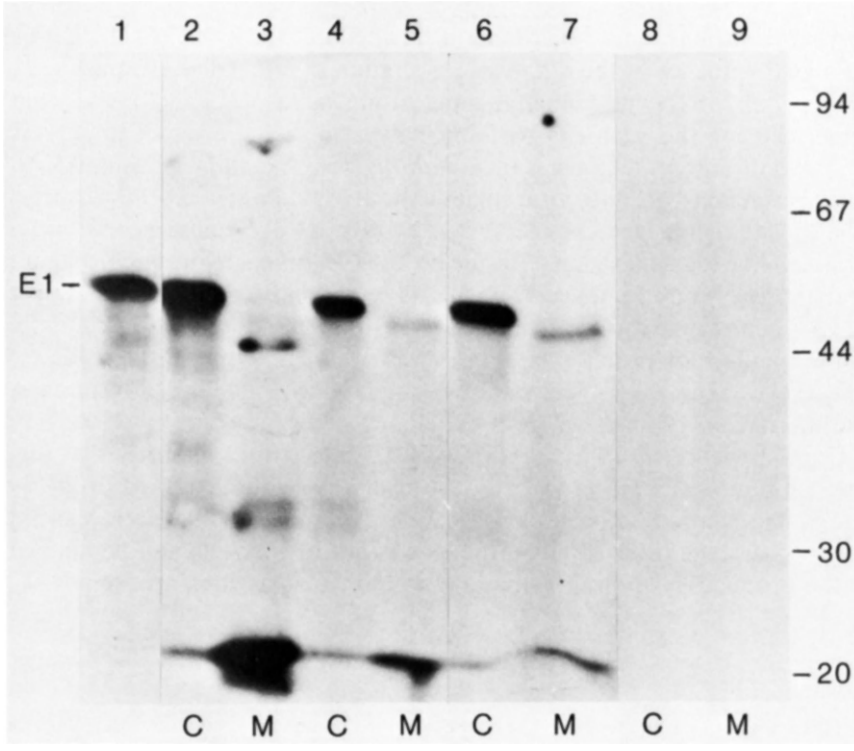


Fig. 4. Immunoblotting analysis of SFV E1 protein synthesized in *B. subtilis*. Cells were transformed with pKTH137 (Fig. 3), and samples from the cells (lanes 2, 4, and 6) and medium (lanes 3, 5, and 7) were fractionated on a 10% SDS-polyacrylamide gel. The synthesis of E1 was analyzed by immunoblotting using specific antiserum against E1 and ^{125}I-labeled protein A from *S. aureus*. (Lane 1) E1 from purified virions; (lane 2) cells; and (lane 3) medium in the absence of protease inhibitors. (Lanes 4 and 6) cells; and (lanes 5 and 7) medium, in the presence of Trasylol and benzamidine, respectively. Samples from cells (lane 8) and medium (line 9) of *B. subtilis* transformed with a hybrid plasmid containing the E1 gene in the wrong orientation. The positions of molecular weight markers are indicated on the right.

proteases. We have also expressed from the cloned genes the E2 protein of SFV (Lundström *et al.*, unpublished) and the G protein of vesicular stomatitis virus (VSV) (Lundström, 1984) with very similar results. It may therefore be that large truncated viral membrane proteins without glycans are extremely sensitive to *B. subtilis* proteases.

VI. CONCLUSIONS

We have described here the construction of a secretion vector that aids the expression of foreign gene products from the cloned genes in *B. subtilis*. The genes are expressed from the promoter of the α-amylase gene of *B.*

amyloliquefaciens, and the product is directed out of the cell by the aid of the signal sequence of the α-amylase. Secretion of β-lactamase from *E. coli* (Palva *et al.*, 1982) and human α_2-interferon (Palva *et al.*, 1983) have been achieved using this vector system. We have also used the vector to express viral membrane protein genes in *B. subtilis* with the aim of being able to use this system to produce viral subunit vaccines. Here we report the expression of one such gene—the membrane protein E1 of Semliki Forest virus. Although the segments coding for the N- and C-terminal hydrophobic amino acid stretches were removed to facilitate secretion of the product, almost all E1—contrary to our expectations—was found to be cell associated. Only in the presence of protease inhibitors was some E1 found in the medium. The level of expression of E1 was low compared to α-amylase, β-lactamase, and interferon, synthesized from the cloned genes.

Our experience with the secretion vector system so far indicates that foreign gene products are produced at a lower level than expected probably due to degradation by bacterial proteases. More research has to be carried out to overcome these serious problems before this system can be applied to the production of viral membrane proteins for vaccine purposes.

ACKNOWLEDGMENTS

This work has been supported by the Finnish National Fund for Research and Development (SITRA), Project 13005, and the Research Laboratory of State Alcohol Company, ALKO.

REFERENCES

Almeida, J. D., Brand, C. M., Edwards, D. C., and Heath, T. D. (1975). *Lancet* **2,** 899–901.

Bachrach, H. L., Moore, D. M., McKercher, P. D., and Polatnic, J. (1975). *J. Immunol.* **115,** 1636–1641.

Beale, J. (1982). *Nature (London)* **298,** 14–15.

Burrell, C. J., Mackay, P., Greenaway, P. J., Hofschneider, P. H., and Murray, K. (1979). *Nature (London)* **279,** 43–47.

Choppin, P. W., Richardson, C. D., Merz, D. C., Hall, W. W., and Scheid, A. (1981). *J. Infect. Dis.* **143,** 352–363.

Chow, M., and Baltimore, D. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 7518–7521.

Cox, J. H., Dietzschold, B., and Schneider, L. G. (1977). *Infect. Immun.* **16,** 743–759.

Davis, A. R., Bos, T., Ueda, M., Nayak, D. P., Dowbenko, D., and Compans, R. W. (1983). *Gene* **21,** 273–284.

Dietzschold, B., Wiktor, T. J., Wunner, W. H., and Varrichio, A. (1983). *Virology* **124,** 330–337.

Dubois, M. -F., Pourcel, G., Rousset, S., Chany, C., and Tiollais, P. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4549–4553.

Emtage, J. S., Tacon, W. C. A., Catlin, G. H., Jenkins, B., Porter, A. G., and Carey, N. H. (1980). *Nature (London)* **283,** 171–174.

Evans, D. M. A., Minor, P. D., Schild, G. S., and Almond, J. W. (1983). *Nature (London)* **304**, 459–462.

Garoff, H., Frischauf, A. -M., Simons, K., Lehrach, H., and Delius, H. (1980a). *Nature (London)* **288**, 236–241.

Garoff, H., Frischauf, A. -M., Simons, K., Lehrach, H., and Delius, H. (1980b). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6376–6380.

Gething, M. -J., and Sambrook, J. (1982). *Nature (London)* **300**, 598–603.

Green, N., *et al.* (1982), *Cell* **28**, 477–487.

Hardy, K., Stahl, S., and Küpper, H. (1981). *Nature (London)* **293**, 481–483.

Heinz, F. X., and Kunz, C. H. (1980). *J. Gen. Virol.* **49**, 125–132.

Heinz, F. X., Berger, R., Tuma, W., and Kunz, C. (1983). *Virology* **126**, 525–537.

Helenius, A., and von Bonsdorff, C. -H. (1976). *Biochim. Biophys. Acta* **436**, 895–899.

Kääriäinen, L., and Söderlund, H. (1978). *Curr. Top. Microbiol. Immunol.* **82**, 15–69.

Kleid, D. G., *et al.* (1981). *Science* **214**, 1125–1129.

Kondor-Koch, C., and Garoff, H. (1982). *J. Gen. Virol.* **58**, 443–448.

Kuismanen, E., Hedman, K., Saraste, J., and Pettersson, R. F. (1982). *Mol. Cell. Biol.* **2**, 1444–1458.

Lee, G. T. -Y., Para, M. F., and Spear, P. G. (1982). *J. Virol.* **43**, 41–49.

Lerner, R. A. (1982). *Nature (London)* **299**, 592–596.

Lundström, K. (1984). *FEMS Microbiol. Lett.* **23**, 65–70.

Matlin, K. S., Reggio, H., Helenuis, A., and Simons, K. (1982). *J. Mol. Biol.* **156**, 609–631.

Minor, P. D., *et al.* (1983). *Nature (London)* **301**, 674–679.

Morein, R., Helenius, A., Simons, K., Pettersson, R., Kääriäinen, L., and Schirrmacher, V. (1978). *Nature (London)* **276**, 715–718.

Morein, B., Sharp, M., Sundqvist, B., and Simons, K. (1983). *J. Gen. Virol.* **64**, 1557–1569.

Morein, B., Sundquist, B., Höglund, S., Dalsgaard, K., and Osterhaus, A. (1984). *Nature (London)* **308**, 457–460.

Oker-Blom, C., Kalkkinen, N., Kääriäinen, L., and Pettersson, R. F. (1983). *J. Virol.* **46**, 964–973.

Oxford, J. J., Hockley, D. J., Heath, T. D., and Patterson, S. (1981). *In* "The Replication of Negative Strand Viruses" (D. H. L. Bishop and R. W. Compans, eds.), pp. 189–193. Elsevier/North-Holland, Amsterdam.

Palva, I. (1982). *Gene* **19**, 81–87.

Palva, I. (1983). Ph.D. Thesis, University of Helsinki.

Palva, I., Pettersson, R. F., Kalkkinen, N., Lehtovaara, P., Sarvas, M., Söderlund, H., Takkinen, K., and Kääriäinen, L. (1981). *Gene* **15**, 43–51.

Palva, I., Sarvas, M., Lehtovaara, P., Sibakov, M., and Kääriäinen, L. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5582–5586.

Palva, I., Lehtovaara, P., Kääriäinen, L., Sibakov, M., Cantell, K., Schein, C. H., Kashiwagi, K., and Weissman, C. (1983). *Gene* **22**, 229–235.

Pettersson, R. F. (1982). *Ann. Clin. Res.* **14**, 245–252.

Pettersson, R. F., Lundström, K., Chattopadhyaya, J. B., Josephson, S., Philipson, L., Kääriäinen, L., and Palva, I. (1983) *Gene* **24**, 15–27.

Rose, J. K., and Bergmann, J. E. (1982). *Cell* **30**, 753–762.

Seigneurin, J. M., Desgranges, C., Paire, J., Renversez, J. C., Jacquemont, B., and Micouin, C. (1983). *Science* **221**, 173–175.

Szmuness, W., *et al.* (1981). *Hepatology* **1**, 377–385.

Takkinen, K., Pettersson, R. F., Kalkkinen, N., Palva, I., Söderlund, H., and Kääriäinen, L. (1983). *J. Biol. Chem.* **258**, 1007–1013.

Väänänen, P. (1982). *J. Virol. Methods* 4, 117–126.

Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G., and Hall, B. D. (1982). *Nature (London)* **298**, 347–350.
van der Werf, S., Dréano, P., Kopecka, H., and Girard, M. (1983). *Gene* **23**, 85–93.
Wild, T. F., Burroughs, J. M., and Brown, F. (1969). *J. Gen. Virol.* **4**, 313–320.
Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981). *Nature (London)* **289**, 366–373.
Yelverton, E., Norton, S., Obijeski, J. E., and Goeddel, D. V. (1983). *Science* **219**, 614–620.
Ziemiecki, A., and Garoff, H. (1978). *J. Mol. Biol.* **122**, 259–269.

5

Production of Hepatitis-Safe Plasma Proteins from Cold Sterilized Plasma

W. STEPHAN

Biotest Pharma GmbH, Frankfurt am Main, Federal Republic of Germany

I. INTRODUCTION

Despite present-day utilization of sensitive "third generation" test methods for the detection of hepatitis B virus (HBV) carriers, and the resulting exclusion of plasma containing detectable hepatitis B surface antigen (HBsAg) from plasma pools, residual HBV and non-A, non-B hepatitis viruses continue to contaminate most lots of the high-risk blood derivatives (Boklan, 1971; Faria and Fiumara, 1972; Hellerstein and Deykin, 1971; Iwarson *et al.*, 1976; Oken *et al.*, 1972; Sandler *et al.*, 1973). For example, Ohlmeier and colleagues found that 71% of prospectively followed cardiac surgery patients who received coagulation factor concentrates prepared from plasma which was free of HBsAg detectable by third-generation methods developed hepatitis (Ohlmeier *et al.*, 1978). The problem is especially acute for non-A, non-B hepatitis virus(es), which presently produce the majority of cases of hepatitis transmitted by transfusion, and for which there is no effective test for identification of carriers (Prince *et al.*, 1974; Alter *et al.*, 1975; Wyke *et al.*, 1979; Sugg *et al.*, 1979).

There is thus a need for sterilization procedures which can be applied to the production of labile blood derivatives. A satisfactory sterilization procedure should effectively inactivate any quantity of HBV, or non-A, non-

ISBN 0-12-429601-7

B hepatitis virus(es) likely to be present in large plasma pools and must preserve the desired labile blood proteins in a biologically active and undenatured state. Furthermore, the resulting products must be nonimmunogenic and noncarcinogenic.

A *combined* β-propiolactone–ultraviolet irradiation (β-PL/UV) procedure was first proposed by LoGrippo and colleagues as an effective means of sterilization of pooled human plasma (Hartman and LoGrippo, 1957; LoGrippo and Hartman, 1958; LoGrippo and Hayashi, 1973).

II. COLD STERILIZATION OF PLASMA

The treatment of plasma by β-PL is performed under standardized conditions between temperatures of 5 and 37°C at constant pH.

The unique properties of β-PL are as follows: (1) It is a strong alkylating agent and inactivates virus infectivity by alkylating viral nucleic acids (Brookes and Lawley, 1964). (2) β-PL reacts very rapidly with water, so that a few hours after addition to plasma it is completely transformed into the nontoxic β-hydroxypropionic acid (Kelly *et al.*, 1957) and is not demonstrable by gas chromatography (Pruggmayer and Stephan, 1976) or in the Ames test (Pincus *et al.*, 1981) in the final products.

The treatment of plasma with β-PL is followed by irradiation with UV light in a rotation-flow apparatus (Fig. 1). The plasma to be irradiated forms a thin film on a rotating cylinder and is irradiated from the center of this cylinder by four UV lamps. The dosage of irradiation is regulated and standardized by variation of the flow rate, to achieve a consistent virucidal effect.

The efficacy of this combined sterilization method has been demonstrated by several studies. LoGrippo and Hayashi reported in 1973 that

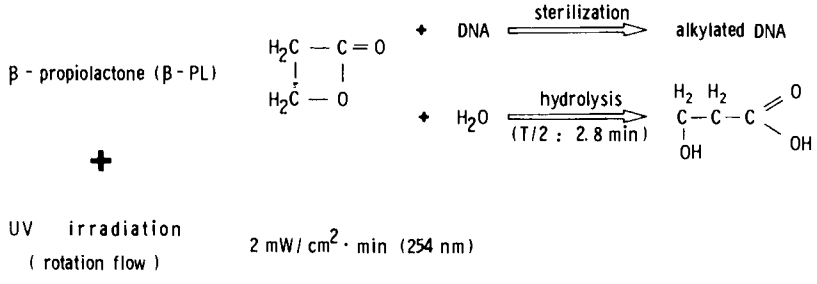


Fig. 1. Cold sterilization technology (5–37°C). From LoGrippo (1956).

HBsAg-positive plasma sterilized with β-PL/UV did not induce clinical hepatitis in 280 patients. Kornhuber showed in 1974 that β-PL/UV-treated serum from a 1000 donor pool showed no hepatitis in 30 high-risk children suffering from leukemia. Studies in chimpanzees demonstrated no hepatitis B and no hepatitis non-A, non-B after the application of β-PL/UV-treated factor IX concentrate (Stephan and Berthold, 1980; Prince *et al.*, 1980). Studies in chimpanzees with HBV-contaminated infectious serum (Stephan *et al.*, 1981a,b) and plasma (Prince *et al.*, 1983) evaluated the efficacy of the β-PL/UV method; it was found that β-PL/UV treatment reduces the HBV infectivity 10^6- to 10^7-fold. This effect is 10^2–10^3 times greater than the effect of pasteurization, for which Shikata *et al.* (1978) found a 10^4-fold reduction of HBV infectivity. It has also been demonstrated that β-PL/UV inactivates non-A, non-B virus infectivity (Prince *et al.*, 1983).

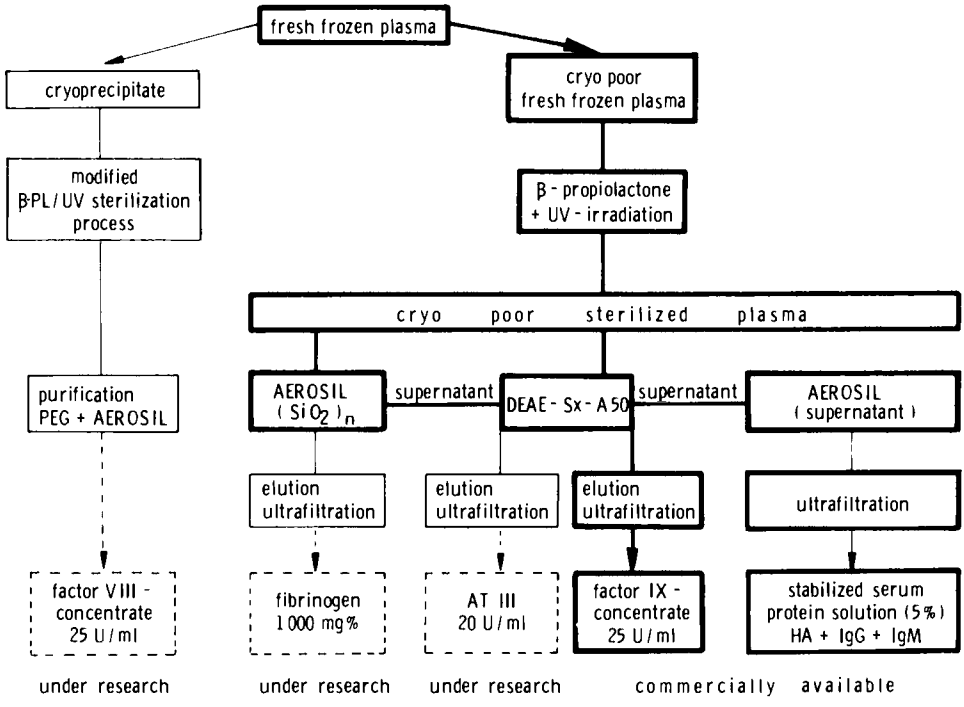


Fig. 2. Fractionation of cold sterilized human plasma given as a production scheme. Sx-A50, Sephadex A50; PEG, polyethylene glycol; HA, human albumin; AT III, antithrombin III.

III. COMBINATION OF COLD STERILIZATION AND ADSORPTION

Colloidal silicic acid in the form of Aerosil 380 is used as an absorbent following the β-PL/UV sterilization process. Aerosil is a special form of high-molecular-weight silicic acid, produced by hydrolysis of silicon tetrafluoride at high temperature (Wagner and Brünner, 1960). Under appropriate conditions, Aerosil adsorbs fibrinogen without any adsorption of the coagulation factors of the PPSB complex (factor IX concentrate); the adsorbed fibrinogen can be eluted with 20% saline at pH 9. Furthermore Aerosil in excess adsorbs quantitatively α- and β-lipoproteins (Stephan and Róka, 1968). This is important for plasma protein solutions which are stored in liquid form, since lipoproteins are highly labile and precipitate within weeks, which interferes with intravenous application.

The process described in Fig. 2 yields the following products: factor VIII, fibrinogen, AT III, factor IX concentrate (PPSB), and a stabilized serum protein preparation which contains, in addition to albumin and other biologically important proteins, especially IgG and IgM, in an intravenously applicable form. In contrast to factor IX concentrate and the stabilized serum preparation called Biseko[1] (BS) which are commercially available, optimal procedures for factor VIII, fibrinogen, and AT III are still under investigation.

The properties of PPSB and the stabilized serum preparation BS are discussed in Section IV.

IV. PROPERTIES OF STERILIZED FACTOR IX CONCENTRATE

Concerning factor IX concentrate derived from cold-sterilized plasma,[1] it has been demonstrated by Schimpf and Westphal (1980) that the cold-sterilized preparation has the same *in vitro* coagulation activities in comparison with other unsterilized commercially available products. These findings have been confirmed by *in vivo* recovery studies on hemophiliacs (Schimpf and Westphal, 1980). Six hemophiliacs received 25 units of factor IX per kilogram body weight by application of cold-sterilized factor IX in comparison with two other products. The recovery was found to be unchanged by β-PL/UV treatment. In the same study the half-life of factor IX was found to be 24 hr for the cold-sterilized product and 23 hr for the other products.

[1]Manufacturer is Biotest Pharma GmbH, Frankfurt am Main, Federal Republic of Germany.

The thrombogenicity of factor IX concentrates was studied in chimpanzees (Kotitschke *et al.*, 1983). For this purpose PPSB isolated from β-PL/UV-treated plasma was injected into chimpanzees at a dosage of approximately 100 U/kg body weight. An FDA-licensed PPSB preparation served as a control. The parameters most relevant for thrombogenicity, i.e., fibrinogen degradation products and platelet count, remained normal in all animals.

To determine whether β-PL/UV-treated factor IX concentrate is immunogenic, three chimpanzees received β-PL/UV-treated material in a dosage of approximately 500 U of factor IX/per animal, 10 times at weekly intervals (Stephan *et al.*, 1981a,b). Fifteen minutes before and after each PPSB application, blood was tested for coagulation factor recovery; at the end of the study, tests for detection of potential antibodies against β-PL/UV-modified proteins were performed using skin tests, PCA tests, and immunodiffusion. These tests showed no signs of sensitization for either of the preparations.

Furthermore, factor IX recovery studies did not reveal any significant difference between β-PL/UV-treated and the untreated material throughout this study. Recoveries of factors II, VII, IX, and X were also unchanged, thus indicating that no antibodies against the coagulation factors had been induced.

The safety of the β-PL/UV-treated factor IX concentrate with respect to hepatitis was investigated in a chimpanzee study performed by Prince *et al.* (1980). Eight chimpanzees received 25 U of factor IX/kg body weight from a mixture of five production lots of PPSB derived from β-PL/UV-treated human plasma from approximately 1000 donors. These animals developed neither hepatitis B nor hepatitis non-A, non-B. This finding is in accordance with a clinical study which shows no hepatitis in volunteers after the application of cold-sterilized PPSB (Heinrich and Kotitschke, 1981).

V. PROPERTIES OF STABILIZED SERUM BISEKO

Biseko (BS) contains, in addition to albumin and other biologically important proteins, IgG and IgM, in an intravenously applicable form (Table I). The justification for such a serum preparation is to be found in clinical practice, where albumin deficiency is frequently accompanied by immunoglobulin deficiency—e.g., as a result of operation and in burn cases. Thus, the rationale for laborious separation of albumin from the immunoglobulins can be questioned.

The biological activity of proteins in BS has been investigated recently. It was found that the bilirubin-binding capacity of albumin in BS is com-

TABLE I
Protein Composition of Biseko

Product	Total protein (g/100 ml)	Cellulose acetate foil electrophoresis				
		Albumin	α_1	α_2	β	γ
Serum	7.25	60.4	3.2	10.9	8.9	16.6
BS 206030	5.4	62.9	4.0	10.7	7.2	15.2
BS 222020	5.7	64.0	4.0	10.0	6.9	15.1
BS 213020	5.7	62.6	4.3	10.4	7.5	15.2

parable with the bilirubin-binding capacity of the albumin in normal serum. The same was true for the hemoglobin-binding capacity of haptoglobin, the iron-binding capacity of transferrin, and the copper-binding of ceruloplasmin (Table II). Fibrin–agar electrophoresis revealed that α_1-antitrypsin and α_2-macroglobulin inhibitor activity in BS was only slightly decreased in comparison with normal serum (Fig. 3). The labile proteins in BS were stable during storage for 4 weeks at 37°C with the exception of α_1-antitrypsin and ceruloplasmin, which show a drop in concentration. Thus, it is desirable to store BS at 5°C. The lability of α_1-antitrypsin was also confirmed by fibrin–agar electrophoresis, which showed a drop of activity during storage at 37°C. Antibody activity was found to be stable. Quantitation of antibody activity showed no effect of storage on antibody activity within the margin of error (Tables III and IV).

To examine possible hepatitis virus infectivity in BS, a safety test in chimpanzees was performed. For this purpose, a mixture of five BS production lots (from 19,000 donors) was given to six chimpanzees in a dosage of 7 ml/kg, equivalent to a human therapeutic dose. No animal developed hepatitis B or non-A, non-B (Prince *et al.*, 1983).

It is to be hoped that application of this approach to fractionation will provide a safe and effective methodology for many other potentially useful blood derivatives.

TABLE II
Transport Proteins in Biseko

Product	Albumin (mg/100 ml)	Haptoglobin (mg/100 ml)	Transferrin (mg/100 ml)	Ceruloplasmin (mg/100 ml)
Serum (5.5 %)	3210	140	257	27
BS 206030	3310	133	250	16
BS 222020	3420	118	262	17
BS 213020	3380	134	260	18

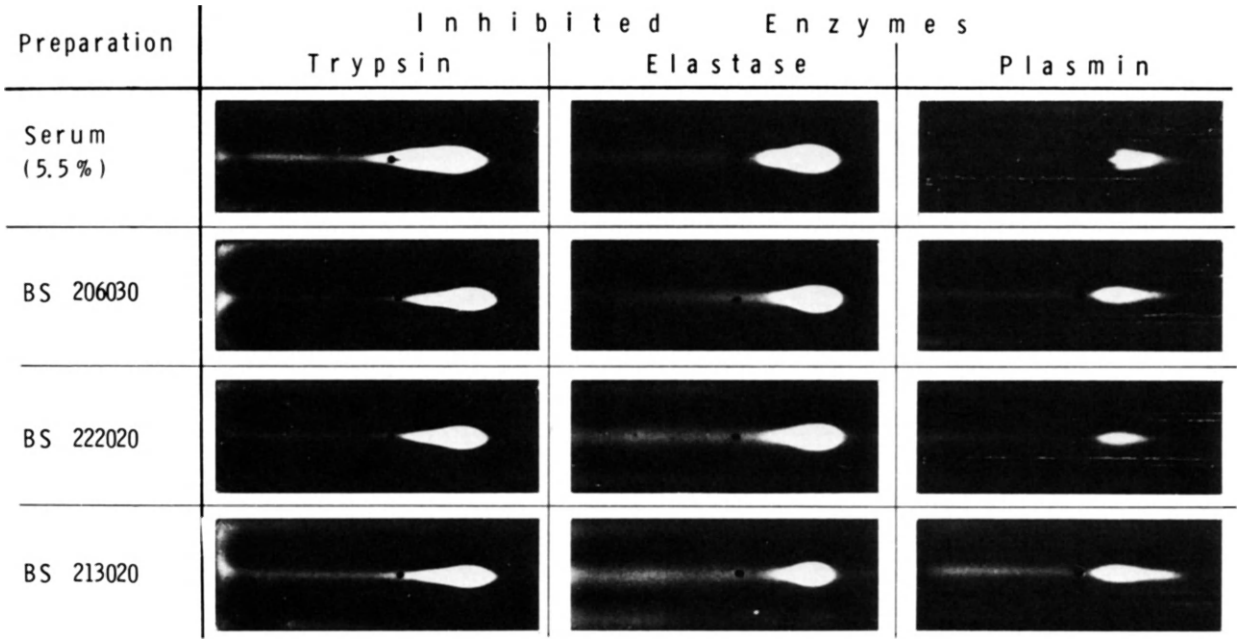


Fig. 3. Determination of inhibitory activity of α_1-antitrypsin and α_2-macroglobulin in Biseko by fibrin-agar gel electrophoresis. The area of undigested fibrin (white) is proportional to the inhibitory activity.

TABLE III
Effect of Storage on Activity of Bacterial Antibodies in Biseko

Product	Antigen				
	E. coli	*Streptococcus pyogenes*	*Klebsiella*	*Streptococcus viridans*	Streptolysin (U/ml)
	Reciprocal titers				
BS 206030, 5°C	320	80	320	40	100
4 weeks, 37°C	160	40	160	40	100
BS 222020, 5°C	160	40	320	40	100
4 weeks, 37°C	160	80	160	40	100
BS 213020, 5°C	320	80	320	80	100
4 weeks, 37°C	160	40	160	40	150

TABLE IV
Effect of Storage on Activity of Viral Antibodies in Biseko[a]

Product	Antigen (reciprocal titers)			
	Rubella (HIT)	Herpes simplex (CF)	HBsAg (RIA)	A_2Taiwan (HIT)
BS 206030, 5°C	64	20	32	40
4 weeks, 37°C	64	10	32	40
BS 222020, 5°C	64	20	64	40
4 weeks, 37°C	64	10	32	40
BS 213020, 5°C	64	40	32	40
4 weeks, 37°C	64	20	32	40

[a]CF, complement fixation; RIA radioimmunoassay; HIT, hemagglutination inhibition test.

REFERENCES

Alter, H. J., Holland, P. V., Morrow, A. G., Purcell, R. H., Feinstone, S. M., and Moritsugu, Y. (1975). *Lancet* **2**, 838–842.
Boklan, B. F. (1971). *Ann. Int. Med.* **74**, 298.
Brookes, P., and Lawley, P. D. (1964). *Br. Med. Bull.* **20**, 91.

Faria, R., and Fiumara, N. J. (1972). *N. Engl. J. Med.* **287**, 358-359.
Hartman, F. W., and LoGrippo, G. A. (1957). *In* "Hepatitis Frontiers" (*Henry Ford Hosp. Int. Symp. 1956*), pp. 407-416. Little, Brown, Boston, Massachusetts.
Heinrich, D., and Kotitschke, R. (1981). *Haemostasis* **10**, Suppl. 1, 213.
Hellerstein, L. J., and Deykin, D. (1971). *N. Engl. J. Med.* **284**, 1039-1040.
Iwarson, S., Kjellman, H., and Teger-Nilsson, A. C. (1976). *Vox Sang.* **31**, 136-140.
Kelly, A. R., Hartman, F. W., and Rupe, C. E. (1957). *In* "Hepatitis Frontiers" (*Henry Ford Hosp. Int. Symp. 1956*), pp. 387-406. Little, Brown, Boston, Massachusetts.
Kornhuber, B. (1974). *Biotest-Mitt.* **35**, 44.
Kotitschke, R., Stephan, W., Prince, A. M., and Brotman, B. (1983). *Thromb. Res.* **30**, 235-244.
LoGrippo, G. A. (1956). *Bacteriol. Proc.*, 101.
LoGrippo, G. A., and Hartman, F. W. (1958). *Bibl. Haematol. (Basel)* **7**, 225-230.
LoGrippo, G. A., and Hayashi, H. (1973). *Henry Ford Hosp. Med. J.* **21**, 181.
Ohlmeier, H., Dahmen, E., and Hoppe, I. (1978). *Dtsch. Med. Wochenschr.* **103**, 1700-1703.
Oken, M. M., Hootkin, L., and DeJager, R. L. (1972). *Am. J. Dig. Dis.* **17**, 271-274.
Pincus, J. H., Mortelmans, K., Tanaka, W., Stephan, W., and Lissner, R. (1981). *Arzneim.-Forsch.* **31** (II), 1924.
Prince, A. M., Grady, G. F., Hazzi, C., Brotman, B., Kuhns, W. J., Levine, R. W., and Millian, S. J. (1974). *Lancet* **1**, 241-246.
Prince, A. M., Stephan, W., Brotman, B., and Van den Ende, M. C. (1980). *Thromb. Haemostasis* **44**, 138-142.
Prince, A. M., Stephan, W., and Brotman, B. (1983). *Rev. Inf. Dis.* **5**, 92-107.
Pruggmayer, D., and Stephan, W. (1976). *Vox Sang* **31**, 191.
Sandler, S. G., Rath, C. E., Wickerhauser, M., Dodd, R. J., and Greenwalt, T. J. (1973). *Transfusion* **13**, 221-223.
Schimpf, K., and Westphal, B. (1980). *In* "Fibrinolyse, Thrombose, Hämostase" (E. Deutsch and K. Lechner, eds.), pp. 335-338. Schattauer, Stuttgart.
Shikata, T., Karasawa, T., Abe, K., Takahashi, T., Mayumi, M., and Oda, T. (1978). *J. Infect. Dis.* **138**, 242.
Stephan, W., and Berthold, H. (1980). *In* "Fibrinogen, Fibrin und Fibrinkleber (K. Schimpf, ed.), pp. 321-326. Schattauer, Stuttgart.
Stephan, W., and Róka, L. (1968). *Z. Klin. Chem. Klin. Biochem.* **6**, 186-190.
Stephan, W., Berthold, H., and Prince, A. M. (1981a). *Vox Sang.* **41**, 134.
Stephan, W., Kotitschke, R., Prince, A. M., and Brotman, B. (1981b). *Thromb. Haemostasis* **46**, 511.
Sugg, U., Schnaidt, M., Schneider, W., and Lissner, R. (1979). *N. Engl. J. Med.* **303**, 943.
Wagner, E., and Brünner, H. (1960). *Angew. Chem.* **72**, 744.
Wyke, R. J., Tsiquaye, K. N., Thornton, A., White, Y., Portmann, B., Das, P. K., Zuckerman, A. J., and Williams, R. (1979). *Lancet* **1**, 520-524.

6

Factors Influencing the Performance of Foot-and-Mouth Disease Vaccines under Field Conditions

THOMAS W. F. PAY

Foot-and-Mouth Disease Vaccine Division,
Wellcome Biotechnology Ltd., Pirbright, Surrey, England

I. INTRODUCTION

Although foot-and-mouth disease (FMD) vaccines have been in use in many regions of the world on a most extensive scale for many years, and although there is a vast literature in existence on most aspects of FMD, it is surprising to find that relatively few publications concern themselves with

APPLIED VIROLOGY

ISBN 0-12-429601-7

the use of vaccine under field conditions and very few of these set out to examine the factors which can influence vaccine performance. As a result, it is not unusual to find that users of FMD vaccines have only a limited understanding of what can be expected of good quality vaccine under certain conditions of field use. In this chapter it is proposed to identify some of the more important factors that determine how well an FMD vaccine appears to perform. All the factors to be described are closely interrelated but, in identifying them and in assessing their relative importance, it is convenient to group them under four main headings: the disease, the vaccine, the host, and the human element.

II. THE DISEASE

A. Pathogenesis

There are several features relating to the pathogenesis of FMD that render it a very difficult disease against which to produce an effective, long-lasting immunity. Thus, its ability to produce fully generalized infection in as short a time as 48 hr after contact exposure means that there is little or no time for the development of the anamnestic postinfection serum antibody response which occurs in primed individuals and is recognized as playing a very significant part in postvaccinal immunity to many diseases with longer incubation periods. Most authors agree that immunity to FMD depends on the maintenance of certain critical levels of serum neutralizing antibody, which may be different for different virus strains.

de Leeuw and co-workers (1979) showed that a level of immunity that is adequate to protect pigs against a moderately heavy challenge can be overwhelmed by the massive challenge that results from close contact with an animal with fully generalized infection. They showed that the constant inhalation of vast quantities of excreted virus in aerosol form could give rise to direct penetration of the lung and the occurrence of an instantaneous and persistent viremia, which could swamp normally protective levels of antibody. It seems possible that a similar effect could occur in cattle.

During a "needle" challenge, animals may develop a primary lesion at the inoculation site, but providing they do not develop secondary, generalized lesions they are classified as immune. However, should such a primary lesion develop by accident as the result of trauma in an animal which is merely in contact with an infected animal, it is classified as nonimmune. Thus the apparent morbidity rates will often be significantly influenced by whether animals showing only partial generalization are classified as infected or protected.

B. Antigenic Variation of the Virus

The seven serotypes of FMD virus are totally distinct immunologically, and there is no cross protection whatsoever between them. It is also well recognized that only partial protection may be obtained between viruses of different subtypes. What is probably not so well recognized is the fact that important one-way antigenic differences can exist between viruses which can nominally be classified within the same subtype. If the vaccine virus is dominant to the field virus there will be no problem, but if the field virus is dominant to the vaccine virus there could be a problem. These unilateral differences are frequently more pronounced in comparisons made using the serum neutralization test (SNT) than the complement fixation test (CFT). The old concept of subtypes forming discontinuous groups of very closely related viruses has now been abandoned (Forman, 1975). In 1977 we put forward the view that the criteria for vaccine strain evaluation, although related, are quite distinct from those used for subtype classification (Rweyemamu *et al.*, 1977). We formulated these concepts: (1) the neutralization test should be the *in vitro* test of choice; (2) antisera to vaccine viruses should form the basis for comparison, *not* antisera to a prototype virus of the subtype to which the vaccine strain belongs; (3) the r value, not the R value, should form the basis of strain selection; and (4) test errors should be considered in arriving at the significance of an estimated r value.

These ideas are becoming widely accepted now and have been endorsed by the FMD Vaccine Strains Committee of the International Association of Biological Standardization. Experience has shown that there is a good correlation between the SNT to a heterologous virus strain and challenge with that strain. Hence, when a cross neutralization r value is available it is possible to predict with reasonable accuracy the likely level of protection that a vaccine of known potency will offer against the heterologous virus strain. A fuller discussion of the effects of antigenic variation on vaccine performance can be found (Pay, 1983).

C. Incidence

It is sometimes said that there is a level of infection of FMD, above which it is very difficult to obtain effective control even with the regular application of good quality vaccines, and below which it becomes progressively easier to do so. If one studies the incidence of FMD in a number of Western European countries subsequent to the introduction of a national FMD vaccination campaign, it can be seen that the rate of reduction of the disease is very approximately exponential. Tens of thousands of outbreaks reduce to thousands, then to hundreds, then to tens, then to units and to zero over periods of 5 to 10 years.

One of the indirect effects of vaccination is to reduce the amount of disease in the field and thereby the force of infection, defined as the probability that a nonimmune animal will become infected within a specified time interval (May, 1982). In Europe, vaccination of cattle is carried out only once a year; as we shall see, this results in a large proportion of the cattle being nonimmune for part of the year. Despite this, however, a high level of vaccination coverage, coupled with good zoosanitary control measures, such as "stamping-out," quarantine, movement control, and ring vaccination, has succeeded in reducing the force of infection and of eventually eradicating the disease in most Western European countries. Now annual vaccination aims not at maintaining an immune population, but at maintaining a primed population, capable of developing a rapid and high level of immunity following an emergency revaccination.

When a high incidence of infection is present, there is a high probability for nonimmune animals to be exposed, sooner or later, to the virus and become infected. Once a nonimmune animal has introduced disease to a farm, there is a strong possibility that the immunity of some vaccinated cattle in close contact will be swamped, as described above, and the resulting morbidity level may not reflect the true original level of herd immunity.

III. THE VACCINE

A. Potency

The immunogenic fraction of FMD virus is the intact virion, the so-called 140 S antigen (Wild and Brown, 1968). A linear relationship has been described between the percentage (probit) of cattle that are immunized and the log dose of antigen contained in an FMD vaccine. The median value of the slopes of the regressions obtained from more than 100 potency tests was approximately 2.0 (Pay and Parker, 1977). More recently, a detailed study of four type A_{24}, four type O_1, and four type C_1 monovalent batches of vaccine, for which values for the mass of 140 S antigen included in each vaccine were available, has given estimates for the antigen dose required to immunize 50% of cattle (PD_{50} values) as follows: type O_1, 166 ng; type A_{24}, 4.5 ng; and type C_1 = 5.4 ng. More than 30 times the quantity of antigen is required to immunize cattle with this strain of type O than is required with these strains of type A or type C (Hingley *et al.*, 1982). From the regression slope of 2.0 it can be calculated that, to obtain a percentage protection level of 95%, a potency value of approximately 6 PD_{50} is required. The equivalent value on the Pb scale, using the regression slope of 2.4 for probit protection versus the logarithm of *vaccine* dose (Stellmann *et al.*, 1977), is 4.8 Pb.

B. Formulation

FMD vaccines can be monovalent or polyvalent in relation to serotype. In some regions, e.g., Brazil, Kenya, Botswana, where a range of serological variants within a serotype may be present in the field, vaccines containing two subtype strains for one or more serotypes may be employed.

The vaccines designed for use in cattle, sheep, goats, and buffalo are almost invariably based on inactivated antigens, adsorbed onto aluminum hydroxide gel and adjuvanted with saponin. Vaccines of this formulation require two vaccinations to produce an adequate IgG response in the pig. Because of this, oil emulsion vaccines of the incomplete Freund adjuvant type have been developed for use in swine. Workers in the Plum Island Laboratory (Cuncliffe and Graves, 1963), the Pan-American FMD Center, Rio de Janeiro (Sutmoller, 1977), and the INTA Laboratory, Buenos Aires (Rivenson, 1979) have all published experimental data which indicated that oil emulsion vaccines give rise to a superior and longer lasting immunity in cattle. Other authors (Mowat, 1974; Favre *et al.*, 1978; Barei *et al.*, 1979) have not found any major difference in the immune responses produced by aqueous or oil emulsion vaccines. In a publication from our laboratories (Rweyemamu *et al.*, 1982) it was reported that both aqueous vaccines containing high antigen payloads and oil emulsion vaccines could produce antibody responses that persisted at protective levels for at least 6 months and were not significantly different from one another.

One important feature in the formulation of vaccines is the method of inactivation used. In the past, formalin was the usual inactivant employed in FMD vaccines. Since the work of Brown and Crick (1959) on acetylethyleneimine (AEI) and of Bahneman (1975) on binary ethyleneimine (BEI), these substances have become more and more widely used, as, unlike formalin, they are first-order inactivants. In 1965, Wellcome found that the formalin inactivation procedure was unreliable and changed first to the use of AEI (Pay *et al.*, 1971) and, more recently, to the use of BEI (Pay *et al.*, 1981). While formalin-inactivated FMD vaccines have been associated with both proven and suspected cases of lack of innocuity in a number of countries, there have been no reports of such problems with AEI- or BEI-inactivated vaccines.

IV. THE HOST

A. Immune Response

Immunity of FMD in cattle appears to be mainly dependent on serum neutralizing antibody levels present at the time of exposure to infection. A linear correlation has been described between the log SNT produced in cat-

tle following a primary vaccination and the log antigen (140 S) dose, with a regression slope value of 0.5 (Pay and Parker, 1977). This means that an average 10-fold (1.0 log) increase in antigen dose is required to produce a 3-fold (0.5 log) increase in serum titer. The rate at which a primary immune response develops and the level at which the primary response levels off after the initial drop in SN antibody level is clearly antigen dose related (Rweyemamu *et al.*, 1982).

In contrast, it has been established that the ability to prime the immune system of cattle was not antigen dose related over an extreme range. In 1971, I showed that cattle could be primed as effectively with vaccines containing only 5% of the normal antigen dose as with the full antigen dose (Pay, 1971). More recently, work in our laboratory has shown that groups of cattle which had received a range of doses between 42 and 330,000 ng of type O 140 S antigen (42 ng = approximately 1% of average normal antigen payload) were equally well primed and responded similarly following revaccination with a fixed antigen dose. The magnitude of the secondary antibody response in these cattle, was, however, related to the antigen mass used for revaccination. It is of interest to note that revaccination even with a 40-ng dose, which was the minimum priming dose, produced a booster antibody response which was higher than the primary response produced by a single vaccination with the highest antigen dose tested (330,000 ng) (Black *et al.*, 1984). In an earlier publication (Berger *et al.*, 1975) we showed that secondary and tertiary immune responses could be obtained in cattle, under field conditions in Kenya, with a range of experimental vaccines containing as little as 1% of the antigen content of the standard vaccine, which gave the same levels and duration of antibody as the standard vaccine.

The animal-to-animal variation in the serum neutralizing antibody responses produced in cattle, even of the same age and breed, following a primary vaccination with a fixed antigen dose is quite large. Values for the animal-to-animal variation displayed by the sera of 1½- to 2-year-old Devon steers gave a pooled estimate for the standard deviation of the mean log SNT of 0.4 (Pay and Parker, 1977). This indicates that on average it can be expected that 95% of SNT values will fall between sixfold lower and sixfold higher than the mean value (i.e., if the mean titer is, say, 1:120, the 95% range will be 1:20 to 1:720). Significant differences have been described for the responses obtained in three genetically distinct strains of Friesian cattle of the same ages and under identical systems of husbandry (Frenkel *et al.*, 1982).

It is probable that nutrition and intercurrent disease may play a role in influencing the magnitude of the antibody response to vaccination. A number of protozoan diseases are known to cause immunosuppression, and trypanosomiasis has been shown to suppress the response of cattle to FMD

vaccines (Scott *et al.*, 1977; Sharpe *et al.*, 1982). Although it was concluded that parasitized cattle that had been revaccinated could achieve antibody levels sufficient to protect them in the short term, it is apparent that their ability to withstand a heterologous challenge or the duration of their immunity would be significantly decreased. Age has been considered to be a factor in the response of cattle to vaccination (Muntiu *et al.*, 1969). However, our experience suggested that calves and piglets over 1 week old, without maternal antibody, can respond as well as adults. The presence of maternal antibody, however, will depress the response of young animals to varying degrees, depending on the level of antibody present and the antigen mass present in the vaccine.

B. Species Difference

Other ruminants will respond well to aqueous aluminum hydroxide/saponin adjuvanted FMD vaccines: sheep and goats (Polydoru *et al.*, 1980; Garland *et al.*, 1981); wild ruminants (Hedger *et al.*, 1980); water buffalo (Awad *et al.*, 1979). However, pigs require at least two inoculations of these vaccines to produce an IgG response.

V. THE HUMAN ELEMENT

A. Storage and Administration of Vaccine

FMD antigens are relatively labile, and their decay rate will be proportional to temperature and time. Although differences in stability may be observed between strains, it is necessary to adopt standard procedures for handling FMD vaccines compatible with the requirements of the least stable strains. This entails maintaining vaccines at 2–8°C up to the moment they are injected.

B. Vaccine Regimen

Both IFFA-Mérieux and Wellcome Foundation, who between them are responsible for the production of more than 50% of the world's FMD vaccine, recommend that a primary course of vaccination should consist of two doses given at an interval of about a month. It is worth noting, therefore, that FMD vaccines are probably unique among major biological and pharmaceutical products in that only very occasionally are they used for primary vaccination in the manner recommended by the manufacturers.

Prophylactic campaigns are based on vaccination either once, twice, or

three times a year. In a number of Western European countries a major campaign is conducted once a year, with a minor campaign 6 months later to vaccinate young stock not included in the main campaign. In a number of countries in Africa and the Middle East, vaccination of all cattle is carried out every 6 months. In most South American countries, vaccination of all cattle is carried out every 4 months.

C. Coverage

The important features relating to coverage are the proportion of the national herd which is regularly vaccinated and the manner in which this proportion is distributed. Thus, it is commonly considered that a "herd immunity" level of around 70% is likely to prevent epidemic spread of many major infectious diseases of man and animals. It can be anticipated, however, that in the case of cattle, where the "vaccination unit" is the farm, not the individual animal, a totally different epidemiological state will exist in a situation where all farms are vaccinated and some 70% of cattle are immune, to one where only 80% of farms are vaccinated and 90% of the cattle are immune on these farms. Even though the total percentage of immune cattle may be approximate to 70% in both cases, in the first example, the disease will in time be controlled and possibly eradicated; whereas in the latter example, the disease will probably maintain a high level of infection in the field and regularly invade the farms in which animals have been well vaccinated.

D. Supporting Zoosanitary Procedures

With any of the possible vaccination regimens, there will always be at any time of the year a proportion of nonimmune animals within a vaccinated herd. These can be young animals not yet vaccinated, or individuals which have responded only poorly to vaccination, or individuals whose immunity has waned. In Section II, C, we discussed how the force of infection can be indirectly reduced by a prophylactic vaccination campaign, but it will also be reduced directly by zoosanitary procedures such as quarantine (with preferably stamping-out of infected animals), control of animal movement, and immediate ring-vaccination around a primary focus of infection. All of these will reduce the force of infection and lessen the probability of nonimmune animals in contiguous areas becoming exposed to infection.

VI. GRAPHIC MODELS

Nearly all the factors described here are capable of operating simultaneously and, thereby, of exerting a cumulative effect on the performance of a vaccine. In order to have a better understanding of these effects, it is of value to construct simple graphic models. Figure 1 shows typical responses in cattle to the recommended vaccination regimen with a type A vaccine of 7 PD_{50} and a SAT 2 vaccine of 14 PD_{50} (Berger *et al.*, 1975).

By combining the regressions of probit protection/log dose = 2.0 and of log SNT/log dose = 0.5, a value for probit protection/log SNT = 4.0, can be derived. Using this regression and assuming an average value for the log SNT equating with 50% protection (PA_{50} value) of 1.3, the values in

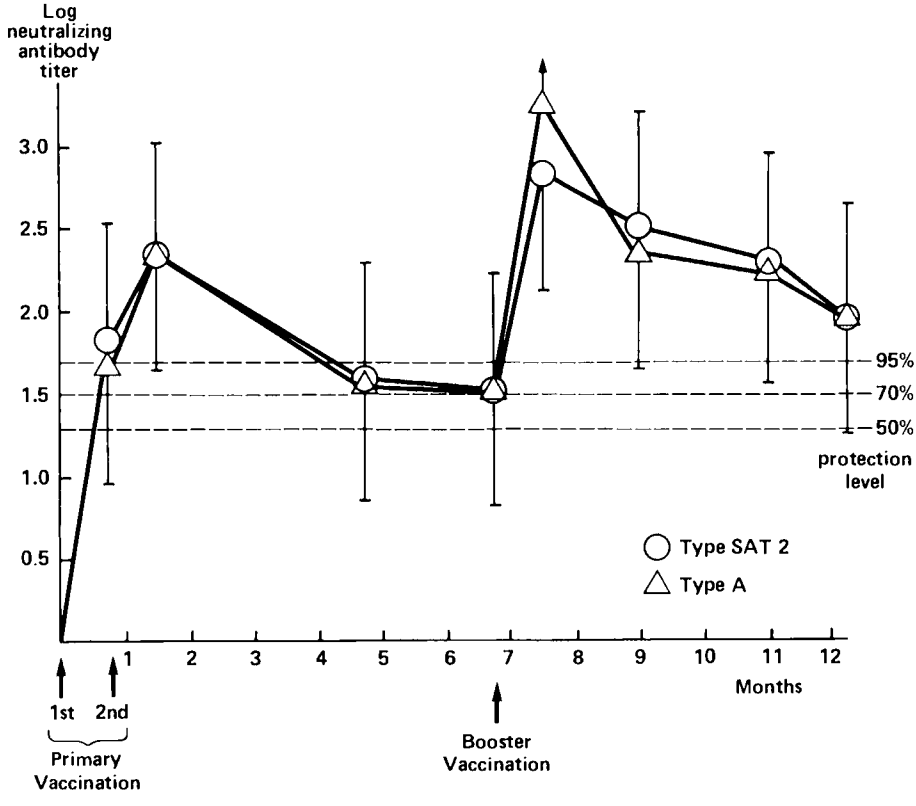


Fig. 1. Vaccination responses of adult cattle under field conditions in Kenya. The type A vaccine had a potency of 7 PD_{50} and was part of a quadrivalent vaccine. The type SAT 2 vaccine was monovalent and had a potency of 14 PD_{50}. Values shown are mean serum neutralizing antibody titers with typical ranges (±2 SD).

Fig. 1 can be transposed into estimated percentage of protection (EPP) values, which are more meaningful (Fig. 2). In constructing the model, it has been assumed that the correlation of serum antibody titer with protection is the same at 6 months after vaccination as at 3 weeks, and also that the level of immunity required to give protection against an average primary field challenge is similar to that required to withstand a heavy laboratory challenge.

Figure 3 shows a model in which EPP values have been calculated for the antibody responses in Fig. 1 against a heterologous virus strain which has a true *r* value of 0.4 against the vaccine strain. This represents a moderate level of serological difference, and such levels can frequently be observed between field viruses within the same subtype group in countries where the disease is endemic.

Figure 4 is a model relating to a heterologous virus strain with an *r* value relationship of 0.1 with the vaccine strain. This represents an average level

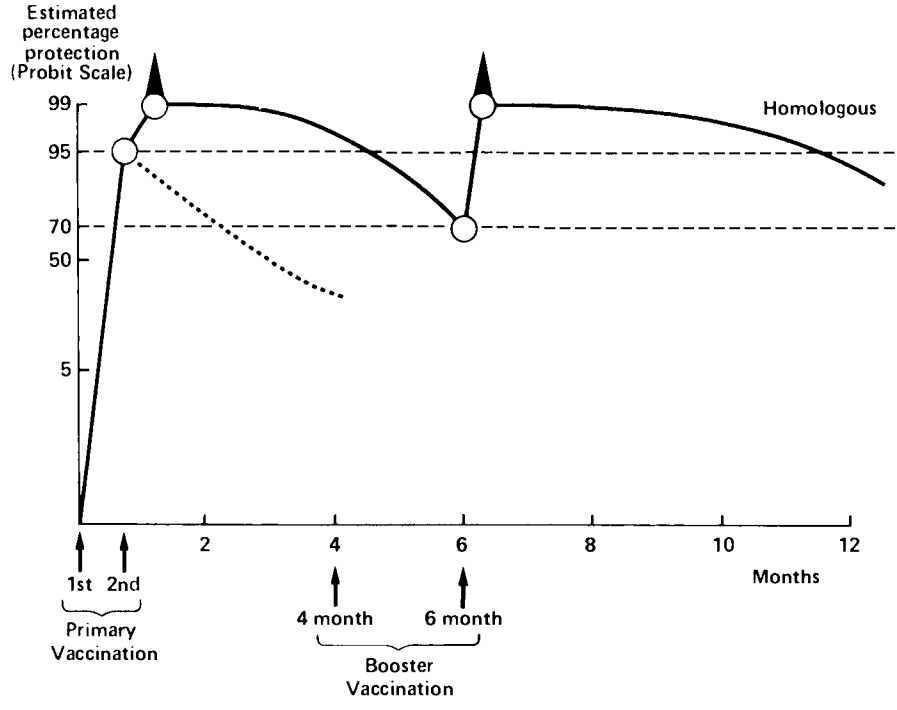


Fig. 2. Graphic model of herd immunity produced by FMD vaccination. Data are based on responses shown in Fig. 1 and show estimated percentage of protection levels against homologous challenge. The dotted line indicates response produced if only one inoculation is given for primary vaccination.

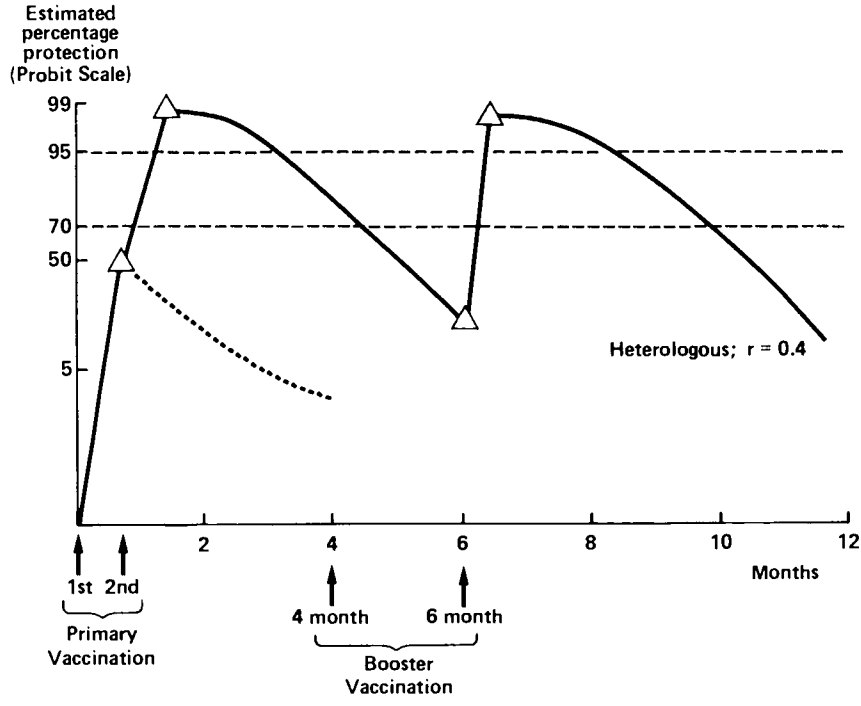


Fig. 3. Graphic model of herd immunity produced by FMD vaccination. Data are based on responses shown in Fig. 1. Model shows estimated percentage of protection levels against a heterologous challenge with a virus showing a moderate antigenic difference ($r = 0.4$).

of full subtype difference, and greater levels of serological subtype differences can occur.

In all three models, the effect of administering only one inoculation during the course of primary vaccination is indicated.

VII. DISCUSSION

We can assume that it may be necessary to maintain herd immunity at levels in excess of 95% protection, if infection is to be kept out of an individual herd, in an endemic region where there is high risk of exposure to infection throughout the year. It can be seen that, even against a truly homologous infection, a primary course of two inoculations followed by booster vaccinations every 4 months may be required to achieve this. Such a regimen may, however, not keep infection out of a herd when the field virus is heterologous to the vaccine virus. Once infection enters a herd, as

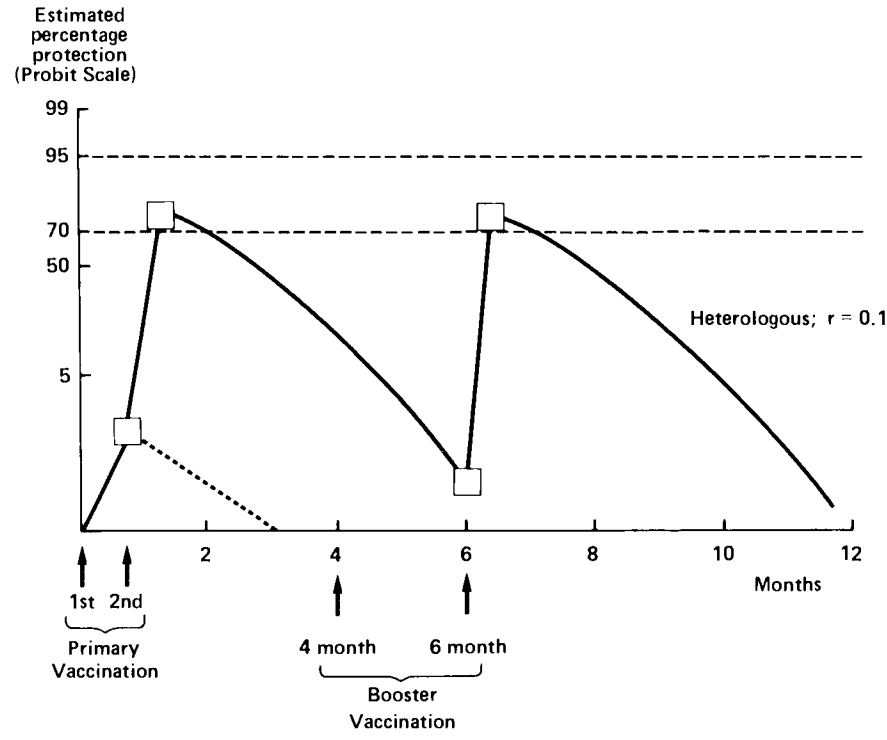


Fig. 4. Graphic model of herd immunity produced by FMD vaccination. Data are based on responses shown in Fig. 1. Model shows estimated percentage of protection levels against a heterologous challenge with a virus showing an average subtype level of antigenic difference ($r = 0.1$).

we have already noted, the immunity of some animals may be swamped by the massive contact challenge they receive.

To obtain the level of herd immunity in excess of 70% protection which will offer effective epizootic control, it can be seen that, while booster vaccinations at 6-month intervals may suffice against an homologous field virus strain, it requires revaccination every 4 months to protect against a field virus showing a moderate level of serological difference.

Vaccination every 4 months may not create an adequate level of herd immunity against endemic viruses showing a full subtype serological difference. However, it must be stressed that experience over the years has shown that, in most situations where a new subtype virus has emerged, it has been possible to prevent a major epizootic spread by immediately revaccinating with the existing vaccine. As the model shows, this may raise the herd immunity to the 70% level for a short period, possibly of only a week or two; but this, if coupled with stringent zoosanitary precautions

aimed at reducing the force of infection, may prevent a brushfire type of epizootic spread.

Even when good serological evidence exists that a new outbreak virus is quite significantly different from the existing vaccine virus, there is nothing to be lost by using the vaccine until such time as a homologous vaccine is available. Experience has shown that, with the exception of the A_{22} variant, most other emerging subtype viruses have been controlled in the short term by two vaccinations with the old vaccine. In the event of a homologous vaccine to the new subtype being made available fairly rapidly, time may be gained in some instances by using the existing vaccine as the priming dose, as experience has shown that, when the viruses of a different subtype are used for revaccination, a secondary level of response can be obtained to both the priming and boosting vaccine viruses.

Should the new subtype virus establish itself and become endemic, it may be necessary to include it in the vaccine, either in combination with or in place of the old vaccine strain. The emerging philosophy for vaccine strain selection is to choose a baseline strain with the broadest overall antigen spectrum against the various viruses in a region, and to add to this other viruses, as and when required, to control a new situation. The baseline strain should never be totally replaced until the new replacement virus has been fully characterized and shown to have the appropriate broad antigenic spectrum and the other properties required of a good vaccine virus. It is of interest that antigenic change does not appear to be one of continuous progressive change, and some recent subtype viruses may have more in common with viruses present in the field 20 years ago than to viruses of more recent isolation.

In conclusion, the special problems arising from the pathogenesis of the disease and the extreme antigenic variability of the virus require that FMD vaccines be used in a much more flexible and tactical manner than most other vaccines. The necessity of maintaining critical levels of circulating antibody makes their administration, in some ways, more analogous to the use of chemotherapeutic pharmaceuticals. However, despite the complexity of the problem, the existing vaccines when properly used not only can control the disease, but can eradicate it, as shown in recent years in Indonesia, Malaysia, Chile, and Botswana.

REFERENCES

Awad, F., Reda, I., Moussa, A. A. M., Mulla, A. A., Daoud, A., Hussein, K., and Marzouk, M. S. (1979). *J. Egypt. Vet. Med. Assoc.* **39**, 97–107.

Bahneman, H. G. (1975). *Arch. Virol.* **47**, 47–56.

Barei, S., Panina, G. F., Orfei, Z., Nardelli, L., and Castelli, S., (1979). *Zentralbl. Veterinaermed., Reihe B* **26**, 454–460.

Berger, J., Schermbrucker, C. G., and Pay, T. W. F. (1975). *Bull. Off. Int. Epizoot.* **83**, 327–336.

Black, L., Rweyemamu, M. M., and Boge, A. (1984). *J. Comp. Pathol.* (in press).

Brown, F., and Crick, J. (1959). *J. Immunol.* **82**, 444–447.

Cunliffe, H. R., and Graves, J. (1963). *Can. J. Comp. Med.* **27**, 193–197.

Favre, H., Brun, A., Duret, C., Roulet, C., and Fargeaud, D. (1978). *6th Int. Meet. Faculty Vet. Sci. Univ. La Plata, Argentina.*

Forman, A. J. (1975). *J. Hyg.* **74**, 227–232.

Frenkel, S., Barendregt, L. G., Kloosterman, E. G., and Talmon, F. P. (1982). *Proc. 16th Conf. Foot-and-Mouth Disease Commission, Office International des Epizooties,* Paris, pp. 211–221.

Garland, A. J. M., Baber, D., Hamblin, C., Rowe, L., and Barnett, I. T. R. (1981). *Br. Vet. J.* **137**, 507–512.

Hedger, R. S., Condy, J. B., and Gradwell, D. V. (1980). *J. Wildl. Dis.* **16**, 431–438.

Hingley, P. J., Black, L., Radlett, P. J., O'Reilly, K. J., and Pay, T. W. F. (1982). Paper presented at Meeting of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Pirbright, England.

de Leeuw, P. W., Tiessink, J. W. A., and van Bekkum, J. G. (1979) *Zentralbl. Veterinaermed., Reihe B* **26**, 98–109.

May, R. M. (1982). *Nature (London)* **300**, 481–483.

Mowat, G. N. (1974). *Bull. Off. Int. Epizoot.* **81**, 1319–1334.

Muntiu, N. (1969). Paper presented at Meeting of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Brescia, Italy.

Pay, T. W. F. (1971). Proc. 2nd Int. Congr. Virol. Budapest; p. 154.

Pay, T. W. F. (1983). *Rev. Sci. Tech. Off. Int. Epizoot.* **2**, 701–723.

Pay, T. W. F., Telling, R. C., Kitchener, B. L., and Southern, J. (1971). Paper presented at Meeting of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Tübingen, Federal Republic of Germany.

Pay, T. W. F., and Parker, M. J. (1977). *Dev. Biol. Stand.* **35**, 369–383.

Pay, T. W. F., Telling, R. C., and Thorne, A. C. (1981). Paper presented at Meeting of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Tubingen, Federal Republic of Germany.

Polydorou, K., Papasolomontos, P., Hadjisavvas T., *et al.* (1980). *Bull. Off. Int. Epizoot* **92**, 863–873.

Rivenson (1979). *Rev. Med. Vet. (Buenos Aires)* **60**, 1–17.

Rweyemamu, M. M., Pay, T. W. F., and Parker, M. J. (1977). *Dev. Biol. Stand.* **35**, 205–214.

Rweyemamu, M. M., Black, L., Nicholls, M. J., Basarab, O., and O'Reilly, K. J. (1982). *Proc. 16th Conf. Foot-and-Mouth Disease Commission, Office International des Epizooties,* Paris, 383–396.

Scott, J. M., Pegram, R. G., Holmes, P. H., Pay, T. W. F., Knight, P. A., Jennings, F. W., and Urquhart, G. M. (1977). *Trop. Animal Health Prod.* **9**, 159.

Sharpe, T. R., Langley, A. M., Mowat, G. M., Macaskill, J. A., and Holmes, P. H. (1982). *Res. Vet. Sci.* **32**, 289–293.

Stellmann, C., Terre, J., Brun, H., Favre, H., and Fontaine, J. (1977). *Arch. Virol.* **54**, 61–74.

Sutmoller, P. (1977). *Dev. Biol. Stand.* **35**, 135–138.

Wild and Brown (1968). *Arch. ges. Virusforsch.* **24**, 86–103.

7

Progress in Antiviral Chemotherapy

G. J. GALASSO[1]

Development and Applications Branch, Microbiology and Infectious Diseases Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

I. INTRODUCTION

Viral diseases remain among the greatest causes of human morbidity and economic loss for both the developed and the developing nations. The leading cause of office visits for medical attention is for infectious disease problems. The average person in developed countries has from two to six viral infections per year. Approximately 6% of all deaths in the United States are due to infectious diseases; this does not include those with underlying diseases such as cancer, which would increase the number. The problem in

[1]Present address: Office of Extramural Research and Training, National Institutes of Health, Bethesda, Maryland.

APPLIED VIROLOGY ISBN 0-12-429601-7

the developing countries is even greater, contributing considerably to mortality—particularly infant mortality. Further, as we become more sophisticated in medicine by doing tissue transplants and as treatment of cancer increases, the importance of infectious diseases will become greater. Viral infections which may be minor in normal patients assume life-threatening proportions in these immunosuppressed individuals. Therefore the concern for infectious diseases, particularly viral diseases, and the need to control them are increasing.

The best way to exercise this control is through prevention. This can take many forms, including good hygiene, control of vectors, and, most important, the use of vaccines.

II. VIRAL VACCINES

Viral vaccines have proved to be invaluable for the control of diseases such as mumps, yellow fever, polio, and measles; they have even achieved the ultimate success of disease eradication, as in the case of smallpox. We are constantly striving for new vaccines, for example, for hepatitis (which will be especially useful in Africa, the Middle East, and the Far East) and varicella and improved vaccines for viral diseases such as influenza.

III. PASSIVE IMMUNIZATION

Another means of protecting the patient is the use of immune plasma for the injection of antibodies to prevent infection. This results in temporary protection useful for a particular "at risk" period. The advantage is immediate protection, particularly when no other treatment regimen is available. The major problems are that this protection is short-lived and the individual may develop clinical reactions such as sensitization, immune complex formation, and cardiovascular shock. The value of immune plasma is evident, for example, when a new highly virulent disease appears for which there is no treatment, but plasma from survivors can be processed and used in subsequent patients, as was done with Lassa fever.

IV. ANTIVIRAL AGENTS

There is no question that the first line of defense against viral diseases is immunization. However, we cannot use vaccines against all possibilities. In many instances vaccines are not available, not practical, or deemed unsafe; for example, there are many who are opposed to whole-virus vaccines

against herpesviruses due to the possibility of oncogenicity. There are also instances where vaccine was not used or there was a failure to develop antibody and treatment is necessary. Therefore, an alternative control measure is needed. This need can be met with the successful development of antiviral agents which will be useful for prophylaxis or therapy of viral diseases.

We have not progressed as rapidly with antiviral agents as with antibiotics because of the nature of the viruses. Bacteria usually replicate extracellularly in body fluids and are easily accessible to the antibiotics. Viruses replicate intracellularly, utilizing the cell's metabolism. Therefore, for an antiviral agent to be effective it must be able to penetrate the cell and disrupt viral replication, but exert no effect on the cell's normal functions. Many investigators felt that this was unlikely and, therefore, all effective agents would be toxic. They believed antiviral agents had no future in clinical medicine. However, the success of agents such as adenine arabinoside, which is highly effective in ongoing serious viral infections, has been most effective in dispelling this attitude, and skepticism has made way for optimism.

Until recently antiviral agents have been developed fortuitously either by large screening programs, in which a number of compounds are tested for their antiviral effect, or by chance in laboratory research. Once a compound has been identified, a series of *in vitro* and *in vivo* studies are done to determine its applicability to the clinic. Such tests include viral spectrum, pharmacology, toxicology, mutagenicity, teratogenicity, and oncogenicity as well as mechanism of action, therapeutic ratios, and drug distribution and excretion. For example, phosphonoacetic acid proved to be a very effective antiviral agent against herpesviruses and caused considerable excitement until pharmacological studies showed that the drug was retained in the bones, making it unsuitable for clinical use.

A. Future Development of Antivirals

The state of the art of the molecular biology of viruses is such that we can now begin to develop targeted and, therefore, less toxic antiviral compounds through a directed approach. We know a great deal about the specific steps in viral replication, and chemists should be able to develop specific inhibitors to virus-specified enzymes and proteins, such as unique RNA-dependent RNA polymerase, virus-specified thymidine kinase, cleavage enzymes for viral capsid protein, and receptor modification.

A major problem is delivery of the drug to the infected sites in sufficient concentration to have the desired effect. Over the past decade many agents have been found to be active in cell culture, but very few are effective in man because either they are too toxic, they are inactivated in the body, the

rate of excretion is too high, or the antiviral cannot get into the cell because of transport problems. Although routine screening should be continued, the directed approach should be emphasized.

There are currently very few antivirals generally accepted as efficacious. These include iododeoxyuridine, adenine arabinoside (ara-A), and trifluorothymidine ointments for herpetic keratitis; acyclovir ointment for genital herpes; oral amantadine against influenza A; intravenous adenine arabinoside against herpes encephalitis; and intravenous acyclovir against mucosal and cutaneous herpes infections in the immunocompromised patient and in very severe cases of initial genital disease in the normal host (Table I).

B. Topical Agents for Herpes Keratoconjunctivitis

The leading cause of corneal blindness is herpesvirus infection; it affects one individual per 1000 worldwide. The incidence in the Middle East and Africa is probably greater, although there are no accurate figures. It is an extremely painful disease which is readily diagnosed by characteristic branching dendritic lesions on the cornea that can be stained with fluorescein. Following the initial infection, the virus may become latent; the exact mechansim is still not fully understood. Recurrences occur in 26% of patients within 2 years of the initial episode and in 43% of patients who have suffered a second attack.

Because the acute episode is limited to the superficial cornea and lends itself to topical treatment, there was less concern for drug toxicity. Thus there was relatively little hesitancy in the early 1960s for approving iododeoxyuridine (IDU) as the first antiviral for treatment of a viral infection

TABLE I

Antivirals Approved for Use in the United States

Drug	Mode	Infection
Iododeoxyuridine ointment, adenine arabinoside ointment, trifluorothymidine ointment	Topical	Herpetic keratitis
Amantadine	Systemic (oral)	Influenza
Adenine arabinoside	Systemic (iv)	Herpetic encephalitis
Acyclovir ointment	Topical	Initial genital herpes
Acyclovir	Systemic (iv)	Mucosal and cutaneous herpes in immunocompromised, severe initial genital herpes

(Kaufman *et al.*, 1962). However, it soon became apparent that IDU was not always clinically effective. The drug can lead to viral resistance, and ocular toxicity has been observed.

Adenine arabinoside ointment was shown to be effective for the treatment of this infection (Pavan-Langston *et al.*, 1975), and its use was approved by the authorities in the United States in 1977. It is at least as effective as IDU, probably more so; it is especially useful in patients in whom IDU has not been beneficial or in whom signs of ocular toxicity have been noted.

Trifluorothymidine (F_3T) has also been approved by national authorities for treatment of this disease. Considerable research on the efficacy of this compound has been performed in the United Kingdom and the United States. Although it is difficult to recommend a drug of choice, some studies indicate that F_3T is the most effective, followed closely by ara-A, with IDU the third choice (Kaufman, 1978).

Other compounds are available for treatment of herpetic keratoconjunctivitis, but they have not received the general acceptance of IDU, ara-A, or F_3T. These include 5-ethyl-2′-deoxyuridine, tromantadine HCl, and iododeoxycytidine. No reliable data on the efficacy of these three compounds are available.

Although acute episodes of this infection can be treated effectively with IDU, ara-A, or F_3T, these compounds are not effective in recurrences. Once the virus has established latency, the currently available antivirals are not able to eradicate the virus. Therefore, new, more effective compounds are needed. Among the new compounds, acyclovir and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine appear to be the most promising, but their effectiveness against the latent virus is doubtful. Currently, treatment with interferon combined with either thermomechanical debridement of the diseased corneal epithelium or F_3T therapy has proved to be more effective than any single therapy tested.

C. Amantadine/Rimantadine

A more interesting story concerns the compound amantadine. The burden of influenza infection on contemporary society remains an important worldwide problem responsible for considerable morbidity and mortality.

There are three types of influenza—A, B, and C. Influenza A is the most important clinically and the one that expresses antigenic variation most readily. The principal antigens in influenza virus are the hemagglutinin and the neuraminidase of the outer coat. These periodically have major changes called shifts, usually resulting in a new pandemic, or minor variations called drifts, which result in considerable morbidity and mortality. Vaccination remains the preferred method of control, but since the antigenic match be-

tween vaccine and circulating virus is not often perfect, alternative methods of control, i.e., antivirals, are important.

One such compound is amantadine HCl, or Symmetrel. It has a simple chemical structure, and its commercial name comes from its structural symmetry. The compound was first reported to have antiviral activity against influenza A virus as early as 1963, and clinical studies conducted with the drug led to approval by the Food and Drug Administration (FDA) in 1966 for use in the prevention of Asian influenza, H2N2. In 1968, Hong Kong influenza appeared (H3N2); although it was also an influenza A, the authorities did not allow Symmetrel to be marketed unless new trials to show efficacy were done. At this point interest waned, and the drug was produced solely for its beneficial effect in parkinsonism. Over the years, data accumulated on the central nervous system effects, and, with the threat of swine flu in 1976, as well as further animal studies, amantadine was approved for prevention and treatment of all influenza A. Since there was still a reluctance to accept it as a treatment of choice, new studies were done in 1978 when a new strain appeared, A/USSR/77 (H1N1). During the initial outbreak in 1978–1979, a double-blind, placebo-controlled trial in 286 college students was performed by Monto *et al.* (1979). The study showed an efficacy rate of 70.7% in the prevention of laboratory-confirmed clinical influenza. Equally important were the data on side effects. The study showed that if side effects do occur, they usually appear during the first 48 hr of treatment; they are usually mild and generally include insomnia, lightheadedness or dizziness, difficulty in concentration, or drowsiness. Careful investigation showed that the side effects were no more severe than those experienced with many of the antihistamines that are freely available. The withdrawal rate in this study was 6.2%.

Although the side effects are insignificant, concern still exists, resulting in increasing interest in an amantadine analog, rimantadine. Laboratory studies have demonstrated that it is just as effective as amantadine, and clinical studies have demonstrated that it is associated with fewer side effects. Studies have been performed comparing the two drugs.

Gordon Douglas studied the therapeutic value of amantadine and rimantadine. When the drug was administered in established influenza A virus infection, the improvement of clinical illness and of pulmonary physiological dysfunction was accelerated. Patients returned to normal activity more quickly than untreated patients (Van Voris *et al.,* 1981). By 48 hr, a large percentage of those on the drug had returned to normal function.

A new study comparing the two drugs was performed in the United States. Four hundred fifty volunteers were enrolled in a double-blind, randomized, placebo-controlled trial of the chemoprophylactic efficacies of rimantadine and amantadine during an outbreak of influenza A in Burlington, Vermont (Dolin *et al.,* 1982). Doses employed were 100 mg orally twice a day for 6

weeks (200 mg/day). Signs and symptoms of illness were monitored, and viral cultures and serum specimens were obtained for each episode of respiratory illness. Influenza-like illness occurred in 41% of placebo recipients, but in only 14% of rimantadine recipients and 9% of amantadine recipients ($p < 0.001$ for either rimantadine or amantadine vs placebo). Illness associated with laboratory-documented influenza A virus infection occurred in 21% of placebo recipients, in 3.0% of rimantadine recipients, and in 2% of amantadine recipients ($p < 0.001$). These latter findings represent efficacy rates of 85.4% for rimantadine and 91% for amantadine when compared to placebo. Significantly more amantadine recipients (13%) withdrew from the study because of central nervous system (CNS) side effects than did those who received rimantadine (6%) ($p < 0.05$) or placebo (4%) ($p < 0.01$). Thus, both rimantadine and amantadine provide highly effective prophylaxis against influenza A in this setting, but rimantadine is associated with significantly fewer CNS side effects than is amantadine. On the basis of this study, rimantadine appears to be the drug of choice for the prophylaxis of influenza A. However, some would argue that the dose for amantadine could be cut in half and still be effective and the side effects would be considerably reduced.

D. Adenine Arabinoside

Adenine arabinoside (9-β-D-arabinofuranosyladenine, ara-A) is of particular interest because it was the first antiviral agent to be approved by any national authority for the systemic treatment of an ongoing life-threatening illness. The efficacy of this drug against brain biopsy-proven herpes encephalitis has been unequivocally demonstrated. More recently this drug was also shown to be effective in the treatment of neonatal herpes, and a study has been completed to evaluate its role against varicella-zoster (shingles) in immunosuppressed patients. The drug is administered by slow drip infusion due to its relative insolubility. The worldwide incidence of herpes encephalitis is not known. It is not a common disease; however, it has a mortality rate as high as 70% and severe residual effects in a large percentage of the survivors. In the initial placebo-controlled study, it was demonstrated that parenteral administration of ara-A reduced mortality from 70 to 28% with a concomitant reduction in the debilitating neurological sequelae in the treated patients. However, patients who were already in a coma did not benefit from therapy.

Early diagnosis is crucial for optimal effects, and it should be pointed out that clinical diagnosis without brain biopsy is difficult. In the second ara-A study, 132 cases were clinically diagnosed; however, only 75 (57%) were biopsy proven. The results of drug efficacy were similar to the first study (Whitley *et al.*, 1980b).

With the increasing incidence of genital herpes, neonatal herpes is becoming of greater worldwide concern. Most of the affected infants acquire the infection from their mother immediately before or at the time of normal delivery. Three possible clinical manifestations usually occur within 1 or 2 weeks of birth. Infection may be disseminated, with multiple organ involvement often including the brain; localized to the central nervous system (CNS); or localized to the skin, eye, and/or mouth. Disseminated and localized CNS disease accounts for over 75% of all neonatal herpes, with mortality rates of 82 and 40%, respectively. In a recently completed study evaluating the efficacy of ara-A, the mortality observed was somewhat higher. Mortality in infants with localized CNS disease was reduced from 50 to 10%; with disseminated disease there was a reduction from 86 to 57%. When combined, the overall mortality was reduced from 74 to 38%. Mortality was not associated with localized skin, eye, or mouth infections; however, severe sequelae occurred in 38% of placebo recipients in this category but in none of those on drug therapy (Whitley *et al.*, 1980a).

Ara-A was also tested in immunocompromised patients with zoster or varicella. The zoster study was performed in 121 patients (63 drug 58 placebo) within 72 hr of onset. The drug was effective in accelerating healing and decreasing cutaneous dissemination, zoster-related visceral complications, and duration of postherpetic neuralgia. Very little drug toxicity was seen (Whitley *et al.*, 1982a). The varicella study was performed in 34 patients (19 drug, 15 placebo) within 72 hr of onset. As with zoster, drug therapy was effective in accelerating cessation of new lesions and healing, in resolving fever more rapidly, and in reducing varicella-related complications (one in the drug group and eight in the placebo group) with minimal evidence of toxicity. These studies indicate that adenine arabinoside is an effective drug for the treatment of zoster and varicella in the immunocompromised patient if given early in the course of infection (Whitley *et al.*, 1982b).

E. Ribavirin

Ribavirin (Virazole) is a compound that has been shown to have a broad spectrum of activity against RNA and DNA viruses in tissue culture and animal model systems. One of the most promising aspects of this agent is its potential against arenaviruses. Laboratory studies in nonhuman primates indicated that it has an effect against the agents of Lassa fever and Bolivian hemorrhagic fever (Machupo virus), and against Pichinde virus. In 1978 a study was done in Sierra Leone in conjunction with the Centers for Disease Control (United States); ribavirin administered orally (1 g/day for 10 days) to patients with Lassa fever had no effect. Subsequent monkey studies demonstrated that intravenous or intramuscular administration at

higher dosages was more effective in saving the animals, even 7 or 8 days after infection with Lassa fever virus. The results were even better when the drug was given in conjunction with immune plasma. A new randomized study is underway in Sierra Leone; patients with aspartate aminotransferase levels greater than 150 U (who are at greater risk of death) are treated intravenously with either ribavirin alone (loading dose of approximately 2 g followed by 1 g every 6 hr for 4 days followed by 0.5 g every 8 hr for an additional 6 days) or ribavirin plus injections of Lassa immune plasma (1 unit of 2250 ml with immunofluorescent antibody titer greater than 1:128). These studies are nearing completion. It is too early to predict the outcome of the study, although the overall results with combined treatment appear to be encouraging. The problem with this regimen is that it is expensive and cumbersome and may prove difficult to administer in countries with limited health resources (J. B. McCormick, 1983, personal communication).

The initial clinical trials with ribavirin were directed against influenza. Although the oral treatment proved to be ineffective, studies are continuing using ribavirin as an aerosol in influenza pneumonia patients. Preliminary experiments have also been done using aerosolized ribavirin in infants with respiratory syncytial virus infection, which can be quite serious in this population. Early results show some promise of efficacy.

F. Bromovinyldeoxyuridine

An equally exciting compound in a much earlier stage of testing is bromovinyldeoxyuridine (DeClercq *et al.*, 1980). It is a very specific antiviral that is more effective against HSV-1 than HSV-2 and appears to be effective also against zoster. The selectivity of the drug appears to depend on its conversion to the 5′-monophosphate by the virus-induced thymidine kinase. Current studies in animals indicate that it stops progression of disease even after symptoms have appeared. This drug has the advantage that it is quite effective by the oral route. Clinical studies have been initiated in Europe; preliminary data indicate good results against zoster.

G. Phosphonoformic Acid

As stated earlier, considerable excitement was engendered in 1974, when animal studies demonstrated that phosphonoacetic acid (PAA) had broad anti-herpes action. The drug was active against ocular and genital herpes, herpes encephalitis, and cutaneous herpes infections. It appeared to be more effective than ara-A, ara-C, IDU, and ribavirin, the four compounds then known to have anti-herpes efficacy. However, safety/toxicological studies revealed that the compound was retained in the bones of treated animals, and interest subsequently waned. A search for equally effective analogs was

initiated, and it was soon demonstrated that phosphonoformic acid (foscarnet) was equally effective and less irritating than PAA when applied topically to the skin.

Animal studies have demonstrated that topical foscarnet has therapeutic effects on cutaneous, genital, and ocular herpes infections (Oberg, 1983). Clinical studies have been performed which support these findings in labial herpes. They indicate that, if treatment is initiated very early, the vesicular period can be shortened and there is a tendency for fewer new vesicles in the treated patients. However there was no difference in time to crusting and healing. Unfortunately, virological studies were not performed. It would have been useful to have determined whether the drug had a beneficial effect on amount and duration of viral shedding. It is hoped that these data can be accumulated from the ongoing studies. However, the problem of bone retention also exists with PFA, but not to the extent of the problem with PAA.

Studies against genital herpes are currently under way in Sweden, and others are planned for the United Kingdom, Finland, and Canada. Data from the Swedish study should be available soon.

H. Acyclovir

One of the most interesting and promising of the antiviral agents currently under clinical study is acyclovir. Its viral specificity has been well characterized, and it selectively inhibits replication of herpes simplex with little effect on the normal cell and should, therefore, be a less toxic drug. It is a synthetic acyclic purine nucleoside analog with *in vitro* and *in vivo* inhibiting activity against HSV, as well as varicella-zoster, Epstein–Barr virus, and cytomegalovirus. HSV-coded thymidine kinase converts acyclovir to the monophosphate nucleotide, which is further converted into the diphosphate by cellular guanylate kinase and into triphosphate by a number of cellular enzymes. The acyclovir triphosphate interferes with HSV DNA polymerase and thereby inhibits viral DNA replication. The cellular DNA polymerase is affected, but to a lesser degree. Cellular thymidine kinase does not convert acyclovir to the monophosphate effectively. Thus acyclovir is preferentially taken up and converted to its active form by herpes-infected cells. This specificity tends to lower the toxicity of the drug because less is being taken up by normal cells, less is converted to the active form, and the cellular α-DNA polymerase is less sensitive to the effects of the active form.

Multicenter, double-blind, placebo-controlled studies against genital herpes were performed with a 5% ACV ointment (Corey *et al.*, 1982). The drug appears to be more effective in initial infection than recurrence. The studies in initial HSV infection provide sufficient statistical evidence that ACV is effective in reducing viral shedding (4 vs 9.5 days; $p < 0.002$),

healing time (8.5 vs 12.5 days; $p = 0.02$), and crusting time (6.6 vs 9.5 days; $p = 0.02$) in females. The result in males is much less substantial. There was a tendency toward less pain.

In recurrent genital HSV, 234 patients were evaluated. The results were less dramatic. Virus shedding was brief in these patients but ceased up to 1 day sooner in the ACV group. There was a tendency toward fewer new lesions in females, but no other benefit was observed. Similar results have been obtained in studies of herpes labialis. There was a decrease in maximum virus titer and shedding, but no clinical effect.

After allogenic bone marrow transplantation, over 80% of patients with HSV antibody will develop mucocutaneous herpes infections. Based on an earlier open trial showing safety and efficacy of intravenous ACV, a double-blind, placebo-controlled trial of intravenous ACV in these patients was performed. The ACV or placebo was given iv for 1 hr every 8 hr for a total of 21 doses (7 days). ACV in 5% dextrose in water was given at a dose of 250 mg/m^2 of body surface (750 mg/m^2 per day). Of 34 patients studied (17 ACV, 17 placebo), 31 had oral lesions (28 HSV isolates all HSV-1), 6 had genital lesions (5 isolates all HSV-2). Patients with ACV had significantly shorter virus excretion, 3 vs 17 days ($p < 0.00005$). Time to first decrease in pain was 6 vs 14 days ($p < 0.05$); cessation of pain 10 versus 16 days ($p < 0.03$); time to crusting, 7 vs 14 days ($p < 0.01$); total healing, 14 versus 28 days ($p < 0.03$). No effect was expected on subsequent recurrent infections, and none was observed (Wade *et al.*, 1982).

A second study was performed in 20 seropositive recipients of bone marrow transplants (Saral *et al.*, 1981). Acyclovir (250 mg/m^2) or placebo was administered iv every 8 hr beginning 3 days before marrow transplantation and continuing for a total of 18 days. Of the 10 placebo patients, 7 developed herpes lesions, but there were no lesions in the treated patients. Some of the treated patients did develop infections after cessation of treatment. Thus acyclovir appears to be effective as a prophylactic drug.

Currently studies are being performed using oral acyclovir. It appears to be effective at dosages of 200 mg every 4 hr, in 3-day cycles alternating with 5 days off drug. This regimen appears to interrupt asymptomatic shedding and delays the appearance of recurrences in immunocompromised patients. Drug absorption by the oral route is slow and variable; the bioavailability is estimated to be in the range of 15 to 30%. However, effective drug levels can be achieved and sustained for several hours (Straus *et al.*, 1982).

I. Arildone

Arildone, 4-[6-(2-chloro-4-methoxy)phenoxyhexyl]-3,5-heptanedione, and chemically related compounds represent a new class of antiviral agent that selectively inhibits replication of some DNA and RNA viruses including herpesviruses, polioviruses, rhinoviruses, picornaviruses, and respira-

tory syncytial viruses. It appears to inhibit viral uncoating, and has been most effective in the model systems tested if present prior to, or at the time of, infection. It is currently undergoing clinical trials in the United States and abroad for its efficacy in the topical therapy of herpes mucocutaneous infections. Although clinical studies have been initiated, it is unlikely that this will prove to be a very exciting drug.

J. Enviroxime

On the basis of extensive studies on the benzimidazoles by Igor Tamm, enviroxime [2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenylketone oxime] has been tested for its efficacy against rhinoviruses in the United Kingdom at the Medical Research Council Common Cold Unit and in the United States. Studies in organ cultures with various serotypes of rhinovirus recently isolated from patients demonstrated this compound to be an efficient inhibitor of viral replication with a high therapeutic ratio. This, together with its virus-specific mode of action, suggests that it may have potential for human chemotherapy against the common cold. Initial clinical studies with an oral formulation showed some efficacy, but drug toxicity was observed. Subsequent studies have been performed administering the drug as a nasal spray (Levandowski *et al.*, 1982). Volunteers were studied with a rhinovirus type 4 challenge for drug prophylaxis or therapy. Although no efficacy was demonstrated, some reduction of symptoms and viral shedding was observed in subjects with higher drug levels. Drug administered as a spray is rapidly removed by nasal mucous and ciliary action; therefore, lack of efficacy may be due to a mechanical problem of drug retention. This is obvious from the inconsistency of drug levels in nasal washes following drug administration. Further studies are warranted to maximize the positive effect, specifically through drug formulation and delivery.

K. Other Chemical Agents of Interest

Several compounds that are in limited use and/or under study should be mentioned. Aedurid, Cebe-Viran, and Viru-Merz are available in Europe for the treatment of ocular herpes infections; however, their clinical efficacy has not been established. 2-Deoxy-D-glucose has been shown to inhibit some enveloped viruses *in vitro,* and some reports have been made on its clinical efficacy against genital herpes, but clinical efficacy has not been adequately demonstrated. A new nucleoside analog, 9-{2-[hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine (Smith *et al.*, 1982), similar in structure to acyclovir has been reported to have potent antiviral activity against HSV-1 and HSV-2 at concentrations well below cytotoxic levels. Its mode

of action appears to be different from that of the other antiviral nucleoside analog, therefore making it a likely candidate for use against viruses resistant to other drugs. Perhaps combination with other drugs could result in synergism. Interesting newly synthesized pyrimidine analogs, 2′-fluoro-5-iodoaracytosine (FIAC), 2′-fluoro-5-iodo-1-β-arabinofuranosyluracil (FIAU), and 2′-fluoro-5-methylarauracil (FMAU), appear to have good antiviral activity against herpesviruses in comparison with the other agents effective against these viruses in *in vitro* tests. Studies are currently under way to evaluate their clinical potential; although there is minimal cytotoxicity, studies to reverse the toxicity with deoxycytidine are also being performed. Other nucleotide and nucleoside analogs with antiviral potential are under study but are too numerous to describe here. Although there has been considerable interest over the years in the value of ascorbic acid in the prevention of the common cold, a careful review of all the studies of its prophylactic and therapeutic effects indicates that no benefit can be ascribed to the high doses advocated.

Two additional agents that should be mentioned are levamisole and methisoprinol (isoprinosine). There has been considerable discussion of these compounds, particularly the latter, which at first was considered to be an antiviral agent; more recently it has been advocated as an immune potentiator. It is assumed that both of these agents can be effective in the treatment of viral disease by stimulating the immune system so as to augment the response. Although these agents, particularly methisoprinol, have been available for a number of years and the subject of considerable publicity, a careful review of the literature does not show any clear evidence of their clinical efficacy against viral disease.

L. Interferon

No discussion of antivirals would be complete without some mention of interferon. It remains an area of great general interest (Baron *et al.*, 1982). Periodically there are still articles in the lay press about the new miracle drug, as it is labeled by the mass media. In reality it is a biological of considerable clinical potential for both viral infectious diseases and cancer, but by no means is it a miracle drug. We have now reached the stage at which there is sufficient material for definitive clinical studies. Eventually it might prove to be excellent for use in conjunction with other chemoprophylactic/therapeutic agents. One of the definite attributes of interferon is that it is a natural product, although not entirely without side effects, but these usually occur within a manageable range.

There are currently a number of clinical trials being conducted with natural, polyvalent interferon and cloned interferon produced through DNA recombinant technology. Such infectious disease trials include studies on

the prevention and treatment of the common cold and other respiratory diseases, chronic hepatitis, cytomegalovirus infections in renal transplant patients, laryngeal papilloma, warts, herpetic keratitis, and initial genital herpes.

There are several other areas in viral diseases or diseases in which viruses may be implicated where interferon may have a role—e.g., in rabies and chronic infections such as Creutzfeldt-Jakob encephalopathy and multiple sclerosis, and in rare but devastating infections such as those induced by Ebola virus. The recently reported syndromes of Kaposi's sarcoma and/or pneumocystis pneumonia with severe immunodeficiency in young homosexual men, drug abusers, hemophiliacs, Haitians, and close contacts may represent another case in which interferon treatment could be beneficial. Both EBV and CMV mononucleosis may be candidates as well as arbovirus (some studies have been done treating dengue patients with interferon), arenavirus, and other exotic agents.

Interferon is not without toxicity and side effects; the toxicity may also be more severe when the drug is administered at very high dosages and in certain conditions. Therefore, it cannot be overly stressed that there is a need for carefully controlled, preferably placebo-controlled, studies prior to any claim for efficacy. Open and preliminary studies are often misleading.

What has not been discussed here are the relative merits of α-, β-, or γ-interferon. There is considerably more experience with the alpha and beta types; they do have some differences, such as tissue tropism, but the differences are slight. The third type, γ-interferon, may have considerably different clinical potential, but this has yet to be demonstrated. Additional questions concern the possibly deleterious effects of interferon in bronchial asthma, rheumatoid arthritis, autoimmune disease, etc. At the current rate of progress, many of these questions will be resolved in the near future.

V. CONCLUSIONS

The demonstration that certain antivirals can be used effectively in ongoing, serious acute and chronic infections has led to the increased interest in the development and testing of new antivirals. The need for such agents is clear, particularly in the developing countries, e.g., for the treatment of Lassa fever, Ebola virus, hemorrhagic fever, dengue, hepatitis, encephalitis, and rabies. The list of viral diseases for which antiviral agents offer hope is already extensive, and the number of potentially useful antiviral agents has been increasing at a satisfying rate. In advancing these agents and these studies, several factors must be taken into consideration. If antiviral agents are to be of value, there must be an increased effort in rapid

viral diagnosis. Early treatment is important; if viral pathogenesis has progressed too far, there is little hope of useful intervention. Once a new agent is identified, great care must be exercised in the performance of efficacy studies. If at all ethically possible, the studies should be done as double-blind, placebo-controlled trials. It is only in this matter that reliable, acceptable data can be obtained. Too often anecdotal studies are put into the literature, and there is a tendency to accept them as proof of efficacy. In some instances, patients can be harmed by using ineffective, and sometimes toxic, drugs. Great care must be exercised in reviewing data to ascertain that claims of efficacy are not made prematurely.

Further considerations when dealing with the less developed countries are convenience of drug application (e.g., oral versus injection), cost, and efficacy rate. It may be perfectly acceptable to use a minimally effective drug for a mild disease in a country which has readily accessible medical care. However, this may be an unaffordable luxury in some countries; in order to be of value, efficacy should be greater than 75% and the drug relatively inexpensive.

In the past, development of antiviral agents has been through serendipity. We have now progressed sufficiently that molecular virologists are able to identify virus-specific enzymes and proteins. The chemists in turn are able to develop specific blocking agents. Thus we can have directed programs for development of specific antivirals. It is also probable that these agents would be less toxic if they are targeted for viral components which are not normal cell constituents.

Another area that has not received sufficient attention is combined therapy using agents that affect different stages of viral replication. Combination of minimally effective drugs or less toxic levels may result in synergism, with the end result of a more effective therapy. What must be guarded against, however, is increased toxicity.

The field has been helped considerably by newly developed techniques. DNA recombinant technology, hybridomas, and monoclonal antibody techniques have revolutionized biological research. These techniques have led to important findings on the synthesis and purification of vaccines and biological agents such as interferon; the monoclonal antibodies may also be of great value in the clinic. All of these advances are important to the worldwide medical community, but they will be of even greater value to the less developed countries. As stated previously, infectious diseases are among the leading causes of mortality and the leading causes of morbidity in developed countries, but they are a much bigger problem in the less developed countries. These techniques have the potential of producing specific pharmaceuticals at a reasonable price, as has been proved with interferon. Considerable progress has been made, but we have barely crossed the threshold. It is fully expected that within the next few years several effective antiviral agents will be available worldwide.

REFERENCES

Baron, S., Dianzani, F., and Stanton, G. J. (eds.). (1982). *Tex. Rep. Biol. Med.* **41.**

Corey, L., Nahmias, A. J., Guinan, M. E., Benedetti, J. K., Critchlow, C. W., and Holmes, K. K. (1982). *N. Engl. J. Med.* **306**, 1313.

DeClercq, E., Descamps, J., Verhelst, G., Walker, R. T., Jones, A. S., Torrence, P. F., and Shugar, D. (1980). *J. Infect. Dis.* **141**, 563.

Dolin, R., Reichman, R. C., Madore, H. P., Maynard, R., Linton, P. N., and Webber-Jones, J. (1982). *N. Engl. J. Med.* **307**, 580.

Kaufman, H. E. (1978). *Invest. Ophthalmol. & Visual Sci.* **17**, 941.

Kaufman, H. E., Nesburn, A. B., and Maloney, E. D. (1962). *Arch. Ophthalmol.* **67**, 583.

Levandowski, R. A., Pachucki, C. T., Rubens, M., and Jackson, G. J. (1982). *Antimicrob. Agents Chemother.* **22**, 1004.

Monto, A. S., Gunn, R. A., Bandry, M. G., and King, C. L. (1979). *J. Am. Med. Assoc.* **241**, 1003.

Oberg, B. (1983). *Pharmacol. & Ther.* **19**, 387.

Pavan-Langston, D., Buchanan, R. A., and Alford, Jr., C. A. (eds.). (1975). "Adenine Arabinsodie: An Antiviral Agent." Raven, New York.

Saral, R., Burns, W. H., Laskin, O. L., Santos, G. W., and Lietman, P. S. (1981). *N. Engl. J. Med.* **305**, 63.

Smith, K. O., Galloway, K. S., Kennell, W. L., Ogilvie, K. K., and Radatus, B. K. (1982). *Antimicrob. Agents Chemother.* **22**, 55.

Straus, S. E., Smith, H. A., Brickman, C., deMiranda, P., McLaren, C., and Keeney, R. E. (1982). *Ann. Intern. Med.* **96**, 270.

Van Voris, L. P., Betts, R. F., Hayden, F. G., Christmas, W. A., and Douglas, Jr., R. G. (1981). *J. Am. Med. Assoc.* **245**, 1128.

Wade, J. C., Newton, B., McLaren, C., Flournay, N., Keeney, R. E., and Meyers, J. D. (1982). *Ann. Intern. Med.* **96**, 265.

Whitley, R. J., Nahmias, A. J., Soong, S. J., Galasso, G. J., Fleming, C. L., Alford, Jr., C. A., and the NIAID Collaborative Antiviral Study Group. (1980a). *Pediatrics* **66**, 495.

Whitley, R. J., Soong, S. J., Hirsch, M. S., Krachmer, A. W., Dolin, R., Galasso, G. J., Dunnick, J. K., Alford, Jr., C. A., and the NIAID Collaborative Antiviral Study Group. (1980b). *N. Engl. J. Med.* **304**, 313.

Whitley, R. J., Hilty, M., Haynes, R., Bryson, Y., Connor, J. D., Soong, S. J., Alford, Jr., C. A., and the NIAID Collaborative Antiviral Study Group. (1982a). *J. Pediatr. (St. Louis)* **101**, 125.

Whitley, R. J., Soong, S. J., Dolin, R., Betts, R., Linnemann, C., Alford, Jr., C. A., and the NIAID Collaborative Antiviral Study Group. (1982b). *N. Engl. J. Med.* **307**, 971.

8

Acyclovir: A Review of the Preclinical and Clinical Status

A. PAUL FIDDIAN

Department of Clinical Immunology and Chemotherapy,
Clinical and Applied Research Division,
Wellcome Research Laboratories, Beckenham, Kent, England

I. INTRODUCTION

Since the first report in the literature by Elion *et al.* 1977), considerable progress has been made in the development of the antiviral agent acyclovir. Previously referred to as 248U or acycloguanosine, the drug is 9-(2-hydroxyethoxymethyl)guanine and has the registered trade name Zovirax. A large number of *in vitro* studies have been performed to elucidate the antiviral activity and mechanism of action of acyclovir. Extensive studies in animals have been undertaken to evaluate the potential toxicity, antiviral activity, and pharmacokinetics of the drug before its wide application to man.

Intravenous, oral, dermal, and ophthalmic formulations of acyclovir have now been studied in man and are licensed in various countries throughout the world. Although currently indicated only for the treatment of herpes

ISBN 0-12-429601-7

simplex virus (HSV) infections data will be presented supporting the efficacy of acyclovir in the prevention of HSV disease and in the treatment of varicella-zoster virus (VZV) infections. This review will include references to all these areas of research but is intended mainly as an update of the clinical applications of the drug. More detailed information about the preclinical and early clinical aspects are contained in previous reviews by Brigden *et al.* (1981) and Fiddian (1982a).

II. *IN VITRO* ACTIVITY

Schaeffer *et al.* (1978) first reported the activity of acyclovir against HSV type 1. Acyclovir is more active than other currently available antiviral agents against this virus. The 50% inhibitory dose (ID_{50}) of acyclovir is generally less than 0.1 μM for most strains of HSV-1. Collins and Bauer (1979) confirmed a similar high degree of activity against HSV-2 with ID_{50} values of around 0.3 μM. It was noted by Schaeffer *et al.* (1978) that the low toxicity against the uninfected host cells compared with the marked antiviral effects against HSV-1 represented a therapeutic index of about 3000.

Schaeffer *et al.* also first reported the antiviral activity against VZV and gave ID_{50} values of about 3 μM. Several others including Biron and Elion (1980) have confirmed this level of sensitivity for VZV, and Boulter *et al.* (1980) showed similar activity against herpes B virus. Colby *et al.* (1980) reported slightly less activity against Epstein–Barr virus (EBV) with an equivalent ID_{50} value of 6 μM. For cytomegalovirus (CMV) there has been more discrepancy between the reports; Tyms *et al.* (1981) and Plotkin *et al.* (1982) demonstrated ID_{50} values of around 44 μM, whereas Crumpacker *et al.* (1979) reported relative insensitivity for most strains of the virus.

It is important to interpret these and other reports of antiviral activity with some care. First, because many factors can influence the values obtained including viral and host cell strains used and study methodology; and, second, because it is not known how to relate *in vitro* activity to plasma or tissue drug levels.

III. MECHANISM OF ACTION

This subject has been reviewed in an excellent paper by Elion (1982). Previously Elion *et al.* (1977) had shown that acyclovir is converted in HSV-infected cells to its monophosphate by specific virus-induced thymidine kinase. Miller and Miller (1980) showed that the monophosphate is phosphorylated to the diphosphate by host cell GMP kinase. Further, cellular

enzymes are then responsible for conversion of the diphosphate to the triphosphate form as demonstrated by Miller and Miller (1982). Furman *et al.* (1979) reported that the triphosphate specifically inhibits viral DNA polymerase by competing with deoxyguanosine triphosphate and also prevents further DNA synthesis by being incorporated as a DNA chain terminator.

For VZV, Fyfe *et al.* (1978) have demonstrated similar mechanisms of action although the VZV-specified thymidine kinase is slightly less effective. Colby *et al.* (1981) have shown that acyclovir monophosphate is produced only at a slow rate in EBV-infected cells, but other cellular enzymes may be more effective and in addition EBV-specified DNA polymerase is very sensitive to acyclovir triphosphate. A lack of virus-induced thymidine kinase in CMV-infected cells probably accounts for the reduced activity of acyclovir against this virus.

In the laboratory it is easy to produce resistance of HSV by serial passage in the presence of drug. Alterations in the thymidine kinase or DNA polymerase may account for the development of this resistance. However, the commonest type of resistance *in vitro,* and the only one reported in man, is a lack of viral thymidine kinase. Field and Darby (1980) demonstrated that such strains are less virulent in mice and so may pose less of a threat to man than the normal strains. Burns *et al.* (1982a) and Crumpacker *et al.* (1982) reported the development of less sensitive strains of HSV following therapy with acyclovir, but these were not clinically significant. It remains to be seen whether this problem will increase, but for the present particular care should be exercised in treating severely immunocompromised patients with chronic HSV infections.

IV. ANIMAL STUDIES

An excellent review of the toxicity testing of acyclovir in animals was reported by Tucker (1982). These studies confirm the general lack of toxicity to uninfected cells at potentially therapeutic doses of acyclovir. A wide range of *in vitro* and animal studies indicate that acyclovir does not pose a genetic risk to man. Pharmacokinetic studies in animals, reviewed by de Miranda *et al.* (1982a) and Good and de Miranda (1982b), have shown that the distribution, metabolism, and excretion of the drug are satisfactory. Using animal models of HSV infections acyclovir has been reported to be effective in the treatment of systemic, cutaneous, genital, or ocular disease in a variety of species. Both local and systemic therapies have been shown to be useful for the treatment of these infections. Useful additional information may be found in papers by Collins and Oliver (1982), Kern (1982), and Bauer (1982a). Antiviral activity has been demonstrated also against

simian VZV in monkeys by Soike and Gerone (1982), simian B virus in rabbits by Boulter *et al.* (1980), and murine CMV in mice by Burns *et al.* (1982b) and Glasgow *et al.* (1982).

It has been proved that early therapy of a primary infection in a variety of animal species can prevent or reduce the development of latent infection. However, once established the latent virus cannot be eradicated, and so the prospect for a once and for all cure for recurrent infections seems remote. For further information, see Park *et al.* (1982), Landry *et al.* (1982), and Klein (1982).

V. PHARMACOKINETICS IN MAN

Several reviews of the clinical pharmacology of acyclovir have been written including those by Lietman (1982), Whitley *et al.* (1982a), and Blum *et al.* (1982). Additional reports concerning the metabolic fate of acyclovir in man by de Miranda *et al.* (1982b), the effects of renal failure on the pharmacokinetics of acyclovir by Laskin *et al.* (1982), and neonatal pharmacokinetics by Hintz *et al.* (1982) also contain useful information. In summary, the mean plasma half-line is about 3 hrs in normal adults, but this is extended to around 20 hrs in end-stage renal disease. During hemodialysis the half-life can be reduced to approximately 6 hrs. Plasma binding is low at around 15%, and most of the drug is excreted unchanged in the urine by glomerular filtration and tubular excretion. Up to 15% of the total dose of acyclovir is usually metabolized, mainly to 9-carboxymethoxymethylguanine, but in renal failure this may be much higher.

For adults the usual dose of intravenous acyclovir is 5–10 mg/kg every 8 hr by intravenous infusion over 1 hr. Peak plasma levels following these doses will tend to exceed 40 and 80 μM, respectively, whereas trough levels will be around 4 and 8 μM. It is suggested that children be given the drug at equivalent doses of 250 to 500 mg/m^2 every 8 hr, but that for neonates a dose of 10 mg/kg every 8 hr be preferred. Where there is evidence of renal impairment, then the interval between doses of acyclovir should be increased.

The standard dose of acyclovir by mouth is 200 mg every 4 hr during the waking day. van Dyke *et al.* (1982) reported that mean peak plasma levels following this dose were 2.5 μM, while peak levels of acyclovir in vaginal secretions and saliva were 1.9 and 0.4 μM, respectively. Brigden *et al.* (1980) demonstrated slightly higher peak plasma levels of 4 μM at this dose and estimated that absorption of the drug was only about 20% of that administered.

VI. CLINICAL EXPERIENCE

Since the natural history of herpes infections may differ depending on whether the patient has an intact or an impaired immune system and whether the infection is the first contact with the virus or a recurrence caused by latent virus, each aspect requires separate evaluation. The results of clinical studies and other experience with acyclovir will be discussed primarily depending on the state of the host's immune system.

A. The Immunocompromised Host

1. HSV Infections

In a large multicenter, double-blind, placebo-controlled study Meyers *et al.* (1982) reported that intravenous acyclovir was effective and safe in the treatment of mucocutaneous HSV infections in immunocompromised patients. Significant effects on the duration of virus shedding from lesions, the duration of pain, the time to scabbing, and the time to healing of lesions were demonstrated. Healing was reduced by almost a week, from median of 20.1 to 13.7 days. Different groups of patients, many of them included in this overall trial report, have been documented separately. Wade *et al.* (1982c) presented similar data for infected patients following bone marrow transplantation, and several renal transplant patients were included in a paper by Mitchell *et al.* (1981). A separate report by Chou *et al.* (1981) showed dramatic effects in a small number of acyclovir-treated patients after cardiac transplantation compared with placebo-treated controls.

Whitley *et al.* (1982b) demonstrated in a controlled trial that topically applied acyclovir was also effective in reducing virus shedding and pain associated with mucocutaneous HSV infections in immunocompromised patients, mostly renal transplant recipients. Numerous uncontrolled series and anecdotal reports of apparently successful topical and intravenous therapy with acyclovir have also been published. More notable among these are papers by Selby *et al.* (1979), O'Meara and Hillary (1981), van der Meer and Versteeg (1982), Spector *et al.* (1982), and Straus *et al.* (1982). In the latter report the authors suggested that orally administered acyclovir is also effective in the treatment of mucocutaneous HSV infections.

If therapy is initiated only after the onset of lesions, then significant tissue damage may still occur and so only partial benefit be achieved. When it is possible to predict a high risk of recurrent HSV infections, as, for instance, following organ or marrow transplantation, prophylaxis may be more appropriate. Saral *et al.* (1981) were the first to show in a controlled trial that intravenous acyclovir could successfuly prevent HSV infections in

susceptible patients immediately after bone marrow transplantation. They used a standard dose of drug every 8 hr which gave complete protection. Hann *et al.* (1982) treated both bone marrow transplant patients and acute leukemics undergoing induction chemotherapy with twice daily intravenous acyclovir or placebo. All the marrow recipients and most of the leukemic patients receiving the drug were prevented from developing HSV infections.

More recently there have been several reports of successful prophylaxis with oral acyclovir in placebo-controlled trials and open studies. Wade *et al.* (1982b) and Devergie *et al.* (1983) conducted formal trials in bone marrow transplant patients demonstrating that doses of 400 mg every 4 hr or 200 mg every 6 hr, respectively, are effective in preventing reactivation of HSV during treatment. Others, including Straus *et al.* (1982), Fiddian *et al.* (1982a), and Prentice *et al.* (1983), reported similarly dramatic results in uncontrolled studies of patients with immunodeficiency syndromes, cardiac transplants, or bone marrow transplants, respectively. It has been noted that recurrences of HSV infections still occur after stopping prophylactic therapy with acyclovir, and so further studies have been initiated to evaluate long-term treatment for periods of up to 6 months or more.

Systemic infections caused by HSV are fortunately not very common, and concerns about the life-threatening nature of such infections have in any case precluded placebo-controlled trials. There are several anecdotal reports in whch intravenous acyclovir was believed to have had beneficial effect in such situations. Among these are reports by Many *et al.* (1980) for HSV esophagitis, Selby *et al.* (1979) and Goldman *et al.* (1979) for HSV pneumonia, and Heaton *et al.* (1981) for HSV encephalitis. It seems likely that the greatest benefits from acyclovir will be achieved by preventing dissemination of infection in patients at risk rather than in treating already widespread disease.

2. *VZV Infections*

So far there have been rather few reports of controlled trials of acyclovir in the treatment of VZV infections in immunocompromised patients. Prober *et al.* (1982) in a small study of chickenpox showed that intravenous acyclovir prevented dissemination of the disease to the lungs. None of 7 acyclovir patients developed pneumonia compared with 5 of 11 placebo recipients. In addition there have been several anecdotal reports of efficacy in the treatment of cutaneous and visceral varicella. Among these, van der Meer and Versteeg (1982), Kinney *et al.* (1981), and Shulman *et al.* (1981) indicate that acyclovir may be useful in the management of primary VZV infections.

A large placebo-controlled trial in herpes zoster has been conducted in the United States, but the results are not yet published. Balfour *et al.* (1981)

presented an interim analysis of this study which indicated effects on the duration of pain and the progression of the skin lesions. They commented that significant differences were detected in the overall analysis but gave no details. Again there are numerous anecdotal reports in the literature. O'Meara and Hillary (1981), Serota *et al.* (1982), Selby *et al.* (1979), Spector *et al.* (1982), and others all treated several patients having localized zoster. In many cases it was felt that acyclovir was beneficial, and most impressive were the rapid effects on pain and the way progression of the disease was arrested. Acyclovir was also thought to be useful in the treatment of disseminated cutaneous zoster, as reported by van der Meer and Versteeg (1982), O'Meara and Hillary (1981), and Cupps *et al.* (1981), and possibly for the visceral complications of zoster, such as pneumonia (Selby *et al.*, 1979), and meningoencephalitis (Nelson *et al.*, 1980; van der Meer and Versteeg, 1982). As with HSV infections it was felt that the greatest impact from acyclovir might be in prevention of dissemination, whether locally or systemically. However, it should be noted that a higher dose of acyclovir was often employed in the treatment of these VZV infections.

3. EBV Infections

There are few published data relating to treatment of EBV infections, and some discrepancy appears with the findings. Hanto *et al.* (1981) reported antiviral and clinical effects in a patient with an EBV-associated lymphoma following renal transplantation. On the other hand, Sullivan *et al.* (1982) showed no such effects in two children with life-threatening EBV infections. Further work is required to determine whether acyclovir will have any role to play in the treatment of EBV disease. At present more emphasis is being placed on evaluating the drug in EBV-infected patients with reasonably intact immune systems.

4. CMV Infections

Finally, for treatment of CMV infections there is also some disagreement among reports from different centers, although this again may be due to differences in the immune status of the patients involved. In a controlled trial reported by Balfour *et al.* (1982), the authors concluded that acyclovir may be useful for treatment of CMV disease in certain immunosuppressed patients, particularly renal allograft recipients. In a single patient case report, Ashraf *et al.* (1982) stated that acyclovir may also be useful for treatment of CMV pneumonia after cardiac transplantation, whereas Wade *et al.* (1982a) reported that acyclovir was not effective for CMV pneumonia after marrow transplantation. It is worth noting that Plotkin *et al.* (1982) demonstrated a transient but definite antiviral effect in congenitally infected children.

B. The Normal Host

1. Genital Herpes

Genital infections caused by HSV, particularly type 2, are an increasing problem in many parts of the world. The primary or initial attack may be quite severe, the patient requiring hospitalization, but the main problem with this disease is its recurring nature. In one placebo-controlled trial of intravenous acyclovir in severe primary genital herpes, Mindel *et al.* (1982) demonstrated significant effects on the duration of viral shedding, duration of symptoms, and progression of lesions. The total duration of the disease was halved from a median of 14 days in the placebo group to only 7 days in the acyclovir patients. Similarly dramatic results were reported in a study by Fife *et al.* (1981) using intravenous acyclovir.

Subsequently Nilsen *et al.* (1982), Bryson *et al.* (1982), and Mertz *et al.* (1982) demonstrated that oral acyclovir too is effective in the treatment of initial genital HSV infections. Topical treatment has been shown to have similar, though perhaps slightly less impressive, effects whether used as the ointment (Corey *et al.*, 1982; Thin *et al.*, 1983) or as the cream (Kinghorn *et al.*, 1983). Oral and dermal formulations are obviously suitable for outpatient treatment of initial disease, but intravenous therapy may be useful for hospitalized patients with severe attacks.

Nilsen *et al.* (1982), Reichman *et al.* (1982), and Salo *et al.* (1983) have reported that oral acyclovir can reduce the duration of recurrent episodes of genital herpes. Since the natural history of such infections is usually fairly short, major effects can be expected only if therapy is initiated as early as possible after the onset of prodromal symptoms. Delayed treatment may still be worthwhile for the more severe episodes, as demonstrated by Fiddian *et al.* (1982b). In patients with frequent recurrences it may be justifiable to attempt prevention of attacks by using continuous therapy. Extensive work is in progress to evaluate such long-term treatment in terms of safety and efficacy, but an early report by Straus *et al.* (1982) in immunodeficient patients suggests that suppression of infection is possible.

Early reports of topical therapy of recurrent infections were not as encouraging. Corey *et al.* (1980) reported only a reduction of viral shedding and new lesion formation with acyclovir ointment compared with placebo. In a larger series Corey *et al.* (1981) demonstrated a significant shortening of the healing time, in male patients only, from 9.7 to 7.6 days. When pooled with results from other centers, the time to crusting in men and the percentage of new lesion formation in all patients were reduced, as reported by Corey *et al.* (1982). More recently an alternative formulation, acyclovir cream, has been evaluated. Using early patient-initiated therapy, Fiddian (1983) has shown that the results achieved with the cream compare favorably with the oral formulation. The apparent superiority of the cream over

the ointment has been demonstrated by Collins and Oliver (1982) using an animal model of cutaneous HSV infections.

2. *Herpes Labialis*

Similar experiences have been reported with topical treatment of orolabial HSV infections. Spruance *et al.* (1982b) showed that acyclovir ointment reduced the virus excretion from lesions of patients with herpes labialis and that this effect was enhanced in those in whom treatment was started early. They demonstrated no significant effects on the clinical course of the episodes, but some trends in favor of acyclovir were seen in the patients treated early. Fiddian and Ivanyi (1983) reported that clinical benefit may be achieved with this formulation following patient-initiated therapy of recurrences in patients experiencing severe attacks. However, using early self-medication and even doubling the concentration of acyclovir in the ointment had no effect on the course of recurrent infections in healthy subjects, as demonstrated by Spruance *et al.* (1982a). The authors concluded that new formulations should be explored which enhance penetration through the skin.

Acyclovir cream has in fact been reported to be effective in the treatment of herpes labialis, presumably because in the aqueous phase acyclovir can pass through the stratum corneum. In one study, Fiddian *et al.* (1983b) showed in healthy subjects that early self-initiated therapy reduced the duration of lesions and, perhaps more important, increased the percentage of episodes that effectively aborted. In another trial van Vloten *et al.* (1983) demonstrated similar effects on the course of established lesions of herpes labialis in patients attending a dermatology clinic. An anecdotal report by Kennedy *et al.* (1981) indicated that prophylactic use of topical acyclovir could prevent most of the frequent recurrences of herpes labialis and the associated attacks of erythema multiforme experienced by one patient. Controlled trials are now in progress evaluating topical and oral therapy in this latter indication. A review of the treatment of herpes labialis with acyclovir has been prepared by Fiddian *et al.* (1983a).

3. *Herpes Keratitis*

Numerous papers attest to the efficacy of acyclovir ophthalmic ointment in the treatment of HSV infections of the eye. Jones *et al.* (1979) first demonstrated in a controlled trial the potential of this formulation for the management of dendritic ulceration of the cornea. They showed that acyclovir completely prevented the early recurrences of lesions following minimal wipe debridement compared with the more than 50% incidence in the placebo group. Since then acyclovir has been compared with the other available antiviral therapies for the treatment of herpes keratitis. Collum *et al.* (1980)

reported that acyclovir was superior to idoxuridine, producing a mean healing time of 4.4 days compared wtih 9.2 days, as did Colin *et al.* (1981); several authors including Coster *et al.* (1980) showed that the drugs had comparable efficacy. Similar results have been demonstrated for acyclovir compared wtih adenine arabinoside, Young *et al.* (1982) showing acyclovir to be more effective whereas McGill *et al.* (1981) and Pavan-Langston *et al.* (1981) reported them to be broadly equivalent. In the only comparative trial with trifluorothymidine, la Lau *et al.* (1982) did not demonstrate any significant differences between the therapies. Patients failing to respond to the other antiviral agents have shown favorable responses to acyclovir in open studies, and this has been documented by Collum and Benedict-Smith (1981). It is particularly in the treatment of the more severe ocular HSV infections that the other existing antivirals are believed to be less than satisfactory, especially when the deeper tissues of the eye are involved. Since acyclovir has been shown by Poirier *et al.* (1982) to penetrate the cornea and produce significant levels of drug in the aqueous humor, whereas the other agents do not, and because of the drug's low toxicity even during long-term use, it is seen to have potential advantages. Collum *et al.* (1983) reported a combination of acyclovir and dilute steroid drops to be effective in the management of disciform keratitis.

4. VZV Infections

Little work has been carried out in the treatment of the primary infection caused by VZV, namely chickenpox, since the disease is not usually severe enough in normal children to warrant therapy. However, in malnourished children in developing countries and in adults that have escaped infection during childhood, the disease can be quite severe with, in some cases, systemic involvement, particularly of the lungs. Al-Nakib *et al.* (1983) reported from a controlled trial in young adults that intravenous acyclovir was effective in reducing the duration of vesicles and fever compared with placebo. Anecdotal reports have also suggested that this formulation might be useful for treatment of chickenpox pneumonia, as in van der Meer *et al.* (1980).

The major application of acyclovir in VZV infections has been in the treatment of herpes zoster or shingles. Papers by Peterslund *et al.* (1981), Bean *et al.* (1982), and McGill *et al.* (1983) have demonstrated in controlled trials the value of intravenous acyclovir in relieving the acute pain and improving the healing rate of herpes zoster. Such a treatment requires admission to hospital and so may be reserved for more severe cases or where patients are at greater risk, as, for instance, with the elderly or if facial disease threatens the eye.

Several trials of acyclovir given by mouth or applied topically as the cream

are in progress, but no results are yet available. Treatment by one or other of these routes may be effective and would obviously facilitate outpatient management of this disease. For involvement of the eye with herpes zoster, McGill (1981) has reported that the ophthalmic ointment is effective, and so perhaps this formulation could be used to prevent spread of infection from the skin as well as for treatment of established disease of the eye.

5. *Other Infections*

The fortunately rare but very serious systemic complications of HSV infections in children and adults, namely neonatal HSV disease and herpes encephalitis, merit some attention. Difficulty in recruiting adequate numbers of patients into controlled trials has meant that it will be some time before definitive results will be available. Reports of case series of treated infections in neonates by Gould *et al.* (1982) and Yeager (1982) and anecdotal observations in herpes encephalitis from Chapman and Brigden (1981) and Harrington *et al.* (1981) are encouraging. Since the mortality of the infections if untreated is so high, it would seem to be justifiable to attempt early treatment with a potentially life-saving drug in the absence of any other known safe or effective therapy. In this regard it should be noted that twice the normal dose of acyclovir is usually given and treatment is continued for 10 days or more.

Another complication of HSV infection is eczema herpeticum, and reports by Gould *et al.* (1982) and Swart *et al.* (1983) suggest that acyclovir is useful in the management of this disease. Several other manifestations of herpesvirus infections require further evaluation, and these include primary HSV stomatitis, and EBV and CMV infections in nonimmunocompromised patients. Despite early enthusiasm expressed by Bauer (1982b) for the effective treatment of chronic plantar warts, a controlled trial by Gibson *et al.* (1983) revealed a lack of effect from topical acyclovir. Yet another DNA virus that has been shown to be susceptible to acyclovir is hepatitis B virus (HBV). Weller *et al.* (1982) reported inhibition of HBV replication in two patients with chronic infection treated with high doses of intravenous acyclovir for 5 or 7 days. Similar effects were seen by Smith *et al.* (1982) in three patients given short courses of therapy. The effects were only transient, and so further work is required to explore the use of longer periods of treatment.

VII. TOLERANCE IN CLINICAL USE

There has now been extensive experience with all the various formulations of acyclovir. In an earlier review by Keeney *et al.* (1982) this subject was discussed. After intravenous injection the major adverse reaction is a

transient impairment of renal function. This is generally avoidable if adequate hydration of the patient is ensured and if the drug is given as a slow infusion over 1 hr rather than as a bolus injection, as reported by Chapman and Brigden (1981). Particularly, care should be exercised in the presence of preexisting renal impairment, when dosage modification should be performed as recommended. Brigden *et al.* (1982) have given detailed information on this topic and demonstrated that, in animals at least, the problem is caused by transient crystal deposition in the collecting tubules of the papilla associated with minimal and reversible histological changes of the epithelial cells.

Another adverse reaction definitely related to administration of acyclovir is local injection site inflammation, but with care this can be kept to a minimum. In addition several other events have been reported in patients receiving intravenous injection; these include occasional nausea and vomiting, reversible neurological reactions, raised liver enzymes, rashes, and decreased hematological indexes. The incidence of these reported adverse events does not cause concern about the toxicity of the drug in normal usage.

As expected since the plasma levels of acyclovir are much lower, the incidence of events occurring during oral therapy is very small. Indeed when the experience gained from treating over 800 patients in controlled trials is pooled, the nature and severity of such events does not differ between the acyclovir and placebo groups. In fact rather more events occurred in patients receiving placebo, particularly elevation of liver enzymes. Nilsen *et al.* (1982) have reported some of this experience.

With dermal applications of acyclovir cream or ointment, the majority of adverse events are the result of physical contact with raw surfaces. Corey *et al.* (1982) reported transient pain or burning on application of the ointment in 24% of initial and 11% of recurrent cases of genital herpes compared with 36 and 16% of placebo recipients, respectively. Since equal numbers of other events have been reported for both acyclovir and placebo groups, the bases rather than the acyclovir are probably responsible. The incidence of side effects in trials of herpes labialis was very low (Fiddian *et al.*, 1983b), or none were experienced (Spruance *et al.*, 1982b).

The use of acyclovir ophthalmic ointment may be associated with transient stinging and the development of superficial punctate keratopathy. This latter adverse effect is found with the administration of other antivirals to the eye, and as discussed by McGill and Tormey (1982) is not considered serious. Otherwise the lack of toxicity of acyclovir to the cornea (Morgan *et al.*, 1980), even after long-term use, reflects the exceptional tolerance of this drug compared with other antivirals. Lass *et al.* (1979) have also shown that acyclovir does not delay corneal wound healing whereas the other antivirals do.

VIII. CONCLUSIONS

In summary, acyclovir seems destined to play an important role in the future management of infections caused by at least some of the herpesviruses. Certainly most HSV infections, whether in the immunocompromised host or in otherwise normal patients, can be satisfactorily controlled with one or other formulation of acyclovir. Much of the initial impact of the drug in HSV infections has been with treatment of established disease, particularly in severely ill patients. In an earlier review by Fiddian (1982b), the potential use of prophylaxis in patients at risk of developing HSV infections was discussed. The wider application of the drug for prevention of HSV disease in all groups of susceptible patients now appears to be emerging as a real possibility as evidence is gained about the efficacy and safety of oral acyclovir.

For treatment of VZV infections, the case for use of intravenous acyclovir is also well on the way to being proved. Alternative methods of treatment are being explored, but since the virus is less sensitive than HSV it is by no means certain that topical or oral therapy will be successful. Research into the enhancement of acyclovir plasma levels by use of orally administered pro-drugs such as the one reported by Good and de Miranda (1982a) may offer a solution to this problem should the need arise. It is also rather less certain whether prophylaxis will be useful in the management of VZV infections, since prediction of those at risk is less easy than with HSV.

Finally, for EBV, CMV and HBV infections it remains to be seen whether acyclovir will have any serious part to play in the treatment or prevention of these diseases. Nevertheless for whichever indication acyclovir is used, it is reassuring to know that the drug will cause little harm to the uninfected host cells because of its targeted activity. It is hoped that the development of acyclovir heralds a new era of safe and effective antiviral chemotherapy.

REFERENCES

Al-Nakib, W., Al-Kandari, S., El-Khalik, D. M. A., and El-Shirbiny, A. M. (1983). *J. Infect.* **6** (1), 49–56.

Ashraf, M. H., Campalani, G. C., Qureshi, S. A., Froud, D. J., and Yacoub, M. H. (1982). Lancet **1**, 173–174.

Balfour, H. H. Jr., Bean, B., Sachs, G. W., and Mitchell, C. D. (1981). *Minn. Med.* **64**, 739–743.

Balfour, H. H. Jr., Bean, B., Mitchell, C. D., Sachs, G. W., Boen, J. R., and Edelman, C. K. (1982). *Am. J. Med.* **73** (1A), 241–248.

Bauer, D. J. (1982a). *Am. J. Med.* **73** (1A), 109–111.

Bauer, D. J. (1982b). *Am. J. Med.* **73** (1A), 313–314.

Bean, B., Braun, C., and Balfour, H. H. Jr. (1982). *Lancet* **2,** 118–121.
Biron, K. K., and Elion, G. B. (1980). *Antimicrob. Agents Chemother.* **18,** 443–447.
Blum, M. R., Liao, S. H. T., and de Miranda, P. (1982). *Am. J. Med.* **73(1A),** 186–192.
Boulter, E. A., Thornton, B., Bauer, D. J., and Bye, A. (1980). *Br. Med. J.* **1,** 681–683.
Brigden, D., Fowle, A., and Rosling, A. (1980). *In* "Developments in Antiviral Therapy" (L. H. Collier and J. Oxford, eds.), pp. 53–62. Academic Press, New York.
Brigden, D., Fiddian, P., Rosling, A. E., and Ravenscroft, T. (1981). *Antiviral Res.* **1,** 203–212.
Brigden, D., Rosling, A. E., and Woods, N. C. (1982). *Am. J. Med.* **73(1A),** 182–185.
Bryson, Y., Dillon, M., Lovett, M., Acuna, G., Taylor, S., Cherry, J., Johnson, L., and Creagh-Kirk, T. (1982). *Clin. Res.* **30(1),** 128A.
Burns, W. H., Saral, R., Santos, G. W., Laskin, O. L., Lietman, P. S., McLaren, C., and Barry, D. W. (1982a). *Lancet* **1,** 421–423.
Burns, W. H., Wingard, J. R., Sandford, G. R., Bender, W. J., and Saral, R. (1982b). *Am. J. Med.* **73(1A),** 118–124.
Chapman, J. R., and Brigden, D. (1981). *Lancet* **2,** 1103.
Chou, S., Gallagher, J. G., and Merigan, T. C. (1981). *Lancet* **1,** 1392–1394.
Colby, B. M., Shaw, J. E., Elion, G. B., and Pagano, J. S. (1980). *J. Virol.* **34,** 560–568.
Colby, B. M., Furman, P. A., Shaw, J. E., Elion, G. B., and Pagano, J. S. (1981). *J. Virol.* **38,** 606–611.
Colin, J., Tournoux, A., Chastel, C., and Renard, G. (1981). *Nouv. Presse Med.* **10,** 2969–2975.
Collins, P., and Bauer, D. J. (1979). *J. Antimicrob. Chemother.* **5,** 431–436.
Collins, P., and Oliver, N. M. (1982). *Am. J. Med.* **73(1A),** 96–99.
Collum, L. M. T., and Benedict-Smith, A. (1981). *In* "Herpetische Augenerkrankungen" (R. Sundmacher, ed.), pp. 323–327. Bergmann, Munich.
Collum, L. M. T., Benedict-Smith, A., and Hillary, I. B. (1980). *Br. J. Ophthalmol.* **64,** 766–769.
Collum, L. M. T., Logan, P., and Ravenscroft, T. (1983). *Br. J. Ophthalmol.* **67,** 115–118.
Corey, L., Keeney, R. E., Remington, M. R., Winter, C. A., Smith, K., Davis, L. G., and Holmes, K. K. (1980). *Interscience Conference on Antimicrobial Agents and Chemotherapy 20th, New Orleans,* Abstr. 515.
Corey, L., Benedetti, J., Critchlow, C., Remington, M., Winter, C. A., Fahnlander, A., Smith, K., and Holmes, K. K. (1981). *Interscience Conference on Antimicrobial Agents and Chemotherapy 21st, Chicago,* Abstr. 31.
Corey, L., Nahmias, A. J., Guinan, M. E., Benedetti, J. K., Critchlow, C. W., and Holmes, K. K. (1982). *N. Engl. J. Med.* **306,** 1313–1319.
Coster, D. J., Wilhelmus, K. R., Michaud, R., and Jones, B. R. (1980). *Br. J. Ophthalmol.* **64,** 763–765.
Crumpacker, C. S., Schnipper, L. E., Zaia, J. A., and Levin, M. J. (1979). *Antimicrob. Agents Chemother.* **15,** 642–645.
Crumpacker, C. S., Schnipper, L. E., Marlowe, S. I., Kowalsky, P. N., Hershey, B. J., and Levin, M. J. (1982). *N. Engl. J. Med.* **306,** 343–346.
Cupps, T. R., Strauss, S. E., and Waldmann, T. A. (1981). *Am. J. Med.* **70,** 882–886.
de Miranda, P., Krasny, H. C., Page, D. A., and Elion, G. B. (1982a). *Am. J. Med.* **73(1A),** 31–35.
de Miranda, P., Good, S. S., Krasny, H. C., Connor, J. D., Laskin, O. L., and Lietman, P. S. (1982b). *Am. J. Med.* **73(1A),** 215–220.
Devergie, A., Gluckman, E., Lotsberg, J., Zao, X. M., Gomex-Morales, M., Mazeron, M. C., and Perol, Y. (1983). *European Bone Marrow Transplantation Meeting, Obersdorf,* Abstr. 93.

Elion, G. B. (1982). *Am. J. Med.* **73(1A)**, 7–13.

Elion, G. B., Furman, P. A., Fyfe, J. A., de Miranda, P., Beauchamp, L., and Schaeffer, H. J., (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5716–5720.

Fiddian, A. P., (1982a). *In* "Transplantation and Clinical Immunology" (J. L. Touraine, J. Traeger, H. Betuel, J. Brochier, J. M. Dubernard, J. P. Revillard, and R. Triau, eds.), Vol XIV, pp. 121–129. Excerpta Medica Found., Amsterdam.

Fiddian, A. P. (1982b). *In* "Transplantation and Clinical Immunology" (J. L. Touraine, J. Traeger, H. Beutel, J. Brochier, J. M. Dubernard, J. P. Revillard, and R. Triau, eds.), Vol. XIV, pp. 130–134. Excerpta Medica Found., Amsterdam.

Fiddian, A. P. (1983). *J. Antimicrob. Chemother.* **12 (B)**, 67–77.

Fiddian, A. P., and Ivanyi, L. (1983). *Br. J. Dermatol.* **109**, 321–326.

Fiddian, A. P., Bye, A., Ashraf, M. H., Froud, D. J. R., Glasby, M. A., and Yacoub, M. H. (1982a). International Congress of Infectious and Parasitic Diseases, 8th, Stockholm, Abstr. 13:5.

Fiddian, A. P., Halsos, A. M., Kinge, B. R., Nilsen, A. E., and Wikström, K. (1982b). *Am. J. Med.* **73(1A)**, 335–337.

Fiddian, A. P., Yeo, J. M., and Clark, A. E. (1983a). *J. Infect.* **6 (1)**, 41–47.

Fiddian, A. P., Yeo, J. M., Stubbings, R., and Dean, D., (1983b). *Br. Med. J.* **1**, 1699–1701.

Field, H. J., and Darby, G. (1980). *Antimicrob. Agents Chemother.* **17**, 209–216.

Fife, K. H., Corey, L., Keeney, R. E., Winter, C. A., Fahnlander, A., Davis, L. G., and Holmes, K. K. (1981). *Interscience Conference on Antimicrobial Agents and Chemotherapy, 21st, Chicago,* Abstr. 29.

Furman, P. A., St. Clair, M. H., Fyfe, J. A., Rideout, J. L., Keller, P. M., and Elion, G. B. (1979). *J. Virol.* **32**, 72–77.

Fyfe, J. A., Keller, P. M., Furman, P. A., Miller, R. L., and Elion, G. B. (1978). *J. Biol. Chem.* **253**, 8721–8727.

Gibson, J. R., Harvey, S. G., Barth, J., Darley, C. R., Reshad, H., and Burke, C. A. (1983). *Br. J. Dermatol.* **109**, 46.

Glasgow, L. A., Richards, J. T., and Kern, E. R. (1982). *Am. J. Med.* **73(1A)**, 132–137.

Goldman, J. M., Chipping, P. M., Agnarsdottir, G., and Brigden, D. (1979). *Lancet* **1**, 820.

Good, S. S., and de Miranda, P. (1982a). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **41(5)**, 1733, Abstr. 8558.

Good, S. S., and de Miranda, P. (1982b). *Am. J. Med.* **73(1A)**, 91–95.

Gould, J. M., Chessells, J. M., Marshall, W. C., and McKendrick, G. D. W. (1982). *J. Infect.* **5**, 283–289.

Hann, I. M., Prentice, H. G., Blacklock, H. A., Ross, M., Brigden, D., Clark, A., Burke, C., Noone, P., and Keaney, M. (1982). *J. Exp. Haematol.* **10**, 2–4.

Hanto, D. W., Sakamoto, K., Purtilo, D., Balfour, H. H., Simmons, R. L., and Najarian, J. S. (1981). *Interscience Conference on Antimicrobial Agents and Chemotherapy, 21st, Chicago,* Abstr. 30.

Harrington, M. G., Durward, W. F., and Simpson, J. A. (1981). *Lancet* **2**, 1281.

Heaton, A., Arze, R. S., and Ward, M. K. (1981). *Lancet* **2**, 875.

Hintz, M., Connor, J. D., Spector, S. A., Blum, M. R., Keeney, R. E., and Yeager, A. S. (1982). *Am. J. Med.* **73(1A)**, 210–214.

Jones, B. R., Coster, D. J., Fison, P. N., Thompson, G. M., Cobo, L. M., and Falcon, M. G. (1979). *Lancet* **1**, 243–244.

Keeney, R. E., Kirk, L. E., and Brigden, D. (1982). *Am. J. Med.* **73(1A)**, 176–181.

Kennedy, C. T. C., Leigh, I. M., Ridgway, H. A., Wansborough-Jones, M. H., and Brigden, D. (1981). *Br. Med. J.* **2**, 1360–1361.

Kern, E. R. (1982). *Am. J. Med.* **73(1A)**, 100–108.

Kinghorn, G. R., Turner, E. B., Barton, I. G., Potter, C. W., Burke, C. A., and Fiddian, A. P. (1983). *Antiviral Res.* **3**, 291–301.

Kinney, T. R., Gutman, L. T., Wilfert, C. M., Keeney, R., de Miranda, P., Rudd, C., and Falletta, J. M. (1981). *Pediatr. Res.* **15** (4), 580.

Klein, R. J. (1982). *Am. J. Med.* **73(1A)**, 138–142.

la Lau, C., Oosterhuis, J. A., Versteeg, J., van Rij, G., Renardel de Lavalette, J. G. C., Creandijk, A., and Lamers, W. P. M. A. (1982). *Br. J. Ophthalmol.* **66**, 506–508.

Landry, M. L., Lucia, H. L., Hsiung, G. D., Pronovost, A. D., Dann, P. R., August, M. J., and Mayo, D. R. (1982). *Am. J. Med.* **73(1A)**, 145–150.

Laskin, O. L., Longstreth, J. A., Whelton, A., Krasny, H. C., Keeney, R. E., Rocco, L., and Lietman, P. S. (1982). *Am. J. Med.* **73(1A)**, 197–201.

Lass, J. H., Pavan-Langston, D., and Park, N.-H. (1979). *Am. J. Ophthalmol.* **88**, 102–108.

Lietman, P. S. (1982). Am. J. Med. **73(1A)**, 193–196.

McGill, J. (1981). *Br. J. Ophthalmol.* **65**, 542–545.

McGill, J., and Tormey, P. (1982). *Am. J. Med.*, **73(1A)**, 286–289.

McGill, J., Tormey, P., and Walker, C. B. (1981). *Br. J. Ophthalmol.* **65**, 610–613.

McGill, J., MacDonald, D. R., Fall, C., McKendrick, G. D. W., and Copplestone, A. (1983). *J. Infect.* **6**, 157–161.

Many, W., Schmidt, R., Whelchel, J., Diethelm, A., Cobbs, G. C., and Whitley, R. (1980). *Interscience Conference on Antimicrobial Agents and Chemotherapy, 20th, New Orleans,* Abstr. 512.

Mertz, G. J., Reichman, R., Dolin, R., Richman, D. D., Oxman, M., Large, K., Tyrrell, D., Portnoy, J., and Corey, L. (1982). *Interscience Conference on Antimicrobial Agents and Chemotherapy, 22nd, Miami,* Abstr. 182.

Meyers, J. D., Wade, J. C., Mitchell, C. D., Saral, R., Lietman, P. S., Durack, D. T., Levin, M. J., Segreti, A. C., and Balfour, H. H. Jr. (1982). *Am. J. Med.* **73(1A)**, 229–235.

Miller, W. H., and Miller, R. L. (1980). *J. Biol. Chem.* **255**, 7204–7207.

Miller, W. H., and Miller, R. L. (1982). *Biochem. Pharmacol.* **31**, 3879–3884.

Mindel, A., Adler, M. W., Sutherland, S., and Fiddian, A. P. (1982). *Lancet* **1**, 697–700.

Mitchell, C. D., Bean, B., Gentry, S. R., Groth, K. E., Boen, J. R., and Balfour, H. H. Jr. (1981). *Lancet* **1**, 1389–1392.

Morgan, K. S., Wander, A. H., Kaufman, H. E., Varnell, E. D., and Creagh-Kirk, T. (1980). *Chemotherapy (Basel)* **26**, 405–408.

Nelson, T. J., Steele, R. W., Keeney, R. E., Moses, E. B., and Barron, A. L. (1980). *Clin. Res.* **28(5)**, 855A.

Nilsen, A. E., Aasen, T., Halsos, A. M., Kinge, B. R., Tjotta, E. A. L., Wikström, K., and Fiddian, A. P. (1982). *Lancet* **2**, 571–573.

O'Meara, A., and Hillary, I. B. (1981). *Ir. J. Med. Sci.* **150 (3)**, 73–77.

Park, N.-H., Pavan-Langstan, D., and de Clercq, E. (1982). *Am. J. Med.* **73(1A)**, 151–154.

Pavan-Langston, D., Lass, J., Hettinger, M., and Udell, I. (1981). *Am. J. Ophthalmol.* **92**, 829–835.

Peterslund, N. A., Seyer-Hansen, K., Ipsen, J., Esmann, V., Schonheyder, H., and Juhl, H. (1981). *Lancet* **2**, 827–830.

Plotkin, S. A., Starr, S. E., and Bryan, C. K. (1982). *Am. J. Med.* **73(1A)**, 257–261.

Poirier, R. H., Kinghorn, J. D., de Miranda, P., and Annel, M. (1982). *Arch. Ophthalmol.* **100**, 1964–1967.

Prentice, H. G., Hann, I. M., and Blacklock, H. A. (1983). *European Bone Marrow Transplantation Meeting, Obersdorf,* Abstr. 94.

Prober, C. G., Kirk, L. E., and Keeney, R. E. (1982). *J. Pediatr.* **101**, 622–625.

Reichman, R. C., Badger, G. J., Richman, D. D., Oxmann, M., Connor, J., Mertz, G. J., Corey, L., Larson, T., Bryson, Y., Tyrrell, D., Portnoy, J., and Dolin, R. (1982). *In-*

terscience Conference on Antimicrobial Agents and Chemotherapy, 22nd, Miami, Abstr. 183.

Salo, O. P., Lassus, A., Hovi, T., and Fiddian, A. P. (1983). *Eur. J. Sex. Transmitted Dis.* 1, 95–98.

Saral, R., Burns, W. H., Laskin, O. L., Santos, G. W., and Lietman, P. S. (1981). *N. Engl. J. Med.* **305**, 63–67.

Schaeffer, H. J., Beauchamp, L., de Miranda, P., Elion, G. B., Bauer, D. J., and Collins, P. (1978). *Nature (London)* **272**, 583–585.

Selby, P. J., Powles, R. L., Jameson, B., Kay, H. E. M., Watson, J. G., Thornton, R., Morgenstern, G., Clink, H. M., McElwain, T. J., Prentice, H. G., Corringham, R., Ross, M. G., Hoffbrand, A. V., and Brigden, D. (1979). *Lancet* **2**, 1267–1270.

Serota, F. T., Starr, S. E., Bryan, C. K., Koch, P. A., Plotkin, S. A., and August, C. S. (1982). *J. Am. Med. Assoc.* **247**, 2132–2135.

Shulman, S., Yogev, R., Keeney, R., and Morgan, E. (1981). *Pediatr. Res.* **15(4)**, 587.

Smith, C. I., Scullard, G. H., Gregory, P. B., Robinson, W. S., and Merigan, T. C. (1982). *Am. J. Med.* **73(1A)**, 267–270.

Soike, K. F., and Gerone, P. J. (1982). *Am. J. Med.* **73(1A)**, 112–117.

Spector, S. A., Hintz, M., Wyborny, C., Connor, J. D., Keeney, R. E., and Liao, S. (1982). *Am. J. Med.* **73(1A)**, 275–280.

Spruance, S. L., Crumpacker, C. S., Schnipper, L. E., Kern, E. R., Marlow, S., Modlin, J., Arndt, K. A., and Overall, J. C. (1982a). *Interscience Conference on Antimicrobial Agents and Chemotherapy 22nd, Miami,* Abstr. 187.

Spruance, S. L., Schnipper, L. E., Overall, J. C. Jr., Kern, E. R., Wester, B., Modlin, J., Wenerstrom, G., Burton, G., Arndt, K. A., Chiu, G. L., and Crumpacker, C. S. (1982b). *J. Infect. Dis.* **146**, 85–90.

Straus, S. E., Smith, H. A., Brickman,C., de Miranda, P., McLaren, C., and Keeney, R. E. (1982). *Ann. Intern. Med.* **96**, 270–277.

Sullivan, J. L., Byron, K. S., Brewster, F. E., Sakamoto, K., Shaw, J. E., and Pagano, J. S. (1982). *Am. J. Med.* **73(1A)**, 262–266.

Swart, R. N. J., Vermeer, B. J, van der Meer, J. W. M., Enschede, F. A. J., and Versteeg, J. (1983). *Arch. Dermatol.* **119**, 13–16.

Thin, R. N., Nabarro, J. M., Parker, J. D., and Fiddian, A. P. (1983). *Br. J. Vener. Dis.* **59**, 116–119.

Tyms, A. S., Scamons, E. M., and Naim, H. M. (1981). *J. Antimicrob. Chemother.* **8**, 65–72.

Tucker, W. E. (1982). *Am. J. Med.* **73(1A)**, 27–30.

van der Meer, J. W. M., Thompson, J., Tan, W. D., and Versteeg, J. (1980). *Lancet* **2**, 473–474.

van der Meer, J. W. M., and Versteeg, J. (1982). *Am. J. Med.* **73(1A)**, 271–274.

van Dyke, R. B., Connor, J. D., Wyborny, C., Hintz, M., and Keeney, R. E. (1982). *Am. J. Med.* **73/1A**, 172–175.

van Vloten, W. A., Swart, R. N. J., and Pot, F. (1983). *J. Antimicrob. Chemother.* **12 (B)**, 89–93.

Wade, J. C., Hintz, M., McGuffin, R. W., Springmeyer, S. C., Connor, J. D., and Meyers, J. D. (1982a). *Am. J. Med.* **73(1A)**, 249–256.

Wade, J. C., Newton, B., Flournoy, N., and Meyers, J. D. (1982b). *Interscience Conference on Antimicrobial Agents and Chemotherapy, 22nd Miami,* Abstr. 184.

Wade, J. C., Newton, B., McLaren, C., Flournoy, N., Keeney, R. E., and Meyers, J. D. (1982c). *Ann. Intern. Med.* **96**, 265–269.

Weller, I. V. D., Carreno, V., Fowler, M. J. F., Monjardino, J., Makinen, D., Thomas, H. C., Sherlock, S. (1982). *Lancet,* **1**, 273.

Whitley, R. J., Blum, M. R., Barton, N., de Miranda, P. (1982a). *Am. J. Med.* **73(1A)**, 165–171.

Whitley, R. J., Barton, N., Collins, E., Whelchel, J., and Diethelm, A. G. (1982b). *Am. J. Med.* **73(1A)**, 236–240.

Yeager, A. S. (1982). *Am. J. Med.* **73(1A)**, 205–209.

Young, B., Patterson, A., and Ravenscroft, T. (1982). *Br. J. Ophthalmol.* **66**, 361–363.

9

Worldwide and Differing Vaccinations against Human Viral Diseases

A. J. CLAYTON

Laboratory Centre for Disease Control, Ottawa, Ontario, Canada

I. INTRODUCTION

In October 1977 a dream became a reality; the last case of smallpox in the world was seen in Somalia in East Africa. A little more than 2 years later, in May of 1980, the World Health Organization certified that smallpox had been eradicated from the world. This probably represents the greatest single event in medical history, perhaps more significant than the introduction of antibiotics or the identification of pathogenic microorganisms some 100 years ago.

It is difficult to realize today, in the 1980s, the enormous toll that smallpox took of the world's population. Essentially it was a disease of children and young adults, a disease with up to 40% mortality, and in essence the adult population of the earth were the survivors of smallpox. The eradication of smallpox has stimulated new dreams—that other diseases can be eradicated also, or at least more effectively controlled.

Immediately one thinks of measles. It has similarities to smallpox, but most important, as far as is known, man is the only host of the virus; and

ISBN 0-12-429601-7

equally important, it can be transmitted only through indirect person-to-person spread. Measles is a highly contagious disease and causes explosive outbreaks. In contradistinction, smallpox was generally spread more slowly and was contained by aggressive outbreak control measures.

While measles does not exact the same toll in terms of mortality, it can nevertheless be extremely serious. It is estimated that there are approximately 900,000 global deaths annually due to measles complications, including encephalitis, otitis media, and pneumonia (Walsh and Warren, 1979). The disease is more severe in malnourished children. It has been reported that measles is a leading cause of death in children 1–4 years of age in several areas of Latin America. Measles outbreaks occur in Africa and Asia, where case fatality rates are 5–20% among children (Foege, 1982).

II. VACCINE-PREVENTABLE VIRAL DISEASES

Measles and smallpox have another common feature, along with certain other viral infections: they are vaccine preventable. This chapter will summarize the current status of vaccination against human viral diseases and use this summary to propose future eradication programs. Certain inconsistencies will be pointed out, and perhaps inaccuracies of immunization principles, and some astonishing variations of immunization regimens throughout the world. These, in some measure, prevent or frustrate attempts to realize the dream of eradication of viral disease. Finally, the chapter points out that steps can be taken, perhaps not to attain the dream, but to go part way toward it.

There are eight viral diseases for which vaccines are commonly or routinely used: measles, rubella, mumps, hepatitis B, poliomyelitis, influenza, rabies, and yellow fever.

It is appropriate to mention at least the existence of other viral diseases for which vaccines are available, but used only in specialized settings. These include a variety of arboviral encephalitides, Argentinian hemorrhagic fever, adenovirus respiratory disease, and vaccinia. In addition, there are several vaccines in the experimental or developmental stage. These include antigens of respiratory syncytial virus disease, cytomegalovirus disease, herpes infections, rotavirus disease, hepatitis A, and many more which one day will play a significant part toward the attainment of the dream.

Finally, one should at least make mention of passive immunizing agents such as varicella-zoster immune globulin, vaccinia immune globulin, rabies immune globulin, hepatitis B immune globulin, and immune serum globulin for hepatitis A.

III. INDIVIDUAL DISEASES

Let us look at some of these diseases individually.

1. *Poliomyelitis.* To a large extent polio has been eliminated and certainly controlled in the Western world by the use of either live trivalent oral polio vaccine (TOPV) or inactivated polio vaccine (IPV). Nevertheless in 1981, the World Health Organization (WHO) estimated 33,870 cases of polio worldwide, almost certainly an underestimation (WHO, 1982).

In 1981 there were six cases of poliomyelitis in the United States, none in Canada, and six in the United Kingdom (Communicable Disease Surveillance Centre, 1982a). It is interesting to note that in Europe 18 of 23 countries use TOPV, 4 employ IPV, and 1 uses IPV and TOPV together. In Canada, where each of the 10 provinces is autonomous in terms of provision of medical care, TOPV is used in 6 provinces and IPV in 4. In the United States TOPV is almost exclusively employed.

As a general rule, TOPV is preferred throughout the world, not only because of the ease of administration of an oral vaccine, but also because it confers immunity in the gastrointestinal tract. "Gut immunity" is clearly preferable to protect against an enteric virus. Further the low frequency of vaccine-associated paralytic cases, which occur once in every 3–4 million doses (Benenson, 1981), is generally considered an "acceptable" complication. Polio still occurs in countries with organized vaccination programs largely because of inadequate vaccine coverage. In order to achieve "herd" immunity using IPV, coverage must be 90% and around 80% for TOPV. A clear example of a country with an established national immunization program using TOPV is Jamaica, yet in 1982 there were 56 cases of polio with 2 deaths in that country, concentrated in one small area (Centers for Disease Control, 1982a).

It is interesting to note the variation in the recommended age of polio immunization in regions throughout the world. Ten countries in Africa start polio vaccination in children between 1 and 2 months of age. In Tanzania, for example, infants receive the first dose at 4 weeks of age. Twenty-five nations give the first dose of vaccine to infants between 3 and 5 months old. In the Americas 15 countries administer polio vaccine before 2 months of age; in Chile infants are immunized at birth, and in Cuba at 1 month. Twelve countries employ the vaccine at 3–5 months. Similar regimens are followed in the Eastern Mediterranean; 3 countries immunize between 1 and 2 months of age, and 16 between 3 and 5 months. In Southeast Asia, 1 and 4 countries in the two age groups, and in Western Pacific 12 and 17 countries during those time periods. In Hong Kong, polio vaccine is administered at birth. In Europe 6 countries employ vaccine between 1 and 2

months, and 24 between 3 and 5 months. Two countries, Greenland and Norway, start their series at 5 months (Velimirovic, 1983).

2. *Measles.* In the case of measles, there is also variation in the time when the vaccine is administered. It varies from 6 to 18 months. At the early end of the scale, Turkey, Colombia, Dominican Republic, El Salvador, and Malagasy vaccinate at 6 months. At the other end, 18 countries vaccinate at 15 months or older; these include the United States, Korea, the Netherlands, Sweden, and Switzerland, the latter two waiting until 18 months of age before administering measles vaccine (Velimirovic, 1983).

Of 82 countries which administer measles vaccine prior to 1 year of age, only 18 provide a booster dose subsequently. The booster dose for such children is extremely important to assure seroconversion, since maternal antibody may interfere with antibody formation in those vaccinated under 1 year of age, and in the developing world measles typically occurs between 12 and 18 months. The administration of vaccine before 12 months is a good policy but must be followed by an appropriate booster.

3. *Rubella.* The major purpose of the administration of rubella vaccine is to prevent congenital rubella syndrome (CRS). The time of vaccination for rubella is controversial as there are two major schools of thought—vaccination of prepubertal females only or vaccination of all children at approximately 1 year of age. Proponents of the former have stated an interesting philosophy, "To prevent flood damage from the raging tide it is perhaps easier to build a dam than attempt to dry up the ocean." However, increasingly, particularly in England, it has been shown that the policy of immunization of prepubertal girls does not prevent occurrence of CRS (I believe this is arguable). More countries are following the second philosophy and administering rubella vaccine at 1 year of age. The advantage of giving rubella at approximately 1 year of age is that it can be, and generally is, given in combination as a measles–mumps–rubella combined vaccine. It is interesting to note that in 1981 there were 5 recorded cases of CRS in the United States (Centers for Disease Control, 1982a) and 3 in Canada (Laboratory Centre for Disease Control, 1982), but in the United Kingdom there were 62 cases in 1979 and 12 in 1980 (Communicable Disease Surveillance Centre, 1982a). The relatively recent introduction of the live attenuated virus strain RA27/3 prepared in human diploid cell culture has strengthened the value of early immunization. The duration of protection is not yet known, but studies indicate that it exceeds 10 years and may be lifelong. Booster doses at this time are not considered necessary.

4. *Mumps.* Mumps is a disease of importance but has a lesser public health impact than those mentioned earlier. The inclusion of mumps with measles and rubella vaccine provided a welcome side effect.

5. *Hepatitis B.* Hepatitis B vaccine, recently introduced, has been shown

to be effective in children under 2 years of age. However, the vaccine is very expensive and is indicated for children only on a very selective basis, i.e., infants whose mothers are chronic carriers of hepatitis B surface antigens. Production costs of the vaccine will continue to be high until it is produced by tissue culture techniques.

6. *Influenza.* Influenza vaccine is given largely to elderly people and to those with chronic cardiovascular, respiratory, or metabolic diseases.

7. *Rabies.* Rabies vaccine is used under certain selected circumstances, namely preexposure for those people who are at occupational risk of rabies exposure, and postexposure for those who have been bitten by rabid or potentially rabid animals.

8. *Yellow Fever.* The last disease of my original list of eight is yellow fever, and this is particularly interesting because it is the only viral vaccine used in conjunction with International Health Regulations. It is at the same time an extremely potent and useful vaccine. For the purposes of international travel, yellow fever vaccine is administered only at yellow fever vaccination centers that have been approved by the WHO, and the vaccination is recorded and validated in the appropriate international certificate of vaccine. This has been the key to the successful containment of yellow fever to South America and Africa. The majority of countries in the world, particularly those in the yellow fever receptive zones, have ascribed to the WHO recommendations and International Health Regulations in this respect. So, here is a vaccine which is controlled at an international level, is extremely effective, and has a duration of immunity of 10 years, and while it has not eradicated yellow fever it has certainly controlled and reduced the disease in terms of geographical areas over the last few years. Notable exceptions are the reemergence in South America and the outbreak of yellow fever in Trinidad in 1979, in which there were 18 cases and 7 deaths due to the sylvatic form (WHO, 1980). Fortunately, increased vaccination and vector control programs prevented spread into the urban cycle.

IV. MEASLES ERADICATION

A. WHO Input

The WHO has the influence to put in place recommendations which would go far toward eradication of viral diseases. Clearly, this would be a long, slow process, and one disease at a time should be attempted. The precedent already exists in that smallpox was successfully eradicated through WHO endeavors and international cooperation. It is encumbent upon WHO (1) to create standardized regimens of vaccine usage, (2) to tie in, where pos-

sible, vaccination requirements of the International Health Regulations, which need to be changed markedly, and (3) to encourage employment of the mechanisms already in existence, that is, the Global Expanded Program of Immunization (EPI).

The EPI is successfully working with national governments and international agencies in an attempt to assure that immunization against six diseases is routinely available to all children in the world by 1990. It is believed that this program can be expanded and concentrated in order to make attempts for global eradication of one disease at a time over the next generation. There now exists a functioning efficient cold chain for storage, transportation, and delivery in order to preserve the viability of live vaccines. There are now heat-stable vaccines that remain potent in the freeze-dried state for 3–4 weeks, even in tropical zones and without refrigeration. Containers to maintain cold temperatures and protect the vaccine have improved, and more important, the cost of measles vaccine, for example, has declined to around 10 cents a dose.

It is clear therefore that measles should be the next target for eradication. It is recommended that the WHO create an acceptable standardized measles immunization scheme and vigorously promote its use throughout the world.

B. West African Campaign

A precedent has been established. In 1967 20 countries of West Africa came together and produced a coordinated program for measles eradication. The vaccine was administered to children 6–36 months of age, and vaccination cycles were scheduled in villages every 12 months. It was estimated that after 1 year there was a 45% reduction in cases in countries representing 95 million people. This represents prevention of an estimated 2.4 million cases of measles and 170,000 deaths (Foege, 1982).

The results of the campaign in West Africa varied for a number of reasons, such as the quality of immunization programs, the degree of dedication to the program at governmental level, and the difficulty of delivering a viable effective vaccine. However, the program in the Gambia, which has a population of 350,000 people, was eminently successful, and for a period of more than 3 years the Gambia experienced almost total elimination of the disease. Sporadic cases after 1967 were deemed to be imported. Unfortunately, resources were not maintained to continue this program, and measles has risen back to its precontrol levels. Nevertheless, this is an important example of a country of limited resources eliminating one viral disease despite a long international border and despite up to 14 importations over a 3-year period. The ultimate failure of the program in the Gambia can be attributed to the fact that maintenance of the successful campaign

was not continued. The eradication of smallpox was in no small way achieved through the "continued surveillance" for cases of smallpox *after* the last one had been reported.

It was estimated that it cost approximately $2.50 to prevent one case of measles in the Gambia and $34.00 to prevent one death from measles, assuming a mortality rate of 7%. At the other end of the scale it is well known that the United States has almost eradicated measles. One realizes that the situation is totally different, but the impact on childhood mortality is just as important. It is estimated that the prevention of measles in the United States yields an annual net saving of $500 million to the health care system each year, with a benefit-to-cost ratio of 10 to 1. Similar benefits in the developing world, where morbidity and mortality for measles is far higher, would be even more significant. It has been estimated in the Ivory Coast that a benefit-to-cost ratio could well exceed 20 to 1 (Foege, 1982).

V. CONCLUSION

The dream could become a reality. Should attempts be made to achieve global eradication of viral diseases? This is perhaps an anachronistic question because it has already happened in the case of smallpox. Even though there are significant differences between smallpox and measles, elimination of the latter is not an impossible task—and if measles can be eradicated, so can other diseases.

In a time of limited global resources, increasing population, and shortages of food, one might ask: Is the exercise worthwhile? The multidisciplined approaches to controlling viral diseases in plants, animals, and humans are valid, and these activities must be prosecuted and continued. The key to controlling the world's population is not to allow diseases to run rampant and ravage populations, but to provide education regarding appropriate birth control and to study population dynamics. The exercise *is* worthwhile.

The drawbacks to achieving success lie in nonuniformity of schedules, poor delivery systems, and lack of dedication of governments, which appear to be willing to spend money to treat, but not to prevent. Control or eradication of viral disease deserves the best effort that we can make; these efforts, however, must be coordinated at the global level, nationally applied, and supported by the international public health community. The WHO has to take the lead in setting the standards for schedules of vaccinations, delivery systems, financial arrangements, and, perhaps the most important, maintenance of the program after success has been achieved. There should be no spurious promises, but only dedication and hard work.

The dream can become a reality if we plan properly and proceed slowly, with full realization of the problems and pitfalls along the way.

REFERENCES

Benenson, A. S. (ed.). (1981). *In* "Control of Communicable Diseases in Man," 13th ed., 268. American Public Health Association, Washington, D.C.

Centers for Disease Control (1982a). *Morbidity and Mortality Weekly Report* **30,** No. 54, 3.

Centers for Disease Control (1982b). *Morbidity and Mortality Weekly Report,* **31,** No. 30, 413–414.

Communicable Disease Surveillance Centre (1982a). *Comm. Dis. Rep. (U.K.)* **36,** 3–4.

Communicable Disease Surveillance Centre (1982b). *Comm. Dis. Rep. (U.K.)* **44,** 3–4.

Foege, W. H. (1982). *Public Health Rep.* **97,** 5, 402–405.

Laboratory Centre for Disease Control (1982). **8** (52), 257–259.

Velimirovic, B. (1983). Personal communication.

Walsh, J. A., and Warren, K. S. (1979). *N. Engl. J. Med.* **301,** 967–974.

WHO (1980). *Weekly Epidemiological Record* **55,** 345–351.

WHO (1982). *Weekly Epidemiological Record* **57,** 305–310.

10

Principal Human Viral Diseases: Present Status of Vaccination

T. A. BEKTIMIROV* AND F. ASSAAD†

**Virus Diseases Unit and †Division of Communicable Diseases, World Health Organization, Geneva, Switzerland*

I. INTRODUCTION

The overall objective of the Virus Disease Program of the World Health Organization (WHO) is the prevention and control of viral disease. Within WHO, those programs considered to be most important are for the following diseases: poliomyelitis, measles, diarrhea, acute respiratory and sexually transmitted diseases, influenza, hepatitis, rabies, hemorrhagic fevers.

All the diseases listed have been recognized as major public health problems throughout the world or in certain WHO regions for many years. The diseases listed above that have worldwide distribution (including the African and Eastern Mediterranean Regions) and are vaccine preventable include poliomyelitis, measles, yellow fever, rabies, and hepatitis B. These diseases are discussed in this chapter.

ISBN 0-12-429601-7

II. MEASLES

Measles is an important cause of morbidity and mortality in the child population of all WHO regions.

The reported annual incidence of measles from 1977 to 1982 is shown in Table I. It is difficult to compare the figures for morbidity over the years because of the different rates of underreporting. Moreover, the reporting is related to action programs and there is gross underreporting where no active immunization programs have been undertaken (Assaad, 1983). Nevertheless, there was a substantial decrease in the global number of reported measles cases in 1981. This was due mainly to the reduced number of cases in countries of the European and Western Pacific Regions, and to a lesser extent in the African countries.

It has been estimated that 900,000 deaths occur yearly in the developing world because of measles (Walsh and Warren, 1978). There is a marked contrast in the severity and outcome of measles between the developed and developing countries. The data available to WHO indicate that the global case fatality rate in the developing world approaches 2%. The case fatality

TABLE I
Reported Annual Incidence of Measles[a]

WHO region	Population 1977–1981 (millions)	Years				
		1977	1978	1979	1980	1981
Africa	327,405 – 358,835	955,755	1,252,390	921,440	964,989	774,647
Americas	575,785 – 609,175	297,494	197,033	268,902	251,229	203,923
Eastern Mediterranean	240,070 – 276,250	185,889	242,306	246,912	170,100	168,840
Europe	815,480 – 832,760	1,014,316	1,039,784	584,626	921,050	203,566
South-East Asia	972,490 – 1,059,180	184,067	213,661	215,983	169,169	216,660
Western Pacific	1,217,921 – 1,327,496	196,666	1,307,564[b]	1,027,253	700,730	570,949
Total	4,149,151 – 4,463,696	2,834,187	4,252,738	3,265,116	3,177,267	2,138,585
Cases/100,000 population	—	91	100	78	70	48

[a]Based on data available to WHO on December 1, 1982.

[b]China has been participating in WHO surveillance systems since 1978, when 1,112,600 cases were reported.

rate in different countries and during different epidemics varies. For example it was 2% in Somalia (WHO, 1981a), but 21% in an outbreak in a village in the Gambia.

Most deaths from measles in tropical countries occur in the second year of life. Measles is severe in underprivileged, malnourished, poor children living in inadequate housing and with no ready access to medical care.

In developed countries today the major importance of measles lies not in mortality, but in the week of acute illness with accompanying absenteeism on the part of the child and work loss on the part of the parents.

Worldwide experience with attenuated live measles vaccine has indicated that it is safe and highly effective and is available within the resources of most of the developing world. Whenever and wherever measles vaccine has been used on a large scale, a marked reduction in the number of cases has been recorded. For example, in the United States during the first few decades of the twentieth century, thousands of fatal measles infections were reported each year. From 1950 through 1959, an annual average of more than 500,000 measles cases and nearly 500 measles deaths were reported. The widespread use of measles vaccine, beginning in 1963, has had a major impact on the occurrence of measles in the United States. In 1982, the reported occurrence of measles reached its lowest level since national reporting of measles began in 1912. A provisional total of 1697 cases was reported (Centers for Disease Control, 1983) giving a record low incidence rate of 0.7 case per 100,000 population of all ages (Fig. 1). This is 99.9% reduction from the prevaccine era and a 45.7% reduction from the 3124 cases in 1981, the previous year of record low incidence.

Efforts to achieve this goal have involved (1) vaccination of all children 15 months old, (2) identification and vaccination of susceptible persons of all ages, (3) the enactment and enforcement of school immunization laws, (4) surveillance systems, and (5) outbreak containment.

Measles vaccination programs have also been very successful in reducing the incidence in Albania, Canada, Czechoslovakia, Japan, the USSR, and Yugoslavia.

Such favorable results are not limited to the developed countries. For example, Costa Rica, Cuba, Chile, and parts of Brazil have achieved significant progress. In fact, the disease had been eliminated in the Gambia for a number of years in the early 1970s when immunization coverage was well over 90%. However, in contrast to the United States and Czechoslovakia, the effort could not be maintained, and the number of cases and deaths have reached the level of the prevaccine era.

Most countries are devoting increased attention to measles and to programs aimed at its control. Vaccine coverage rates ranging from 2 to 99% have been reported from various countries. Even in the European Region,

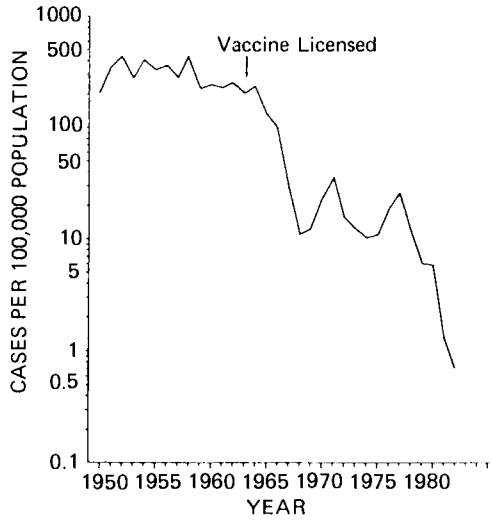


Fig. 1. Reported incidence of measles in the United States (1950–1980), U.S. Department of Health and Human Services/Public Health Service. The widespread use of measles vaccine, beginning in 1963, has had a major impact on the occurrence of measles in the United States. In 1982, the reported occurrence of measles reached its lowest level (0.7 cases per 100,000 population) since national reporting of measles began in 1912.

which consists mainly of developed countries, vaccine coverage varies from <10 to 99% (WHO/EURO, 1983), and measles immunization is not yet well accepted in most of the countries of the region.

So far, vaccine coverage rates are not available for most countries. In the Eastern Mediterranean Region in 1979, an estimated 10,548,000 children were born and survived to the age of 6 months. In that year, 3,064,393 doses of measles vaccine were given; of these, 1,660,894 doses were given to infants less than 12 months old, representing a 15.7% coverage (WHO, 1981b).

Five years after the launching of the Expanded Program on Immunization in Africa, the percentage of coverage varied substantially (Table II) from 5 to 103%. The total vaccine coverage for the 13 countries listed is 27%, and this is insufficient to stop transmission and outbreaks of measles. It is evident that the permanent elimination of measles requires at least a 95% level of herd immunity.

Proper scheduling is essential if immunization is to be successful. In the developed world measles infection during the first year of life is unusual. However, in many other countries, 30% or more of the children will already have developed measles by 12 months of age. For example, in Kenya 2–3%

TABLE II

Immunization Coverage Rates in Relation to Target Population in the African Region[a]

Country	Survivors at the end of first year of life (target population in thousands)	Coverage rate by vaccine (%)
Burundi	170	5
Ethiopia	1343	7
Gambia	25	90
Liberia	59	103
Mauritania	65	45
Niger	230	19
Sao-Tome	4	25
Sierra Leone	132	23
Uganda	480	22
Cameroon	392	16
Tanzania	737	82
Upper Volta	244	23
Zaire	1147	20
Total	5028	27

[a]Data available as of October, 1982.

of children contract measles before they reach 6 months of age, and 25–30% before the age of 12 months (Hayden, 1974). Therefore, it is important to determine the earliest possible age for vaccination against measles. Maternal antibody usually confers protection against measles to infants during the first few months of life. At 7–8 months of age, 90% of children in tropical countries no longer have maternal antibody, and the incidence of measles begins to rise sharply. Several studies revealed that more than 90% of infants beyond 7½ months of age seroconverted after the administration of measles vaccine (WHO, 1977b). Taking into account the above data in tropical countries, the optimal age for measles immunization appears to be about 9 months.

In the United States it is recommended that children be vaccinated at 15 months of age, and in other countries after the first birthday. The participants of an International Symposium on Measles Immunization (in Washington, D.C. 1982) noted that, although it may initially be advisable to immunize infants less than 1 year old in order to reduce epidemic transmission and disease, eventual control by immunization at or after the first birthday is a desirable goal. They also found no convincing evidence at present of a need for booster doses in children immunized successfully.

III. POLIOMYELITIS

The WHO Expanded Program on Immunization (EPI) recommends starting antipoliomyelitis vaccination at the age of 3 months (WHO, 1977a). The child should receive three doses of vaccine. The minimum interval between two doses is 4 weeks. Thus, the child is vaccinated against poliomyelitis by the age of 6 months. The program does not provide for any booster dose after that age.

With regard to the vaccination method currently in use, most countries now use live vaccine (Sabin) administered orally. In Denmark, Norway, the Netherlands, and Sweeden, killed vaccine (Salk) is administered by injection. In Canada and France, both types of vaccine are used. Both the inactivated and live poliomyelitis vaccines have demonstrated their safety and efficacy (Melnick, 1978). Countries which have been able to set up and sustain effective immunization programs, whether using the live or inactivated poliomyelitis vaccines, have succeeded in dramatically reducing to zero or near zero the incidence of paralytic poliomyelitis.

In the developed world, with an overall high vaccination coverage, outbreaks still occur from time to time in small groups of the population not vaccinated because of religious or other reasons (Bijkerk, 1979; Furesz, 1979). For example, poliomyelitis outbreaks occurred in the Netherlands and Canada, where vaccine coverage is within the range of 90%.

It would seem, therefore, that in the developed world there is still a problem with occasional outbreaks. Nevertheless, in these countries the health services are well equipped to detect cases very early and to ensure prompt and extensive use of the vaccine in the affected areas. It would also appear that in countries with good vaccination programs the choice of vaccine, whether inactivated or live, plays a minor role (Assaad, 1979). The obvious way to prevent outbreaks is to ensure that every person in the community is fully immunized. There is no reason for countries using the inexpensive and efficient oral vaccine to change to inactivated vaccine, nor is there reason for those countries using the inactivated vaccine to change to the other form. Inactivated vaccine has not yet been used on large scale in any developing country. Its efficacy in preventing clinical poliomyelitis in developing countries is unknown. However, small-scale controlled trials in India, using inactivated vaccine, have demonstrated good serological responses in vaccinees, starting as early as 6 weeks of age, with better antibody response than with oral polio vaccine (Krishnan *et al.*, 1982).

In light of current knowledge, oral vaccine can be seen as being preferable to inactivated vaccine for general use in countries which either are intending to institute poliomyelitis immunization or are already using oral vaccine.

The arguments in favor of the application of live polio vaccine in tropical

and subtropical countries are that (1) it has proven value in reducing the incidence of the disease; (2) it may reduce the circulation of wild polioviruses; (3) there is no need for the further development of manufacturing methods; (4) it is cheaper per dose than the inactivated vaccine by a factor of at least 25; and (5) it requires oral application, i.e., no injection is needed.

Working toward the reduction of morbidity and mortality from poliomyelitis, measles, and four bacterial diseases, the WHO Expanded Program on Immunization has predicted that all countries will achieve an immunization coverage of at least 80% of their susceptible populations during, or before, 1990 (WHO, 1982c). So far, in most of the developing world, vaccination coverage of the target population is generally low; some sectors of the population may not even be included in the national vaccination activities.

Table III shows that immunization coverage varies from 3% in South-East Asia to 82% in the European Region. The estimated percentage of children receiving three doses of oral poliomyelitis vaccine in the first year of life in the Eastern Mediterranean Region is 24. Data from the African Region are not available.

WHO international surveillance indicates that poliomyelitis remains a serious problem in a large part of the developing world, where the disease presents a constant threat to the child population.

If we analyze a mean annual number of cases reported in 1971–1975 and 1976–1980 (Table IV), a significant decrease of poliomyelitis incidence may be seen only in Europe and North America. In some other regions the incidence of the disease has increased. There are several reasons for the increasing incidence of the disease in tropical countries, for example, the high rate of population growth, falling infant mortality rates, and rapidly increasing urbanization. Better reporting also plays some part. In fact, in

TABLE III

Estimated Percentage of Children Receiving Three Doses of Oral Poliomyelitis Vaccine in the First Year of Life[a]

WHO region	Percentage of population covered by reports	Estimated percentage of children immunized
Africa[b]	—	—
Americas	60	34
Eastern Mediterranean	99	24
Europe	26	82
South-East Asia	98	3
Western Pacific	5	70

[a]Data from 1979 to 1980 (WHO, 1982d).

[b]The information system in the African Region is still in the course of development.

TABLE IV
Poliomyelitis in the WHO Regions (1971–1981)

WHO region	Mean annual number of cases reported	
	1971–1975	1976–1980
Africa	4,442	4,662
Americas	10,470	3,871
Eastern Mediterranean	6,378	6,815
Europe	1,315	786
South-East Asia	10,479	16,174
Western Pacific	1,715	9,992
Total	34,799	42,300

many developing countries, official reporting of poliomyelitis does not provide a realistic picture of the magnitude of this disease.

Lameness surveys in Burma (1979), Cameroon (1980), India (1978), Indonesia (1979), the Ivory Coast (1980), the Philippines (1981), Thailand (1979), and Yemen (1981) have reported polio prevalence rates of from 1 to 8.2 per 1000 children.

Experience from these and other countries has made it clear that official statistics (reported cases) grossly underestimate the number of cases, since these statistics are limited, for the most part, to hospitalized cases. For example, the lameness survey in Rangoon (Schonberger *et al.*, 1980) showed that 4.1 cases per 1000 appeared to be a reasonable estimate of the prevalence of poliomyelitis lameness in primary school children. It was noteworthy that 43% of the cases were never seen at the two major acute care hospitals for poliomyelitis in Rangoon, the major institutions that took care of patients with post-poliomyelitis problems.

Over the past 5–6 years, in every WHO region only a small number of countries were responsible for the majority of the cases, as shown in Table V. These countries show a high endemicity with periodic peaks of even higher incidence. They comprise, but are not composed exclusively of, the most populous countries in their regions.

In 1981, 118 countries reported cases of poliomyelitis (Table VI) to the World Health Organization (WHO, 1982d). These countries represent 78% of the world's population. The considerable world total of 33,870 cases certainly underestimated the true incidence.

In 1981, poliomyelitis continued to be endemic in most of Africa, with occasional localized epidemics. A notable epidemic occurred in the Central African Republic.

The situation in South and Central America was somewhat patchy, only some countries achieving good control. Brazil reduced the number of cases

TABLE V

Number of Countries Responsible for the Majority of Poliomyelitis Cases in 1976–1981

WHO region	Total number of reporting countries	Countries accounting for majority of cases	
		No. of countries	Percentage of total cases
Africa	46	6	50–55
Americas	47	4	79–93
Eastern Mediterranean	24	5	56–87
Europe	37	3	64–86
South-East Asia	11	3	94–98
Western Pacific	32	3	87–99

from an average of 8562 per annum in 1971–1975 to 123 cases in 1981 by a supplementary mass immunization campaign with OPV in July and August, 1980. However, Bolivia, Colombia, the Dominican Republic, El Salvador, Guatemala, Honduras, Paraguay, Peru, Mexico, and Nicaragua had a rather high incidence of disease.

Of the 23 countries in the eastern Mediterranean Region participating in the international surveillance of poliomyelitis, Cyprus is the only country where the disease has been controlled over a long period of time. Afghanistan, Egypt, and Yemen accounted for almost 80% of the total number of cases reported in 1981 (>400 cases each). Countries with a very low reported number of cases (<10) included Bahrain, Cyprus, Kuwait, and Qatar.

In the South-East Asia Region poliomyelitis remained endemic in 1981.

TABLE VI

Poliomyelitis in the WHO Regions in 1980–1981

WHO region	Population in 1981	Number of cases reported	
		1980	1981
Africa	358,835	4,822	3,081
Americas	609,175	2,965	651
Eastern Mediterranean	276,250	9,976	3,562
Europe	832,760	598	18
South-East Asia	1,059,180	16,976	21,173
Western Pacific	1,327,496	10,407	5,385
Total	4,463,696	45,744	33,870

Only Nepal, Mongolia, and the Maldives reported <10 cases, and Indonesia 941 cases during the year. In the Western Pacific also, poliomyelitis generally remained an uncontrolled problem in 1981, especially in Laos, the Philippines, and Vietnam. In Australia and New Zealand, Singapore, Malaysia, and Japan, a high degree of control was maintained. In the European Region, no major episodes of poliomyelitis were reported in 1981.

However, at the time of writing, many countries had not yet completed their reporting for 1981, and data for 1980 suggest that infection remains endemic in most of North Africa and Turkey. Bulgaria, Romania, and the USSR each reported >10 cases in 1980.

The incidence of poliomyelitis for 1982 is not yet available, but is should be mentioned that five outbreaks of poliomyelitis have been reported to WHO by five countries: South Africa, 270 cases; Dominican Republic, 150 cases; Guatemala, 29 cases; Jamaica, 50 cases; and Paraguay, 42 cases.

In tropical and semitropical countries outbreaks of over 500 reported cases are no longer unusual, and in some countries 5000 cases or more have been reported in a single year. From 1980 to 1981, 14 countries reported more than 500 paralytic cases (Table VII). The developing countries with the largest populations reported the most cases. For example, China re-

TABLE VII

Countries Reporting More Than 500 Paralytic Cases of Poliomyelitis in 1980 and/or 1981

Country	Number of cases	
	1980	1981
India	15,740	19,360
Indonesia	607	941
Brazil	1,342	123
Mexico	682	NA[a]
Lao People's Democratic Republic	1,165	NA
China	7,442	4,634
Philippines	790	326
Vietnam	961	408
Afghanistan	481	837
Egypt	2,006	1,605
Iraq	997	NA
Pakistan	1,039	NA
Sudan	4,151	NA
Yemen	664	410

[a]NA, data not available.

ported 7442 cases in 1980 and 4634 cases in 1981; India reported 15,740 cases in 1980 and 19,360 cases in 1981.

One particular feature of epidemic poliomyelitis in tropical countries is the occurrence of paralytic disease in young children. This phenomenon has been described by Wahdan *et al.* (1977) in Egypt, where a survey in 1976 showed that 10% of children under 10 years of age with residual paralysis had been affected in the first 4 months of life. The same low age of onset has been noted in areas of low socioeconomic status in India (Ratnaswamy *et al.*, 1973). Serological studies in a number of countries have shown that infection with all three serotypes of poliovirus may have occurred before the end of the first year of life in 50% or more of children.

These findings are surprising in light of the common belief that maternal antibody will protect the young infant. Although generally this may be true, there are conditions in which this mechanism may fail: (1) the maternal antibody may have fallen to unprotective levels before the first challenge (Fig. 2); (2) neonates receive inadequate transplacental antibody because the mother's titer of circulating antibody is not high enough. In both circumstances the infants become susceptible to infection earlier than their peers. These conditions are likely to occur in developing countries moving from a relatively stable endemic state to the epidemic state reached by the developed countries prior to the introduction of the vaccine. At present it is clear that an active immunity to poliovirus infection in infants should be induced in the first few months of life.

Bearing in mind the above conditions, EPI recommends starting anti-poliomyelitis vaccination at the age of 3 months. Recently the age has been changed to 6 weeks. Several studies in tropical countries have shown that three doses of poliomyelitis oral vaccine may not give an equally high serological response against the individual poliovirus types as that encountered in temperate zones (Dömök *et al.*, 1974), though only a small proportion of the vaccinees may remain serologically triple negative, i.e., negative against all three types. The practical significance of this observation has yet to be determined in terms of providing individual protection and of reducing the circulation of wild viruses in the community.

Coordinated research should be continued to determine the optimum methods of preventing poliomyelitis, including optimum dosage, and administration schedules of both live and inactivated vaccines.

Among the scientific problems related to vaccine immunization against poliomyelitis with live oral vaccine (OPV) is paralysis associated with poliomyelitis vaccine. In 1969, on the recommendation of a group of consultants, WHO organized a collaborative study to obtain definitive information on the possible risks associated with the use of OPV and on

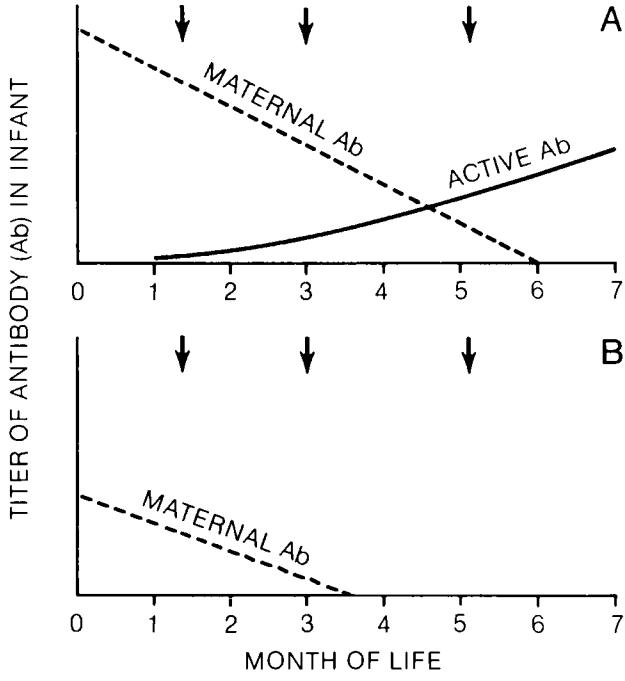


Fig. 2. Poliomyelitis virus infection: level of maternal antibody and poliomyelitis disease. (A) Neonates receive a high level of transplacental antibody. The first challenge takes place before the antibody level falls to an unprotective level and the infant develops active antibody. Despite infection, the infant does not develop any symptoms. (B) Neonate receives inadequate transplacental antibody, because the mother's titer of circulating antibody is not high enough or the maternal antibody falls too rapidly. In this circumstance the infant becomes susceptible to infection earlier than peers and may develop disease before 6 months of age. Arrows show repeated infection with wild poliovirus.

measures to reduce these risks if they were clearly shown to exist. The findings from the 13 participating countries for the first 10 years of the study have been reported (WHO Consultative Group, 1982). The results of the study confirm that OPV is one of the safest vaccines in use and that both OPV and IPV protect the individual and the community. Rigorous monitoring of the vaccine has brought the risk from OPV, in all but one of the participating countries, to less than one recipient case for every two million doses of vaccine distributed. The study also shows that parents without a definite history of immunization should be given OPV at the same time as their children.

The study also indicates the need for precise methods for strain characterization of polioviruses isolated from patients. The laboratory techniques for intratypic differentiation of wild and attenuated strains are very important for epidemiological surveillance and control of the safety of vaccines. Until recently strain-specific absorbed sera and oligonucleotide mapping have been used in the WHO Collaborative Study. International collaborative studies, organized through WHO, to examine the value of strain-specific monoclonal antibodies in strain identification were proposed and this project is in progress (Minor *et al.*, 1982).

In conclusion, practical experience has shown that the administration of vaccine to a high proportion of the presumed susceptible population in an epidemic area is as effective a means of cutting short an epidemic in a warm climate as it is in countries with temperate climates (Cockburn and Drozdov, 1970; Drozdov, 1982). Thus, when live poliovirus vaccine is properly used, it gives good results also in tropical countries. The main reason so far for high morbidity in many developing countries is the failure to establish effective regular vaccination programs. The discussion of explanations for failure is not included in this chapter, but we would like to mention that experience gained in EPI evaluations has shown that even a well-organized field program that reaches a high percentage of the target population will be ineffective, and may even undermine public confidence in the whole health care system, if the vaccine is not potent as a result of improper refrigeration or handling somewhere along the cold chain.

Also disquieting is the fact that a number of cases are beginning to occur again in areas where vaccination programs were instituted with considerable publicity and enthusiasm several years ago, but where the enthusiasm has waned and the proportion of susceptible children has again increased.

In May 1982, the Director-General of WHO presented a progress and evaluation report on EPI to the thirty-fifth World Health Assembly. He warned that the progress achieved so far is not enough to reach all children of the world by 1990. In order to accelerate the achievement of the EPI goals, the Director-General made these recommendations (WHO, 1982c): (1) promote EPI within the context of primary health care; (2) invest adequate human resources in EPI; (3) invest adequate financial resources in EPI; (4) ensure that programs are continuously evaluated and adapted so as to achieve high immunization coverage and maximum reduction in target disease, death, and cases; (5) pursue research efforts as part of program operations.

The above action program was endorsed by the Health Assembly in Resolution WHA 35.31 and will serve as a guide for national and international efforts for the remainder of the decade.

IV. VIRAL HEPATITIS

At least three forms of viral hepatitis are recognized: hepatitis A, hepatitis B, and hepatitis non-A, non-B. All three forms of hepatitis may exist in a community, without being differentiated by clincial syndromes, as the icteric phase is similar for all three types. There are also similarities in biochemical test values. Therefore the epidemiological data and the specific serological tests for infection with both hepatitis A and B viruses has assumed great importance. The diagnosis of non-A, non-B hepatitis remains one by exclusion (Deinhardt and Gust, 1982).

The distribution of hepatitis agents appears to be worldwide, but the patterns of diseases and the morbidity rate may vary from continent to continent and from country to country. The exact prevalence of different forms of hepatitis is difficult to estimate because of the lack of uniformity in surveillance systems and diagnostic reagents. Studies of the relative importance of hepatitis A, B, and non-A, non-B viruses indicate that hepatitis A may account for 50%, hepatitis B for 30%, and hepatitis non-A, non-B for up to 20% of clinical hepatitis cases.

Concerning vaccines against the various forms of hepatitis, the situation is entirely different. At present, the nature of the non-A, non-B agents remains unclear. Cross-challenge studies in chimpanzees showed the existence of two distinct forms of non-A, non-B hepatitis. The need for a safe, effective hepatitis A vaccine is universally recognized. The feasibility of production of an inactivated vaccine has been proved in marmosets using a heat- and formalin-treated antigen derived from marmoset liver.

The successful adaptation of hepatitis A virus to growth in cell cultures and recent advances in recombinant DNA technology suggest that it will be feasible to produce either killed or live vaccines. It is likely, because of similarities in epidemiology, that strategies for the use of hepatitis A vaccine will be similar to those used for the control of poliomyelitis.

Hepatitis B has been studied very extensively, and therefore we shall concentrate on this clinical entity, especially on the establishment of the chronic carrier state and its prevention.

Hepatitis B infection may be inapparent or may result in acute or chronic disease. In about 10% of patients with acute hepatitis, the disease progresses to a chronic form, and 3% develop chronic active hepatitis which may or may not be associated with antigenemia. Chronic antigenemia, or the carrier state, may develop especially in immunologically immature or impaired hosts to HBs antigen, and specific immunological unresponsiveness to HBs antigen occurs. Therefore, the coexistence of HBV without immune elimination from the host is induced in the host exposed to HBV. In populations where HBV is endemic (Asia, Sub-Saharan Africa, and

Oceania) there is substantial evidence that many of the chronic carriers acquire HBV as a result of infection transmitted from the mother early in life (at the time of delivery, during the period after birth when mother and child have considerable close contact, or perhaps prenatally). That is, if the mothers themselves are chronic carriers, offspring born when the mother is infectious are likely to become chronic carriers.

There is a high correlation between the infectivity of the mother and maternal HBeAg positivity. Approximately 80–95% of babies born to HBeAg-positive carrier mothers establish a persistent carrier state within 6 months of birth. Such babies, like their mothers, constitute a reservoir of HBV for further spread in the community.

In Asia up to 40% of HBsAg carrier women of childbearing age are HBeAg positive. By contrast, HBsAg positivity and perinatal transmission is rare in the Caucasian population, and perinatal transmission is of intermediate frequency in other racial groups. In Africa too, mother to infant transmission is important, but because HBeAg positivity is less common (up to 20%) than in Asia, maternal–infant transmission often occurs during early childhood.

According to information on hepatitis issued by WHO, the HBV carrier rate is significantly different in the different continents. As shown in Table VIII, among the overall world population of 4266 million, approximately 200 million are estimated to be HBV carriers. A total of 168 million (78% of all HBV carriers in the world) exist in Asia, according to the above estimation, and more than 26 million (12%) in Africa.

It is estimated that at least 20% of the 200 million HBV carriers in the world are likely to have contracted the perinatal infection. Close contact with HBV carriers in childhood is also considered to be one of the main

TABLE VIII

Estimated Number of Carriers of HBV in the World

Region	Population (millions)	Carriers of HBV	
		%	Number (millions)
Africa	441	6.0	*26.5*
United States and Canada	338	0.5	1.8
South America	228	0.6	1.4
Asia	2437	6.9	*168.0*
Europe	480	1.0	4.8
Oceania	22	2.0	0.4
USSR	263	?	?
Total	4209	3.0	202.9

routes of infection (Table IX). The immunosuppressive effect due to virus infections, such as measles, rubella, and other diseases, might cause immunological unresponsiveness to the exposed HBV, resulting in the induction of HBV persistent infection. Within a population, persons infected at birth or during the first year of life will have been chronic carriers of HBV longer than persons who are infected later in life. There are some lines of evidence to indicate that babies who acquired HBV from their mothers are at high risk of developing hepatocellular carcinoma in later years. Exposure in developed countries occurs predominantly in adults among persons whose occupation, illness, or personal habits, bring them into frequent contact with blood, blood products, or other body fluids.

The control of HBV infection is clearly justified as a public health measure for the prevention of acute and chronic hepatitis. The only method to achieve widespread, effective control of hepatitis B is active immunization. The priorities for immunization against hepatitis B are not the same for each geographical region or country.

Recently, the participants of an informal WHO meeting (Munich, May 26–28, 1982) and of a WHO Scientific Group on the Prevention and Control of Liver Cancer convened in Geneva from January 31 to February 4, 1983, reviewed the problem, and made recommendations on vaccination policies (Table X). All the investigators agreed that priority should be given to children born from HBsAg-positive mothers, and especially HBeAg positive ones, and adults at high risk (hospital personnel and contacts of all hepatitis B-positive cases).

TABLE IX
Geographical Patterns of Hepatitis B[a]

Hepatitis prevalence	Geographical areas	Pattern of prevalence (%)		Neonatal infection	Childhood infection
		HBsAg	Anti-HBs		
Low	North America, Western Europe, Australia	0.2–0.5	4–6	Uncommon	Uncommon
Intermediate	Mediterranean, Eastern Europe, USSR, Middle East	2–7	20–55	Uncommon	More common, neonatal infection uncommon
High	South-East Asia, China, tropical Africa	8–15	70–95	Common	Common

[a]From Deinhardt and Gust (1982).

TABLE X
Strategies for Vaccine Prophylaxis[a]

Hepatitis prevalence	Target groups (high risk)	
	Preexposure	Postexposure
High	All infants	Infants of HBsAg-positive mothers
Intermediate	All infants	Infants of HBsAg-positive mothers
Low	Health personnel; dialysis patients; institutionalized patients; drug addicts; male homosexuals	Accidental percutaneous exposure; infants of HBsAg-positive mothers; sexual contact of acute cases

[a]From Dienhardt and Gust (1982).

Hepatitis B vaccine induces a rather slow immune response. Of over 400 healthy young men treated with three 40-μg doses of the vaccine, only 31% had antibodies within 1 month, and 77% within 2 months. Only after the second booster, given at a 6-month interval, could antibody be detected in 96% of those vaccinated (Szmuness *et al.*, 1980). In most investigations, the vaccine has been injected subcutaneously, two or three times monthly. A booster injection is often administered 6–12 months after the first injection. One human dose usually contains 10–50 μg of HBsAg. The anti-HBs titers were rather high, approaching the level of 50–400 international mU. Two years after the booster, the antibody content was about 2–3 mU. Taking into account these data, Maupas *et al.* (1981) suggested that vaccine-induced HBs antibodies would persist at a protective level for 5–6 years after the first injection of vaccine.

Szmuness *et al.* (1981) reported that passively acquired antibody did not interfere with an active immune response to the vaccine. Therefore, the best way of protecting people already exposed would be to combine passive with active immunization. Tada *et al.* (1982) could prevent the mother-to-baby transmission of HBV by combined passive and active immunization in 90% of babies born to HBeAg-positive carrier mothers.

Numerous studies in at least seven countries are now under way to evaluate the immunogenicity and efficacy of HBV vaccine alone or in combination with hepatitis B immunoglobulin (HBIG). Studies of the immunogenicity in the newborn showed that more than 90% of them develop antibodies by 6 months of age in response to only two doses of vaccine.

There is hope that vaccination without HBIG would be a satisfactory alternative to an HBIG–vaccine combination, but a decision must await the results of the studies now in progress. Field studies are needed to determine

the best way of administering vaccine (i.e., dose, timing of infections, value of HBIG, etc.) and cost vs effectiveness of the vaccine.

The intensive progress of hepatitis B studies with the use of recombinant DNA technology has opened new perspectives for the development of inexpensive, safe, and effective vaccines. It is quite possible that the development of new strains and cell lines, producing sufficient quantities of HBsAg polypeptides, would permit the start of mass production of cheap and effective vaccine *in vitro*. It seems to us, however, that these approaches belong to the next decade.

At present, for human plasma-derived vaccines, the key to large quantity and low-cost production is the production of vaccine on a regional basis. The establishment of integrated facilities for simultaneous production of vaccine, hepatitis diagnostics, and immunoglobulins in hyperendemic areas should serve the extension of those vaccination programs aimed at the immunization of a large number of the susceptible population.

V. RABIES

Rabies is found all over the world and in all climates—in the Tropics and in the Arctic. However, at present some regions report mostly animal rabies and only single cases of human rabies, whereas others have a relatively high incidence of human and animal rabies. Australia, Bulgaria, Finland, Greece, Ireland, the Netherlands, New Zealand, Panama, Portugal, the United Kingdom, and others appear to have been free of rabies for several years, and in the case of Australia and the United Kingdom for a long time.

In the American Region during 1970–1979 an average of 280 cases of human rabies were reported annually (PAHO, 1982).

In the European Region, from 1972 to 1976, of the 621 cases reported in man, the overwhelming majority (598 or 96.3%) occurred in countries with canine rabies (Table XI). Of these, 116 (18.7%) occurred in Algeria (recently, Algeria has been transferred to the African Region), 22 (3.6%) in Morocco, 1 (0.16%) in Spain, 230 (37%) in Turkey, 200 (32.2%) in the USSR, and 29 (4.7%) in Yugoslavia. Only 9 cases after exposure were reported from three countries with sylvatic rabies, and 14 persons (2.2%) died of rabies acquired abroad. In the period 1977–1981 only 3 or 4 cases of human rabies were reported annually.

In the American Region a small variation in human rabies was recorded (Fig. 3). A reduced number of cases in 1972 and 1973 was due to increased control activities in severely affected areas in some countries. It should be noted that the incidence of human rabies in the Americas is reported differently in the various countries of the region. For example, Belize, Costa Rica, Grenada, Chile, Cuba, and Panama have reported very few human

TABLE XI

Human Cases of Rabies in the European Region; 1972–1976[a]

Situation	No. of cases in man		Country (No. of cases)
Sylvatic rabies	9	(1.4%)	Czechoslovakia (1) Federal Republic of Germany (3) Poland (5)
	10[b]	(1.6%)	Federal Republic of Germany (4) France (6)
Canine rabies	369	(96.3%)	Algeria (116) Morocco (22) Spain (1) Turkey (230)
Canine rabies with local foci of wildlife rabies	229		USSR (200) Yugoslavia (29)
Arctic rabies	—		—
Free of rabies	4[b]	(0.7%)	Sweden (1) United Kingdom (3)
Total	621		

[a]From Wachendörfer (1977).
[b]Imported 1977–1981, 3–4 cases annually (excluding Turkey).

cases. At the same time, Brazil reported 1140 cases, Mexico 647 cases, and Ecuador 209 cases, representing more than 70% of all cases reported in the region.

Although very few cases of human rabies were reported from European and North American countries, rabies in these countries was established as a widespread infection among wildlife. In Europe and North America foxes are the chief carriers of rabies among wild animals (Table XII).

Rabies in wild animals in Europe and North America continued to pose a significant risk both to humans and domestic animals, but it should be stressed that the danger to man occurs mainly when the rabies in wildlife spreads to dogs and cats: i.e., dogs and cats were responsible for most human exposures on all continents, irrespective of whether the reservoir hosts are wild animals or dogs. Few cases reported in the world originated in wild animals. Statistical data on the incidence of rabies in African, Eastern Mediterranean, and other regions, are lacking.

Rabies, particularly canine rabies, is increasing in case frequency and spreading to new areas in many developing countries, owing to increases in the density and mobility of both human and animal populations (e.g., in East Africa) (WHO, 1982e).

In recent years, almost all the African countries have reported cases of

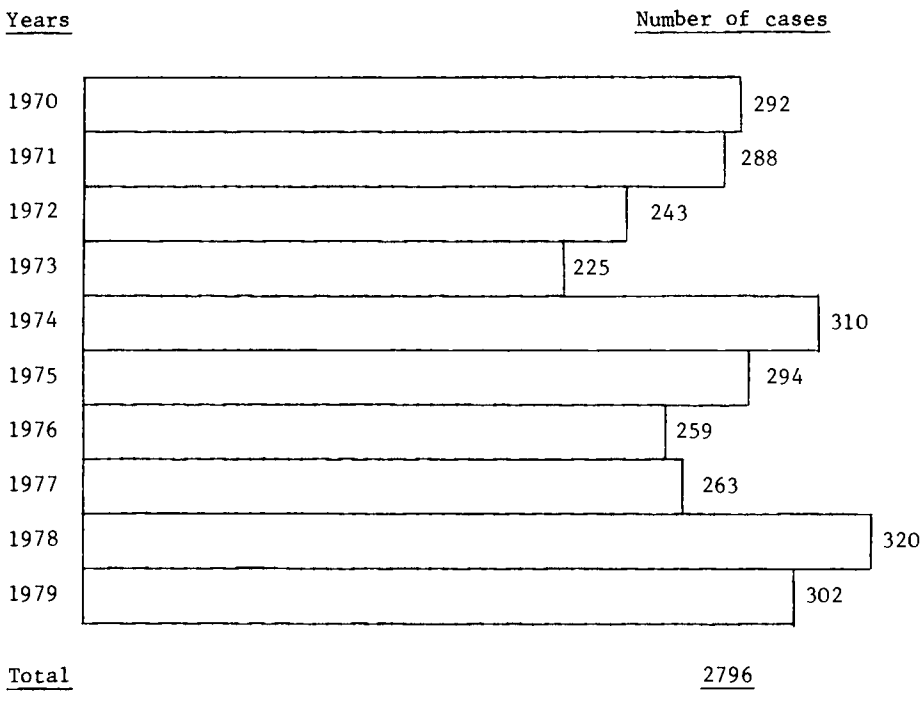


Fig. 3. Human rabies in the Americas, 1970–1979.

rabies, especially in dogs (Turner, 1976; Pekadu, 1982; Schneider and Bögel, 1984) (Table XIII). Dogs continue to be the major source of transmission of rabies to man, followed in descending order of importance by cats, farm animals, and wild animals. The long incubation period, the presence of virus in saliva, and the wandering of a rabid dog are all factors which are woven into a living epidemiological network connecting the rabies reservoirs in dogs, and occasionally in wildlife, with rabies in domestic animals and man.

It is reported that in most countries urban cases exceeded those in rural localities. A high incidence of rabies in humans is consistent with a large

TABLE XII

Animal Rabies in Europe, 1979–1981

	Proportion of infected animals (%)		
Animal	1979	1980	1981
Domesticated	20.6	23.4	24.5
Wild	79.4	76.6	75.5
(Foxes)	70.1	68.6	67.8

TABLE XIII

Rabies Cases in Africa by Country and Species[a]

Country	Period (years)	Dog	Cat	Farm animals	Wildlife	Human	Totals
Algeria	1964–1967	30	10	5	4	70	219
Tunisia	1964–1965, 1977, 1979	430	26	52	1	38	547
Morocco	1958, 1965–1977	856	15	219	1	132	1223
Egypt	1958, 1964–1966	188	16	19	7	64	294
Ghana	1970, 1977–1979	446	—	3	—	113	562
Kenya	1958–1965, 1968–1975, 1977–1979	198	11	52	40	13	314
Nigeria	1928–1978	2453	75	32	3	340	2903
Angola	1953–1968	935	32	33	18	1	1019
Botswana	1977–1979	54	4	68	9	5	140
Cameroon	1964–1967	148	2	—	1	9	160
Chad and Central African Republic	1964–1968, 1970, 1975	326	4	10	6	72	418
Congo (Brazzaville)	1964–1967	173	2	—	1	7	183
Formerly Belgium Congo	1935–1958	563	19	8	3	8	601
Kinshasa	1964–1967	581	3	7	1	—	592
Ethiopia	1965–1966, 1977–1979	330	7	18	2	81	438
Madagascar	1964–1968	136	5	5	—	4	150
Malawi	1964–1968	646	32	52	9	29	768
Mali	1967, 1977–1979	41	—	3	—	19	63
Rwanda-Burundi	1954–1961	356	9	49	5	19	438
Sierra Leone	1966–1968	21	—	2	—	10	33
Sudan	1935–1945	638	—	77	71	155	941
Tanzania	1966–1968	236	1	1	3	25	266
Uganda	1965–1966	78	1	3	4	22	108
Zambia	1964	48	1	3	—	6	58
South-West Africa, Namibia	1967–1976, 1977–1979	283	109	664	1234	4	2299
Rohdesia/Zimbabwe	1966–1967, 1977–1979	392	14	125	279	36	846
Total		10,686	398	1510	1702	1282	15,578

[a]From Schneider and Bögel (1984).

population of stray dogs. A large number of free-roaming dogs is a common sight in many cities in developing countries. Thus, people in these areas, especially in urban areas, live under a high risk of rabies infection.

The main objective of rabies control in urban areas is the interruption of dog-to-dog transmission, which can be achieved by the elimination of unvaccinated stray dogs and cats and the vaccination of dogs. Without the removal of stray dogs there may be no resultant substantial decrease in human rabies. When dog rabies is controlled, the number of cases in people drops correspondingly. Strict import requirements may further contribute to rabies control.

As mentioned above, the nationwide rabies control programs in many countries have provided for the almost complete elimination of rabies.

The incidence of human rabies can be substantially reduced by human pre- or postexposure vaccinations. At present four types of rabies vaccine are used in various countries: (1) suckling mouse brain vaccine, used frequently in the Americas; (2) duck embryo vaccine, still used in some countries; (3) cell culture vaccines, used frequently in Europe and North America; and (4) goat, sheep, and rabbit brain vaccines (Semple, Fermi), used in many countries in Africa and Asia. Various cells are used in the production of cell culture vaccines: (1) human diploid (WI-38 or MRC-5), (2) hamster kidney, (3) fetal bovine kidney, and (4) chick embryo fibroblast.

WHO recommends that no vaccine that contains living virus should be employed in man, and that those countries which are still producing nervous tissue vaccine should change to cell culture vaccine. Postvaccination neurological complications have been reported for all types of vaccine, except for cell culture vaccines, and particularly for human diploid cell vaccine, which is both highly effective and safe.

It should be pointed out that most of the people reported to have died of rabies might have been saved if they had been treated in time. The persistence of the disease is not due to a lack of tools to eliminate it but to the failure to decide to use them. Although rabies is not one of the major causes of mortality in man, the number of cases, especially those caused by dogs and cats, could be reduced considerably and even eliminated completely. National programs for elimination of dog rabies need particular attention in view of the increasing costs of human postexposure treatment. There seems to be nothing more urgent in the field of rabies than the development of national programs for elimination of dog rabies.

Worldwide control of rabies among wild animals is not yet technically or economically practical. However, WHO-coordinated research on oral immunization of foxes in Switzerland has proved to be effective in halting the spread of rabies under suitable topographical conditions. This work is still an experimental procedure, and field trials of oral immunization should not be considered as routine measures.

VI. YELLOW FEVER

Yellow fever continues to be a problem in endemic areas in both Africa and South America. Although the reporting of yellow fever cases is an obligation for WHO member states, the statistical data available are largely underestimated. The total number of yellow fever cases in the world in 1980–1982 is presented in Table XIV. The number of cases was higher in 1981 than in 1980 and in 1982. The number of deaths was higher in 1980.

A. South America

Since 1969, cases of yellow fever have been reported in 10 countries in the Americas Region (Fig. 4). Some outbreaks had occurred in places near urban areas infested by *Aedes aegypti*. The advance of human groups into jungle areas have favored contact with the reservoirs and vectors of the disease. Many countries of the Americas Region had succeeded in eradicating the mosquito from their territory about 30 years earlier. However,

TABLE XIV

Yellow Fever in 1980–1982

	1980		1981		1982[a]	
Country	Cases	Deaths	Cases	Deaths	Cases	Deaths
Africa						
Ghana	9	7	4	1	2	2
Ivory Coast	—	—	—	—	25	25
Nigeria	1	0	—	—	3	1
Senegal	—	—	3	1	—	—
United Republic of Cameroon	7	2	—	—	—	—
Totals	17	9	7	2	30	28
South America						
Bolivia	46	39	102	29	96	35
Brazil	27	22	22	21	24	24
Colombia	11	11	6	6	2	2
Ecuador	2	—	2	—	—	—
Peru	30	26	98	47	17	17
Venezuela	4	4	—	—	—	—
Total	120	102	230	103	139	78
Grand total	137	111	237	105	169	106

[a] Preliminary data.

Fig. 4. Yellow fever in South America, 1969–1981. The shaded areas on the map indicate the endemic territories. The figures indicate a total number of reported cases for the 13-year period (WHO).

most of the countries have been reinfested, and at present only Argentina, Chile, Ecuador, Peru, and Uruguay are still free of *A. aegypti*. Some countries of South America have never eradicated the mosquito (Table XV). Over the last 3 years, most cases were reported from Bolivia. The majority of infected persons (more than 70%) consists of male workers, about 90% of cases occurring in the 15–50 year age group.

In South America, control measures have concentrated upon preventing

TABLE XV
Infestation of *A. aegypti* in the American Region

Freer	Reinfested	Never eradicated
Argentina	Bolivia	French Guyana
Chile	Brazil	Guyana
Ecuador	Colombia	Surinam
Peru	Paraguay	Venezuela
Uruguay		

the urbanization of yellow fever through control of *A. aegypti,* the vector of urban yellow fever. As far as the vaccination policy is concerned, this has consisted primarily of vaccinating workers, visitors, immigrants, and other exposed persons in enzootic areas. However, information on the number of persons vaccinated, by region or by country, is not available. About 3 million vaccinations are performed annually in Brazil. The increasing levels of *A. aegypti* and the reinfestation of countries calls for increased surveillance and prevention of the spread of mosquitoes.

In view of the risk of the urbanization of yellow fever and the heightened risk of the spread of an epidemic, in June 1982 the WHO Regional Office for the Americas convened a meeting of a group of experts to study the problem and proposed possible alternative courses of action on a regional basis for eradicating *A. aegypti,* and other means for controlling dengue and dispelling the threat of the urbanization of yellow fever.

The group concluded that it was technically feasible to eradicate *A. aegypti,* but factors impeding the achievement of this objective existed at the regional level.

As a result of difficulties in controlling mosquitoes, it was recommended that group immunity should be maintained through regular and systemic vaccination of children and immigrants. Enlarging the population to be immunized was also recommended, to include (1) residents of communities located in areas with active jungle yellow fever, and (2) residents of cities and urban centers infested with *A. aegypti* near foci of jungle yellow fever and/or having frequent contact with a jungle yellow fever area.

B. Africa

In Africa, yellow fever cases are exceptionally detected as jungle yellow fever. The endemic area in Africa lies between parallels 15°N and 10°S. Since 1965, epidemics have been reported from 11 countries (Fig. 5), mostly from west Africa. The most important recent outbreaks occurred in Ghana, Gambia, and the Ivory Coast. In Africa the multiplicity of vector mosquito

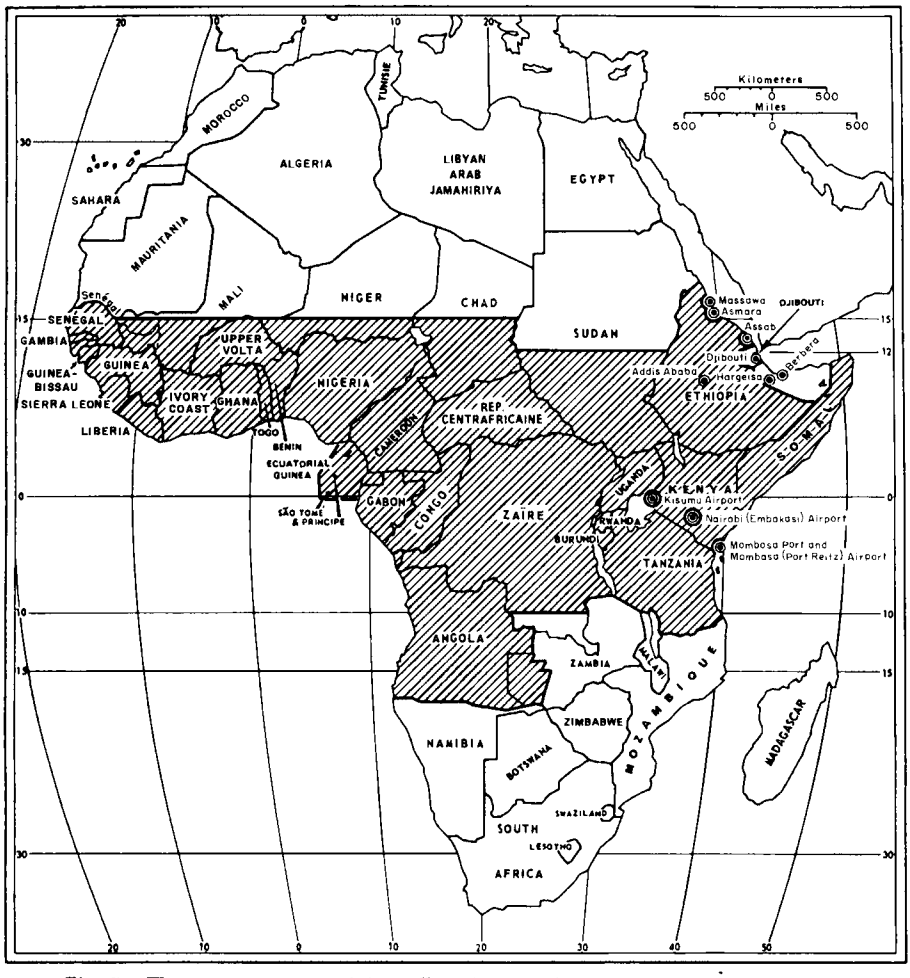


Fig. 5. The present status of the yellow fever endemic zone in Africa (WHO).

species makes their control difficult. Vector control of mosquitoes transmitting the virus from monkeys to man, and man to man, is limited to temporary abatement of vector populations during an outbreak. The use of vaccine is an alternative for the prevention of yellow fever.

WHO policy in Africa has been the "fire-fighting" vaccination strategy. All age groups in the risk zone of an outbreak have been vaccinated by emergency teams regardless of prior immunization. However, the "fire-fighting" policy has some advantages and disadvantages and has usually been initiated only after the epidemic peak has already occurred. This policy does not require large quantities of vaccine and permanent vaccination

teams, but its main drawback is that it ensures immunity only after a delay of about 7 days after vaccination, and it requires vigilant surveillance.

At present, some countries carry out regular vaccination programs on their own, but often intermittently and with a low percentage of coverage. Because of financial constraints, only part of the eligible population is vaccinated. Moreover, herd immunity raised by vaccination becomes diluted by birth and movement of population. The cost-to-benefit ratio of routine yellow fever immunization needs to be compared with that of epidemic control measures.

REFERENCES

Assaad, F. (1979). *In* "Immunization Benefit versus Risk Factors." *Dev. Biol. Standard.* **43,** 141–150.

Assaad, F. (1983). *Rev. Inf. Dis.* **5,** 452–459.

Beasley, R. P., *et al.* (1981). *Lancet* **2,** 388.

Bijkerk, H. (1979). *In* "Immunization Benefit versus Risk Factors." *Dev. Biol. Standard.* **43,** 195–206.

Centers for Disease Control (1977). Measles Surveillance Report No. 10, 1973–1976.

Centers for Disease Control (1983). Measles—United States, 1982. *Morbidity and Mortality Weekly Report* **32,** (4), 49–51.

Cockburn, W. C. and Drozdov, S. G. (1970). *Bull. W.H.O.* **42,** 405–417.

Deinhardt, F., and Gust, I. (1982). *Bull. W.H.O.* **60,** 661–691.

Dömök, I., *et al.* (1974). *Bull. W.H.O.* **51,** 333–347.

Drozdov, S. G. (1982). *Zh. Microbiol. Epidemiol. Immunobiol.* No. 1, 8–18.

Furesz, J. (1979). *Can. Med. Assoc. J.* **120,** 905–906.

Hayden, R. J. (1974). *East Afr. Med. J.* **51,** 199–205.

Kaplan, C. (1977). The world problem. *In* "Rabies: The Facts" (C. Kaplan, ed.), pp. 1–28. Corgi Books, Falmouth, England.

Krishnan, R., *et al.* (1982). *Br. Med. J.* **284,** 164.

McGregor, J. A. (1964). *West Afr. Med. J.* **13,** 215–257.

Maupas, P., *et al.* (1981). "Hepatitis B Vaccine" (P. Maupas and P. Gueszy, eds.), p. 117. Elsevier/North-Holland, Amsterdam.

Maupas, P., *et al.* (1982). *Lancet* **1,** 289.

Mazzur, S., *et al.* (1974). *Nature (London)* **247,** 41.

Melnick (1978). *Bull. W.H.O.* **56,** 21–38.

Minor, P. D., *et al.* (1982). *Nature (London)* **299,**(5879), 109–110.

Okada, K., *et al.* (1976). *N. Engl. J. Med.* **294,** 746.

PAHO (1982). Human rabies in the Americas, 1970–1979. *Epidemiological Bulletin, PAHO,* **3,**(1), 1–3.

PAO/WHO (1970–1979). Epidemiological surveillance of rabies for the Americas. *Monthly* Reports *(Pan American Zoonoses Center, PAHO/WHO)*

Pekadu, M. (1982). *Am. J. Epidemiol.* **115,** 266–273.

Ratnaswamy, L., *et al.* (1973). *Indian Pediatr.* **10,** 443–447.

Schneider, L. G., and Bögel, K. (1984). *In* "Guidelines for Dog Rabies Control." WHO, Geneva.

Schonberger, L. B., *et al.* (1981). *Dev. Biol. Standard.* **47,** 282–292.

Snyder, J. D., *et al.* (1981). *Am. J. Trop. Med. Hyg.* **30**, 426–430.
Szmuness, W., *et al.* (1980). *N. Engl. J. Med.* **303**, 833–841.
Szmuness, W., *et al.* (1981). *Lancet* **1**, 575.
Tada, H., *et al.* (1982). *Pediatrics* **70**, 4, 613–619.
Turner, G. S. (1976). *Trans. R. Soc. Trop. Med. Hyg.* **70**, 175–178.
Wachendörfer, G. (1978). Pp. 36–49. *In* "Surveillance and Control of Rabies." Copenhagen.
Walsh, J. H., and Warren, K. S. (1978). *New. Engl. J. Med.* **301**, 967–974.
Wahdan, M. H., *et al.* (1977). *J. Egypt. Pub. Health Assoc.* **2**, 305–316.
WHO (1977a). "Expanded Programme on Immunization Manual," Vol. 1, Section 5.
WHO (1977b). Measles immunity in the first year after birth and the optimum age for vaccination in Kenyan children. *Bull. W.H.O.* **55**, 21–31.
WHO (1981a). Expanded Programme on Immunization. Epidemiology of measles in a rural community. *Weekly Epidemiological Record* **56**, 85–87.
WHO (1981b). Expanded Programme on Immunization in the Eastern Mediterranean Region, *Weekly Epidemiological Record* **56**, 153–160.
WHO (1982a). Report of consultations on oral and enteric mass immunization of wildlife. WHO, Geneva, Sept. 20–22, 1982.
WHO (1982b). The optimal age for measles immunization. *Weekly Epidemiological Record* **57**, 89–91.
WHO (1982c). Expanded Programme on Immunization Global Five-Point Action Programme. *Weekly Epidemiological Record* **57**(33), 249–253.
WHO (1982d). Poliomyelitis 1981, *Weekly Epidemiological Record* **57**(40), 305–312.
WHO (1982e). Baterial and Viral Zoonoser. *W. H. O. Tech. Rep. Ser.* **n682**
WHO Consultative Group (1982). *Bull. W.H.O.* **60**, 231–242.
WHO/Euro. (1983). Possible programme for elimination of measles in Europe. WHO/EURO Informal *ad hoc* Consultation, Copenhagen, Jan. 17–18, 1983.
WHO/WPRO (1982). Report of a Scientific Group on viral hepatitis B and its related liver diseases. WHO/WPRO, Nagasaki, Japan, Sept. 29–Oct. 2, 1982.

11

Present Status of Vaccination against Poliomyelitis in the African Region of the World Health Organization

C. ALGAN[1]

Regional Office for Africa, World Health Organization, Brazzaville, Congo (popular Republic)

I. INTRODUCTION

Recognition of the worldwide importance of poliomyelitis led the twenty-second World Health Assembly in 1969 to place this disease under international surveillance (WHO, 1969). Despite the deficiencies, the epidemiological data provided to the World Health Organization by the member states made it possible in 1976 to divide the countries into three groups according to the degree of success attained in controlling the disease (WHO, 1977): (1) highly industrialized countries with effective immunization programs and long-established control of the disease, (2) developing countries which have instituted immunization programs and succeeded in controlling the disease, and (3) developing countries with or without vaccination activities which have not been able to bring the disease under control.

[1]Present address: WHO Program Coordinator, Antananarivo, Madagascar.

ISBN 0-12-429601-7

The African Region was in the third group with the Middle East, South-East Asia, and some countries in Central and South America.

The twenty-seventh World Health Assembly (1974) established the expanded program on immunization (EPI) (WHO, 1974) and requested all the member states to develop and maintain immunization and surveillance programs against childhood diseases, including poliomyelitis, which can be prevented by means of vaccination.

The first medium-term EPI program (WHO Program, 1978–1983) was formulated by the Regional Office for Africa (AFRO) in 1978. However, implementation of the program had already begun in the field in 1977.

The purpose of this chapter is to outline, 6 years after EPI effectively commenced, the present state of poliomyelitis vaccination in the African Region of WHO. It has been drawn up on the basis of an evaluation of national programs and WHO. Although it has been deliberately simplified, it is hoped that it will lead to discussion among the participants.

II. EPIDEMIOLOGY OF POLIOMYELITIS

Poliomyelitis is the cause of serious public health and socioeconomic problems in the African Region due to the disabilities that it causes. Indeed, although it is not a particularly deadly disease, it is one of the principal causes of paralysis, especially infantile paralysis.

According to notifications, which however fall far short of the reality (Table I), the average annual incidence is constant whereas prevalence is on the increase. In surveys carried out in some countries on the paralytic sequels of poliomyelitis, the prevalence of infantile paralysis varies from 2 to 12% in children aged 6 to 10 years (Table II). On the basis of these data, a relatively accurate estimate puts the average annual rate at between 17 and 46 cases of paralysis for 100,000 inhabitants (Table III). It should be noted that the annual rate of incidence of poliomyelitis in the United States between 1945 and 1954 was between 15 and 30 per 100,000 inhabitants (La Force *et al.*, 1980). Moreover, comparison between the notified incidence and the incidence estimated on the basis of surveys shows that official notifications cover only a twentieth part of the real number of cases in view of the fact that such statistics in general record hospitalized cases only.

Although the spread of the virus in our climates and environment is almost continuous, the seasonal recrudescence of the infection in Africa follows two reflective curves, as it were; an increase in morbidity from July to October in the north between latitudes 10°N and 35°N and from January to May in the south between latitudes 10°S and 25°S, whereas the virus

TABLE I

Poliomyelitis in the African Region. Average Annual Number of Cases in 1951–1955, 1966–1970, 1971–1975, and 1976–1980 and Number of Cases in 1976, 1977, 1978, 1979, 1980, and 1981

Country	1981 Estimated population (millions)	Average annual number of cases				Number of cases					
		1951–1955	1966–1970	1971–1975	1976–1980	1976	1977	1978	1979	1980	1981
Angola	6.8	314	9	4	28	18	10	50	29	32	0
Benin	3.7	4	98	120	102	112	81	109	52	155	182
Botswana	0.9	4	4[a]	4	3	7	5	4	0	1	1
Burundi	4.4	—[b]	22	22	42	38	36	59	32	43	102
Cape Verde	0.3	—	—	—	3	1	5	2	2	3	10
Central African Republic	2.4	—	—	—	61	147	44	49	44	21	477
Chad	4.6	0	19	37	47[a]	44	78	20	—	—	—
Comores	0.4	—	—	—	—	—	—	—	—	3	—
Congo	1.6	78	298	193	115	46	129	117	146	136	16
Equatorial Guinea	0.3	1	9[a]	74[a]	—	—	—	—	30	—	—
Ethiopia	30.5	8	85	268[a]	145	25	200	120	147	234	322
Gabon	0.7	7	46	55	44	18	38	45	47	70	9
Gambia	0.6	0	1[a]	4	10	1	3	39	5	1	1
Ghana	12.4	9[a]	53	187	243	313	157	155	445	145	53
Guinea	5.3	2	14[a]	31	—	2	4	—	—	—	—
Guinea-Bissau	0.8	4	22	7	3	1	4	3	5	3	53
Ivory Coast	8.8	2	135	104[a]	91	94	60	212	43	47	34
Kenya	17.9	204	355	290	407	210	290	1020	59	455	19
Lesotho	1.4	5	22[a]	27	24	16	6	6	—	36	—
Liberia	2.0	—	1[a]	2[a]	8	2	8	24	3	2	2
Madagascar	9.2	9	36	120	130	1	0	406	176	68	—
Malawi	6.6	13	194[a]	160	65	58	31	96	87	51	12

(*Continued*)

TABLE I (*Continued*)

Country	1981 Estimated population (millions)	Average annual number of cases				Number of cases					
		1951–1955	1966–1970	1971–1975	1976–1980	1976	1977	1978	1979	1980	1981
Mali	7.1	19	379	432	501	581	527	685	479	234	276
Mauritania	1.7	0	22[a]	30	53	46	10	18	34	159	—
Mauritius	1.0	80	2	0	0	0	0	0	0	0	0
Mozambique	12.7	39	39	40	42	8	22	65	48	65	38
Niger	5.8	0	57	179	264	232	250	189	336	311	279
Nigeria	82.3	4	120	384	601	627	437	657	469	816	233
Reunion	0.5	7	6	1	0	0	0	0	1	0	0
Rwanda	5.4	—	17[a]	9	82	8	15	15	12	362	38
Sao Tome and Principe	0.1	—	—	2	11	5	6	1	1	44	7
Senegal	5.9	90	80	159	194	161	175	354	144	138	100
Seychelles	0.1	—	—	—	0[a]	—	0	—	0	0	0
Sierra Leone	3.7	2	9[a]	18	92	192	87	110	60	11	21
South Africa	30.0	610	360	533	144	328	92	50	132	116	—
Swaziland	0.6	3	9	6	66	2	5	—	—	192	—
Togo	2.8	13	53	24	117	33	22	19	316	196	5
Uganda	13.7	113	21	40	97	98	66	222	62	37	124
United Republic of Cameroon	8.9	15	57	26	92	48	12	220	—	87	38
United Republic of Tanzania	19.9	112	232	36	88[a]	14	157	90	—	91	—
Upper Volta	6.7	15	82	129	190	113	405	150	137	145	150
Zaire	30.3	815	592	545	356	531	343	396	248	263	407
Zambia	6.0	39	248	66	86	83	74	42	215	17	44
Zimbabwe	8.0	127	134	44	15	3	11	6	24	32	28
Total	374.8					4267	3905	5825	4070	4822	3081

[a]Covers less than the 5-year-period.
[b]Data not available.

TABLE II

Prevalence of Paralytic Poliomyelitis Based on Lameness Surveys in Nine Countries[a,b]

Country	Polio prevalence per 1000 children
Cameroon	3.6–8.7
Gambia	4.7
Ghana	6–12
Ivory Coast	8–12
Madagascar	3.5
Malawi	6.5
Niger	6
Nigeria	2–4
Swaziland	3.2

[a]The prevalence of residual poliomyelitis can be arbitrarily described as being low: less than 1 per 1000; moderate: 1–4 per 1000; high: more than 4 per 1000.

[b]For methodology, see La Force *et al.* (1980).

TABLE III

Estimated Reporting Completeness of Poliomyelitis based on Lameness Survey and Reported Incidence in Six Countries, 1973–1981

Country	Incidence of poliomyelitis per 100,000 population		
	Incidence estimated from lameness surveys[a]	Reported incidence	Reporting completeness (%)
Cameroon[b]	24	1.2	5
Ghana[b]	31	2.3	7
Ivory-Coast[b]	34	1.2	4
Malawi[b]	28	1.2	4
Niger[c]	46	6	13
Nigeria[d]	17	0.6	4

[a]For methodology, see La Force *et al.* (1980).

[b]WHO/OMS, WER. 1982, No. 47, p. 362.

[c]WHO/OMS, WER. 1982, No. 1, p. 4.

[d]WHO/OMS, WER. 1982, No. 2, p. 14 (based on a report from the National Institute for Medical Research, Lagos, Nigeria).

appears to be permanently active throughout the year in the equatorial belt (10°N–10°S). Thus it can be seen that both curves are almost straight (WHO, 1979).

According to a study carried out in Ghana (Böttinger *et al.*, 1981) between 1976 and 1978, the results of titrations of poliomyelitis antibodies provide grounds for believing that the circulation of the uncontrolled virus in infants (3–8 months old) is more intensive than in older children (9–14 years old).

In the African Region the disease attacks at a very early age. Some 75% of the children have it before they are 3 years old, and 99% before they are 5 (WHO, 1982). According to surveys (WHO, 1982; Rey, 1980) the median age is about 16 months, and the most frequent age is 1 year. In light of these observations, we agree with Rey's conclusion (1980) that poliomyelitis in the Third World deserves to continue under the more conventional name *infantile paralysis,* since most cases are noted before the age of 3 years (Said, 1959; Rey, 1980).

The disease undoubtedly has a decisive impact on the development of the countries. It is extremely costly in social terms. No therapeutic can check it. Modern methods of symptomatic and palliative treatment, which are highly specialized and costly (resuscitation, physiotherapy, and rehabilitation), are beyond the reach of poor persons, so that vaccination is the only effective control measure available at present.

III. EXPANDED PROGRAM OF IMMUNIZATION (EPI)

For want of immunization, in the African Region each year approximately one million children will die from EPI target diseases (measles, tetanus, pertussis, poliomyelitis, diphtheria, and tuberculosis), and as many again will remain physically and mentally handicapped, at least one-fifth of them being victims of poliomyelitis. The most effective way of preventing poliomyelitis concurrently with the other above-mentioned diseases is to vaccinate every child in its first year of life. EPI, which was adopted by all the member states as an essential component of primary health care (PHC), should accomplish this task by 1990 (WHO, 1981).

At the regional level, the medium-term EPI (WHO Program, 1978–1983) covering the period 1978–1983 set out the problems relating to the EPI target diseases (their extent and severity) and to the communities concerned. The program's general and specific objectives were also formulated. One of the main aspects of the strategy chosen has been to integrate EPI as far

as possible into the permanent health services. Such services provide continuity of action in a multisectoral framework. The field of action of WHO's cooperation was stated specifically in the medium-term program 1978–1983. The aim was to launch EPI in 35 countries at the rate of 5 countries per year starting in 1977, so that they could provide coverage to at least 60% of the target population within the EPI zones. To this end a certain number of activities were designated. Six fields of action were identified: planning, implementation, evaluation, manpower training, research, and vaccine production and quality control. Regarding planning and implementation, WHO set itself the task of collaborating with the countries in planning EPI as part of comprehensive health services. As to implementation, in collaboration with the countries, WHO would promote the basic immunization of children and mothers (against tetanus) as part of maternal and child health services and give direct support to national programs in the form of personnel, equipment, and vaccines. As to evaluation, WHO would participate in strengthening or setting up, where necessary, mechanisms for the epidemiological surveillance of all communicable diseases of local epidemiological importance, among them the EPI target diseases including poliomyelitis. WHO should further participate in evaluating activities connected with immunization in the countries. WHO should also strengthen manpower training in program management and evaluation. With regard to research, WHO would contribute to the development of appropriate methodologies for this program. Finally, it would participate in feasibility studies and identify funds wherever this was economically desirable so as to provide for vaccine production and quality control in the member states of the region.

At the country level, the program was received by all the member states of the region with enthusiasm and a notable sense of resolve. This took on concrete form with the medium-term program forecast for 1978–1983. However, in the planning and execution of national programs, some operational approaches adopted by certain countries of the region are considered to be the fundamental cause of the shortcomings noted. For example, some countries begin their operations in the capital city and formulate very costly programs which they are unable to extend to the country as a whole with the available financial resources. Nor is it unusual to find certain countries setting up or strengthening independent vertical structures to administer vaccinations without taking any steps whatever to ensure integration. In some countries, too, it is clear that programs are too dependent on the initiative of collaborating agencies that limit their activities to restricted areas in which they are sure of outstanding success. These are a few of the many examples that go to show that further efforts will have to be made

to improve the adequacy of planning and implementation of certain national programs.

IV. PRINCIPAL EPI ACHIEVEMENTS

A. Extent of Achievements

Six years after the start of the program, the objectives of the medium-term program have been achieved as follows: (1) In the field of planning, 40 countries, not only the expected 35, have formulated their plans of operation, which represents an achievement of 114%. (2) In the field of implementation, at least 41 countries, not only 35 as expected, have launched EPI in accordance with the standards laid down, an achievement rate of 117% (it should, however, be pointed out that very few of them have integrated EPI into PHC). (3) In the field of evaluation, 27 countries out of an expected 35 are on record as having already made at least one evaluation, an achievement rate of 77%. (4) Seventy-eight surveys carried out in 27 countries indicate that at least 60% vaccinal coverage (in EPI zones) has been achieved by 20 countries for BCG, 10 for measles, 7 for DTP (3 doses), 5 for poliomyelitis (3 doses), and 3 for tetanus (pregnant women, 3 doses). According to data provided by 13 countries, estimated average vaccinal coverage of children of 0–11 months in the countries of the region is 31% for BCG, 18% for DTP III, 17% for poliomyelitis III, 27% for measles, and, for pregnant women, 18% for neonatal tetnus (second dose). (5) Ten countries out of 35 sent in monitoring reports on a regular basis, an achievement rate of 29%. (6) Twelve countries out of 35 have made surveys in order to pinpoint the incidence and prevalence of a few target diseases, an achievement rate of 34%.

It is not easy to evaluate the degree of attainment of objectives in the field of training, research, and vaccine production and quality control, given that the medium-term program did not formulate quantified objectives. Nonetheless, if we take as output indicator the number of countries out of 35 expected to organize the participation of central, intermediate, and peripheral level EPI senior-grade staff in EPI training courses, we find that 41 countries (117%), not 35 as expected, arranged for their health personnel to participate in senior level courses. Twenty-two out of 35 (63%) organized intermediate level courses. Five out of 35 (14%) organized courses in logistics and cold-chain technology, and, finally, two out of 35 (6%) at the end of 1982 organized a course on the repair of refrigerators. As to research, 13 countries out of 35 (35%) carried out research projects to develop

strategies adapted to local conditions. No new activities (0%) have been recorded in the field of vaccine production and quality control.

B. Impact

Furthermore, it has proved to be quite difficult to measure the effectiveness and impact of EPI due to the lack and/or irregularity of data sent in by the countries. In point of fact, using the data relating to the six EPI target diseases, it has not been possible to monitor the evolution of mortality and morbidity indicators on the basis of the number of vaccinations administered to the target populations. The member states were advised to improve their data collection system at country level by supplementing it with surveys of prevalence and incidence and to inform AFRO regularly of vaccinal coverage in EPI geographic zones. As a result, detailed data are now coming in from some countries. Thus in March 1982, for instance, we were aware that EPI in Ivory Coast covered 61% of the population and that vaccinal coverage for three doses of poliomyelitis vaccine was almost 70% in several areas. In Abidjan, morbidity due to poliomyelitis in hospitals fell by 72% and mortality by 95% between 1978 and 1981. In Cameroon, 20 zones, representing 50.2% of the population, were provided with vaccination services. Vaccinal coverage for poliomyelitis exceeded 70% in some areas, and the incidence of poliomyelitis fell by approximately 88% (from 62.1 per 100,000 in 1974–1975 to 7.5 per 100,000 in 1981) in the Yaoundé area (WHO, 1982). In the Gambia, EPI has covered the entire country since September 1980. Poliomyelitis vaccinal coverage increased from 53 to 66% in 1981. A fall in the incidence of poliomyelitis of about 75% was recorded (Western Division).

V. CONCLUSION

Poliomyelitis is still endemic in the African Region. The number of countries that reported cases (although quite incomplete and very far below the reality) is encouraging. According to the results of surveys of the prevalence of claudication due to poliomyelitis, the incidence of the disease appears to be higher in several countries than that observed in Europe and North America before vaccines were developed. From certain local indices, it would appear that the quality of notification has improved since the expanded program on immunization started. A large number of studies for the purpose of improving the information and monitoring system is under way in several countries.

Vaccination of all children by the year 1990 with an effective vaccine in their first year of life is one of the priorities of EPI. This program, which began in 1977, is developing quite satisfactorily. Except for a few small countries which already have full EPI coverage, most of the member states plan to extend it by stages, achieving total coverage within a few years (2–7 years).

Average vaccinal coverage is as yet too inadequate to have had a marked effect on the incidence of poliomyelitis in the region. Nonetheless, assessment of the situation in certain EPI geographic zones leads to very encouraging conclusions regarding the trends of morbidity due to poliomyelitis. WHO provides support for comparative studies of the development of diseases in different countries (and regions within each country) and for surveys of prevalence and vaccinal coverage, and we hope to be better documented within a few years so as to be in a position to evaluate the program's effectiveness in reducing the problem of poliomyelitis throughout the region.

However, in the light of the objectives determined as part of the medium-term program (1978–1983), it may be said that those relating to planning and manpower training have been achieved, whereas this is only partly the case with respect to implementation, evaluation, and research.

It is difficult at present, and in view of the data available, to evaluate the impact of EPI on health development and on allied socioeconomic development. However, it has had a moderate impact on the formulation and implementation of national health policies and programs. Nearly all the countries have drawn up their plans of operation, EPI. In most cases, EPI uses a horizontal and multisectoral approach, thus enhancing promotion of the other primary health care components. Training courses in planning and management give concrete form to technical cooperation between several developing countries, on the one hand, and, on the other, between the countries and multi- and bilateral organizations as well as between these organizations themselves.

REFERENCES

Böttiger, M., Litvinov, S., Assaad, F., Lundbeck, H., Heller, L., and Beausoleil, E. G. (1981). Vaccine field trials in Ghana, 1976–1978. Virus Diseases Unit, WHO, Geneva.

La Force, F. M., Lichnevski, M. S., Keja, J., and Henderson, R. H. (1980). *Bull. W.H.O.* **58**(4), 609–620.

Rey, M. (1980). *In* "Vaccination," p. 73. Masson, Paris.

Said, G. (1959). *Sem. Hop.* **35**, 1362/SP. 272.

WHO (1969). Offic. Ac. No. 176, p. 23. Resolution WHA22.47.

WHO (1974). Resolution WHA27.57.

WHO (1977). *Weekly Epidemiological Record* **51,** 405–410.
WHO (1979). *World Sanitary Statistics.* **33, No. 3,** 203.
WHO (1980). *Weekly Epidemiological Record* **No. 52,** 402.
WHO (1981). Resolution AFRO/RC31/R14.
WHO (1982). SHDS/AFRO Mid-year Progress Report.
WHO Program EPI/AFRO (1978–1983). Regional Office for Africa, Brazzaville, Congo.

12

Hepatitis B Virus: Approaches to Control and Vaccination

COLIN R. HOWARD AND ARIE J. ZUCKERMAN

Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London, England

I. INTRODUCTION

Conventional viral vaccines contain a number of complex antigenic determinants which induce protective humoral and cellular immune responses. These are usually prepared after attenuation or fixation of intact virus or, less generally, separating a portion of the virus, such as the coat protein or its subunits, after growth *in vitro*. The latter approach requires selection of virus-specific proteins which contain those antigenic sites required for the induction of a protective antibody response. Either of these vaccine sources may contain contaminating antigenic components, proteins, and other material derived from the virus, the host cell in which the virus has been grown, or the substrate and its media, which are not relevant to protect immunity and may lead to undesirable side effects (Wilson, 1967). Vaccines are subject to strict manufacturing requirements and control to ensure safety and freedom from contamination and untoward complications.

The historical perspective of hepatitis B is unique in virology in that development and application of genome sequencing precedes the isolation and study of the intracellular replication cycle.

The failure by numerous investigators to propagate hepatitis B virus in

ISBN 0-12-429601-7

the laboratory *in vitro* has prevented the development of conventional vaccines from virus grown in cell cultures. Attention has therefore been directed to the use of alternative sources of viral antigen for active immunization, including the use of hepatitis B surface antigen, the noninfectious surplus protein coat of the virus, purified from the plasma of asymptomatic human carriers and subsequently subjected to an inactivation procedure. Since hepatitis B surface antigen (HBsAg) leads to the production of neutralizing or protective surface antibody, as shown by serological surveys and experimental transmission studies to human volunteers and chimpanzees susceptible to hepatitis B infection, purified 22-nm spherical surface antigen particles have been developed as vaccines. It is generally accepted that the 22-nm particles, when pure, are free of nucleic acid and therefore noninfectious, but the fact that the starting material is human plasma obtained from persons infected with hepatitis B virus means that extreme caution must be exercised to ensure that the preparations of antigen are free of all harmful contaminating material, including host components. Some concern has been expressed about the possible induction of harmful immunological reactions to human host components, including preexisting structures of liver cells, which may be either present as an integral component of the surface antigen or intimately associated with the antigen as a contaminant derived from cells or from plasma (Zuckerman, 1975; Melnick *et al.*, 1976), but neither reactions of this type nor autoimmune reactions have been observed with the highly purified 22-nm particle vaccines in chimpanzees and the many individuals immunized.

Nevertheless, there are advantages in the use of chemically characterized antigen since many studies have shown that considerable variation exists among protein analyses of purified hepatitis B surface antigen obtained from different sources and of different serological subtypes (reviewed by Howard and Burrell, 1976). Four approaches are currently being pursued in the development of hepatitis B vaccines. In addition to isolation of antigen from human sources, cloning and subsequent expression of the HBsAg gene are now feasible in prokaryote cells. This has been rapidly extended to include expression in higher cells and complements the availability of HBsAg from hepatoma cells transformed *in vivo*. Finally, knowledge of the HBV genome sequence now allows the manufacture of specific protein sequences which mimic areas of importance on the HBsAg polypeptide believed to be responsible for eliciting a protective antibody response.

II. IMMUNOCHEMICAL PROPERTIES OF HEPATITIS B SURFACE ANTIGEN

Preparations consisting of the separated 22-nm particles, which constitute the bulk of the hepatitis B surface antigen material in the sera of most carriers, have been analyzed both chemically and serologically in several

laboratories, but with varying results. Although in most studies at least two major polypeptides were found in the molecular weight range of 20,000–30,000, variable amounts of larger components were frequently present (see, e.g., Howard and Zuckerman, 1974; Shih and Gerin, 1975, 1977; Dreesman *et al.*, 1975; Skelly *et al.*, 1978). It is possible that some of these polypeptides represent integral host-coded proteins which may play a role maintaining and preserving surface antigenic reactivity.

Although the two major polypeptides of purified hepatitis B surface antigen of molecular weight 22,000–25,000 and glycoprotein of molecular weight 28,000–30,000 have been frequently designated p22 or p23 and gp28, respectively, Peterson (1981) has proposed that these should be more properly designated p25 and gp30 based upon the probable true molecular weight of the protein as deduced from the DNA sequence of the viral *S* gene. The fact that treatment of HBsAg with anhydrous hydrofluoric acid converted gp30 into p25 reaffirms the conclusion that these two major proteins differ only by the presence of carbohydrate in gp30.

The advantages of a polypeptide vaccine, derived from any source, include precise biochemical characterization, exclusion of genetic material of viral origin, and exclusion of host- or donor-derived substances (Zuckerman, 1975, 1976). Disadvantages of polypeptide vaccines include low yield if strong ionic detergents are used for separation, although the yield has been substantially improved by the use of nonionic detergents (Skelly *et al.*, 1979a, 1981a.)

Various studies have shown that individual HBsAg polypeptides are immunogenic after inoculation into guinea pigs. For example, Dreesman *et al.* (1975) prepared antisera to five polypeptides derived by solubilization of hepatitis B surface antigen with sodium dodecyl sulfate in the presence of urea. Although some variation was found in the responses obtained against individual polypeptides prepared from subtypes *adw* and *ayw*, antibody cross-reacting with (HBsAg) was induced in animals inoculated with either the 24,000, 35,000, or 40,000 molecular weight polypeptides, respectively. Antisera to 6 or 7 polypeptides of molecular weight 23,000 to 97,000 separated from purified 22-nm particles of subtypes *adw* and *ayw* were raised in guinea pigs by Gold *et al.* (1976). Most or all of the polypeptides stimulated antibodies to the *d* or *y* subdeterminants, demonstrating that these antigens are part of the constituent structure of each polypeptide. Although the nature of the minor components remains unclear, two polypeptides with molecular weight 23,000 and 29,500 were found as the major components of all subtypes with the remaining polypeptides varying within each subtype both in number and in relative concentration (Shih and Gerin, 1977). Shih *et al.* (1978) later studied the immunogenicity of the major polypeptides separated from purified surface antigen using double antibody radioimmunoprecipitation. Each of three polypeptides, 23,000, 29,500, and 72,000 molecular weight, respectively, were trace-labeled and used as the

radioligand in the assay. Each of these polypeptides contained both the group-specific determinant and the subtype-specific determinant as shown by precipitation by antiserum to the native surface antigen and by antisera prepared against the separated polypeptides, thereby indicating a high degree of serological relationship between these three components.

The two major polypeptides of HBsAg have essentially identical amino acid composition to each other and to those present in intact HBsAg particles (Peterson *et al.*, 1977; Gerlich *et al.*, 1980). The amino-terminal and carboxy-terminal sequences of the amino acids in these two major polypeptides, together with tryptic peptide mapping, show that the surface *S* gene product of the virus consists of a single major polypeptide chain. The difference in the molecular weight of the two virus-specific HBsAg components is due entirely to the carbohydrate moiety of the glycoprotein of the second band. Inoculation into guinea pigs of the 22,000 molecular weight polypeptide emulsified in Freund's complete adjuvant elicited antibodies to the group-specific determinant *a* and to one of the major subdeterminants *d*. The 28,000 molecular weight polypeptide, however, did not induce an antibody response, and it was suggested that the discrepancy in the immunogenicity of the glycosylated peptides reported in other studies could have been due to contamination of the two major polypeptides.

Although the above findings suggest that each HBsAg-associated polypeptide may contain amino acid sequences which are essential for immunoreactivity, results obtained after separation of components with the strongly anionic detergent sodium dodecyl sulfate are difficult to interpret. Renaturation of polypeptides into a native conformation may be inaccurate or incomplete, and since antigenicity in globular proteins is dependent on three-dimensional conformation the antigenic determinants of such molecules may differ markedly from those of the original state. A further consideration is that substantial losses in protein yield frequently occur when individual polypeptides are extracted from polyacrylamide gels. Alternatively, nonionic detergents and bile salts have been used extensively for the dissociation of viruses into soluble complexes which retain biological activity. For example, Simons *et al.* (1973) disrupted the virus envelope of Semliki Forest virus into soluble protein and lipid complexes by treatment with Triton X-100. The protein and lipid complexes were then separated by density gradient centrifugation in the presence of the detergent. Hayman *et al.* (1973) separated successfully the surface glycoproteins of both influenza and mouse mammary tumor viruses by affinity chromatography using columns of immobilized phytohemagglutinin equilibrated with buffer containing sodium deoxycholate. A considerable advantage in these studies was the retention of the biological activities of the developed glycoproteins after disruption of the virus. Skelly *et al.* (1979a) disrupted purified HBsAg with

2% Triton X-100 in the presence of salt to yield a product with an estimated sedimentation coefficient of 3.9 S. Fractionation of radiolabeled antigen by passage through columns of immobilized concanavalin A resulted in the separation of the two major polypeptides into two fractions. The first fraction, which did not bind to the lectin, contained exclusively a 64,000 molecular weight polypeptide. This component reacted with serum albumin antibodies and produced peptide maps similar to those for albumin after treatment with trypsin. The second fraction was obtained by eluting bound material for the immobilized lectin column with α-methyl D-mannoside and was found to contain 28,000 (p25) and 23,000 (gp30) molecular weight HBsAg polypeptides. Since p25 was not found previously to be glycosylated, this polypeptide probably remained bound to gp30 by a protein–protein linkage after detergent treatment. The reactivity of this material with antibodies to HBsAg, in the absence of any detectable reactions with antibodies to normal serum components, indicates that hepatitis B surface antigen reactivity resides with this subunit preparation. The technique of Triton X-100 solubilization followed by affinity chromatography offers a considerable advantage for the preparation of milligram quantities of immunologically reactive material not possible previously by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoretic techniques. Hepatitis B polypeptide vaccine material containing both polypeptides p25 and gp30 have been successfully examined for safety, immunogenicity, and protective efficacy in susceptible chimpanzees (Dreesman *et al.*, 1981; Tabor *et al.*, 1982; Howard *et al.*, 1982). The results show that, at least for polypeptides in micellar form, a rapid and high-titer surface antibody develops with kinetics comparable to the use of intact 22-nm HBsAg particles. Antibody to the core component (Anti-HBc) has been consistently absent in animals immunized with separated HBsAg polypeptides, and there has been no evidence of liver injury associated with the use of these materials.

All the available evidence suggests that protection is conferred by induction of antibody to the group *a* determinant present on the HBsAg polypeptides. However, the location of this antigen and the possible role of carbohydrate in preserving the structural integrity of this or adjacent epitopes have yet to be clearly distinguished. For example, Burrell *et al.* (1976) found that combined treatment of HBsAg particles with SDS and trypsin resulted in the release of a small 5000–15,000 MW glycoprotein bearing the group-specific *a* determinant. Although the serological reactivity of this peptide resisted heat at 100°C, the antigen was destroyed by exposure to reducing agents indicating the presence of disulfide bonds. Neurath *et al.* (1978) obtained slightly different results after treatment of HBsAg with SDS and chymotrypsin. The released material from the *ad* subtype contained both *a* and *d* antigenic determinants and was found to consist of several

polypeptides with molecular weights of 8500 and 7800, respectively. However, when trypsin was substituted for chymotrypsin, serologically reactive material was not released. There was no evidence of interpeptide disulfide bonds in the serologically active SDS–chymotrypsin cleavage fragments obtained from the *ad* subtype.

Further evidence that the *a* determinant may be associated with a glycopeptide has been provided by Karelin and Zhdanov (1981). Linkage between the p25 and gp30 polypeptides appears to be mediated via disulfide bridges, and maintenance of covalent bonding may be of essence in maximizing the immunogenic potential of separated HBsAg polypeptides. For example, Mishiro *et al.* (1980) treated purified 22-nm HBsAg particles with 1% SDS at 37°C for 2 hr in the absence of a reducing agent, e.g., 2-mercaptoethanol. This treatment resulted in the solubilization of the outer coat of the particle, yielding a polypeptide with a molecular weight of 49,000. This polypeptide carried the two common determinants of the surface antigen, *a* and *Re*, as well as four subdeterminants, *d, y, w,* and *r*. This material was found to be a potent immunogen in mice, even though the recovery of the polypeptide on the basis of optical density at 280 nm was 2% of the starting surface antigen material. When the 49,000 molecular weight polypeptide was reduced in the presence of 2-mercaptoethanol, it split into two polypeptides with molecular weights of 22,000 (p25) and 27,000 (gp30). These latter two polypeptides were considerably less antigenic and immunogenic. Mishiro *et al.* (1980) proposed that the 49,000 molecular weight polypeptide dimer formed the basis of a suitable polypeptide vaccine and is compatible with the observations of Skelly *et al.* (1981a) whereby solubilization occurred in the absence of a reducing agent.

Purified HBsAg particles were treated with 0.1% Pronase in the presence of 1% sodium dodecyl sulfate at 37°C overnight, and the resulting antigenic fragments found to contain the group-specific antigen *a* (Karelin and Zhdanov, 1981). About 30% or less of subdeterminant activity *y* was also preserved. The electrophoretic mobility of the fragments, referred to collectively as gp12, in SDS–polyacrylamide gels corresponded to globular proteins with an average molecular weight of 12,000. The antigen, gp12, differed from the native hepatitis B surface antigen in its isoelectric points and UV-absorption spectrum. This antigenic material was resistant to heat, but it was inactivated after treatment with neuraminidase free of any other glycosidase activity. It is possible, therefore, that the terminal neuraminic acid residue plays a role in the composition of the *a* antigenic determinant. Aggregates of gp12 were immunogenic for guinea pigs, and antibodies to *a* and *y* were produced, but not against proteins of normal human plasma. These results collectively suggest that the *a* determinant may be isolated in an intact state, is not dependent on disulfate linkage for antigenicity, and

may preferentially be associated with one of the two major HBsAg polypeptides. The single peptide fragment containing a glycosylated site at position 146 in the molecule found by Peterson (1981) may indicate that the group determinant is located toward the carboxyl terminus of gp30. The purification of viral coat subunits in large quantities presents considerable problems, particularly with viruses possessing a lipoprotein envelope, in which the immunogenic components are integral membrane proteins, highly hydrophobic, insoluble in aqueous media, and requiring detergent solubilization. The extraction of the antigenic polypeptides by the nonionic detergent Triton X-100 resolved one of the problems. However, polypeptides maintained as monomers in the presence of detergent are not a suitable form of vaccine and remain weakly immunogenic. Detergent removal is thus critical for the reassociation of polypeptides into a suitable immunogenic form. A method of detergent removal that allows the gp30 and p25 complex of HBsAg to reassociate into water-soluble protein micelles has been described (Skelly *et al.*, 1981a). Protein micelles are aggregates of polypeptides ordered so that the hydrophobic regions are sequestered in the interior of the particles with the hydrophilic residue on the surface, thereby rendering the polypeptides water soluble.

The buoyant density of the micelles in cesium chloride was 1.25 g/ml compared to density of 1.10 g/ml for intact 22-nm particles, an increase consistent with the removal of most of the lipid by the solubilization procedure. Electron microscopy showed that the micelles were pleomorphic and fluffy in appearance, with diameters in the range of 60–200 nm (mean 120 nm). Polyacrylamide gel electrophoresis showed that both gp30 and p25 were present in the same proportions as in the original Triton X-100 detergent extract and together constituted 40% of the total protein of the purified 22-nm particles used as starting material. Ninety percent of the gp28–p23 complex was recovered from a concanavalin A–Sepharose column, and the final yield of the two polypeptides in micellar form was estimated to be 60–70% of the amount originally present in the intact HBsAg particles.

The micelles competed effectively with intact 22-nm surface antigen particles for surface antibody in a radioimmunoprecipitation test. Their immunogenicity was compared in a mouse potency test, since it is considered that the serological response in mice is a useful indicator of the immunogenic potential of candidate hepatitis B vaccines. Consistently higher levels of surface antibody were induced in animals receiving micelles, possibly also with a higher affinity. Several factors may, singly or in combination, account for the greater immunogenicity of the micelles, including their large size, altered distribution of antigenic sites, or the absence in them of host-derived serum proteins such as albumin. The results show that the antigenic

complex of gp30 and p25 can be readily isolated in high yield from intact 22-nm particles and that this complex can be reassociated into a micellar form as has been described for other viral envelope proteins. In addition, micelles may be obtained using plasma from asymptomatically infected chimpanzees or human carriers (Young *et al.*, 1982). The chemical purity, specific serological activity, and immunogenicity of the micelles, taken together with the ease of their preparations on a large scale, strongly favor their development as an alternative "second generation" hepatitis B vaccine.

Formaldehyde is widely used for the inactivation of viral vaccines, yet there is little published information on the effects of formaldehyde on hepatitis B surface antigen. Skelly *et al.* (1981b) reported that treatment with formaldehyde under conditions generally used for viral inactivation had little effect on the antigenicity or immunogenicity of the intact 22-nm surface antigen particle or on that of the protein micelles. Pretreatment with formaldehyde had no effect on the ability of Triton X-100 to disrupt the particles, nor did it alter the behavior of the disrupted material in affinity chromatography. Polypeptides in a micellar form may also be a suitable preparation of vaccines from antigenic proteins produced by the expression of cloned hepatitis B virus DNA in prokaryotic and eukaryotic cells and from peptides produced by chemical synthesis (Howard *et al.*, 1982).

III. NATURE OF THE HEPATITIS B GENOME AND MOLECULAR CLONING STUDIES

Undoubtedly the biggest advance in our understanding of the hepatitis B virus genome has resulted from the application of gene cloning technology. In addition, eukaryotic cells expressing hepatitis B surface antigen proteins as a result of cloning of fragments of hepatitis B virus DNA are particularly attractive sources of antigenic material and propagation in *Escherichia coli* using plasmids and derivatives of lambda bacteriophages as vectors has been reported by a number of groups (Burrell *et al.*, 1979; Edman *et al.*, 1981; Charnay *et al.*, 1980; MacKay *et al.*, 1981). This work has resulted in the determination of the nucleotide sequence of the viral DNA and the organization of the viral genome and the prediction of amino acid sequence of the major gene products (Valenzuela *et al.*, 1979; Galibert *et al.*, 1979; Pasek *et al.*, 1979). The important features of this work with respect to potential vaccine development stem from the identification of the gene (*S*) coding for the major HBsAg polypeptide present in the circulation of infected individuals. The genome contains four major polypeptide reading

frames, which theoretically may be available for transcription within a naturally infected hepatocyte from the long DNA strand of the double-stranded genome. One of these codes for the 21,000 MW polypeptide component of the viral core (*C* gene). Another codes for a protein of 25,000 MW which closely resembles in predicted sequence the unglycosylated major HBsAg polypeptide identified by electrophoretic analysis of purified 20-nm particles. A third reading frame overlaps the *S* gene, and the fourth partially overlaps the *C* gene. The genome is characterized by the presence of many potential ribosomal binding sites in RNA transcripts, but the number of reading frames is severely limited by many stop codons and relatively few initiation sites.

The amino acid sequence of the *S* gene product may be predicted from the nucleotide sequence and was proposed by comparison with the limited data available for the two major HBsAg polypeptides which had previously been shown to possess identical amino and carboxy-terminal sequences (Peterson *et al.*, 1981). The full amino acid sequence predicted by these studies is shown in Fig. 1. The 226-amino acid long polypeptide contains a relatively high content of aromatic and hydrophobic amino acids, in accord with previously published total amino acid studies of HBsAg particles. The se-

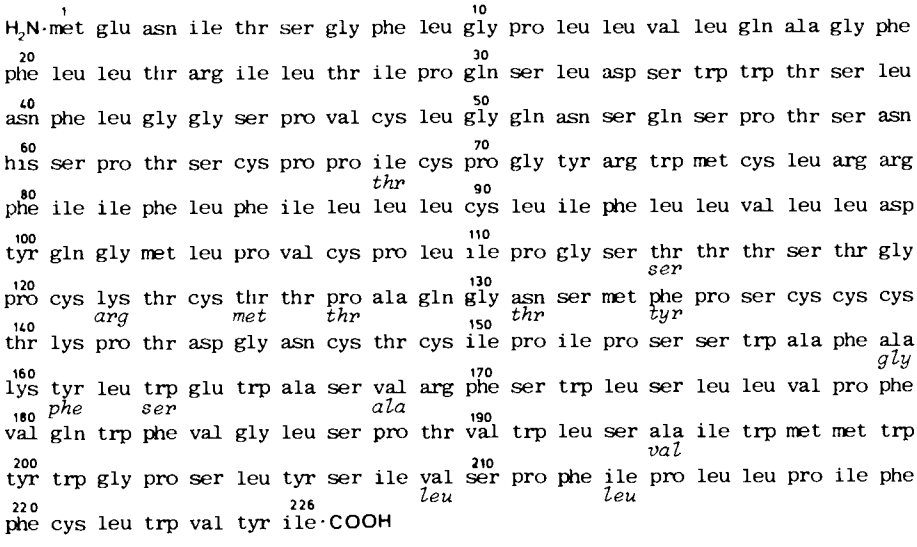


Fig. 1. Amino acid sequence of the HBsAg 25,000 MW polypeptide is predicted from the nucleotide sequence of the hepatitis B virus *adw* subtype *S* gene (Valenzuela *et al.*, 1979). A total of 16 amino acid changes are predicted from a similar analysis of the *ayw* subtype gene (Galibert *et al.*, 1979).

quence also predicts a globular configuration with a 19-amino acid long hydrophobic region. Galibert *et al.* (1979) reported the corresponding sequence for the *ayw* subtype HBsAg gene, which suggested a total of 16 amino acid changes, 6 of which are clustered in a region immediately on the carboxyl side of the conserved hydrophobic region. Although there are thus some similarities and differences between DNA cloned from sources containing different subtypes, differences between preparations expressing HBsAg of the same subtype in the original host have been reported (Siddiqui *et al.*, 1979). However, it is reasonable to predict that the most variable region is between residues 120 and 144. This may subsequently be borne out by peptide mapping of HBsAg polypeptides of different subtypes, although Peterson (1981) found that peptides in a preparation of *ayw* antigen were not completely identical with those predicted from the DNA sequence of a genome from a different virus of similar *ayw* subtype. Again, this demonstrates that at least some changes may not necessarily be reflected in antigenic variation of the *S* gene product.

Direct confirmation of a number of structural properties predicted from nucleotide sequencing of the *S* gene has been reported (Peterson, 1981). The results showed that p25 and gp30 separated by SDS–polyacrylamide gel electrophoresis indeed contained similar amino acid compositions and shared the same sequences at both NH_2 and COOH termini. In addition, these matched exactly with those predicted from the nucleotide sequence data. Furthermore, polypeptides derived from HBsAg particles of either *adw* or *ayw* gave identical results at either terminal. One interesting feature of this study was the finding that trypsin digestion led to the appearance of only two digestion products as a result of cleavage to the right of the lysine residue at position 122. This was so despite the presence of other suitable sites, which presumably were not available to the enzyme. After aminoethylation, trypsin digestion proceeded further and allowed the identification of glycopeptides derived from gp30. Analysis indicated that carbohydrate attachment proceeds with asparagine-*N*-acetylglucosamine linkages, probably at position 146. The type of linkage is consistent with previous chemical analyses reported by Shiraishi *et al.* (1977).

Expression of cloned hepatitis B DNA encompassing the viral *S* gene has been achieved in *E. coli* following conventional procedures involving fusion of HBV DNA sequences to the NH_2-terminal part of β-galactosidase (Charnay *et al.*, 1980). *Escherichia coli* lacking a portion of the gene *lacZ* was infected with lambda phage into which was previously inserted a major portion of the *S* gene DNA adjacent to the *lacZ* gene. Infected cells synthesized a hybrid protein of molecular weight 138,000, which possessed antigenic determinants of both HBsAg and β-galactosidase. Although the hybrid accounted for only 0.05% of total protein in cell lysates, this was enhanced

fivefold by genetic manipulation of the infecting phage genome. These results emphasize that certain HBsAg determinants are not dependent on glycosylation mediated by eukaryote metabolism and that there are no introns to interrupt the processing of mRNA transcripts. Although *E. coli* has generally been used as the host cell for expressing cloned genes (Sninsky and Cohen, 1982), there are advantages in using other bacteria, such as *Bacillus subtilis*, for the production of viral and other polypeptides. For example, *B. subtilis* is nonpathogenic and does not produce endotoxin, and it excretes several extracellular proteins in large amounts. Furthermore, *Bacillus* strains are widely used commercially for producing antibiotics and enzymes and are often used as a source of food for human consumption in the Far East. Hepatitis B core antigen and the major antigen of foot-and-mouth disease virus have been produced in *B. subtilis* after the insertion of the appropriate viral DNA into a plasmid vector (Hardy *et al.*, 1981). It is therefore reasonable to expect that expression of HBsAg could also be achieved in gram-positive cells.

Expression of cloned genes in yeast may be expected to offer considerable advantages for HBsAg expression, including the possession of Golgi-like secretory apparatus for protein maturation and the potentially much higher yield of the required gene product owing to the availability of a eukaryote codon usage system for RNA recognition during translation. With these advantages in mind, Valenzuela *et al.* (1982) brought about the expression of HBsAg in the yeast *Saccharomyces cerevisiae* using an autonomously replicating plasmid containing the HBV *S* gene linked to an alcohol dehydrogenase promoter. Correct orientation of the *S* gene sequence with respect to the promoter was essential for HBsAg production, which occurred at an approximate rate of 10–25 μg per liter of culture fluid. The HBsAg synthesized in yeast was particulate, allowing its purification and subsequent examination by electron microscopy. Spherical particles approximately 20 nm in diameter were visualized, although only the unglycosylated p25 polypeptide was present. These particles were fully immunogenic, suggesting that glycosylation of the HBsAg polypeptide is not a prerequisite for immunogenicity.

The genetic map of the HBV genome indicates the possible presence of a "leader" or precursor nucleotide sequence antecedent to the *S* gene, suggesting the theoretical possibility of posttranscriptional and/or posttranslational modification of the *S* gene product. However, this sequence was absent from the plasmid used in the studies of Valenzuela *et al.* (1979, 1982), suggesting that these processes are not required for the formation of HBsAg spherical lipoprotein particles.

Expression of hepatitis B proteins of eukaryote cells has also been achieved, either by transformation of cell lines with cloned DNA (Dubois

et al., 1980; Hirschman and Garfinkel, 1982) or by the use of SV40 as a vector (Moriarty *et al.*, 1981). The approach used by Dubois was to transform mouse L cells, deficient in thymidine kinase, simultaneously with plasmid DNA containing HBV DNA and a plasmid containing the thymidine kinase gene of herpes simplex virus. The first plasmid contained two copies of HBV DNA in a tandem head-to-tail arrangement in order to facilitate transcription of the *S* gene. Detectable amounts of HBsAg were found both in the cytoplasm and in cell culture fluids of cotransformed cells. This material resembled serum-derived HBsAg 22-nm particles in many respects, including the apparent glycosylation of the primary *S* gene product. The authors have estimated production at a rate approaching 4×10^4 particles per cell per 24 hr, making the use of eukaryote cells containing HBV DNA suitable for HBsAg vaccine if an alternative diploid cell line could be similarly transfected. Hirschman and Garfinkel (1982) employed essentially the same technique, save that there was no attempt to select transfected cells from inoculated HeLa cell monolayers, and expression of viral markers was mediated by exposure to circular cloned HBV DNA.

Cytotoxicity became apparent in these cells at approximately the same time as HBsAg was first detected. Variable cytopathology was observed, including the presence of large cells with multiple nuclei and ground-glass cytoplasm. Unlike the results of Dubois *et al.* (1980), however, HBcAg was also detected, making this particular set of cells less suitable for potential vaccine material.

The introduction of the *S* gene DNA into the SV40 genome by Moriarty *et al.* (1981) led to expression of HBsAg in monkey kidney cells. Particles resembling the 22-nm lipoprotein form of HBsAg were produced at a rate of approximately 1 $\mu g/10^7$ cells per day and found to contain both p25 and gp30 surface antigen-specific polypeptides. The presence of gp30 indicate that the glycosylation and modification of the p25 primary gene product may occur in cells other than infected hepatocytes.

IV. CHEMICALLY SYNTHESIZED HEPATITIS B VACCINES

The development of synthetic polypeptide vaccines offers many advantages in attaining the ultimate goal of producing uniform and safe viral immunogens to replace many current vaccines, which often contain large quantities of irrelevant microbial antigenic determinants, proteins, and other material additional to the essential immunogen required for the induction of a protective antibody response. The preparation of antibodies against viral proteins using fragments or chemically synthesized peptides mimicking amino acid sequences of interest is now an acceptable alternative approach

for viral immunoprophylaxis. The feasibility of synthetic vaccines was first demonstrated in studies with tobacco mosaic virus after the identification of an antigenic determinant and its amino acid sequences responsible for the immunogenic activity of the virus (reviewed by Arnon, 1980). Such amino acid moieties can be synthesized and, when coupled to a carrier protein, induced the production of high-titer neutralizing antibody in experimental animals. More recent studies include the induction of an immune response to an intact strain of type A human influenza virus using a synthetic peptide analogous to a sequence of the hemagglutinin of type A H3N2 influenza virus (Muller *et al.*, 1982). The peptide was covalently linked to several macromolecular carriers, and the conjugate with tetanus toxoid was used for the immunization of rabbits and mice with the production of specific antibodies. These were found to protect against infection with a relatively low viral challenge in mice with the A/Texas/77 mouse-adapted influenza virus. These results indicate the potential of synthetic material for eliciting antiviral immunity against an important and common human pathogen. Veterinary vaccines are also possible. For example, Bittle *et al.* (1982) have shown that synthesized peptides corresponding to two regions of the major VP1 polypeptide of foot-and-mouth disease virus coupled to a protein carrier produced high titers of specific neutralizing antibody in several animal species including cattle, and immunized guinea pigs were fully protected against virulent virus after receiving only a single dose of one peptide.

Much information regarding the use of synthetic peptides is becoming available from attempts to stimulate antibodies against bacterial toxin. Diphtheria toxin is a single polypeptide chain of 62,000 molecular weight with two disulfide bridges. There is evidence that the loop of 14 amino acids subtended by the disulfide bridge near the NH_2 terminus is implicated in the toxicity and immunological specificity of the molecule. A synthetic tetradecapeptide mimicking a portion of this active site linked covalently to two different carriers by Audibert *et al.* (1981) elicited in guinea pigs antibodies which bind specifically with the toxin and neutralize the dermonecrotic and lethal effects. Another example is the induction of type-specific protective immunity by Beachey *et al.* (1981) using a synthetic peptide of *Streptococcus pyogenes* M protein which contained only 12 amino acid residues. The immunogenicity of such small peptides indicates a way to the development of safe vaccines against streptococcal infections which cause rheumatic fever and rheumatic heart disease, and in more general terms the efficacy of very small peptides would permit the disposal of a large portion of the protein molecule. This approach should also reduce the chances of eliciting immunological cross-reactions against host tissues.

Similar approaches to the development of chemically synthesized hepatitis B vaccines were encouraged a few years ago by several investigators

(Rao and Vyas, 1973; Anonymous, 1973; Zuckerman and Howard, 1973; Zuckerman, 1975, and others), and current progress suggests that such synthetic peptide vaccines are now within reach.

The strategy for producing synthetic material adopted by all groups to date consists of four stages. First, the prediction of antigenic portions of the *S* gene product directly by analysis of its amino acid sequence. Computer analysis is used to locate the points of greatest hydrophilicity in the polypeptide chain. Next, selected sequences are synthesized, generally using the classical solid-phase method (Erickson and Merrifield, 1976). Finally, the antigenic and immunogenic properties of synthesized fragments are assessed, either directly or after coupling to a carrier protein.

Using this approach, three groups have independently reported the successful mimicking of HBsAg determinants using chemically synthesized peptides. Hopp and Woods (1981) selected the sequence between residues 138 and 149 of the surface antigen, which contained a putative dominant epitope in positions 141–146. The four cysteinyl residues were replaced by α-aminobutyric acid in order to prevent polymerization and other side reactions common to sulfhydryl-containing peptides, and glycine was added to the COOH terminus to aid radioimmunoassay directly on the solid phase used for synthesis. The prediction that the synthetic peptide contains a major epitope of hepatitis B surface antigen was confirmed, this sequence of amino acids eliciting antibodies directed to group-specific determinant *a* and the subdeterminant *d,* but not the epitope of subdeterminant *y* or that of albumin added as a control. When the peptide was attached to aldehyde-stabilized human erythrocytes and injected into mice, it induced the formation of hepatitis B surface antibody with and without the use of Freund's complete adjuvant (Prince *et al.,* 1982). Vyas (1981) previously reported that a synthetic oligopeptide of 13 amino acids between the sequence 136–147 represented a partial analog of the *a* determinant of the surface antigen.

Dreesman *et al.* (1982) reported the results of a slightly different approach using two predicted hydrophilic regions of the HBsAg molecule. Two cyclic peptides containing disulfide bonds in the region between the amino acid sequences 117 and 137 were synthesized. The two synthetic peptides with sequences 117–137 and 112–137 were incorporated into several adjuvants including Freund's complete adjuvant, alum, and multilamellar liposomes with and without muramyl dipeptide. Groups of BALB/c mice were immunized intraperitoneally with each of the preparations. Hepatitis B surface antibody was induced 7–14 days after inoculation in approximately 50% of the mice in each group, and in four or five out of six mice when the immunizing preparation of the 117–137 peptide was emulsified in Freund's complete adjuvant. On day 21, however, the peak levels of antibody decreased in most groups of mice. It should be noted that antibody

response was elicited in mice after a single injection without covalent linkage to a carrier protein, and the choice of adjuvant did not significantly affect the primary antibody response. It is also worth noticing that this study found the anti-HBs response in mice inoculated with p25 solubilized with SDS was similar to that observed with the synthetic peptides. This is of interest considering the author's intention to generate synthetic structures that may reasonably be believed to conserve probable β turns at residues 129–132 and 135–137.

Lerner *et al.* (1981) examined a more extensive array of synthetic peptides, but avoided the region 110–140 because of published differences in nucleotide sequences in this region. Instead, the 13 peptides included sequences at the amino and carboxyl termini, peptides corresponding to hydrophilic domains of the protein, and peptides likely to contain exposed folds in the peptide chain indicated by proline-containing junctions between hydrophilic and hydrophobic regions.

Seven out of the 13 free or protein carrier-linked synthetic peptides elicited an antipeptide response in rabbits. Where used, the carrier protein was keyhole limpet hemocyanin in complete and subsequently incomplete Freund's adjuvant. Antisera against four out of the six soluble peptides, ranging from 10 to 34 amino acid residues in size, reacted with the native antigen and also precipitated the 25,000 (p25) and 30,000 (gp30) molecular weight major polypeptides of HBsAg. These findings again illustrate that antibodies to certain HBsAg determinants may be produced using short peptides; this is somewhat at variance with work on intact HBsAg 22-nm particles, which has previously suggested that the relevant antigens are conformationally dependent. Lerner and colleagues suggested that short peptides may be immunogenic when free of constraints imposed by neighboring amino acids that are manifested by denaturation of the native molecule, citing the theoretical agreement that the short peptides are free to assume all possible configurations at any one time, one of which resembles the native molecule and thus stimulates antibody production (Sachs *et al.*, 1972).

Synthetic peptides may well be employed in due course as vaccines, although mixtures of more than one of the peptides may be required, in particular if protection is required against several serotypes. Although conventional vaccines may continue to be of value against many viral diseases, synthetic peptides will supplement these in instances where, for example, particular serotypes are difficult to cultivate *in vitro* or whole viruses are particularly susceptible to temperature. Of the many questions which remain to be answered, the critical issues are whether antibodies induced by synthetic immunogens will be protective and whether protective immunity will persist. Some of the carrier proteins and some of the adjuvants which had been linked to the synthetic molecules cannot be used in man,

and it is therefore essential to find acceptable and safe material for covalent linkage, or, alternatively, to synthesize sequences which do not require linkage. It is clear that we have entered the era of antigen and antibody engineering, and the prospect of synthetic vaccines against a variety of microbial agents including hepatitis A and B viruses appears to be within reach.

V. HEPATITIS B SURFACE ANTIGEN FROM HETEROPLOID CELL LINES

Antigen sources from other than human carriers of markers of hepatitis B virus are becoming available from heteroploid surface antigen secreting cells derived from primary hepatocellular carcinoma (see, e.g., Aden *et al.*, 1979; Skelly *et al.*, 1979b; Copeland *et al.*, 1980; Das *et al.*, 1981; Wen *et al.*, 1981). The cell cultures and fluids are noninfectious (Daemer *et al.*, 1980; Tabor *et al.*, 1981), the cell lines can be characterized, techniques are available to ensure freedom from contaminating nucleic acid, and potent inactivating agents are available. However, the cell lines are transformed and show heterotransplantability, and therefore development of such vaccines must proceed with caution. This topic has been discussed and reviewed by Mann *et al.* (1982).

Serial medium samples from the PLC/PRF/5 hepatoma cell line show that HBsAg progressively accumulates during growth and maintenance, yield being maximal toward the end of the maintenance period when nutrients have been depleted from the culture medium (Copeland *et al.*, 1980). This would suggest that HBsAg production is a terminal event, increasing yield being accompanied by sloughing of cells and cytological changes in adherent cells.

There would appear to be large cytoplasmic pools of HBsAg within the cytoplasm of PLC/PRF/5 cells, probably in polypeptide form, as radioimmunoassay results are consistently low despite intense immunofluorescence, and HBsAg particle morphogenesis is not readily distinguishable by electron microscopy. These cells may therefore offer an alternative source of HBsAg polypeptide, made more attractive by the apparent absence of serum albumin, a major contaminant of HBsAg particles present in plasma of chronically infected carriers (Skelly *et al.*, 1979b).

ACKNOWLEDGMENTS

The work on hepatitis in progress at the London School of Hygiene and Tropical Medicine is supported by generous grants from the Medical Research Council, the Department of Health and Social Security, the Wellcome Trust, and the World Health Organization.

The hepatitis B vaccine development project is generously supported by the Department of Health and Social Security, the British Technology Group (formerly the National Research Development Corporation), and the Wellcome Trust.

REFERENCES

Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B. B. (1979). *Nature (London)* **282,** 615–616.

Anonymous (1973). *Nature (London)* **241,** 499.

Arnon, R. (1980). *Annu. Rev. Microbiol.* **34,** 593–618.

Audibert, F., Jolivet, M., Chedid, L., Alouf, J. E., Boquet, P., Rivaille, P., and Siffert, O. (1981). *Nature (London)* **289,** 593–594.

Beachey, E. H., Seyer, J. M., Dale, J. B., Simpson, W. A., and Kang, A. H. (1981). *Nature (London)* **292,** 457–459.

Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G., Lerner, R. A., Rowlands, D. J., and Brown, F. (1982). *Nature (London)* **298,** 30–33.

Burrell, C. J., Leadbetter, G., MacKay, P., and Marmion, B. P. (1976). *J. Gen. Virol.* **33,** 41–50.

Burrell, C. J., MacKay, P., Greenaway, P. J., Hofschneider, P. H., and Murray, K. (1979). *Nature (London)* **279,** 43–47.

Charnay, P., Gervais, M., Louise, A., Galibert, F., and Tiollais, P. (1980). *Nature (London)* **286,** 893–895.

Copeland, J. A., Skelly, J., Mann, G. F., Howard, C. R., and Zuckerman, A. J. (1980). *J. Med. Virol.* **5,** 257–264.

Daemer, R. J., Feinstone, S. M., Alexander, J. J., Tully, J. G., London, W. J., Wong, D. C., and Purrell, R. H. (1980). *Infect. Immun.* **30,** 607–611.

Das, P. K., Nayak, N. C., Tsiquaye, K. N., and Zuckerman, A. J. (1981). *Br. J. Exp. Pathol.* **61,** 648–654.

Dressman, G. R., Chairez, R., Suarez, M., Hollinger, R. B., Courtney, R. J., and Melnick, J. L. (1975). *J. Virol.* **16,** 508–515.

Dressman, G. R., Hollinger, F. B., Sanchez, Y., Oefinger, P., and Melnick, J. L. (1981). *Infect. Immun.* **32,** 62–67.

Dreesman, G. R., Sanchez, Y., Ionescu-Matiu, I., Sparrow, J. T., Six, H. R., Peterson, D. L., Hollinger, F. B., and Melnick, J. L. (1982). *Nature (London)* **295,** 158–160.

Dubois, M. F., Pourcel, C., Pousset, S., Chany, C., and Tiollais, P. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **17,** 4549–4555.

Edman, J. C., Hallewell, R. A., Valenzuela, P., Goodman, H. M., and Rutter, W. J. (1981). *Nature (London)* **291,** 503–506.

Erickson, B. W., and Merrifield, R. B. (1976). In "The Proteins" (H. Neurath and R. Hill, eds.), pp. 257–528. Academic Press, New York.

Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P., and Charnay, P. (1979). *Nature (London)* **281,** 646–650.

Gerlich, W. H., Feitelson, M. A., Marion, P. L., and Robinson, W. S. (1980). *J. Virol.* **36,** 787–795.

Gold, J. W. M., Shih, J. W. -K. Purcell, R. H., and Gerin, J. L. (1976). *J. Immunol.* **117,** 1404–1406.

Hardy, K., Stahl, S., and Kupper, H. (1981). *Nature (London)* **293,** 481–483.

Hayman, M. J., Skehel, J. J., and Crumpton, M. J. (1973). *FEBS Lett.* **29,** 185–188.

Hirschman, S. Z., and Garfinkel, E. (1982). *Hepatology* **2** (Suppl) 795–845.
Hirschman, S. Z., Price, P., Garfinkel, E., Kristman, J., and Acs, G. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5507–5511.
Hopp, T. P. and Woods, K. R. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3824–3828.
Howard, C. R., and Burrell, C. J. (1976). *Prog. Med. Virol.* **22**, 36–103.
Howard, C. R., and Zuckerman, A. J. (1974). *Intervirology* **4**, 31–44.
Howard, C. R., Skelly, J., Tabor, E., Gerety, R. J., Kremastinou, J., Tsiquaye, K. N., and Zuckerman, A. J. (1982). *In* "Viral Hepatitis 1981" (International Symposium) (W. Szmuness, H. J. Alter, and J. E. Maynard, Eds.), pp. 411–423. Franklin Inst. Press, Philadephia, Pennsylvania.
Karelin, V. P., and Zhdanov, V. M. (1981). *Mol. Virol.* **18**, 237–244.
Lerner, R. A., Green, N., Alexander, H., Liu, F. -T., Sutcliffe, G., and Shinnick, T. M. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3403–3407.
MacKay, P., Pasek, M., Magazin, M., Kovacic, R. T., Allet, B., Stahl, S., Galibert, W., Schaller, H., Bruce, S., and Murray, K. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4510–4514.
Mann, G. F., Copeland, J. A., Skelly, J., Howard, C. R., and Zuckerman, A. J. (1982). *In* "Viral Hepatitis, 1981" (International Symposium) (W. Szmuness, H. J. Alter, and J. E. Maynard, eds.), pp. 69–80. Franklin Inst. Press, Philadelphia, Pennsylvania.
Melnick, J. L., Dreesman, G. R., and Hollinger, F. B. (1976). *J. Infect. Dis.* **133**, 210.
Mishiro, S., Imai, M., Takahaski, K., Machida, A., Gotanda, T., Miyakawa, Y., and Mayumi, M. (1980). *J. Immunol.* **124**, 1589–1593.
Moriarty, A. M., Hoyer, B. H., Shih, J. W. -K., Gerin, J. L., and Hamer, D. H. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2606–2610.
Muller, G. M., Shapira, M., and Arnon, R. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 569–573.
Neurath, A. R., Strick, N., and Huang, C. Y. (1978). *Intervirology* **10**, 265–275.
Pasek, M., Goto, T., Gilbert, W., Zink, B., Schaller, H., MacKay, P., Leadbetter, G., and Murray, K. (1979). *Nature (London)* **282**, 575–579.
Peterson, D. L. (1981). *J. Biol. Chem.* **256**, 6975–6983.
Peterson, D. L., Roberts, I. M., and Vyas, G. N. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1530–1534.
Prince, A. M., Ikram, H., and Hopp, T. P. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 579–582.
Rao, K. R., and Vyas, G. N. (1973). *Nature (London) New Biol.* **241**, 240–241.
Sachs, D. H., Schechter, A. N., Eastlake, A., and Anfinsen, C. B. (1972). *J. Immunol.* **109**, 1300–1310.
Shih, J. W. -K., and Gerin, J. L. (1975). *J. Immunol.* **115**, 634–639.
Shih, J. W. -K., and Gerin, J. L. (1977). *J. Virol.* **21**, 347–357.
Shih, J. W. -K., Tan, P. L., and Gerin, J. L. (1978). *J. Immunol.* **120**, 520–525.
Shiraishi, H., Kohama, T., Shirachi, R., and Ishida, N. (1977). *J. Gen. Virol.* **36**, 207–210.
Siddiqui, A., Sattler, F., and Robinson, W. S. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4664–4668.
Simons, K., Helenius, A., and Garoff, H. (1973). *J. Mol. Biol.* **80**, 119–133.
Skelly, J., Howard, C. R., and Zuckerman, A. J. (1978). *J. Gen. Virol.* **41**, 447–457.
Skelly, J., Howard, C. R., and Zuckerman, A. J. (1979a). *J. Gen. Virol.* **44**, 679–689.
Skelly, J., Copeland, J. A., Howard, C. R., and Zuckerman, A. J. (1979b). *Nature (London)* **282**, 617–618.
Skelly, J., Howard, C. R., and Zuckerman, A. J. (1981a). *Nature (London)* **290**, 51–54.
Skelly, J., Howard, C. R., and Zuckerman, A. J. (1981b). *J. Virol. Methods* **3**, 51–59.
Sninsky, J. J., and Cohen, S. N. (1982). *Hepatology* **2** (Suppl), 725–785.

Tabor, E., Copeland, J. A., Mann, G. F., Howard, C. R., Skelly, J., Snoy, P., Zuckerman, A. J., and Gerety, R. J. (1981). *Intervirology* **15,** 82–86.

Tabor, E., Howard, C. R., Skelly, J., Snoy, P., Goudeau, A., Zuckerman, A. J., and Gerety, R. J. (1982). *J. Med. Virol.* **10,** 65–74.

Valenzuela, P., Gray, P., Quiroga, M., Zaldivan, J., Goodman, H. M., and Rutter, W. J. (1979). *Nature (London)* **280,** 815–819.

Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G., and Hall, B. D. (1982). *Nature (London)* **298,** 347–350.

Vyas, G. N. (1981). *In* "Hepatitis B Vaccine" (INSERM Symposium No. 18) (P. Maupas and P. Guesry, eds.), pp. 227–236. Elsevier/North-Holland, Amsterdam.

Wen, Y. -M., Copeland, J. A., Mann, G. F., Howard, C. R., and Zuckerman, A. J. (1981). *Arch. Virol.* **68,** 157–163.

Wilson, G. S. (1967). "The Hazards of Immunization." Oxford Univer. Press (Athlone), London and New York.

Young, P., Vaudin, M., Dixon, J., and Zuckerman, A. J. (1982). *J. Virol. Methods* **4,** 177–185.

Zuckerman, A. J. (1975). *Nature (London)* **255,** 104–105.

Zuckerman, A. J. (1976). *Lancet* **1,** 1396–1397.

Zuckerman, A. J., and Howard, C. R. (1973). *Nature (London)* **246,** 445–447.

13

Vaccination of Newborns against Hepatitis B

F. DEINHARDT, W. JILG, R. ZACHOVAL, J. ABB, M. ROGGENDORF, AND G. ZOULEK

Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, Munich, Federal Republic of Germany

I. DEVELOPMENT OF VACCINES AGAINST HEPATITIS B

The most significant advance in the prophylaxis against hepatitis B (HB) was the development of a vaccine, a development which began with Krugman's studies in the late 1960s and resulted in the production of safe, effective, nonliving vaccines against HB (Krugman *et al.*, 1970, 1971; Hilleman *et al.*, 1982; Krugman, 1983; for general review see Deinhardt and Deinhardt, 1983). These vaccines consist of purified HBsAg extracted from the plasma of HBsAg carriers; although they contain no complete virus, no detectable DNA, HBcAg, or DNA polymerase, the vaccines are nevertheless inactivated as an additional precaution against occasional contamination with hepatitis B virus (HBV) particles, hepatitis non-A, non-B virus (HNANBV), or other viruses which could be present in human plasma. In addition to the purification procedures, one manufacturer (Merck, Sharp & Dohme, West Point, PA) uses three independent inactivation steps proved to inactivate all known viruses including slow viruses. The inactivation steps consist of treatment with pepsin at pH 2, 8 *M* urea, and 1:4000 formalin at 36°C for 72 hr (Hilleman *et al.*, 1982). Another manufacturer

ISBN 0-12-429601-7

(Institut Pasteur, Paris) relies on the use of human plasma free of HBeAg, the purification procedure, and inactivation with formalin (Adamowicz *et al.*, 1981). The HB vaccines licensed in various countries do not induce systemic side effects, and only minor local reactions occur in about 5% of the persons vaccinated. Local reactions observed in the past in adults, small children, or newborns consisted of reddening, tenderness, and swelling at the infection site, but normal activities of the vaccinees were not inhibited. The production of the vaccines must adhere to guidelines established by the World Health Organization (WHO) in 1981 and reconfirmed by a WHO informal expert consultation in May 1982 (WHO, 1981; Deinhardt and Gust, 1982). Although the production of the currently licensed hepatitis B vaccines is rigidly controlled, and it is generally accepted that these vaccines are probably the safest vaccines ever developed, questions have been raised about the adequacy of chimpanzee safety tests or the formalin inactivation. These doubts have been considered by various groups of experts and have been dispelled, and the lack of induction of hepatitis or other diseases including the acquired immune deficiency syndrome (AIDS) by the vaccines in almost a million persons vaccinated is a convincing confirmation of these opinions (Centers for Disease Control, 1982; Deinhardt and Gust, 1982; Brede *et al.*, 1983; Gerety and Tabor, 1983; Macek, 1983; Tabor *et al.*, 1983; Zuckerman *et al.*, 1983).

II. EFFICACY OF VACCINES

In various efficacy trials in healthy adults, 95–98% of vaccinees developed anti-HBs, and it was shown that persons developing anti-HBs as a result of vaccination were protected against infections with HBV. Immunization with vaccines containing only one HBsAg subtype always induced antibodies against the common antigenic component *a* besides antibodies against the subtype-specific antigens, and the vaccines protected against actual infection with a heterologous HBV subtype (Maupas and Guesry, 1981; Szmuness *et al.*, 1981a; Deinhardt and Gust, 1982; Krugman, 1983; see also "Worldwide Control of Hepatitis B," 1983). In these studies, three or four inoculations were given over a period of 6 to 12 months, respectively, and preliminary observations suggest that immunity lasts for at least 3–5 years. In general, women reacted somewhat faster and with higher antibody titers than man, and young individuals better than older persons over 50 years of age, but even individuals over 70 years were vaccinated successfully. Less effective immunization has so far been achieved in immunologically compromised persons, particularly dialysis patients and children with hematopoietic malignancies. Using standard vaccination protocols, only 50–70%

of dialysis patients seroconvert (develop anti-HBs). In contrast, children with osteogenic sarcomas, thalassemia patients, hemophiliacs, and drug abusers react normally.

III. INDICATIONS FOR VACCINATION

The hepatitis B vaccine is not a vaccine for mass vaccination of entire populations; its use currently is restricted to individuals with a high risk of HBV infection. Recommendation for the type of high-risk groups to be vaccinated which have been formulated in various countries are very similar and include the following: medical and dental personnel including all health care workers with frequent blood contact; hemodialysis patients and staff; recipients of certain blood products; patients receiving large amounts of blood transfusions; patients before major surgery; patients and staff of institutions for the mentally retarded; newborns of HBV-carrier mothers; household contacts of HBV carriers; long-term prisoners; illicit injectable drug users; individuals with a frequent change of sexual partners; travelers to areas in which hepatitis B has a high prevalence if close contact with the local population can be expected or if a prolonged stay is planned.

IV. PASSIVE–ACTIVE IMMUNIZATION

A. Adults

In some instances it would be desirable to achieve an immediate immunity, for example, for patients newly admitted to dialysis units, personnel transferred into high-risk situations after accidental infections, and particularly newborns of HBsAg-positive mothers.

Immediate protection can be achieved by passive–active immunization. Several studies have shown that the simultaneous administration of hepatitis B immunoglobulin (HBIG) at one site and hepatitis B vaccine at the contralateral site induces passive immunity within a few hours and does not suppress the active response to the vaccine (Deinhardt 1981; Deinhardt *et al.*, 1981; Szmuness *et al.*, 1981a,b; Goudeau *et al.*, 1982). Representative results of one study are given in Fig. 1. Healthy young adults were vaccinated with 20 μg of HBsAg in adjuvant (H-B-VAX; Merck, Sharp & Dohme) at time 0 and at 1 and 6 months. Group 1 received no HBIG, group 2 received 3 ml of HBIG (200 IU anti-HBs/ml) at a contralateral site at the time of the first vaccination, and group 3 received the same dose of HBIG at the time of the first and the second vaccination. As can be seen, there

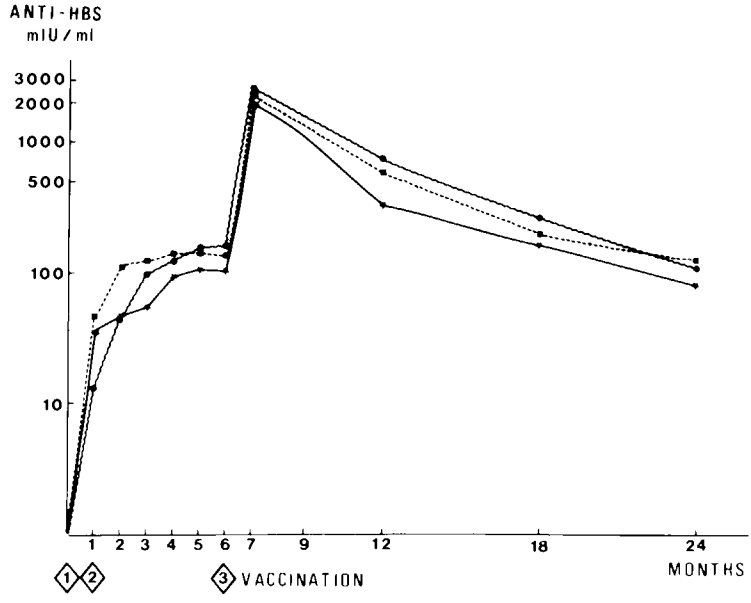


Fig. 1. Anti-HBs after active and passive-active immunization. (●) Group 1 (active); (*) group 2; (■) group 3 (passive-active). Based on unpublished data of F. Deinhardt, W. Jilg, R. Zachoval, G. Zoulek, M. Roggendorf, J. Abb, and B. Lorbeer.

was no difference in immune response between the three groups (Zachoval *et al.*, 1981; Deinhardt *et al.*, 1983).

B. Newborns

Passive-active immunization is particularly important in areas with a medium to high prevalence of HB; transmission occurs often from HBV-positive mothers to their offspring at birth or during the first years of life. These early infections become chronic more frequently than infections acquired later in life, and these individuals have a high risk of developing hepatocellular carcinomas (HCC). According to a recent statement of a WHO scientific group, about 80% of human HCCs are attributable to infection with HBV (Maupas and Melnick, 1981; Zuckerman *et al.*, 1983). Therefore identification of pregnant women who are HBsAg carriers and vaccination of their babies immediately after birth should be encouraged. It has already been shown in several studies that almost 100% of vaccinated babies produce anti-HBs and that this prevents the development of an HBV carrier state even if HBV infection is not prevented; i.e., newborns and infants in whom vaccination was started at birth, 3, 6, or 12 months of age

appear to react equally well to the vaccine, and, as in adults, there is no interference between the passively administered anti-HBs and the vaccine. However, it has not been shown whether the administration of HBIG is needed or if active vaccination alone would prevent clinical HB and the development of a carrier state. This question is of considerable economic importance, and current and future studies will decide between the choices of passive–active or only active immunization (Barin *et al.*, 1982; Beasley, *et al.*, 1981, 1983; Maupas *et al.*, 1981; Prozesky *et al.*, 1983; Rosendahl, 1983; Stevens, 1982, personal communication).

Considering the frequency of HCC and the HBV carrier rates among childbearing women, particularly in some of the countries of the Middle East and Africa, vaccination of newborns should have one of the highest priorities in any program of vaccination against HB. The interruption of the chain of infection from mothers to their offspring would be most effective in efforts to control HB in these countries because vaccination of newborns prevents not only disease, but also the establishment of new carriers who would serve as further sources for the spread of HBV. Such programs are most urgently needed in those countries of the Sub-Saharan region of Africa with a very high incidence of HB, but they are also indicated in the countries of the Middle East with a medium or medium-to-high prevalence of HB.

The public health authorities in these countries should therefore be encouraged and supported in establishing such vaccination programs as have been suggested recently by scientific groups meeting under the auspices of the WHO (Tables I and II) (Deinhardt and Gust, 1982; Zuckerman *et al.*, 1983).

TABLE I

Patterns of Hepatitis B Prevalence[a]

Low	Intermediate	High
HBsAg, 0.2–0.5%	HBsAg, 2–7%	HBsAg, 8–20%[b]
Anti-HBs, 4–6%	Anti-HBs, 20–55%	Anti-HBs, 70–95%
Childhood infection infrequent	Childhood infection frequent, neonatal infection frequent	Childhood infection highly frequent, neonatal infection highly frequent
Australia, Central Europe, North America	Eastern Europe, Japan, Mediterranean South-West Asia, USSR, parts of the Middle East	Some parts of China, southern Asia, tropical Africa, parts of the Middle East

[a] Reprinted from Deinhardt and Gust, (1982).

[b] Prevalence up to 50% has been identified in some isolated Pacific islands.

TABLE II

Recommendations for Hepatitis B Vaccine Prophylaxis according to Prevalence of HBV[a]

Low prevalence		Intermediate or high prevalence	
Preexposure	Postexposure	Preexposure[b]	Postexposure[b]
High-risk groups (health care personnel, dialysis patients, drug addicts, male homosexuals, military recruits)	Accidental percutaneous exposure, infants of HBsAg-positive mothers, sexual contacts of acute cases, and carriers	All infants	Infants of HBsAg-positive mothers

[a]Reprinted from Deinhardt and Gust (1982).
[b]Depending on availability of vaccine.

REFERENCES

Adamowicz, P., Gerfaux, G., Platel, A., Muller, L., Vacher, B., Mazert, M. C., and Prunet, P. (1981). *In* "Hepatitis B Vaccine" (P. Maupas and P. Guesry, eds.), pp. 37–50. Elsevier/North-Holland, Amsterdam.

Barin, F., Goudeau, A., Denis, F., Yvonnet, B., Chiron, J. P., Coursaget, P., and Diop Mar, I. (1982). *Lancet* **1**, 251.

Beasley, R. P., Lin, C. C., Wang, K. Y., Hsieh, F. J., Hwang, L. Y., Stevens, C. E., Sun, T. S., and Szmuness, W. (1981). *Lancet* **2**, 388.

Beasley, R. P., Hwang, L. Y., Stevens, C. E., Lin, C. C., Hsieh, F. J., Wang, K. Y., Sun, T. S., and Szmuness, W. (1983). *Hepatology* **3**, 135.

Brede, H. D., Deinhardt, F., Habermehl, K. O., Mass, G., and Abb, J. (1983). *Dtsch. Med. Wochenschr.* **19**, 754.

Centers for Disease Control (1982). Hepatitis B virus vaccine safety: Report of an interagency group. *Morbidity and Mortality Weekly Report* **31**, 465.

Deinhardt, F. (1981). *In* "Progress in Liver Diseases" (H. Popper and F. Schaffner, eds.), pp. 451–468. Grune & Stratton, New York.

Deinhardt, F., and Deinhardt, J. (eds.) (1983). "Viral Hepatitis: Laboratory and Clinical Science." Dekker, New York.

Deinhardt, F., and Gust, I. D. (1982). *Bull. W. H. O.* **60**, 661–691.

Deinhardt, F., Zachoval, R., Schmidt, M., Frösner, H., and Frösner, G. (1981). *In* "Hepatitis B Vaccine" (P. Maupas and P. Guesry, eds.), Elsevier/North-Holland, Amsterdam.

Deinhardt, F., Zachoval, R., Jilg, W., Lorbeer, B., and Roggendorf, M. (1983). *J. Infect.* 7, Suppl. 1, 21–25.

Gerety, R. J., and Tabor, E. (1983). *J. Am. Med. Assoc.* **249**, 745.

Goudeau, A., Coursaget, P., Barin, E., Dubois, F., Chiron, J. B., Denis, F., and Diop Mar, I. (1982). *In* "Viral Hepatitis" (W. Szmuness, H. J. Alter, and J. E. Maynard, eds.) (1981 International Symposium), pp. 509–526. Franklin Inst. Press, Philadelphia, Pennsylvania.

Hilleman, M. R., Buynak, E. B., McAleer, W. J., McLean, A. A., Provost, P. J., and Tytell,

A. A. (1982). *In* "Viral Hepatitis (W. Szmuness, H. J. Alter, and J. E., Maynard, eds.) (1981 International Symposium), pp. 385–397. Franklin Inst. Press, Phildelphia, Pennsylvania.

Krugman, S. (1983). *In* "Viral Hepatitis: Laboratory and Clinical Science" (F. Deinhardt and J. Deinhardt, eds.), pp. 257–263. Dekker, New York.

Krugman, S., Giles, G. P., and Hammond, J. (1970). *J. Infect. Dis.* **122,** 432.

Krugman, S., Giles, G. P., and Hammond, J. (1971). *J. Am. Med. Assoc.* **217,** 41.

Macek, C. (1983). *J. Am. Med. Assoc.* **249,** 685.

Maupas, P., and Guesry, P. (eds.). (1981). "Hepatitis B Vaccine." Elsevier/North-Holland, Amsterdam.

Maupas, P., and Melnick, J. L., eds. (1981). *Prog. Med. Virol.* **27,** 1–210.

Maupas, P., Barin, F., Chiron, J. P., Coursaget, P., Goudeau, A., Perrin, J., Denis, F., and Diop Mar, I. (1981). *Lancet* **1,** 575.

Prozesky, O. W., Stevens, C. E., Szmuness, W., Rolka, H., Harley, E. J., Kew, M. C., Scholty, J. E. R., and Mitchell, A. D. (1983). *J. Infect.* 7, Suppl. 1, 53–55.

Rosendahl, C. (1983). *In* "Impfung gegen Hepatitis B: Erfahrungen 10 Monate nach der Zulassung und Voraussage für die Zukunft" (F. Deinhardt and H. Spiess, eds.), pp. 55–61, Med. Verlagsges. Marburg, West Germany.

Szmuness, W., Stevens, C. E., Zang, E. A., Harley, E. J., and Kellner, A. (1981a). *Hepatology* **1,** 377.

Szmuness, W., Oleszko, W. R., Stevens, C. E., and Goodman, A. (1981b). *Lancet* **1,** 575.

Tabor, E., Buynak, E., Smallwood, L. A., Snoy, P., Hilleman, M., and Gerety, R. J. (1983). *J. Med. Virol.* **11,** 1.

"Worldwide Control of Hepatis B " (1983).(2nd International IABS Symposium on Viral Hepatitis) *J. Infect.* 7, Suppl. 1.

WHO. (1981). *W. H. O. Tech. Rep. Ser.* **658,** 1–156.

Zachoval, R., Frösner, G., and Deinhardt, F. (1981). *Münch. med. Wschr.* **123,** 1506–1508.

Zuckerman, A. J., Tsung-Tang, S., Linsell, A., and Stjenswärd, J. (1983). *Lancet* **1,** 463.

14

Hepatitis B Vaccine and the Developing World

ALFRED M. PRINCE

Laboratory of Virology, The Lindsley F. Kimball Research Institute of The New York Blood Center, New York, New York

I. INTRODUCTION

Hepatitis B virus (HBV) infections occur throughout the world, but in radically differing frequency. This is best appreciated by comparison of the frequency of chronic carriers in different regions. In Europe and North America, the chronic carrier state occurs in 1–2 per 1000 inhabitants. In marked contrast, in most parts of Africa and Asia and in many parts of southern Europe and the Middle East, 5–30% of the population are chronic carriers of this virus (Sobeslavsky, 1978).

The epidemiology of HBV infection also differs markedly in different parts of the world. In low carrier-prevalence regions, a high proportion of infections occur among adults in high-risk groups such as multiply transfused persons, renal dialysis patients and staff, sexually promiscuous persons, illicit drug users, and medical staff. By contrast, in high-prevalence regions, infections tend to occur in childhood and derive largely from contact with carriers in the family or with other infected infants and children. In West Africa, the annual incidence of new HBV infections is approximately 25% in each of the first 3 years of life (Prince *et al.*, 1981). As a

ISBN 0-12-429601-7

consequence of the different epidemiology of HBV infection in different parts of the world, strategies for prevention must also differ widely. In the lowest prevalence regions, active immunization is likely to be limited to high-risk groups. However, in the highest prevalence regions, all infants are clearly at high risk, and therefore universal immunization is required.

In this review, I pose essentially three questions: (1) Is mass immunization in high prevalence regions, assuming it to be economically feasible, really needed? (2) Is mass immunization feasible with a vaccine derived from human plasma? (3) What alternatives to a plasma-derived vaccine are on the horizon?

II. BASIS FOR A NEED FOR MASS IMMUNIZATION IN HIGH-PREVALENCE REGIONS

In most high-prevalence regions acute hepatitis B infections are rarely recognized, occurring subclinically during infancy or childhood. Thus, if the goal of mass immunization is to prevent acute hepatitis, its importance could well be questioned. However, a high proportion of subclinical infections by HBV occurring in childhood progress to the chronic carrier state, which in turn may lead to serious disease in later life: chronic active hepatitis, cirrhosis, and most important, hepatocellular carcinoma (HCC). The evidence implicating the HBV chronic carrier state as an etiologic factor in the development of HCC can be summarized as follows.

1. *Geographical association.* The incidence of HCC varies widely throughout the world and in general tends to parallel the prevalence of the HBV carrier state. The incidence of HCC is lowest (2–5/100,000 males per year) in regions such as Western Europe and North America with carrier rates of 1–2/1000, and is highest (50–100/100,000 males per year) in regions of Africa and Asia having carrier rates of 10–30%.

2. *Association with disease.* It has been found that 60–70% of African cases of HCC, 40–50% of Asian cases, and 40–50% of United States cases are HBV carriers (reviewed in Prince, 1980). In all studies, including those with carefully matched controls (Prince *et al.*, 1975), the frequency of HBV infection in HCC cases is much higher than that of controls.

3. *Evidence that HBV infection precedes HCC.* In Africa, HBV infections, as mentioned above, occur predominantly in infancy, yet HCC usually occurs in adults between the ages of 30 and 50. Furthermore, a high proportion of mothers of HCC cases have been found to be chronic HBV carriers, suggesting that many cases acquired their infections by perinatal transmission from their mothers (Blumberg *et al.*, 1975). The association

between HBV infection and HCC thus cannot be explained by a higher susceptibility of HCC cases to this infection.

4. *Prospective studies.* The above conclusion has been further substantiated in prospective follow-up studies designed to determine the risk of development of HCC in HBV carriers. The largest of these studies, carried out by Beasley and his colleagues in Taiwan, is particularly important. In this study, 3454 male HBV carriers over the age of 40, and 19,253 males free of the hepatitis B surface antigen (HBsAg) were followed prospectively with serial determinations of α-fetoprotein and annual medical examinations for an average of 2½ years (Beasley *et al.,* 1981). Forty cases of HCC occurred among the 3454 HBV carriers as compared to only 1 among the 19,253 noncarriers. The relative risk, 223:1, is the highest ever determined for a potential carcinogenic factor in man. A second dramatic finding in this study was the fact that, of all deaths occurring in the male carriers during the follow-up period, 38% were due to HCC and 16% to cirrhosis. This suggests that these conditions are the major causes of death in male HBV carriers in this age group.

5. *Woodchuck hepatitis virus (WHV).* A virus closely resembling HBV morphologically, immunologically, and biochemically has been found to be prevalent in woodchucks in the eastern United States (Summers *et al.,* 1978). In one large captive colony of these animals, 29% of animals over the age of 4 died with HCC. All the animals which developed this tumor were found to be chronic carriers of WHV.

6. *Molecular biological studies.* Molecular hybridization studies with cloned ^{32}P-labeled HBV DNA have revealed that most cases of HCC have HBV DNA sequences, integrated into host cellular DNA (reviewed in Prince, 1981). Integration appears to be at a limited number of sites. Integrated HBV DNA has also been detected in cases of HCC occurring in the absence of serum HBsAg, or in the presence of anti-HBs suggesting that in older carriers, HBsAg levels in serum may fall to undetectable levels while integrated HBV DNA remains present in the genome (Brechot *et al.,* 1982). The molecular biological findings resemble those of known oncogenic DNA viruses such as SV40 and polyoma, thus further strengthening the conclusion that HBV is an oncogenic agent in man.

The accumulating evidence linking the HBV chronic carrier state to the etiology of HCC, the most common cancer occurring in males in many parts of the world, is a strong indication for attempts to prevent development of the HBV carrier state by mass immunization. If such mass immunization eventually results in a parallel fall of HBV carrier-state prevalence and HCC incidence, this would provide the last link in the chain of evidence indicating HBV to be a causative factor in the etiology of this cancer.

III. APPROACHES TO PREVENTION

A. Vaccines Derived from Human Plasma

Krugman *et al.*, in a pioneering study reported in 1971, were the first to show that an immunogen providing protection against HBV infection was present in the plasma of a chronic HBV carrier (Krugman *et al.*, 1971). The demonstration that antibody to the hepatitis B surface antigen (anti-HBs) could aggregate the characteristic 20-nm surface antigen particles together with the complete virion—the 42-nm Dane particle—indicated that the protective immunogen was in all likelihood the surface antigen (HBsAg). Thus, to provide more potent antigenic material, procedures for large-scale purification of the surface antigen were developed. This resulted in 1975 in the first experimental vaccines composed of substantially pure HBsAg particles inactivated by formaldehyde (Purcell and Gerin, 1975; Hilleman *et al.*, 1975). These procedures were first adapted to large-scale manufacture by Merck, Sharp & Dohme in the United States (Hilleman *et al.*, 1982) and by Institut Pasteur in France (Adamowicz *et al.*, 1981). Both of the resulting vaccines have been proved safe and highly effective in controlled clinical trials (Szmuness *et al.*, 1980; Crosnier *et al.*, 1981) and both are now licensed and available for use.

Unfortunately, these vaccines are costly: presently more than $100 for immunization with three 20-μg doses for the Merck, Sharp & Dohme vaccine. Thus, application of the present vaccines to mass immunization in developing countries is not yet practical.

Undoubtedly, a major contribution to the present high cost of this vaccine relates to the need to recover the considerable investment made by the producers in research and development, clinical trials, and construction of plant and facilities for production. This should soon be amortized.

An additional portion of the high cost of present vaccines derives from the complex purification methodology employed. Both manufacturers make extensive use of rate zonal and isopycnic centrifugation steps in large-volume Electronucleonics K-2 ultracentrifuge rotors.

In an attempt to achieve greater economy in vaccine manufacture, we have developed a purification methodology in which a combination of polyethylene glycol preparation steps and hydroxyapatite adsorptions permits the bulk of the purification process to be completed prior to a single final isopycnic centrifugation in which the antigen derived from as much as 100 liters of plasma can be processed (Prince and Vnek, 1983). So far, limited clinical trials of this vaccine have shown it to be safe and highly immunogenic (Kaufmann *et al.*, 1983).

Another factor affecting economy is the type of source plasma used. The

Institut Pasteur vaccine uses HBeAg-negative plasma, as this is known to be less infective (Shikata *et al.*, 1977). However, the HBsAg content of such plasma pools is usually one-tenth that of HBeAg(+) plasma which we and Merck, Sharp & Dohme have used. Thus, 10-fold more plasma must be processed for a given yield of purified HBsAg. Because of the centrifugation steps used to remove Dane particles, and subsequent virus inactivation procedures, it has been shown that a safe product can be made from the initially more infective HBeAg(+) plasma. In the case of the Merck vaccine, an impressive safety margin is achieved by the use of three different inactivation steps: pepsin digestion at pH 2.0, exposure to 8 *M* urea, and treatment with formalin, each of which has been found to inactivate at least 10^5 chimpanzee infectious doses of HBV (Tabor *et al.*, 1983).

A particularly economical approach to vaccine manufacture has been proposed by workers at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Brummelhuis *et al.*, 1981). This vaccine is made by extracting plasma with fluorocarbon, fractional precipitation with polyethylene glycol, and a single pelleting of HBsAg in the ultracentrifuge. The vaccine is inactivated by exposure to 101°C for 90 sec, adsorbed to alum, and then further inactivated by pasteurization. This vaccine differs from all others in that no attempt has been made to achieve complete purity; indeed only about 0.5% of the protein in the vaccine is HBsAg, the remainder being human serum protein. In clinical trials, this vaccine has been found to be effective and highly immunogenic (Reerink-Brongers *et al.*, 1983). Even doses of less than 1 μg of HBsAg have produced a high rate of seroconversion. No side effects due to the presence of serum proteins (about 600 μg per dose) have been detected. If the safety of such a partially purified vaccine is further confirmed by additional experience, it would bring into question the requirement for rigid and expensive purification methodologies.

A major factor in the cost of plasma-derived vaccines is the cost of the source plasma itself. In the United States, high-titer HBsAg containing plasma obtained by plasmapheresis can cost $300–1000 per liter. If plasma-derived vaccines are to be used in developing countries, it seems obvious that the plasma source should be derived from the countries which will use the vaccine. This would result in significant reductions in cost.

An additional factor in the present high cost of plasma-derived vaccines is the requirement that each production lot be safety tested in chimpanzees. It is to be hoped that regulatory authorities will allow this requirement to be dispensed with when adequate data for inactivation process efficacy are available and when consistent safety has been shown in experience with successive lots.

Perhaps the ultimate factor in the cost equation is the dose required for

effective immunization. If plasma-derived vaccines are to be used for mass immunization in the developing world, it is critical that *minimal* quantities of HBsAg and minimal frequencies of administration be investigated. It is encouraging in this respect that the immune response to HBsAg seems to increase with decreasing age (Coursaget *et al.*, 1983). It is thus likely that effective immunization of infants can be accomplished with much lower doses than those currently used for adults. Furthermore, present dosages and regimens have been aimed at producing high levels of anti-HBs and complete immunity to HBV infection. One may question whether this is really necessary for prevention of carrier-state infections in infancy. It is possible that even a single small dose of vaccine given at birth, or during the first few months of life, would produce sufficient immunological memory to convert subsequent naturally acquired infections into mild and transient subclinical infections resulting in lasting immunity.

Three adult doses of HBV vaccine have been found to be effective in preventing the establishment of carrier-state infections in studies carried out by Maupas and his colleagues in Senegal (Maupas *et al.*, 1981). It is now necessary to determine whether this can be accomplished with doses and regimens that are affordable and practical in the developing world.

One approach for dose reduction which should also be kept in mind is an improvement in the ratio of immunogenicity to antibody binding capacity as a result of improved antigen presentation. An intriguing example of this is the significantly improved specific immunogenicity achieved by dissociation of HBsAg particles by detergents and then reassociation into larger and more immunogenic micelles (Skelly *et al.*, 1981).

There are thus a number of approaches available which could markedly reduce the cost of plasma-derived vaccines and perhaps make this available for mass immunization. If this does not prove to be feasible, reliance will have to be placed on alternative vaccines. Fortunately, progress in this direction is rapidly being made.

B. Alternative Vaccines

1. Recombinant DNA Approaches

The genome of HBV has now been cloned and sequenced (Pasek *et al.*, 1979; Valenzuela *et al.*, 1979; Charnay *et al.*, 1979). Furthermore, the coding region of the surface antigen polypeptide has been identified. This protein has a molecular weight of about 25,000 and occurs in a glycosylated and a nonglycosylated form (Peterson *et al.*, 1978). The gene for the surface antigen has been inserted into plasmids adjacent to known bacterial promoters, in *E. coli*, and limited expression of HBsAg has been achieved

(Charnay *et al.*, 1980; MacKay *et al.*, 1981; Edman *et al.*, 1981). The yields and immunogenicity have so far been disappointing.

Another perhaps more promising approach has been to introduce the gene for HBsAg into mammalian cells *in vitro*. This was first accomplished in 1980 by Dubois *et al.* (1980), who contransfected mouse L cells with plasmids containing two HBV genomes in tandem head-to-tail arrangement and plasmids containing the herpes simplex thymidine kinase gene. Stable productive cell clones were derived in which HBV DNA was integrated into the host cell genome. Culture fluids from these cells contained about 150 ng of HBsAg per milliliter. The surface antigen produced by these cells was in the form of 22-nm lipoprotein particles similar to those found in the plasma of chronic carriers, and has been found to be highly immunogenic (Pourcel *et al.*, 1982). Others have introduced the surface antigen gene in the form of a recombinant with portions of the SV40 virus genome (Moriarty *et al.*, 1981; Liu *et al.*, 1982). Cells infected with these recombinant viruses have produced substantial quantities of HBsAg in the form of 22-nm lipoprotein particles.

Another potentially practical source of HBsAg for vaccine production has been provided by the demonstration that the HBsAg gene can be expressed in yeast (Valenzuela *et al.*, 1982; Miyanohara *et al.*, 1983). Surprisingly, the antigen synthesized in yeast is assembled into 20-nm lipoprotein particles similar in density, morphology, and immunogenicity to those in carrier plasma. The yields so far reported are in the range of 2–5 μg of HBsAg per 10^8 yeast cells. As yeast can be inexpensively grown in unlimited quantity, this is an exciting potential source for inexpensive HBV vaccines.

2. *Synthetic Vaccines*

The availability of the complete nucleotide sequence for the gene coding for the HBsAg polypeptide has permitted its translation to yield the complete amino acid sequence. As the typical antibody combining site commonly compromises a sequence of only about six amino acids, several groups have begun to synthesize portions of this sequence in the hope of locating an epitope which could induce protective antibody (Table I). Lerner and colleagues synthesized and tested 13 different peptides derived from the HBsAg sequence: 7 of these gave rise to antibodies to the synthetic peptides when injected into rabbits after coupling to a protein carrier and 4 of these antisera reacted also with native HBsAg and immunoprecipitated the 25,000-dalton HBsAg polypeptide (Lerner *et al.*, 1981). Hopp and Woods (1981) utilized a theoretical approach for locating putative immunodominant epitopes to predict that amino acids 141–146 constitutes such a site. A synthetic peptide including this region was found to absorb 50–

TABLE I
Synthetic Antigenic HBsAg Peptides

Amino acids	Reference
48–81	Lerner *et al.* (1981)
2–16	Lerner *et al.* (1981)
22–35	Lerner *et al.* (1981)
95–109	Lerner *et al.* (1981)
138–149	Hopp and Woods (1981)
139–147	Bhatnagar *et al.* (1982)
139–158	Bhatnagar *et al.* (1982)
140–158	Bhatnagar *et al.* (1982)
122–137 (cyclic)	Dreesman *et al.* (1982)
135–155	Neurath *et al.* (1982)

75% of the predominant pooled human anti-HBs and was immunogenic in mice after coupling to carrier erythrocytes (Prince *et al.*, 1982). Bhatnagar synthesized peptides covering this same region and reported that the elicited antibodies were neutralized by all known subtypes of HBsAg (Bhatnagar *et al.*, 1982). This suggested that these peptides contained a common *a* determinant thought to be the target of protective antibody (McAuliffe *et al.*, 1980.

Dreesman *et al.* synthesized a cyclic peptide containing the amino acids 122–137 and found this to be immunogenic even in the absence of protein carriers (Dreesman *et al.*, 1982), although immunogenicity was considerably enhanced when this peptide was coupled to tetanus toxoid or transformed into micelles and adsorbed to aluminum hydroxide (Sanchez *et al.*, 1982). This group had suggested that this peptide contains a critical group determinant, since it has been found to inhibit an anti-idiotypic antibody to human anti-HBs (Kennedy and Dreesman, 1983). It is likely that there may be several group determinants, i.e., antigenic determinants common to all subtypes of HBsAg. It is not yet clear which site or sites will give rise to virus-neutralizing antibody.

Much remains to be learned before synthetic peptides become potential hepatitis B vaccines. The immunogenicity of most HBsAg peptide carrier combinations tested so far has been rather low. Neurath has also pointed out that the antibodies produced are of extremely low affinity in comparison to those elicited by the natural antigen (Neurath *et al.*, 1982). Furthermore, he found that, although anamnestic IgG anti-peptide antibody resulted from booster injections, antibody active with native HBsAg remained in the IgM subclass and was not boosted in titer.

There are also theoretical dangers in the use of synthetic peptide immunogens. Antibodies elicited by such restricted immunogens may, like monoclonal antibodies, cross-react with a variety of proteins carrying sim-

ilar amino acid sequences. Indeed a cross-reaction between a normal serum protein and the synthetic HBsAg peptide containing amino acids 135–155 has been reported (Neurath *et al.*, 1982). Thus, the possible induction of autoimmune complications must be carefully evaluated.

Moderate-sized synthetic polypeptides can be produced inexpensively on a large scale. Furthermore, it is possible that multiple epitopes, specific for a variety of different infectious agents, could be coupled into a single protein carrier to provide polyvalent vaccines. Peptides which induce protection against foot-and-mouth disease virus in animals have been reported (Bittle *et al.*, 1982). Similar success with other agents may be anticipated. Such polyvalent vaccines may provide an ideal approach to mass immunization in the developing world.

IV. CONCLUSIONS

The accumulated evidence implicating the HBV chronic carrier state as an etiologic agent of hepatocellular carcinoma justifies energetic efforts to provide for mass immunization against HBV infection in high prevalence regions of the world.

Although present vaccines derived from human carrier plasma are too costly for this purpose, it is possible that, through improvements in production technology, plasma sources, and immunogenicity, and most important by determination of minimal doses required for immunization in infancy, such vaccines may still be practical for mass use. If not, reliance will have to be placed on alternative vaccines. For this purpose, the achievement of HBsAg expression in yeast and mammalian cells *in vitro* through recombinant DNA technology, and the chemical synthesis of immunogenic HBsAg epitopes, offer exciting possibilities for the future.

REFERENCES

Adamowicz, P. H., Gerfaux, G., Platel, A., *et al.* (1981). *In* "Hepatitis B Vaccine" (P. Maupas and P. Guesry, eds.), pp. 37–50. Elsevier/North-Holland, Amsterdam.

Beasley, R. P., Hwang, L. -Y., Lin, C. -C, and Chien, C. -S. (1981). *Lancet* **2**, 1129–1133.

Bhatnagar, P. K., Papas, E., Blum, H. E., *et al.* (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4400–4404.

Bittle, J. L., Houghten, R. A., Alexander, H., *et al.* (1982). *Nature (London)* **298**, 30–33.

Blumberg, B. S., Larouzé, B., London, W. T., *et al.* (1975). *Am. J. Pathol.* **81**, 669–682.

Brechot, C., Nalpas, B., Couroucé, A. -M., *et al.* (1982). *N. Engl. J. Med.* **306**, 1384–1387.

Brummelhuis, H. G. J., Wilson-DeStürler, L. A., and Raap, A. K. (1981). *In* "Hepatitis B Vaccine" (P. Maupas and P. Guesry, eds.), pp. 51–56. Elsevier/North-Holland, Amsterdam.

Charnay, P., Pourcel, C., Louise, A., *et al.* (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2222–2226.

Charnay, P., Gervais, M., Louise, A., *et al.* (1980). *Nature (London)* **286**, 893–895.

Coursaget, P., Chiron, J., Barin, F., and Goudeau, A. (1983). *Dev. Biol. Standard.* **54**, 118–122.

Crosnier, J., Jungers, P., Couroucé, A. -M., *et al.* (1981). *Lancet* **1**, 455–459.

Dreesman, G. R., Sanchez, Y., Ionescu-Matiu, I., *et al.* (1982). *Nature (London)* **295**, 158–160.

Dubois, M. -F., Pourcel, C., Rousset, S., *et al.* (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4549–4553.

Edman, J. C., Hallewell, R. A., Valenzuela, P., *et al.* (1981). *Nature (London)* **291**, 503–506.

Hilleman, M. R., Buynak, E. B., Roehm, R. R., *et al.* (1975). *Am. J. Med. Sci.* **270**, 401–404.

Hilleman, M. R., Buynak, E. B., McAleer, W. J., *et al.* (1982). *In* "Viral Hepatitis" (1981 International Symposium) (W. Szmuness, H. J. Alter, J. E. Maynard, eds.) pp. 385–397, Franklin Inst. Press, Philadelphia, Pennsylvania.

Hopp, T. P., and Woods, K. R. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3824–3828.

Kaufmann, R., Mondorf, A., Uthemann, H., *et al.* (1983). *Dev. Biol. Standard.* **54**, 229–235.

Kennedy, R. C., and Dreesman, G. R. (1983). *J. Immunol.* **130**, 385–389.

Krugman, S., Giles, J. P., and Hammond, J. (1971). *JAMA, J. Am. Med. Assoc.* **217**, 41–45.

Lerner, R. A., Green, N., Alexander, H., *et al.* (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3403–3407.

Liu, C. -C., Yanzura, D., and Levinson, A. D. (1982). *DNA* **1**, 213–221.

McAuliffe, V. J., Purcell, R. H., and Gerin, J. L. (1980). *Rev. Infect. Dis.* **2**, 470–492.

MacKay, P., Pasek, M., Magazin, M., *et al.* (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4510–4514.

Maupas, P., Goudeau, A., Dubois, F., *et al.* (1981). *In* "Hepatitis B Vaccine" (P. Maupas and P. Guesry, eds.), pp. 117–132, Elsevier/North-Holland, Amsterdam.

Miyanohara, A., Toh-e, A., Nozaki, C., *et al.* (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1–5.

Moriarty, A. M., Hoyer, B. H., Shih, J. W.-K., *et al.* (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2606–2610.

Neurath, A. R., Kent, S. B. H., and Strick, N. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7871–7875.

Pasek, M., Goto, T., Gilbert, W., *et al.* (1979). *Nature (London)* **282**, 575–579.

Peterson, D. L., Chien, D. Y., and Vyas, G. N. (1978). *In* "Viral Hepatitis" (G. N. Vyas, S. N. Cohen, and R. Schmid, eds.), pp. 569–573. Franklin Inst. Press, Philadelphia, Pennsylvania.

Pourcel, C., Sobzack, E., Dubois, M. -F., *et al.* (1982). *Virology* **121**, 175–183.

Prince, A. M. (1980). *In* "The Role of Viruses in Human Cancer" (G. Giraldo and E. Beth, eds.), pp. 141–155, Elsevier/North-Holland, Amsterdam.

Prince, A. M. (1981). *Hepatology* **1**, 73–75.

Prince, A. M., and Vnek, J. (1983). *Dev. Biol. Standard.* **54**, 13–22.

Prince, A. M., Szmuness, W., Michon, J., *et al.* (1975). *Int. J. Cancer* **16**, 375–383.

Prince, A. M., White, T., Pollack, N., *et al.* (1981). *Infect. Immun.* **32**, 675–680.

Prince, A. M., Ikram, H., and Hopp, T. P. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 579–582.

Purcell, R. H., and Gerin, J. L. (1975). *Am. J. Med. Sci.* **270**, 395–399.

Reerink-Brongers, E. E., Lelie, P., Kuipers, H., *et al.* (1983). *Dev. Biol. Standard.* (in press). Presented at the Second International IABS Symposium on Viral Hepatitis.

Sanchez, Y., Ionescu-Matiu, I., Sparrow, J. T., *et al.* (1982). *Intervirology* **18**, 209–213.

Shikata, T., Karasawa, T., Abe, K., *et al.* (1977). *J. Infect. Dis.* **136,** 571–576.
Skelly, J., Howard, C. R., and Zuckerman, A. J. (1981). *Nature (London)* **290,** 51–54.
Sobeslavsky, O. (1978). *In* "Viral Hepatitis" (G. N. Vyas, S. N. Cohen, and R. Schmid, eds.), pp. 347–355. Franklin Inst. Press, Philadelphia, Pennsylvania.
Summers, J., Smolec, M. J., and Snyder, R. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 4533–4537.
Szmuness, W., Stevens, C. E., Harley, E. J., *et al.* (1980). *N. Engl. J. Med.* **303,** 833–841.
Tabor, E., Buynak, E., Smallwood, L. A., *et al.* (1983). *J. Med. Virol.* **11,** 1–9.
Valenzuela, P., Gray, P., Quiroga, M., *et al.* (1979). *Nature (London)* **280,** 815–819.
Valenzuela, P., Medina, A., Rutter, W. J., *et al.* (1982). *Nature (London)* **298,** 347–350.

15

Measles: A Continuing Problem or Eradication?

RAYMOND G. MARUSYK

Neurovirology Research Unit, Department of Medical Microbiology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada

I. INTRODUCTION

Measles, the disease, was first clinically described by Abu Becr (Rhazes), who in his reports also referred to the earlier descriptions of Al Yehudi. Abu Becr used the descriptive Arabic work "Hasbah" in his writings, and from that specific use we know that the measles of today is not unlike that of the ninth or tenth century (Black, 1982).

No one need be told that measles is still very much a problem disease of children, particularly in the developing countries. Unfortunately, measles virus is ubiquitous, persistent, and highly infectious. The disease is easily recognized by the clinically distinct exanthema, and one can assume that, unless vaccine protected, almost all individuals will contract the disease before adulthood. Measles is also frequently associated with such serious complications as otitis media, pneumonia, encephalitis, prolonged diarrhea, and, rarely, subacute sclerosing panencephalitis. The associated complications are described more frequently in the malnourished child.

APPLIED VIROLOGY

ISBN 0-12-429601-7

It has been reported that more than 900,000 measles-related deaths occur yearly (Walsh and Warren, 1979), but more recent unofficial World Health Organization (WHO) reports have estimated that the actual number of such deaths may be as high as 1.5 million (F. Assaad, personal communication).

II. THE VIRUS

Measles is a member of the Paramyxoviridae family, genus *Morbillivirus* (Fenner, 1976). The genus also includes the canine distemper, rinderpest, and peste des petits ruminants viruses. Members of this genus do not possess detectable neuraminidase activity (Choppin and Compans, 1974). Measles virus is composed of an outer lipoprotein envelope that contains two peplomers, the hemagglutinin (designated H) and the hemolysin (F). The internal nucleocapsid contains a negative-sense, single-stranded RNA genome of unit length. The size of the RNA genome has been accepted to be approximately 6.2 million daltons and to have a sedimentation coefficient of 50 S (Hall and Martin, 1973; Hammarskjold and Norrby, 1974). More recent studies, however, have suggested that the RNA genome is somewhat smaller (45–48 S, 5.2 million daltons; W.-C. Leung and D. L. J. Tyrrell, personal communication). The differing values being reported for the genome size of measles virus may be strain related (A. Salmi, personal communication).

Analytical electrophoresis techniques have revealed the presence of seven polypeptides within the measles virion. These polypeptides range in size from 36,000 daltons to greater than 200,000 daltons (Tyrrell and Norrby, 1978). Several of the components/polypeptides have associated biological activity, e.g., H (hemagglutinin), F (cell membrane fusion), and P (RNA polymerase?; Seifried *et al.*, 1978).

Little is known concerning the replication processes of measles virus other than that all events appear to take place intracytoplasmically (Portner and Bussell, 1973). Of interest is the observation that production of measles virus defective-interfering particle is temperature dependent, and that the presence of such particles enhances the development of persistent infections at 37°C (Tsang *et al.*, 1981). As current measles vaccine strains are attenuated, temperature-sensitive mutants and the presence of defective-interfering particles in such preparations should be closely monitored.

Although data are available concerning the possible antigenic variation (drift?) of measles virus without added selective pressure through the use of monoclonal antibody, there is no strong evidence to indicate that such changes occur in antigenic sites responsible for the induction of neutralizing antibody (L. Chui and R. G. Marusyk, unpublished observations).

III. THE DISEASE

Measles is clinically defined as (1) a generalized maculopapular rash persisting for 3 or more days; (2) a temperature of 38.3°C or higher; and (3) a cough and/or coryza and/or conjunctivitis (Centers for Disease Control, 1983a). Such cases need not be confirmed virologically or serologically if good clinical or epidemiological data are available.

American authorities have further suggested that measles cases should be classified as suspect cases (any rash illness with fever), probable cases (meets the clinical definition but there are no epidemiological or comfirmatory data), or confirmed cases. In the United States and certain other world areas, there is also a need to classify measles cases further as indigenous or imported (international or regional). Such classification schemes have been ruled necessary to the analysis of the source of infection.

Although usually associated with a typical skin rash, other body tissues may be involved in virus multiplication. A 10- to 12-day incubation period usually follows infection with measles virus but may, in some instances, be as short as 2–3 days. Virus can be isolated from the throat, tears, nasal secretions, and urine during the prodromal period and has been isolated from the blood. As the prodromal period ends, typical Koplik spots may be seen within the oral cavity. The Koplik spots have been reported to contain viral structural components (Surinaga *et al.*, 1970). As the typical measles rash becomes evident, IgM and IgG titers rise, the levels peaking at 10 days and 30 days, respectively. The immunity developed against measles virus is, fortunately, lifelong.

Occasionally, measles virus infection can lead to clinically unusual forms of disease. Atypical measles was described in partially immunized individuals by Rauk and Schmidt (1965). Seen only in patients who have received killed vaccine then been exposed to live virus, the disease is associated with a variable skin rash—macropapular becoming petechial or hemorrhagic. The rash usually begins peripherally (unlike typical measles) and spreads centripetally. Individuals diagnosed as having atypical measles are often more seriously ill than if having had typical measles infection. This particular manifestation of measles infection is not often seen because of the direct association with the use of killed vaccine preparations, but more than 2000 cases were reported during a measles epidemic in Western Canada in 1979 (D. L. J. Tyrrell, personal communication).

Of particular interest is that only one isolate of measles virus was obtained during the above epidemic (more than 10,000 cases of confirmed measles infections). The isolate obtained, designated VSC, has been shown to display unusual biological activity patterns (infectivity and hemagglutination; Marusyk and Tyrrell, 1984).

Other unusual host reponses such as measles encephalitis and subacute sclerosing panencephalitis are seen relatively infrequently, and a description of such is not within the context of this review.

IV. THE PROBLEM

Outside of the developed and major undeveloped countries, and removed from the reaches of highly efficient virus eradication programs, the populace still passively accepts measles infection as an inevitable part of childhood. No one questions that the disease is serious, but, in most instances, little if anything has been offered to change the existing circumstances.

In large villages and rural populations of the developing world, measles epidemics occur every 2–3 years (if not more often; Pereira, 1979). In such areas, many measles cases are seen during the first year of life. The highest incidence is in children between the ages of 1 and 2 years. Most children are infected with measles by the age of 3 to 4.

Previous isolation from sources of infection may prove to be more harmful than beneficial in the event that highly infectious measles is introduced to such a community. This is well documented and perhaps best illustrated by the introduction of measles to the island of Tristan da Cunha in 1959. All, save four, of the islanders contracted the disease (Gear, 1962).

When comparing measles infection in developed and nondeveloped countries, the most striking contrast seen involves the severity and outcome of the disease. The global case fatality rate in developing countries has been estimated to approach 2% (1.5 million deaths per year; F. Assaad, personal communication). Measles is found to be most severe in socioeconomically depressed areas, where there is little, if any, access to proper medical care. Unfortunately, public health officials in many of these areas are simply not made aware of cases of measles because of failure of the populace to bring such to their attention.

V. THE CONTROL OF MEASLES

Measles virus infections can be controlled through the use of vaccines (Table I). Killed vaccines were found to confer temporary immunity to measles, but unknown to the distributors of the vaccine was the fact that the inactivation procedure used had destroyed the antigenicity of the hemolysin (F) component of the virus (Norrby and Gollmar, 1975). Upon exposure to measles virus, individuals having received the killed virus vaccine may de-

TABLE I
Vaccine-Related Decrease in Measles Incidence

Year	≈ Cases/100,000 population	
	United States[a]	World (WHO)[b]
1950	200	—
1955	400	—
1960	200	—
(1963: vaccine → U.S.)		
1965	150	—
1970	20	—
1975	10	90
1980	5	70
(1982: first 37 weeks)	0.7	50

[a]From Centers for Disease Control (1983c).
[b]From F. Assaad, personal communication.

velop atypical measles (described above), with an associated anamnestic response to the hemagglutinin (as measured by hemagglutination inhibition antibody titers) and nucleocapsid (complement-fixing antibody titers). It is apparent that an immunological response to the hemolysin component is necessary for development of long-term immunity.

Currently available attenuated measles vaccines have none of the problems associated with the first such available products (Katz *et al.*, 1960). Little if any virus is spread via excretion from vaccine recipients. Evidence to date suggests that there is no need for revaccination programs other than in individuals who earlier received only the killed virus vaccine (Black, 1982) or in very young children who may have been selected for immunization.

Recent outbreaks of measles virus in the United States among college students have led authorities in a number of jurisdictions to begin area-wide immunization programs to increase the level of measles immunity among the very large population of college and university students. The high susceptibility of certain segments of this particular age group of individuals is probably related to four factors: (1) many children may not have been immunized with measles vaccine during the period immediately following its approval; (2) many children/students may have entered schools without the immunization required by laws now in effect; (3) similarly, many institutes of higher learning lacked immunization requirements for new registrants; (4) many children/students may have escaped natural measles infection because of decreasing transmission (Centers for Disease Control, 1983b).

In other countries as well, reported cases of measles have been in older age groups, confirming the observation that the average age of infection

has moved upward in partially, or improperly, immunized populations. In recent cases reported in Alberta, the mean age was 12 years with many young adults presenting with measles (Marusyk and Tyrrell, 1984). This observation is in strong contrast to the mean age of 3–4 years in areas where vaccine programs have not been introduced.

Different countries have varying regulations concerning the administration of virus vaccines. Generally, however, it is agreed that measles vaccine should be given, if at all possible, to young children between the ages of 9 and 15 months (even as young as 6 months), the exact age to be determined by particular circumstance.

VI. THE ERADICATION OF MEASLES

In those countries where measles vaccine has been introduced, there has been a profound effect on the incidence of measles and its associated complications (Hinman *et al.,* 1979). In the United States the occurrence of measles had decreased from more than 500,000 cases per year in 1960 to fewer than 1500 cases in the first half of 1982 (Table I) (Centers for Disease Control, 1982). Figures supporting the success of the United States measles eradication program have, because of the recent outbreaks described above, been subject to reexamination and are not yet available.

Based on the success of the global eradicaton of smallpox, the U.S. Public Health Service undertook a program to eliminate indigenous measles from the United States by October, 1982. The program has been largely successful, notwithstanding the recent localized outbreaks. The measles elimination strategy has three major aspects: (1) achievement and maintenance of high immunization levels; (2) development of strong and effective surveillance systems; and (3) aggressive response to the occurrence of the disease. Such goals are not difficult to set and attain in a country with a highly efficient health delivery and protection system.

The goal of global eradication of measles is highly desirable, and in light of the success of the smallpox eradication program, claims have been made that such is possible (Hopkins *et al.,* 1982). These authors, representing the Centers for Disease Control of the U.S. Public Health Service, have proposed that, through an accelerated implementation of the World Health Organization's Expanded Program on Immunization (EPI), measles eradication is feasible on both technical and epidemiological grounds.

Hopkins *et al.* (1982) pointed out that though there are several similarities between measles and smallpox injections, e.g., both viruses produce rashes, confer life-long immunity, have no animal reservoir, and do not induce a

chronic carrier state, there are sufficient points of difference which must be considered before drawing parallels between eradication of these two viruses. Two major differences are the more highly infectious nature of measles virus (Hope-Simpson, 1952) and the average age of infection with the two viruses (4 to 5 years for smallpox and less than 2 years for measles).

Based on the assumption of Hopkins *et al.* (1982), what are the prospects for the eradication of measles? One serious constraint to the success of such a program previously was the lack of a heat-stable vaccine and the inherent cost of cold-handling and delivery of the available vaccines. The development of relatively heat-stable, lyophilized vaccines and relatively inexpensive low-temperature storage containers has meant that the cost of measles vaccine has decreased to as little as $0.10 (US$) per dose (or 300 doses per barrel of oil at current market prices). Luckily, measles vaccine is capable of inducing immunity in more than 90% of the recipients. Thus far, no serious side effects or contraindications have been associated with the currently available vaccines.

Recent experiences in establishing measles-free areas would indicate that if the eradication proposal guidelines set forth by the U.S. Public Health Service (see above) and certain other governments are stringently followed, then measles eradication *should* be possible (Centers for Disease Control, 1982).

Henderson (1982) pointed to the fact that the proposal of Hopkins *et al.* (1982) to eradicate measles through the current delivery strategy of the WHO EPI is counter to the stated policy of that organization. The EPI calls for progressive expansion of childhood immunization rather than a concerted effort to eradicate one disease at a time. When one considers Henderson's arguments (1982) in context with the claims of Hopkins *et al.* (1982) that measles vaccination efforts must be directed toward a defined age group of 9 months to 5 years, it is difficult to envisage how that goal could be easily attained. Near universal immunization of those less than 5 years of age would, according to Henderson, be an almost impossible task if geopolitical problems are considered. One cannot ignore the fact that the majority of individuals to whom such a program would be directed are localized in areas subject to periods of political turmoil and irrational decision making. There is no doubt, however, that the successful implementation of a worldwide measles eradication program would be of enormous benefit to those most concerned—the young children of the developing countries.

If the problem of vaccine delivery in certain countries is problematic, no less serious is a similar situation in developed countries. Many medically qualified individuals still question the advisability of immunization against a seemingly innocuous childhood disease. If such problems of vaccine de-

livery exist in supposedly aware societies, one can hope that the apparent and effective use of measles vaccine will quickly change the attitudes of recipients and delivery personnel alike.

VII. COMMENTS

Measles has been called a universal disease (Wilson, 1962). With the introduction of such amibitious programs as that of the World Health Organization, and the increase in the number of countries developing, or advocating, mass programs for measles vaccination, it can only be hoped that Wilson's definition will become obsolete. The introduction of a stable vaccine at an affordable price would indicate that implementation of such programs could be realistically pursued. Great care must be taken, however, to ensure that for programs implemented the success rate is high. Questions concerning the exact age at which to immunize and whether very young recipients should be considered for revaccination must be successfully answered before such programs can be judged. Failure of any vaccine program, for whatever reason, will quickly discredit any attempts to reintroduce a similar or new vaccine.

Fortunately, as Nathanson *et al.* (1978) have indicated, measles requires a large population for survival and, if forced through vaccine pressure to retreat to remote or isolated pockets limited in number, may very well be amenable to the ultimate goal of eradication—so we hope.

REFERENCES

Black, F. (1982). *In* "Viral Infections of Man" (A. S. Evans, ed.), pp. 397–418. Plenum, New York.

Centers for Disease Control (1982). *Morbidity and Mortality Weekly Report* **31**, No. 38.

Centers for Disease Control (1983a). *Morbidity and Mortality Weekly Report* **31**, No. 52.

Centers for Disease Control (1983b). *Morbidity and Mortality Weekly Report* **32**, No. 8.

Centers for Disease Control (1983c). *Morbidity and Mortality Weekly Report* **32**, No. 4.

Choppin, P., and Compans, R. (1974). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. Wagner, eds.), Vol. 4, pp. 95–178. Plenum, New York.

Fenner, F. (1976). *Intervirology* **7**, 1–116.

Gear, J. (1962). *Am. J. Dis. Child.* **103**, 261–264.

Hall, W., and Martin, S. (1973). *J. Gen. Virol.* **19**, 175–182.

Hammarskjold, B., and Norrby, E. (1974). *Med. Microbiol. Immunol.* **160**, 99–104.

Henderson, D. (1982). *Lancet* **2**, 208.

Hinman, A., Brandling-Bennett, A., and Nieberb, P. (1979). *J. Am. Med. Assoc.* **242**, 1157–1162.

Hope-Simpson, R. (1952). *Lancet* **2**, 549–552.

Hopkins, D., Hinman, A., Koplan, J., and Lane, J. (1982). *Lancet* **1,** 1396–1398.
Katz, S., Kempe, C., Black, F., Lepow, M., Krugman, S., Haggerty, R., and Enders, J. (1960). *New Engl. J. Med.* **273,** 180–184.
Marusyk, R. G., and Tyrrell, D. L. (1984). *In* "Control of Virus Diseases" (E. Kurstak and R. G. Marusyk, eds.), pp. 67–78. Dekker, New York.
Nathanson, N., Yorke, J., Pianigiani, G., and Martin, J. (1978). *In* "Persistent Viruses" (J. Stevens, G. Todaro, and C. Foy, eds.), pp. 75–100. Academic Press, New York.
Norrby, E., and Gollmar, Y. (1975). *Infect. Immun.* **11,** 231–239.
Pereira, S. (1979). *Health and Population* **2,** 32–37.
Portner, A., and Bussell, R. (1973). *J. Virol.* **11,** 46–53.
Rauk, L., and Schmidt, R. (1965). *Am. J. Dis. Child.* **109,** 232–237.
Seifried, A., Albrecht, P., and Milstein, J. (1978). *J. Virol.* **25,** 781–787.
Surinaga, D., Bank, L., and Ackerman, A. (1970). *New Engl. J. Med.* **283,** 1139–1142.
Tsang, L., Chang, N., and Marusyk, R. (1981). *J. Gen. Virol.* **56,** 195–198.
Tyrrell, D. L., and Norrby, E. (1978). *J. Gen. Virol.* **39,** 219–229.
Walsh, J., and Warren, K. (1979). *New Engl. J. Med.* **301,** 967–974.
Wilson, G. (1962). *Am. J. Dis. Child.* **103,** 219–223.

16

Rubella Revaccination: Will It Be Necessary?

JENNIFER M. BEST, SIOBHAN O'SHEA, AND J. E. BANATVALA

Department of Virology, St. Thomas' Hospital and Medical School, London, England

I. INTRODUCTION

Rubella vaccines, which make congenitally acquired rubella a preventable disease, have been licensed for 13 years in the United Kingdom and United States. However, the two countries have different vaccination policies. In the United States the vaccine program aims to establish herd immunity and was initially directed toward prepubertal children of both sexes. This program has resulted in a decline in the incidence of both postnatally and congenitally acquired rubella, and there have been no major epidemics since 1969 (Centers for Disease Control, 1981). However, during 1978 and 1979 the relative attack rates increased among adolescents and young adults. Efforts were then directed toward this age group, and the incidence rate showed a dramatic decline in 1980 and 1981. In 1981, only 2077 cases of rubella per 100,000 population were reported in the United States, 83% less than in 1979 (Centers for Disease Control, 1982).

In Britain, the rubella vaccination program was initially directed toward

ISBN 0-12-429601-7

11- to 14-year-old schoolgirls. A year or two later rubella vaccination was also offered to seronegative adult women. This policy is not expected to influence the incidence of congentially acquired rubella until the mid-1980s. In 1978–1979 there was an extensive epidemic of rubella in Britain, and although the increase in the incidence of congenitally acquired rubella was somewhat less than expected, this was probably due to a large proportion of rubella-infected pregnancies being terminated (Goldwater *et al.*, 1978).

Several rubella vaccines are available. HPV77.DE5 was used in the United States until 1979, when it was replaced with RA27/3. Cendehill and RA27/3 are licensed in the United Kingdom.

The success of rubella vaccination policies depends not only on adequate uptake of vaccine, but also on the persistence of adequate immunity throughout childbearing years—approximately 30 years in the United Kingdom and even longer in the United States. Although Herrmann and colleagues (1982) have reported the persistence of HAI antibodies in 97% vaccinees, Horstmann (1975) and Balfour and Amren (1978) showed that 29 of 342 (8.5%) and 58 of 159 (36%) children, respectively, lacked HAI antibody when tested 3–9 years after vaccination with HPV77.DE5. Subsequent studies by Balfour and colleagues (1981) showed that these children had residual immunity since they developed accelerated IgG responses, little or no rubella-specific IgM, and no viremia.

We obtained sera in 1981 from adult female volunteers given one of four rubella vaccines 6–16 years earlier and tested them for rubella antibodies by single radial hemolysis (SRH) and radioimmunoassay (RIA), both of which are more sensitive than hemagglutination inhibition (HAI) for detecting low levels of antibody (O'Shea *et al.*, 1982). We also challenged volunteers with low levels of rubella antibody (< 15 IU) either naturally acquired or vaccine induced and looked for viremia and virus excretion following challenge.

II. MATERIALS AND METHODS

A. Persistence of Rubella Antibodies after Vaccination

We obtained sera from two groups of volunteers. The first group of 123 volunteers had been vaccinated with RA27/3, Cendehill, HPV77.DE5, or a Japanese vaccine, To-336, 6–8 years previously at St. Thomas' Hospital, London (Best *et al.*, 1974). Nine volunteers who had experienced naturally acquired rubella at the same time were also followed up. The second group of 38 volunteers had been given RA27/3 or Cendehill vaccines while student nurses at the Hospital for Sick Children, London (Marshall *et al.*, 1971).

Sera were tested for rubella antibodies by SRH (Kurtz *et al.*, 1980) and for rubella-specific IgG by RIA (Best *et al.*, 1980).

B. Detection of Viremia after Rubella Challenge

Fifty-one volunteers were challenged intranasally with a high-titer preparation of the vaccine strain RA27/3 ($10^{4.69}$ PFU/dose). Ten volunteers were seronegative by SRH, HAI, and RIA; 10 had antibody levels of >15 IU; and 31 had levels of <15 IU when tested by RIA. Of the 31 low-titered volunteers 12 had no history of vaccination and must therefore have had naturally acquired antibody; 19 had a history of vaccination (Table IV). Four girls had been vaccinated at school, and their prior immune status and the vaccine received were unknown. Informed consent was obtained from all volunteers. Volunteers were asked to record symptoms daily for 30 days after vaccination on a self-evaluation chart. Samples of blood and pharyngeal and nasal swabs were taken on three occasions between days 6 and 14 after vaccination in order to test for rubella viremia and virus excretion. Serological studies were carried out on sera taken between days 6 and 14 and 4 and 8 weeks after challenge.

1. *Serological Techniques*

Sera were tested for rubella-specific IgG by RIA and for rubella-specific IgM by an M-antibody capture RIA (MACRIA) (Mortimer *et al.*, 1981; Tedder *et al.*, 1982).

2. *Virus Isolation*

Attempts were made to isolate rubella virus from heparinized blood, purified peripheral blood lymphocytes, and pharyngeal and nasal swabs after rubella vaccination or challenge. Specimens were inoculated into Vero cell cultures and incubated for 10 days before passage into RK13 cells grown on Lab-Tek tissue culture slides. Rubella virus was identified by immunofluorescence (Schmidt *et al.*, 1978).

III. RESULTS

A. Persistence of Rubella Antibodies after Vaccination

Low concentrations of antibody (<15 IU) were detected in 19 of the 123 (15.4%) vaccinees by SRH and in 13 (10.6%) by RIA (Table I). Antibody concentrations less than 15 IU occurred most frequently among subjects given Cendehill vaccine. Eight of the 123 (6.5%) had no detectable antibody

TABLE I
Persistence of Rubella Antibodies 6–16 Years after Vaccination[a]

Group	No. tested	No. (%) Seronegative		No. (%) ≤ 15 IU	
		SRH (no zone)	RIA (<20)	SRH	RIA
Natural infection	9	0	0	0	0
RA27/3	46	0	1 (2.2)	3 (6.5)	2 (4.3)
Cendehill	45	5 (11.1)	1 (2.2)	12 (26.7)	8 (17.8)
HPV77.DE5	18	2 (11.1)	0	3 (16.7)	2 (11.1)
To-336	14	1 (7.1)	0	1 (7.1)	1 (7.1)
Total	123	8 (6.5)	2 (1.6)	19 (15.4)	13 (10.6)

[a]Volunteers had been vaccinated 6–16 years previously at St. Thomas' Hospital and the Hospital for Sick Children.

when tested by SRH, but when tested by RIA only two of the 123 (1.6%) were seronegative.

Six to eight years after vaccination, RA27/3 and HPV77.DE5 induced the highest geometric mean titers (GMTs) and Cendehill the lowest (Table II). In contrast, volunteers vaccinated 11–16 years previously with RA27/3 had a lower GMT than those given Cendehill (Table III).

B. Detection of Viremia after Rubella Challenge

1. Viremia

Viremia was detected in 80% of the primary vaccinees following intranasal challenge (Table IV). Viremia was not detected in any of the 10 seropositive volunteers, in the 12 low-titered volunteers with naturally acquired

TABLE II
Geometric Mean Titers 6–8 Years after Vaccination[a]

Group	No. tested	Range	GMT
Natural infection	9	1280–5120	2764
RA27/3	33	320–5120	915
HPV77.DE5	18	80–5120	1185
To-336	14	80–5120	780
Cendehill	20	<20–5120	355
Total vaccinees	85	<20–5120	740

[a]Volunteers had been vaccinated 6–8 years previously at St. Thomas' Hospital. Antibody titers were measured by radioimmunoassay.

TABLE III
Geometric Mean Titers 11–16 Years after Vaccination[a]

Group	No. tested	Range	GMT
RA27/3	13	<20–1280	464.7
Cendehill	25	80–5120	821
Total vaccinees	38	<20–5120	617.6

[a]Volunteers had been vaccinated 11–16 years previously at the Hospital for Sick Children. Antibody titers were measured by radioimmunoassay.

immunity, or in 18 of the 19 vaccinees. However, virus was detected 12 days after challenge in the whole blood of one volunteer who had been vaccinated at school. She showed an accelerated immune reponse following challenge, and a low level of rubella-specific IgM was detected 14 days after challenge (Table V).

2. *Virus Excretion*

Virus excretion was detected in 8 of the 10 seronegative volunteers after challenge and in one seropositive volunteer who had been vaccinated 6 years previously at school (Table IV). Among the low-titered volunteers, none of those with naturally acquired immunity excreted virus, but virus excretion was detected in one Cendehill vaccinee and in two whose primary vaccine was unknown, in one of whom we had detected a viremia.

3. *Rubella-Specific IgG*

After challenge, a significant (>fourfold) rise in antibody titer was seen in all 19 low-titered volunteers with a history of vaccination, but in only 8 of the 12 with naturally acquired immunity (Table VI). Booster responses

TABLE IV
Detection of Viremia and Virus Excretion after Challenge with RA27/3

Prechallenge antibody status	No. tested	Viremia	Virus excretion
Seronegative	10	8 (days 10–14)	8 (days 6–14)
Seropositive, >15 IU	10	0	1 (day 7)
Low titer, ≤ 15 IU			
Natural infection	12	0	0
Cendehill	6	0	1 (days 7 and 8)
RA27/3	7	0	0
HPV77.DE5	2	0	0
Primary vaccine unknown	4	1 (day 12)	2 (days 6 and 9)
Total	31	1	3

TABLE V

Detection of Viremia, Virus Excretion, and Antibody Response in One Volunteer Who Had a History of Vaccination at School

Parameters	Prechallenge	Postchallenge			
		Day 6	Day 12	Day 14	8 weeks
Serology HAI	10–20[a]	10	160	160	160
SRH (mm)	5	5	10	11.5	11.5
RIA IgG	80	80	1280	1280	1280
IgM	Neg.	Neg.	Neg.	Pos.	Neg.
Viremia		Neg.	Pos. whole blood	Neg.	
Excretion		Pos. TN/S	Neg.	Neg.	
Vaccine reaction		Lymphadenopathy days 11–13			

[a]Confirmed by flotation centrifugation.

TABLE VI

Volunteers Showing a Significant Rise in Rubella-Specific IgG as Measured by Radioimmunoassay after Intranasal Challenge with RA27/3

Prechallenge antibody status	No. showing ≥ fourfold rise in titer/ No. tested	No. showing rise/No. tested Interval postchallenge		
		6–14 days	4 weeks	8 weeks
Seronegative	10/10 (100%)	0/10	—	10/10
Seropositive, >15 IU	3/10 (30%)	1/10	1/1	1/10
Low titer, ≤15 IU				
Natural infection	8/12 (66.6%)	6/12	1/4	1/2
Cendehill	6/6 (100%)	5/6	0/3	1/6
RA27/3	7/7 (100%)	4/7	1/3	2/7
HPV77.DE5	2/2	1/2	0/1	1/2
Primary vaccine unknown	4/4	4/4		
Total	27/31 (87%)	20/31 (64.5%)	2/11 (18.1%)	5/17 (18.5%)

were detected within 14 days of challenge in 20 of the 31 low-titered volunteers, whereas seronegative volunteers had no rubella antibodies at this time. Of the 10 seropositive volunteers, 3 developed a significant rise in IgG titer; 2 of these had a history of vaccination.

4. *Rubella-Specific IgM*

Low levels of rubella-specific IgM (1–2.3 MACRIA units/ml) were detected using MACRIA in 4 of the 31 low-titered volunteers between 12 days and 11 weeks after challenge (Table VII). All 4 volunteers had a history of vaccination. Higher levels of rubella-specific IgM (1–18 MACRIA units/ml) were detected after primary vaccination. No rubella-specific IgM was detected after challenge of seropositive volunteers.

5. *Vaccine Reactions*

Arthralgia occurred in 1 seronegative volunteer between days 9 and 12, and a rash developed in another 7 days after vaccination (Table VIII). Arthralgia occurred in 5 of the 31 (16.1%) low-titered volunteers between 5 and 12 days after challenge (mean 8 days).

IV. DISCUSSION

Our studies on the persistence of rubella antibody after vaccination have shown that nearly 90% of adult vaccinees had adequate levels of antibody (>15 IU) when tested 6–16 years later. Similar results have been published by Zealley and Edmond (1982) and Herrmann *et al.* (1982). In our study, 13 of 123 vaccinees (10.6%) had a low level of antibodies (<15 IU) 6–16 years after vaccination when tested by RIA, but 2 of 123 (1.6%) had no detectable antibody.

We have previously shown that Cendehill induces not only lower antibody concentrations than RA27/3, but also a less durable local antibody response (Best *et al.*, 1979). Furthermore, after intranasal challenge with RA27/3, reinfection as measured by a boost in antibody titers and production of rubella-specific IgM occurred more commonly among Cendehill vaccinees (Harcourt *et al.*, 1980). Macdonald *et al.* (1978) also showed a higher incidence of naturally acquired reinfection in subjects given Cendehill vaccine. Such booster antibody responses may account for the higher GMTs of rubella antibody seen in sera obtained 11–16 years after Cendehill vaccination.

We detected viremia in one volunteer after experimental challenge, 6 years after rubella vaccination (O'Shea *et al.*, 1983). We were not able to take daily samples from our volunteers, and it is therefore possible that we may

TABLE VII

Detection of Rubella-Specific IgM after Challenge with RA27/3

Prechallenge antibody status	Total volunteers positive/No. tested	No. positive/No. tested Interval postchallenge			MACRIA units/ml (range)
		6–14 days	4 weeks	8 weeks	
Seronegative	8/10	1/10	3/3	7/10	1–15
Seronegative[a]	7/7	—	7/7	—	1–18
Seropositive, >15 IU	0/10	0/10	0/1	0/10	
Low titers, ≤15 IU					
Natural infection	0/12	0/12	0/4	0/12	—
Cendehill	1/6	1/6	0/3	0/5	1.0
RA27/3	0/7	0/7	0/4	0/7	—
HPV77.DE5	1/2	0/2		1/2 [b]	2.3
Primary vaccine unknown	2/4	1/4[c]	1/4[c]	0/4	1.2–2.1
Total	4/31 (12.9%)	2/31	1/15	1/30	1–2.3

[a] Serum samples taken from seven volunteers 4 weeks after vaccination.
[b] Samples taken at 11 weeks.
[c] Also positive by sucrose density gradient.

TABLE VIII

Vaccine-Associated Reactions after Challenge with RA27/3

Prechallenge antibody status	No.	Numbers experiencing	
		Arthralgia	Rash
Seronegative	10	1	1
Seropositive, >15 IU	10	1	0
Low titers, ≤15 IU			
Natural infection	12	3	0
Cendehill	6	1	0
RA27/3	7	0	0
HPV77.DE5	2	1	0
Primary vaccine unknown	4	0	0
Total	31	5 (16.1%)	0

have missed a transient viremia in other volunteers and may thus have underestimated the frequency of viremia. In earlier collaborative studies we also detected viremia after challenge with RA27/3 in a volunteer with a very low level of naturally acquired rubella antibody (Balfour *et al.*, 1981).

Viremia and virus excretion were detected in 8 of the 10 primary vaccinees following intranasal vaccination. Thus, rubella virus was isolated significantly more frequently from seronegative volunteers than from low-titer volunteers ($p < 0.001$), suggesting that preexisting antibody limits virus replication in the majority of low-titered volunteers. Virus excretion was detected between 6 and 14 days after intranasal vaccination. The virus recovered could not have been residual vaccine virus since when daily swabs were taken we failed to recover virus before day 5.

Arthralgia occurred less frequently among low-titered volunteers (5/31, 16%) than was reported among RA27/3 primary vaccinees in our previous study (42%) (Best *et al.*, 1974). However, arthralgia occurred earlier (5–12 days after vaccination) in those with preexisting antibody than in primary vaccinees (13–21 days).

In addition to the two reports of rubella viremia following experimental challenge, there have been a small number of reports of naturally acquired reinfection with such features as rash, arthralgia, and fetal infection, suggesting that a viremia had occurred (Best *et al.*, 1981). Three such reports have been of reinfection in women with a history of successful vaccination (Forrest *et al.*, 1972; Enders *et al.*, 1984; M. Forsgren, personal communication). Of the 36% of children vaccinated with HPV77.DE5 in the United States, many have very low antibody levels, undetectable by the standard HI test, while approximately 10% Cendehill vaccinees have antibody levels < 15 IU when tested within 10 years of vaccination. Further challenge studies are required in order to determine whether such persons are susceptible to reinfection and whether mass revaccination should therefore be recommended. We do not yet know whether rubella immunity will persist for the required 30–40 years after rubella vaccination, since most follow-up studies have been in progress for only 10 years. The ideal vaccination policy may be to vaccinate preschool children of both sexes, to eliminate the risk of seronegative pregnant women catching rubella from their own children. All girls might then be revaccinated between the ages of 10 and 14 to ensure that they are immune during their childbearing years. It must be remembered that revaccination is accepted for such virus infections as polio, smallpox, yellow fever, and hepatitis B. Although such a policy appears successful in Sweden (Christenson *et al.*, 1983), it is unlikely to be successful in the United Kingdom, since measles vaccine is accepted by only 50% of children at 15 months of age. Acceptance of rubella vaccine by less than 80% of preschool children would result in an increased incidence of rubella among adults

(Knox, 1980; Anderson and May, 1983), as occurred in the United States in 1978/1979.

REFERENCES

Anderson, R. M., and May, R. M. (1983). *Lancet* **2**, 1416–1417.

Balfour, H. H. Jr., and Amren, D. P. (1978). *Am. J. Dis. Child.* **132**, 573–577.

Balfour, H. H., Jr., Groth, K. E., Edelman, C. K., Amren, D. P., Best, J. M., and Banatvala, J. E. (1981). *Lancet* **1**, 1078–1079.

Best, J. M., Banatvala, J. E., and Bowen, J. M. (1974). *Br. Med. J.* **3**, 221–224.

Best, J. M., Harcourt, G. C., O'Shea, S., and Banatvala, J. E. (1979). *Lancet* **2**, 690–691.

Best, J. M., Harcourt, G. C., Druce, A., Palmer, S. J., O'Shea, S., and Banatvala, J. E. (1980). *J. Med. Virol.* **5**, 239–247.

Best, J. M., Harcourt, G. C., Banatvala, J. E., and Flewett, T. H. (1981). *Br. Med. J.* **282**, 1235.

Centers for Disease Control (1981). *Morbidity and Mortality Weekly Report* **30**, 412–417.

Centers for Disease Control (1982). *Morbidity and Mortality Weekly Report* **31**, 568–575.

Christenson, B., Böttinger, M., and Heller, L. (1983). *Brit. Med. J.* **287**, 389–391.

Enders, G., Calm, A., and Schaub, J. (1984). *Infection* **12**, 96–98.

Forrest, J. M., Menser, M. A., Honeyman, M. C., Stout, M., and Murphy, A. (1972). *Lancet* **2**, 399–400.

Goldwater, P. N., Quiney, J. R., and Banatvala, J. E. (1978). *Lancet* **2**, 1298–1300.

Harcourt, G. C., Best, J. M., and Banatvala, J. E. (1980). *J. Infect. Dis.* **142**, 145–155.

Herrmann, K. L., Halstead, S. B., and Wiebenga, N. H. (1982). *J. Am. Med. Assoc.* **247**, 193–195.

Horstmann, D. M. (1975). *Ann. Intern. Med.* **83**, 412–417.

Knox, E. G. (1983). *Int. J. Epidemiol.* **9**, 13–23.

Kurtz, J. B., Mortimer, P. P., Mortimer, P. R., Morgan-Capner, P., Shafi, M. S., and White, G. B. B. (1980). *J. Hyg.* **84**, 213–222.

MacDonald, H., Tobin, J. O'H., Cradock-Watson, J. E., and Lomax, J. (1978). *J. Hyg.* **80**, 337–347.

Marshall, W. C., Peckham, C. S., Darby, C. P., Dudgeon, J. A., and Hawkins, G. T. (1971). *Practitioner* **207**, 632.

Mortimer, P. P., Tedder, R. S., Hambling, M. H., Shafi, M. S., Burkhardt, F., and Schilt, U. (1981). *J. Hyg.* **86**, 139–153.

O'Shea, S., Best, J. M., Banatvala, J. E., Marshall, W. C., and Dudgeon, J. A. (1982). *Br. Med. J.* **285**, 253–255.

O'Shea, S., Best, J. M., and Banatvala, J. E. (1983). *J. Infect. Dis.* **148**, 639–647.

Schmidt, N. J., Dennis, J., and Lennette, E. H. (1978). *J. Clin.* Microbiol. **7**, 576–583.

Tedder, R. S., Yao, J. L., and Anderson, M. J. (1982). *J. Hyg.* **88**, 335–350.

Zealley, H., and Edmond, E. (1982). *Br. Med. J.* **284**, 382–384.

17

Rabies Enigma: Human and Animal Disease Control

ROBERT E. SHOPE

Department of Epidemiology and Public Health, Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, Connecticut

I. INTRODUCTION

Rabies is an enigma. On the one hand, we understand so little about its true pathogenesis, where it hides during a long incubation period, and by what pathophysiological mechanism it induces illness and death. On the other hand, for decades we have known how to control rural and urban rabies. Simply put, since over 90% of rabies exposure is to dogs, one vaccinates and controls dogs. Now for various reasons, control is not so simple. This chapter examines some of these reasons. It reviews the present status of rabies as it affects the Middle East and African countries and estimates the size of the problem and the success and failure of past efforts to control rabies in this area.

APPLIED VIROLOGY

ISBN 0-12-429601-7

I describe here the WHO Program for Global Elimination of Rabies for which the World Health Organization (WHO) is now trying to muster world and local country support. It represents an experiment which we shall all watch with interest.

Finally, I outline some of the timely important considerations that may have a profound impact on the success or failure to control the disease. These include the rabies-related viruses of Africa, strain variations among "classical" rabies isolates, human diploid cell vaccines, present and future development of veterinary vaccines, and prospects for more sensitive and easily applied virus surveillance techniques.

II. PRESENT PROBLEM OF RABIES IN THE MIDDLE EAST AND AFRICA

Rabies is a current or a potential problem for every country in the Middle East and Africa. Dogs are the principal reservoir of rabies in most of the Middle East and in all of Africa except perhaps for the southernmost part of the continent. In Iran, the wolf, and in South Africa and Zimbabwe, other carnivores such as the genet, civet cat, yellow mongoose, and especially the jackal are major reservoirs, as well as domestic cats and dogs.

The following countries of the Middle East reported no cases of rabies in 1981 according to WHO informal statistics: Bahrain, Cyprus, Iraq, Oman, Qatar, and United Arab Emirates. Rabies was diagnosed in sheep in Kuwait in 1982 (*Kuwait Monthly Epidemiological Report,* 1982). The African countries that reported no cases were Libya, Djibouti, Congo, Equatorial Guinea, and Lesotho. The lack of reporting in some of these areas may represent a low level of surveillance rather than absence of disease.

Surveillance data are inadequate. For instance, in Algeria during a 3-year period an average of 33 dog cases and 18 human cases per year were reported; it is reasonable to assume that for every human case there should be many more infections than this in dogs. However, it is clear that rabies is increasing in prevalence. Israel, which since 1954 has had a high degree of control of rabies in dogs, now is rabies free only in districts along the Mediterranean Sea. Western Kenya, once free of rabies, is now reinfected. and during 1981 and 1982 rabies spread relatively uncontrolled through the country. In Zimbabwe, where internal problems have interrupted veterinary public health services, rabies is on the increase in the eastern and southern zones. In Botswana new outbreaks were reported in 1979 in the northwest and in 1981 near the border of Namibia, where rabies was epidemic; and in Natal, South Africa, a rabies outbreak in dogs was in progress in 1980

and has since spread southward (Meredith and Standing, 1981). The general worldwide increase is explained according to Schneider and Bogel (1983) by a combination of (1) increasing human and dog populations with a greater chance for active transmission because of higher densities and (2) diminished rabies control because of decreasing resources per capita and failure to enforce rabies control laws. Unsettled political conditions and regional wars contribute also to diminished control.

III. HISTORY OF RABIES CONTROL IN THE MIDDLE EAST AND AFRICA

It has been shown that human rabies *can* be prevented by control of dog rabies. Mass vaccination campaigns in the past were satisfyingly successful in a technical sense. During the early 1950s, with the development of avianized live attenuated vaccines, the WHO organized campaigns in Israel and Southern Rhodesia (Zimbabwe). In Israel, there were between 50 and 333 cases in dogs per year between 1932 and 1947. A vaccination campaign was initiated in 1951 and continued into 1952 until 90% of the dogs over 6 months of age were vaccinated with Flury or Delev chicken embryo vaccines. The campaign was highly successful (Kaplan *et al.*, 1954). In 1948 there were 99 cases; in 1949, 194 cases; and in 1950, 68 cases. Since 1951 no more than 11 cases were reported annually until recent years, when there has been increased prevalence in the border areas. In 1979 with 85% of dogs vaccinated, there were only two rabid dogs reported.

In Zimbabwe, where vaccination was also undertaken, numbers of dog cases decreased from 129 to 2 per year between 1952 and 1961 (Adamson, 1954). As with all public health campaigns which involve continuing action, it is difficult to maintain the control (short of eradication as with smallpox). When a public health measure becomes routine, it loses its appeal, workers slacken, and the disease tends to return.

For a campaign to be cost effective it must be thorough and widespread. Paradoxically, if rabies control is not thorough, the cost may be higher than with no control. Human postexposure vaccination, which is done at great expense, must be continued even when only a few cases in dogs remain, and as numbers of infected dogs increase, the disease tends to move back into a controlled area. The aim of the vaccination scheme therefore must be rapid and complete coverage over a wide area.

Rabies does not respect national borders. International cooperation is thus essential, and a campaign should take advantage of physical barriers such as water, deserts, and mountains which may help to prevent reintroduction from outside the control area.

IV. WORLD HEALTH ORGANIZATION PROGRAM FOR GLOBAL ELIMINATION OF RABIES

The WHO program is based on a series of WHO-assisted national projects, the first two of which are being initiated as demonstrations respectively in Tunisia and Tanzania. Six basic assumptions were made: (1) 95% of exposure of people is to dogs; (2) dogs can be vaccinated with a single inoculation of a killed vaccine which will protect for at least 2 years; (3) a killed vaccine can be produced locally or regionally at a cost which national veterinary services can afford; (4) technical assistance from the international community will become available to aid regional vaccine production, to implement surveillance, and to help with rapid mass vaccination methods such as the dermojet apparatus; (5) other countries which border the initially targeted country will follow with their own control programs so that reintroduction will not occur; and (6) revaccination of dogs later will be maintained at a high level (over 70%).

The success of these WHO-assisted national programs will depend on the assumptions being correct, as well as on support initially at the local country level.

V. CONSIDERATIONS RELATED TO RABIES CONTROL

A. Rabies-Related Viruses

When several of us first reported the existence of viruses related to rabies (Shope *et al.*, 1970), we really did not know enough to predict the impact on disease in Africa. Since then we have learned a great deal. Table I shows the rabies-related viruses and their distribution as we know it today. Mo-

TABLE I
Distribution and Hosts of Rabies-Related Viruses

Virus	Distribution	Host
Mokola	Nigeria	*Crocidura flavescens*, man
	Cameroon	*Crocidura* sp.
Lagos bat	Nigeria	*Eidolon helvum*
	Central African Republic	*Micropteropus pusillus*
	South Africa	*Epomophorus wahlbergi*
Duvenhage	South Africa	Man, bat
Obodhiang	Sudan	*Mansonia uniformis*
Kotonkan	Nigeria	*Culicoides* spp.
Not identified	Zimbabwe	Cats, dog

kola virus has been isolated twice from people and subsequently once from shrews in Cameroon; Lagos bat virus, in addition to the original isolation from *Eidolon helvum* bats in Lagos, Nigeria, was subsequently found in the dwarf epauletted bat, *Micropteropus pusillus,* 110 km north of Bangui, Central African Republic (Sureau *et al.,* 1977). In a specimen from South Africa sent to our laboratory by C. D. Meredith, Crick *et al.* (1982) confirmed by complement fixation (CF) and cross-challenge tests in mice that a strain isolated near Durban, Natal, from *Epomophorus wahlbergi,* the common epauletted fruit bat, was closely related to Lagos bat virus. In another set of bat specimens by B. J. H. Barnard, collected near the site of original isolation of Duvenhage virus in South Africa, my colleagues (G. H. Tignor and M. Harrington, personal communication, 1983) identified a virus very closely related to Duvenhage virus. Dog isolates from the Natal region were classic rabies strains.

There are at least five rabies-related agents, each serologically quite distinct. Although some cross-neutralization is usually demonstrable, the immunization challenge test in mice indicated that rabies vaccine would not likely protect animals against infection with Mokola or Duvenhage viruses (Tignor and Shope, 1972; Tignor *et al.,* 1977). These two viruses infect man and produce severe, sometimes fatal, encephalitis or myelitis. Obodhiang and kotonkan from biting insects are so far curiosities; they are very distant relatives of rabies, and there is no evidence that they infect man, nor does laboratory inoculation of monkeys or dogs make them sick. Lagos bat virus is pathogenic for monkeys and dogs, and its disease should be sought in man. With rabies-related viruses, like rabies, people are infected tangentially as dead-end hosts. Until recently, it was believed that the rabies-related viruses were transmitted only among wildlife. From a public health point of view, the real hazard would emerge if the viruses set up a domestic or peridomestic cycle in dogs or cats. A very interesting report was published by Foggin (1982) in which he recorded four cases of rabies in cats and one in a dog in Zimbabwe. Each animal yielded an isolate which cross-reacted by immunofluorescence with rabies virus but was not neutralized by rabies antibody; rabies vaccine did not protect against the dog isolate on challenge in mice. It is important to explore further the identification of these agents and their epizootiology in cats and dogs.

B. Variation among Strains of Rabies Virus

Antigenic variation among strains of rabies virus had been noted in early studies of rabies vaccine viruses. The development of the monoclonal antibody technique and its application to the study of rabies strains on a worldwide basis by Tadeus Wiktor and his co-workers (Charlton *et al.,* 1982)

have led to reexamination of this question: Do rabies strains vary antigenically to a sufficient degree that present vaccines do not protect? Rabies monoclonal antibodies are products of mouse hybridoma cells formed by fusion of murine myeloma with splenic lymphocytes from mice immunized with rabies virus. The resulting antibodies are highly specific and can detect single epitopes on the rabies nucleocapsid and glycoprotein surface antigens. Since the composition of a single epitope can be modified rapidly during evolution (apparently without changing the major antigenic composition of rabies virus), it follows that minor antigenic differences among rabies strains can be detected with a battery of monoclonal antibodies. In fact, such differences have been induced (Wiktor and Koprowski, 1980) by selection of progeny from viruses passaged in the laboratory in the presence of neutralizing monoclonal antibody.

A collaborative study is now in progress to map the worldwide distribution of antigenic variants of rabies virus. Several hundred strains were examined with a battery of monoclonal antibodies (Charlton *et al.*, 1982). Most Old World strains had similar patterns of reaction with monoclonal antibodies, and these strains were uniformly protected against by vaccine prepared with the Pitman Moore (PM) strain, the same strain used in the human diploid cell vaccine. A few strains from Iran and Thailand in Asia and from Madagascar in Africa differed to a greater degree in monoclonal antibody tests, and the PM-vaccinated mice were only partly resistant to challenge with these viruses. It remains yet to be confirmed that these differences are responsible for vaccine failures in man or animals.

C. Postexposure Prophylaxis in Humans

Only inactivated vaccines are recommended for use in people. The human diploid cell vaccine was developed through adaptation of the Pasteur-derived PM strain of rabies virus to human diploid cells and then partial purification and concentration of the antigen by physical methods. This vaccine is produced in France and Germany. The WHO recommends that preparations having antigenic potency of 2.5 IU or greater be administered as soon after exposure as possible, on days 0, 3, 7, 14, 30, and 90. WHO also advises use of 20 IU of human rabies immune globulin per kilogram intramuscularly. If human immune globulin is not available, then 40 IU/kg of equine, rabbit, or other species origin should be administered. At this writing, only three deaths have been reported in persons receiving the vaccine. One was a 29-year-old American woman who was bitten by a dog in Rwanda (Devriendt *et al.*, 1982). She died 2 months later with high-titer rabies antibody in serum and cerebrospinal fluid. She had received the vaccine immediately after the bite but was not given immune globulin until

after onset of illness. The other two cases occurred in Thailand and included a 35-year-old woman who was severely bitten by a dog, but was not started on the vaccine until 2 days later (Wattanasri *et al.*, 1982). This patient did not receive immune globulin. In all cases there was a failure to administer promptly the recommended immune globulin. Thus the human diploid cell rabies vaccine plus immune globulin regimen has been remarkably successful. This success argues against the hypothesis that strain variation will result in human postexposure prophylaxis failures.

D. Impact of Human Diploid Cell Vaccine in the Third World

Ironically, the remarkably successful research efforts during the past 20 years in France, Germany, England, and the United States have created a new problem for the Third World nations. With the advent of the human diploid cell vaccine we now have a safe inactivated rabies vaccine which, when given with rabies immune globulin, is effective in the postexposure treatment of human rabies. This vaccine is readily available throughout the world, but it is costly. It is at least one order of magnitude more expensive than older commercial vaccines or than Semple vaccines produced locally, and its expense together with the high cost of rabies immune globulin has created problems in the Third World and even in relatively affluent areas of the United States. The lay and scientific press have told the world that rabies can now be treated postexposure with little risk or discomfort. This is true. It is not, however, a practical solution where the health system of a country cannot afford the human diploid cell vaccine and human immune globulin.

The world needs a less expensive human vaccine. The Fuenzalida-type vaccine, prepared in mice less than 10 days old and inactivated with β-propiolactone, is produced and used at the Institut Pasteur, Dakar. It is administered postexposure daily for 7 days followed by injections on days 11, 15, 30 and 60. Antirabies serum is also administered immediately postexposure if the wound is deep or on the face, neck, head, or hands; serum is also given for wild animal bites. This regimen (slightly modified when antirabies serum is included) has been remarkably effective (J. P. Digoutte, personal communication, 1983). An inactivated vaccine developed in Japan (Kondo, 1978) and produced in chicken embryos with HEP Flury virus offers prospects of an affordable product. The Japanese inactivated vaccine has antigenic values ranging between 1.1 and 12.7; it was tested in Thailand in eight persons in a postexposure trial without subsequent disease, but larger scale testing has not yet been done. Another new vaccine prepared in rhesus monkey diploid cells (Berlin *et al.*, 1982) has been tested preex-

posure in volunteers with antibody responses as good as that of the human diploid cell vaccine. The rhesus cell vaccine has not yet been tested postexposure.

E. Veterinary Vaccines

Vaccines for dogs and cats are produced in a variety of ways in the Middle East and Africa. Most of the vaccine is made in sheep brain (Fermi or Semple methods); however, LEP Flury live attenuated vaccine is manufactured in Kenya and South Africa. A recent adaptation at the National Rabies Research Center in Malzeville/Nancy, France, of Pasteur virus to suckling lamb brain appears to offer a potent, cheap alternative for a locally produced product. This vaccine is inactivated with β-propiolactone and adsorbed to aluminum hydroxide. Such a locally made vaccine is an essential prerequisite for a successful campagin to eliminate rabies from many zones in Africa and the Middle East.

Theoretically, it should be possible to develop a very inexpensive animal vaccine using rabies glycoprotein. The glycoprotein constitutes the surface spike layer on the rabies virion and induces neutralizing antibodies and protection to challenge. Purified glycoprotein fragments were shown to induce virus-neutralizing antibody (Dietzschold *et al.*, 1982) in mice. The mRNA corresponding to the glycoprotein was purified, and cDNA was prepared. This cDNA was inserted into plasmid pBR322 in *E. coli* (Curtis *et al.*, 1980). It has not yet been shown that the protein can be produced in quantity, nor that such a protein, which would perhaps need to be glycosylated, is antigenic. The approach, however, seems logical. Alternatively, the glycoprotein or its essential polypeptides may be subject to synthesis. It remains to be determined whether a cost-effective vaccine will result from this research. Such a vaccine may be available soon or may be several years away, but its prospects should not deter us from initiating control programs now with conventional biologics.

F. Laboratory Techniques for Surveillance

The immunofluorescence test brought speed and reliability to the diagnosis of rabies. Unfortunately, however, some countries in Africa either do not yet do this test or have only recently adopted it. The high cost of equipment, the paucity of proper service personnel, and the skill needed for reliable results argue for attempts to develop new methods. One with great promise is the enzyme-linked immunosorbent assay (ELISA), in which the antiserum is conjugated to an enzyme which develops the color of an added substrate (Nicholson and Prestage, 1982). The color can be read visually

without special equipment, or the results may be quantified spectrophotometrically. The use of monoclonal antibodies to coat the solid phase to capture the antigen is an attractive possibility with this test. A further sophisticated method, which has not yet been tried, is the use of rabies cDNA labeled with biotin to probe for RNA (Langer-Safer *et al.*, 1982).

If these newer techniques can be developed and applied in national laboratories or as kits for local physicians and veterinarians, then surveillance should be improved, and chances for control enhanced.

G. Prospects for Control or Elimination

I have not considered here control of wildlife rabies. Wildlife rabies occurs in Iran and prominently in parts of eastern and southern Africa. Most human exposures are to dogs, and we will not completely understand the role of wildlife rabies until dog rabies is under control. It is clear, however, that where wildlife rabies exists the presently proposed methods will not rid the area of rabies. Considerable research effort has been expended on control of rabies in wildlife through oral vaccination with live SAD vaccine. The success in foxes in Switzerland has been highly satisfactory. I believe, however, that wider application of this technique should await the development of a safer vaccine. This might be a genetically defined strain with deletion mutations; or it might even be a genetically engineered product analogous to the proposed use of vaccinia in humans to vector an insert of DNA capable of elaborating the immunogen (Mackett *et al.*, 1982).

There is ample evidence from past history that control of dog rabies (and thus human rabies) is quite feasible. With inexpensive dog vaccines, improved surveillance, new and adequate financial resources, and, most important, cooperation between governments and within governments, the control (and even elimination in some areas) of rabies is an exciting prospect.

ACKNOWLEDGMENT

I am indebted to Dr. Konrad Bogel of the World Health Organization Veterinary Public Health Unit for much of the data and some of the concepts about control.

REFERENCES

Adamson, J. S. (1954). *Bull. W. H. O.* **10**, 753–759.

Berlin, B. S., Mitchell, J. R., Burgoyne, G. H., Oleson, D., Brown, W. E., Goswick, G., and McCullough, N. B. (1982). *J. Am. Med. Assoc.* **247**, 1726–1728.

Charlton, K. M., Casey, G. A., Boucher, D. W., and Wiktor, T. J. (1982). *Comp. Immunol. Microbiol. Infect. Dis.* **5**, 113–115.

Crick, J., Tignor, G. H., and Moreno, K. (1982). *Trans. R. Soc. Trop. Med. Hyg.* **76**, 211–213.

Curtis, P. J., Anilionis, A., and Wunner, W. H. (1980). *In* "Proceedings of the 4th International Symposium of Negative Strand Viruses" (D. H. L. Bishop and R. W. Compans, eds.), pp. 721–725. Elsevier, Amsterdam.

Devriendt J., Staroukine, M., Costy, F., and Vanderhaeghen, J.-J. (1982). *J. Am. Med. Assoc.* **248**, 2304–2306.

Dietzschold, B., Wiktor, T. J., Macfarlan, R., and Varrichio, A. (1982). *J. Virol.* **44**, 595–602.

Foggin, C. M. (1982). *Vet. Rec.* **110**, 338.

Kaplan, M. M., Goor, Y., and Tierkel, E. S. (1954). *Bull. W.H.O.* **10**, 743–752.

Kondo, A. (1978). *Dev. Biol. Standard.* **40**, 147–153.

Kuwait Monthly Epidemiological Report (1982). **9**, 9–11.

Langer-Safer, P. R., Levine, M., Ward D. C. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4381–4385.

Mackett, M., Smith, G. L., and Moss, B. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7415–7419.

Meredith, C. D., and Standing, E. (1981). *Lancet* **1**, 832–833.

Nicholson, K. G., and Prestage, H. (1982). *J. Med. Virol.* **9**, 43–49.

Schneider, L., and Bogel, K. (1983). "Third Interamerican Meeting at the Ministerial Level on Animal Health, April 11–14." Pan American Health Organization, Washington, D.C.

Shope, R. E., Murphy, F. A., Harrison, A. K., Causey, O. R., Kemp, G. E., Simpson, D. I. H., and Moore, D. L. (1970). *J. Virol.* **6**, 690–692.

Steck, F., Wandeler, A., Bichsel, P., Capt, S., Hafliger, U., and Schneider, L. (1982). *Comp. Immunol. Microbiol. Infect. Dis.* **5**, 165–171.

Sureau, P., Germain, M., Herve, J.-P., Geoffrey, B., Cornet, J.-P., Heme, G., and Robin, Y. (1977). *Bull. Soc. Pathol. Exot. Ses. Fil.* **70**, 467–470.

Tignor, G. H., and Shope, R. E. (1972). *J. Infect. Dis.* **125**, 322–324.

Tignor, G. H., Murphy, F. A., Clark, H. F., Shope, R. E., Madore, P., Bauer, S. P., Buckley, S. M., and Meredith, C. D. (1977). *J. Gen. Virol.* **37**, 595–611.

Wattanasri, S., Boonthai, P., and Thongcharoen, P. (1982). *Lancet* **2**(8303), 870.

Wiktor, T. J., and Koprowski, H. (1980). *J. Exp. Med.* **152**, 99–112.

18

Rabies Vaccine Produced in Cell Culture: Production Control and Clinical Results

M. ROUMIANTZEFF, N. AJJAN, R. BRANCHE, P. FOURNIER, B. MONTAGNON, P. TROTEMANN, AND J. C. VINCENT-FALQUET

Mérieux Institute, Lyon, France

I. INTRODUCTION

Rabies is an infectious disease transmitted between animals or from animals to man which still terrorizes our planet. The unavoidable fatal outcome of the disease in man has made preventive means essential; sanitary

ISBN 0-12-429601-7

policy regarding contamination can limit the risks of rabies virus transmission from animals to man, and prophylaxis can halt the propagation of the infection in a person accidentally bitten by a rabid animal. Early historical successes in preventive policy and the introduction of the first veterinary and human vaccines pointed toward an optimistic future, with a progressive control of the disease. However, today we must recognize that these successes were limited; rabies is still endemic in the majority of countries, even in developed countries where the most well-established and costly means of defense have managed only to limit the number of human cases. The disease has particularly dramatic implications in tropical and developing countries, and its importance in these regions was long underestimated or minimized in official reports. Today, rabies in both animals and humans is studied and analyzed more widely so as to determine the most effective action and with the aim of controlling and then eliminating the human disease.

Antirabies vaccination is now 100 years old. Since the landmark of Louis Pasteur's research, there has been a proliferation of attempts to improve the safety and efficacy of rabies vaccines for immunization in animals and man. An important stage was reached 20 years ago with the development of rabies vaccine produced in cell culture.

In this chapter, we would like to review cellular vaccine progress, with special emphasis on the human diploid cell strain (HDCS) vaccine developed at the Wistar Institute. Despite its qualities of safety and efficacy, use of this vaccine has not supplanted that of traditional vaccines. We would like to clarify the technical and economic reasons for this slow evolution and to consider the opportunities provided by recent advances in vaccine technology which will perhaps enable the prevention of rabies throughout the world.

II. TRADITIONAL VACCINES

A. Nervous Tissue Vaccines

Nearly 100 years ago, in 1885, Louis Pasteur demonstrated that rabies virus could be attenuated by repeated passages through the rabbit nervous system. On July 6, 1885, he decided to attempt the first human treatment for rabies, by giving 13 consecutive and increasingly virulent doses of desiccated spinal cord from a rabid animal (Pasteur, 1885).

After the controversy regarding the possibility of "laboratory rabies," Fermi (1908) improved on Pasteur's method by treating the brain suspension with phenol at room temperature maintaining, however, some residual virulence. In 1911, Semple improved the innocuity of the rabid brain sus-

pension by fixing the inactivation temperature at 30°C. An ether treatment was added by Hempt (1925) in order to improve even further the vaccine's innocuity (Table I).

Since then, many variations of these original vaccines have been introduced by vaccine-producing institutes (Seligmann, 1973a; Lépine *et al.*, 1973). Different choices of animals to be cerebrally inoculated were made (rabbit, goat, or sheep); the original 5% concentration of the cerebral suspension was sometimes reduced to 4%; the dosage volume was reduced from 5 to 2 ml; the inactivation method changed in terms of time, temperature, and agent (phenol, ultraviolet, and more recently, β-propiolactone);

TABLE I
Rabies Vaccines for Human Use

Vaccine	Year	Innocuity	Methodology	Present recommendation
Pasteur	1885		Rabbit cord method	−
Fermi	1908	"Inactivated"	Phenol, 22°C	±
Semple	1911	Inactivated	Phenol, 37°C	+
Hempt	1925	Inactivated	Ether/phenol/glycerol	+
Koprowski	1954	Live	Egg-HEP high passage	−
Fuenzalida and Palacios	1955	Inactivated	Suckling mouse brain	+
Peck *et al.*	1956	Inactivated	Duck embryo vaccine	±
Fenje	1960	Inactivated	SAD-primary hamster kidney cells	−
Selimov *et al.*	1966	Inactivated	Vnukovo-primary hamster kidney cells	+
Wiktor *et al.*	1970	Inactivated	PM-human diploid cells	+
Atanasiu *et al.*	1972	Inactivated	PV-primary fetal calf kidney cells	+
Kondo	1972	Inactivated	HEP-primary chick embryo cells	+
Van Wezel and Van Steenis	1978	Inactivated	PM-primary dog kidney cells	+

and lyophilization was added to the production process to increase the vaccine's stability from 6 to 18 months.

This type of vaccine is now kept specifically for postexposure treatment and cannot be envisaged for preventive use. The number of daily doses is still between 14 and 21 injections, with 2 or 3 boosters. Injections are administered subcutaneously in the skin of the abdomen, and different injection sites are chosen each time.

Many cases of intolerance or accidents have been reported with these vaccines, but systematic monitoring on a worldwide basis is still lacking. From 1953 (Appelbaum *et al.*, 1953) to the latest report in 1981 (Kuwert *et al.*, 1981), the rate of severe neuroparalytic accidents reported following the use of Semple-type vaccine has remained consistently high, at between 1 in 500 and 1 in 2000 treatments.

Although Semple vaccines have been widely recommended, certain reports have shown the limits to the activity of these vaccines. In 1955, Baltazard *et al.* reported that 3 out of 4 patients severely bitten on the head died in spite of having received daily doses of vaccine. The addition of rabies serum has significantly improved success rates in this treatment. Other studies have demonstrated failure in the protection provided by the vaccines, e.g., in India (Shah and Jaswal, 1976) or in Thailand, where death occurred in 1 in 2000 cases following completed treatment courses (Thongchaoren *et al.*, 1982a).

Some studies have attempted to evaluate the protective capacities of these vaccines. Kuwert (1967) showed that virus-specific antigenicity was low when measured against the antibody conversion induced by the use of serum from vaccinated humans and animals. He pointed out that the testing methods used for this vaccine were insufficient. It is only the advent of HDCS vaccine which has enabled definitive comparisons showing the limits of the vaccine's efficacy.

Despite the considerable drawbacks encountered with these vaccines, 15 countries reported officially in 1981 to the World Health Organization (WHO, 1982) on the production of brain-type rabies vaccine, to a total of more than 13 million doses. In India alone, 12 institutes produced about 8 million doses of Semple vaccines enabling the treatment of from 1 to 3 million rabies-suspected subjects each year (Menon *et al.*, 1976; Sehgal and Gupta, 1983).

B. Suckling Mouse Brain Vaccines

Jervis (1954) assembled data proving the etiology of neuroparalytic accidents following antirabies treatment in man, and suggested that solutions to the problem were either the elimination of the encephalitogenic factor or the use of a vaccine which did not contain brain tissue.

Following this line of reasoning Fuenzalida and Palacios (1955) in Chile suggested the production of vaccine by cerebrally inoculating suckling mice aged from 3 to 5 days and then harvesting the brains 4 days later. At this stage, the myelin would be found only in small quantities in the central nervous tissue and the vaccine suspension would show a greatly reduced amount of encephalitogenic factor. This original Chilean vaccine (Table I) was made up from the supernatant of a 1% suspension of suckling mouse brain (SMB) tissue containing 10^5 MLD_{50} of rabies virus, inactivated by ultraviolet light, and supplemented with phenol (1:1000) and thimerosal (1:10,000) as preservatives (Fuenzalida, 1973). Later, different producers in Latin America altered the preparation methods, e.g., by changing the brain suspension preparation or replacing ultraviolet inactivation by β-propiolactone or phenol inactivation (Held and Lopez Adaros, 1972).

Svet-Moldavskij *et al.* (1965) suggested the use of suckling albino rats, having shown that their brains did not become encephalitogenic until after the day 18 of life. Fuenzalida *et al.* (1964) later demonstrated that the suckling mouse brain suspension did not provoke experimental allergic encephalitis when the animals were used before day 10 of life.

The preparation was later improved by the addition of centrifugation and chromatography to the production process (Sikes and Larghi, 1967), providing better concentration and specific purification of the SMB vaccine. Lavender (1970) added stages of zonal centrifugation precipitation and a 1:8000 β-propiolactone inactivation.

No standardized production techniques have been published for this vaccine, and so today there is a wide variety of SMB vaccines. All these variations were introduced in an attempt to reduce the risk of neuroparalytic accidents (Svet-Moldavskij *et al.*, 1965; Held and Lopez Adaros, 1972; Varela-Diaz *et al.*, 1974; Larghi *et al.*, 1976) while not reducing the vaccines' antigenicity (Lavender, 1970; Larghi and Fuenzalida, 1972; Mathew *et al.*, 1981). These vaccines were basically intended for postexposure treatment, and the inoculation of 14–21 daily doses was originally recommended for this purpose. Since 1970, however, a reduction in the number of injections has been proposed, and today the different protocols recommend 5–10 daily doses followed by 1–5 boosters depending on the severity of the bite (WHO, 1982). Despite the risks to subjects, some countries recommend the use of SMB vaccine for preventive immunization by administering 3 doses at varying intervals followed by a booster (Instituto Nacional de Salud, Bogota, Colombia, 1981).

Insufficient data have been gathered on a worldwide basis to evaluate the efficacy of these vaccines. However, in their general report to the Tunis Congress on Rabies in the Tropics, Acha and Arambulo (1984) showed that in Tropical America in 1981, 296,759 postexposure treatments were officially reported, with 14 cases of postvaccinal complications. From 1970 to

1981, the Pan American Health Organization (PAHO) reported for the WHO Americas Region on between 225 and 354 rabies cases each year, followed by between 7 and 30 postvaccinal complications. Despite these difficulties, 17 countries provided official production statistics concerning 4,778,425 doses of SMB vaccine (WHO, 1982).

C. Duck Embryo Vaccine (DEV)

In 1955, the same year as Fuenzalida and Palacios' publication, Peck *et al.* suggested another approach to vaccine production which might reduce the risk of neuroparalytic accidents (Table I). The rabies virus was grown in chicken or duck embryos, and the vaccine was constituted by a suspension of the embryonic tissues inactivated with β-propiolactone. As in the case of suckling mouse brain material, it was shown that duck embryos contained a very small quantity of immunoencephalitogenic factor (MacFarlane and Culbertson, 1954). The minimal postexposure treatment course consisted of 14 daily subcutaneous doses completed by 3 booster injections 10, 20, and 90 days after the 14-day course.

The low risk of neurological side effects made it possible to recommend preexposure immunization for those with a high risk of rabies contact. Different protocols, including 3 or 4 doses plus boosters, were suggested. Different researchers tried to improve the original vaccine by zonal centrifugation purification (Lavender and Van Franck, 1971) or concentration (Schell *et al.*, 1980).

The DEV vaccine was widely used for 27 years in the United States and many other countries. Serious side effects were rarely observed, but a large majority of those preventively immunized presented local or general reactions, as did nearly all those receiving the complete postexposure treatment (Rubin *et al.*, 1973). Comparisons made with HDCS vaccine when it became available showed the limited efficacy of the DEV vaccine (Ellenbogen and Slugg, 1973). Thus, in 1981, the main worldwide producer, based in the United States, announced that it would gradually cease the promotion and then production of DEV vaccine (Centers for Disease Control, CDC, 1981a).

III. HUMAN DIPLOID CELL STRAIN VACCINE

A. Production

Human diploid cell strain (HDCS) vaccine was first described by Wiktor *et al.* (1964) at the Wistar Institute where, at the same time, Koprowski (1966) was comparing different virus strains and culture systems for the

production of experimental vaccines. Adaptation of fixed rabies virus to a diploid cell strain followed, and it was measured for viral adsorption, penetration, release, and cytopathic effect (Wiktor, 1966). A satisfactory production system was eventually defined using the Pitman Moore (PM) rabies strain, derived from the virus used for Semple vaccine production and adapted to WI-38 cells (Hayflick and Moorhead, 1961) using subcultivation of infected cells and a cell mixing technique (Wiktor *et al.*, 1964). The original technique has been fully described by Koprowski (1973) in the WHO monograph entitled "Laboratory Techniques in Rabies."

The new vaccine proposed by Wiktor and associates has been under development at the Institut Mérieux since 1967 (Peterman *et al.*, 1967), and it was first licensed in 1974; routine commercialization and use started in 1978. The viral strain has remained the same since 1967 (Fig. 1). The virus was originally grown in WI-38 strain human diploid cells (Hayflick and Moorhead, 1961) but is now developed in MRC-5 cells (Jacobs *et al.*, 1970) (Fig. 2).

1. The Viral Strain

In their recommendations, the WHO Rabies Expert Committee (WHO, 1980) pointed out the paramount importance of using a seed virus system, and that the selected production strain should meet basic requirements and

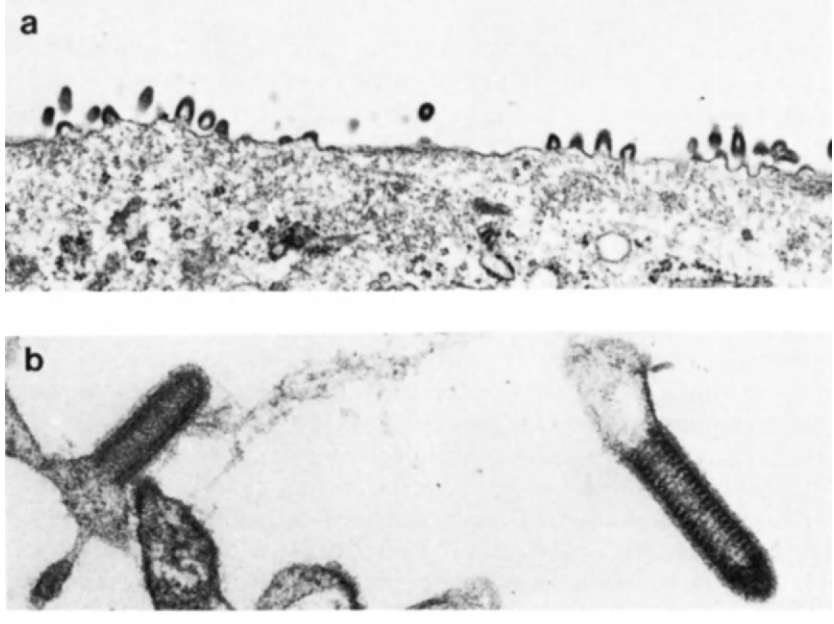


Fig. 1. (a) Rabies virions budding from cell surface. ×24,000. (b) Two virus particles. ×90,000.

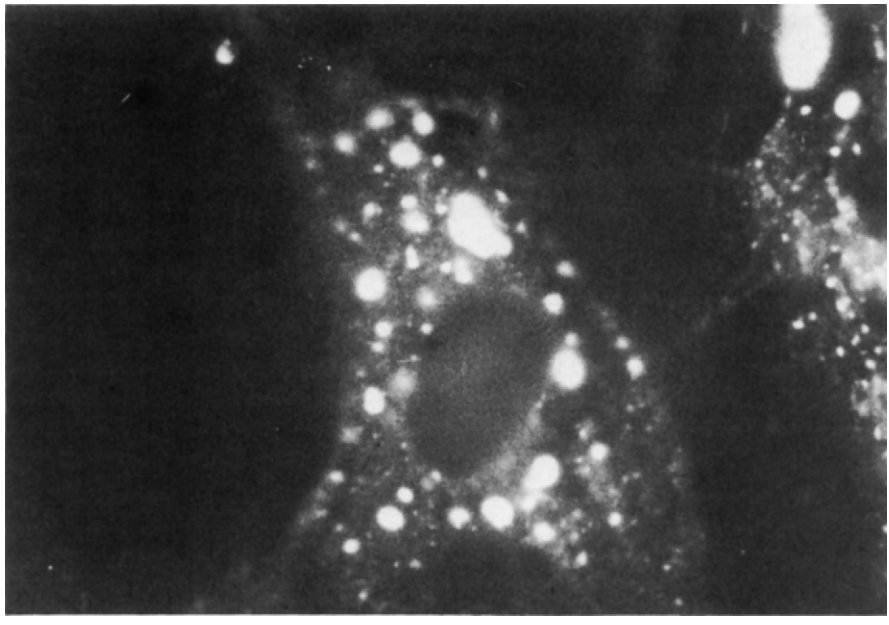


Fig. 2. Human diploid cell with fluorescent intracytoplasmic viral inclusions, 72 hr postinfection. ×800.

be checked regularly for stability. The virus strain is a fixed strain of rabies virus called Pitman Moore. The deep-frozen original strain is stored in sealed ampules at −70°C. A seed lot is prepared from the original ampules and aliquot parts are deep-frozen at −70°C. Each aliquot of seed lot enables the inoculation of diploid cells with the same virus for each production batch.

2. *Culture of Diploid Cells and Virus*

The cells necessary for rabies virus propagation are obtained by serial culture from a cell seed lot. For MRC-5 diploid cells, production is carried out using a deep-frozen ampule from a homogeneous cell seed lot established at the sixteenth passage (working seed lot). Figure 3 shows the serial passages giving monolayers of increasing surface between doublings 16 and 27; 20 days are necessary to get the cultures from doubling 16 to doubling 27. Cell growth is ensured in Eagle's basal medium, supplemented with 10% fetal calf serum. This provides 192 Roux bottles of culture ready for inoculation with the rabies virus. After inoculation, two subcultures are carried out to level 29, producing 768 Roux bottles of culture. Viral propagation is then made using Eagle's medium without calf serum but with human albumin, which makes it possible to keep the cells in good condition

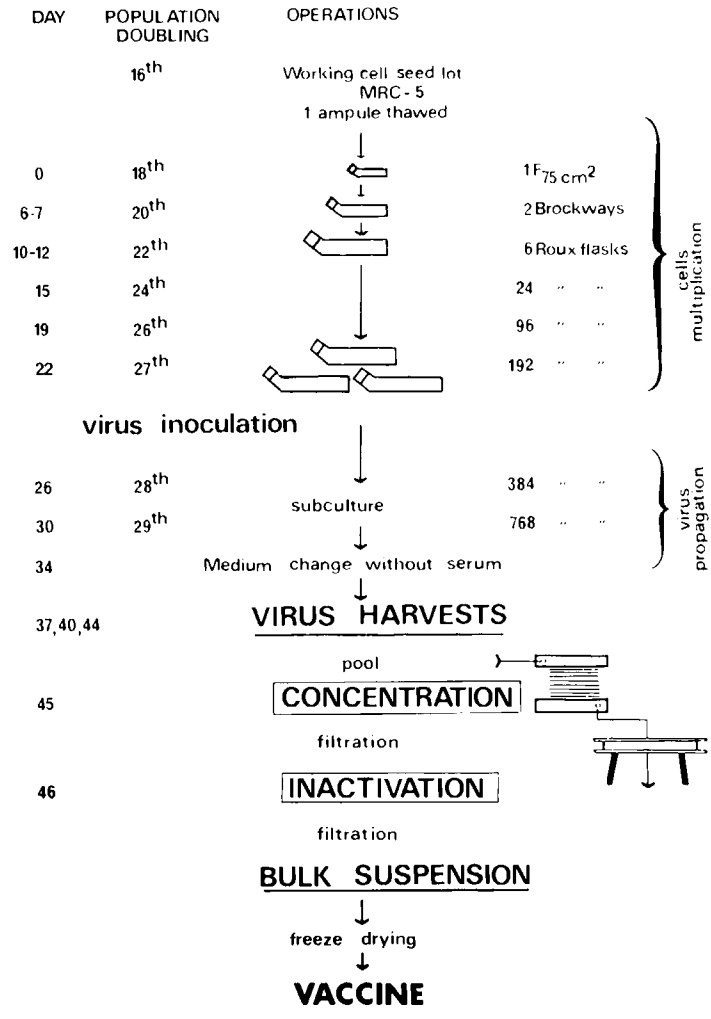


Fig. 3. Flow sheet for production of HDCS vaccine.

and to ensure the stability of the rabies virus (Koprowski, 1973). The supernatants are then harvested several times at intervals of 3–4 days and pooled to constitute the bulk suspension (see Fig. 3). Despite optimal adaptation of the PM strain to WI-38 and then to MRC-5 cells, production yields of the virus are still limited in quantity.

3. *Vaccine Preparation*

The bulk viral suspension is concentrated by a system of ultrafiltration which eliminates all molecules of less than 10,000 daltons and then is clarified by filtration through a 0.45-μm porosity membrane.

Inactivation is ensured by a combination of heat and β-propiolactone, an inactivating chemical agent whose concentration is fixed at 1:4000 of the bulk concentration (Koprowski, 1973). Inactivation is performed for 24 hr at 4°C and then 2½ hr at 37°C. Finally, the concentrated and inactivated liquid bulk suspension is clarified once more through a 0.45-μm porosity membrane before distribution into vials and freeze-drying.

All stages of virus culture, harvest, and treatments (including fundamental inactivation) must respect the immunogenic component of the virus, the glycoprotein. It has been proved that this viral glycoprotein induces the formation of virus neutralizing antibodies and thus protects animals against subsequent challenge (Wiktor *et al.*, 1973a).

Unfortunately, the low yield of virus, the length and risks of human diploid cell culture, the need for a heavy viral antigen concentration, and the complicated and lengthy controls (Roumiantzeff *et al.*, 1984) account for the high production costs of HDCS rabies vaccine.

B. Controls and Requirements

The first WHO monograph "Rabies Laboratory Techniques" was published in 1955. The WHO Rabies Expert Committee has regularly revised this monograph to agree with the progress realized in production procedures for rabies vaccines, titration, and diagnostic methods, issuing two subsequent editions in 1967 and 1973. This latest edition (WHO, 1973) forms the major reference work regarding main control techniques.

The first formal requirements regarding rabies vaccine for human use were formulated less than 10 years ago (WHO/IABS, 1978). The rapid technical development of different human diploid cell lines, such as WI-38 (Hayflick and Moorhead, 1961) and MRC-5 (Jacobs *et al.*, 1970), their acceptance as approved substrates for the production of viral vaccine (Fondation Mérieux, 1968; Gucic, 1970; Jacobs *et al.*, 1970), and their utilization for the production of rabies vaccines, first in Europe and a few years later in the United States, justified revision of these requirements in 1981 to cover all types of rabies vaccine (WHO, 1981).

1. Testing of the Cells

The utilization of human diploid cell cultures for vaccine manufacture is based on the cell seed system which represents a quantity of cells derived from a single tissue source and stored frozen at −70°C or below in aliquots, one of which is used for the production of a manufacturer's working cell bank of which one ampule gives a single harvest.

The cell seed used for the production of viral vaccine should be approved by and registered with the National Control Authority. The working cell

bank is checked with respect to lack of tumorigenicity, normal karyology during approximately the first two-thirds of its normal life span, and identity. It is also necessary to ensure that the vaccine is free of extraneous agents by testing the cells in animals (suckling mice, adult mice, guinea pigs, rabbits, and eggs) and the cell culture supernatant in cells (monkey kidney cells, primary rabbit kidney cells). Checks are also made for freedom from cultivable *Mycoplasma* and sterility.

2. *Testing of the Virus Strain*

The preparation of rabies vaccine is based on the virus seed lot system. A master seed lot represents a quantity of virus of uniform composition, processed together, and stored frozen at −70°C or below in aliquots, one of which is used for the preparation of a working seed lot. The latter should not be more than five passages away from the master seed lot and should be kept continuously frozen in aliquots at a temperature below −70°C.

The virus strain to be used for vaccine production should be identified by historical records and be a "fixed strain"; i.e., one which has a short, stable, and reproducible incubation period when injected intracerebrally into suitable animals and does not form Negri bodies (Netter and Perkins, 1973).

The virus strain used should be approved by and registered with the National Control Authority. The working seed lot is tested for freedom from extraneous agents in animals (suckling mice, adult mice) and in cells (human diploid cells, monkey kidney cells). Checks are also made for the absence of *Mycobacterium tuberculosis* in guinea pigs, freedom from cultivable *Mycoplasma,* and sterility.

3. *Production Controls*

a. Cell Controls. At least 5% of the cell suspension (not less than 500 ml), at the concentration employed for seeding the vaccine production culture, is used to prepare a control culture. Cells for control should be treated in the same way as production cells, but should not be inoculated, thus enabling the detection of extraneous viruses. They are incubated under the same conditions as the inoculated culture for at least 2 weeks and observed during this period for cytopathic change. At the end of the observation period, 25% of the control cells should be tested, using guinea pig red cells, for the presence of hemadsorbing viruses. At the final virus harvest, supernatant samples are collected from each group of control cell cultures, pooled, and checked for freedom from extraneous agents in cells (human diploid cells, primary human cells, primary monkey kidney cells), sterility, freedom from cultivable *Mycoplasma,* and karyology (identity test).

b. Controls of Bulk Material. The following controls are made on the viral harvest pool before inactivation: determination of the infectious titer, sterility test, and a test for the detection of cultivable *Mycoplasma.*

c. Final Bulk Controls. After concentration and inactivation, the final bulk is stored in quarantine at 4°C until control results are available. Checks are then made for effective inactivation by amplification and direct inoculation in mice. In amplification, a minimum of 25 ml of the bulk suspension (25 human doses) is inoculated in the human diploid cell cultures in order to test for the presence of live virus. The cells are observed for at least 21 days. At days 14 and 21, supernatant samples are taken, and 0.03 ml of each is inoculated intracerebrally into 20 mice, which are then observed for 21 days. For inoculation in mice, 20 mice are inoculated intracerebrally with 0.03 ml of the final bulk and then observed for 21 days. In either case, these tests are satisfactory if none of the mice show any evidence of infection with live rabies virus. Further checks are made for sterility and for freedom from cultivable *Mycoplasma.*

d. Controls of the Final Batch. Each final batch undergoes a series of physicochemical tests for aspect, residual moisture, and total protein content, then tests for sterility, general safety and potency at 4°C and after 1 month at 37°C to demonstrate stability. The final containers are then inspected.

4. Potency Determination of Rabies Vaccine

The evaluation of rabies vaccine efficacy is of major importance to those concerned with vaccine manufacture and control. In order to measure protective immunogenic activity, the NIH test (Seligmann, 1973b) is more commonly used; this is one of the officially recommended tests for the determination of rabies vaccine potency (WHO, 1981). The test is carried out using mice which are immunized and subsequently challenged with rabies virus. The reproducibility and safety of the tests depend in part on the rabies virus strain used for the challenge, and also on the use of a reference preparation. A suitable challenge strain, CVS, should be used; it is available from the World Health Organization (Atanasiu, 1973).

The vaccine's potency in international units (IU) is then determined by comparison of its activity with that of the reference preparation. The reference preparation proposed in 1965 was a freeze-dried suspension of rabbit's brain infected with fixed rabies virus and inactivated by ultraviolet light. Sweet (1966) suggested that a standard derived from animal nervous tissue or embryonated eggs was not acceptable for measuring the efficacy of the newly developed tissue culture vaccines. It was only in 1978 that an International Rabies Vaccine Reference preparation became available in the

form of ampules containing freeze-dried rabies vaccine prepared on human diploid cells, inactivated with β-propiolactone, and with activity of 10 IU per ampule. This reference preparation is distributed by the Serum Institute in Copenhagen. Since only a limited amount is available, an "in house" reference preparation, calibrated in IU against the third international reference rabies vaccine preparation, can be used for the NIH test (Wiktor *et al.*, 1978b).

Ten years of industrial production and worldwide marketing have proved the value of these testing procedures (Kuwert *et al.*, 1984).

The problem of vaccine potency is a major one, as seen by the final recommendations of the Tunis Congress on "Rabies in the Tropics": good potency is essential for all rabies vaccines, especially those intended for use in tropical countries (Baer, 1984 a,b).

Although the NIH test effectively evaluates *in vivo* protective activity, many researchers have tried to find a simpler way of measuring *in vitro* the quantity of glycoprotein present in rabies virus particles, and responsible for the stimulation of neutralizing antibodies (Cox *et al.*, 1977). Several different tests have been proposed: antibody binding test (Arko *et al.*, 1973), radioimmunoassay (Wiktor and Koprowski, 1978), ELISA test (Van Der Marel and Van Wezel, 1981), single radial immunodiffusion (Schild and Ferguson, 1982).

Even if these *in vitro* tests do not necessarily reflect the protective activity of vaccines with total accuracy, a comparison of 78 HDCS rabies batches (Vincent-Falquet *et al.*, 1984) showed that 3 of them classified the vaccines in the same order. These tests do not present a perfect correlation with the NIH test, but they enable a sufficiently accurate indication of the future NIH potency of the vaccine and would thus be useful for the evaluation of potency during vaccine manufacture.

5. *Measurement of Humoral Immunity*

In order to determine the vaccine's activity after administration, it is important to be able to measure the level of rabies antibody in a subject. The *in vivo* test in mice, developed more than 40 years ago (Webster and Dawson, 1935), used to be the most common procedure for determining the level of rabies neutralizing antibody. The introduction of an International Standard for Antirabies Serum calibrated in IU has enabled the expression of a serum's potency (Atanasiu, 1973). However, this *in vivo* test in mice has two major drawbacks: potency values show considerable variability within a laboratory and from one laboratory to another, and it is time consuming and expensive. Thus in recent years, several laboratories have developed *in vitro* techniques for the testing of rabies virus neutralizing antibody, e.g., the passive hemagglutination test (Dierks and Gouch, 1973), or the radioimmu-

noassay for rabies binding antibody (Wiktor, 1973). The most commonly used and officially recognized technique is the rapid fluorescent focus inhibition test (RFFIT) (Smith *et al.*, 1973). This technique is quick (it can be performed in 24 hr) and inexpensive, and results can be correlated with those obtained with the *in vivo* test in the mouse. A standard reference calibrated in IU makes it possible to give the serum's titer in IU for each test. The reference serum is a desiccated horse hyperimmune serum, corresponding to 86.6 IU per ampule, distributed by the Serum Institute, Copenhagen.

C. Purity and Stability

The use of diploid cells in the preparation of rabies vaccines has represented a major step toward an improvement in their quality. This can be considered with respect to two main criteria; the purity and the stability of the final product.

1. Purity of HDCS Vaccine

The cell culture method, particularly in diploid cells, has enabled the obtention of an extremely pure viral preparation with a very high antigen titer. This purity can be qualitative and quantitative.

From a qualitative point of view, HDCS vaccine prepared from diploid cell culture does not contain any neurotoxic factors and is free from the encephalitogenic risk present with the repeated injection of classical vaccines produced from crushed animal brains or duck embryos containing nervous cell elements.

Quantitatively, purity can be estimated from the protein content. The protein concentration of the Mérieux vaccine is mainly provided by human albumin introduced during the production process. In spite of this addition, the protein content of the final HDCS vaccine is about $\frac{1}{20}$th that of SMB vaccines, $\frac{1}{70}$th that of DEV vaccine, and $\frac{1}{250}$th that of classical Semple vaccines made using sheep brains. More important, the HDCS vaccine has no trace of foreign animal contaminants, thus explaining its good tolerance after even repeated injections.

2. Stability of HDCS Vaccine

One of the more important qualities for a vaccine is its stability. This is particularly important for rabies vaccine, which must ensure good preexposure prevention or postexposure treatment for a high-risk disease. Stability is all the more important when it becomes clear from published data (Acha and Arambulo, 1984) that in 1981 almost all deaths after rabies exposure (99.4%) occurred in the tropics, and that 94.2% of all antirabies treatments were given in tropical regions.

Traditional vaccines, as well as their weak immunogenic ability, usually present very poor stability, and lyophilized modern vaccines prepared from cell culture have proved to be much better in this respect. Our increasing knowledge of the rabies virus structure and its antigenic components (Cox *et al.*, 1977) has thrown light on the stability of rabies virus and vaccines. Rabies virus grown in cell culture may lose up to 90% of its infectivity in 24 hr at 37°C; the virus is relatively stable between pH 5 and 10, but, because of its lipid content, is easily disrupted by treatment with organic solvents, soap, or detergent (Crick, 1983).

When the WHO Rabies Expert Committee defined its requirements for good rabies vaccines, they imposed the use of an accelerated stability test where, according to the NIH method (Seligmann, 1973b), two aliquots of the product should be tested, one kept at 4°C and the other for 28 days at 37°C (Netter and Perkins, 1973). Each final lot should be tested for potency in the final container immediately after lyophilization.

Early in its development, the HDCS vaccine was seen to have a good capacity for stability, and after some years of industrial production Nicolas *et al.*, (1978) reported a surprisingly high stability rate. These results were confirmed by Chippaux *et al.* (1984). Table II summarizes the data on stability observed between 1980 and 1983 for batches of HDCS vaccine tested according to WHO requirements. These show that the average titer of all lots at 4°C is 4.78 IU per dose, and after 1 month at 37°C it is 4.74 IU per dose. Statistical analyses demonstrate that there is no significant difference between the results obtained at 4 and 37°C.

Table III shows the stability of vaccine in real time at 4°C. The normal variations found with the biological NIH test and analyzed in Table II are in fact the main reason for the apparent differences seen between some of

TABLE II
Comparison of NIH Potency Tests Carried out on Pair Samples[a]

Parameter	Stored at 4°C	After 1 month at 37°C
Number of batches	127	127
Antigenic value IU/ml (arithmetic mean)	4.68	4.74
Standard deviation	1.93	2.40
Range	2.5–16.7	2.5–13.6
Difference	0.06	
Difference standard deviation	2.64	
Paired *t* statistic	0.26	
P	Not significant	

[a]One sample is kept at 4°C and the other was tested after 1 month at 37°C for 127 batches of HDCS vaccine.

TABLE III
Stability of HDCS Vaccines[a]

Vaccine batch	Storage	Titer (IU per dose)	
		Initial titer	After conservation
1	3 Years, 7 months	4.3	4.6
2	3 Years, 7 months	5.3	2.7
3	3 Years, 7 months	6.6	3.1
4	3 Years, 7 months	5.6	3.0
5	3 Years, 8 months	4.3	4.0
6	4 Years	9.7	3.2

[a]Vaccines were stored at 4°C, and stability was estimated by potency comparisons using the NIH test. Normal value for satisfactory potency = 2.5 IU per dose.

the six lots. It has been shown that there are very few variations in potency during a storage period of more than 3½ years, and the final potency of each lot was always superior to 2.5 IU per dose.

This stability and long shelf-life demonstrated in the laboratory have also been proved under field conditions. Nicholson *et al.* (1983) monitored the stability of a batch of HDCS vaccine during its distribution and unsuitable storage in Pakistan. Despite continuous exposure to high temperatures for up to 11 weeks the three laboratory tests showed that the vaccine retained its antigenicity. A substantial antibody response was noted in all the immunized subjects, injected subcutaneously or intradermally, whatever the storage conditions. These excellent field results were confirmed by those obtained in Thailand (Wasi *et al.*, 1983) or more recently during an investigation in the United States and Kenya (CDC, 1983b).

But despite this good stability, no risks should be taken which might diminish the rabies vaccine's activity or affect the absolute security expected of it; users should always be urged to respect the recommended storage temperature of 4–8°C.

D. Experimental Investigations in Animals

The first trial batches of rabies vaccine produced on WI-38 diploid cells were used for experimental investigations in animals. From 1963 to 1966, the new vaccine was extensively investigated in mice using the Habel test and then the NIH test (Wiktor *et al.*, 1978a). These early results were used some years later as a basis for the proposal of new potency test requirements for rabies vaccines prepared in cell culture. In particular, the NIH test was to become mandatory with a definition of a minimum level of 2.5 IU per dose.

The new vaccine also demonstrated a better comparative immunogenicity in the monkey (Wiktor and Koprowski, 1965). The level of neutralizing antibodies measured over a period of 135 days in a group of 10 monkeys vaccinated with DEV or HDCS vaccines was higher and remained at a significant level longer after three doses of HDCS than after seven doses of DEV vaccine (Koprowski, 1966). Preexposure experiments in rhesus monkeys showed that neutralizing antibody levels were very high after a single injection of different batches of Mérieux HDCS vaccine. This was in contrast with the very low response achieved after one human dose of DEV or SMB vaccine, which was undetectable 36 days after immunization (Sikes *et al.*, 1971). In the same preexposure studies, the good results obtained with two out of three experimental lots made it possible to define the optimum production method for HDCS vaccine. These two lots afforded excellent protection against a street virus challenge administered 73 days after vaccination. However, virtually no protection was obtained with either a commercial DEV or the official NIH reference vaccines (Sikes *et al.*, 1971).

These and other experimental investigations on animals have demonstrated the excellent tolerance of the new HDCS vaccine. Traditional vaccines never underwent such exhaustive safety trials. All these excellent results obtained in laboratory animals opened the way to early trials regarding safety and activity in human volunteers (Wiktor *et al.*, 1973b; Dureux, 1974).

E. Safety and Tolerance

1. Traditional Vaccines

The high level of adverse reactions and the potential danger of postvaccinal nervous complications following the administration of traditional rabies vaccines are well recognized. Numerous investigations in the past have assessed these risks, which are known to be due essentially to the high content of contaminating agents.

a. Nervous Tissue Vaccines. In Fermi, Semple, or Hempt vaccines, the rate of reported severe neurological accidents varied between 1:400 and 1:8500 cases (Greenwood, 1945; Sellers, 1948; Pait and Pearson, 1949; Sikes and Larghi, 1967; Vejjajiva, 1967; Thongcharoen *et al.*, 1982a). Neither the inclusion of ether treatment in the Hempt procedure used in Eastern Europe, nor the further addition of total inactivation by β-propiolactone, significantly reduced these risks. Before the introduction of HDCS vaccine, Hempt-type vaccines had postvaccination neurological accident rates of 1:765 in the Federal Republic of Germany. The most recent report following a review of the literature and analysis of official statistics has confirmed

this high risk with rates of neurocomplications with brain-type vaccines approaching values of between 1:500 and 1:5000 (Kuwert *et al.*, 1981).

b. Suckling Mouse Brain Vaccines. The SMB vaccines certainly represented an improvement, but the rate of postvaccinal neurological accidents in the different countries producing the vaccine was still estimated at 1:15,953 treatments, with a large majority of severe Guillain-Barré syndrome (Held and Lopez Adaros, 1972) or 1:17,891 calculated from official 1972 WHO figures (Lery *et al.*, 1975), with a high proportion of fatal outcomes (Ofsanpan, 1976). In spite of different manufacturing improvements, the risk was still appreciable in certain producing countries. PAHO reported annually from 1970 to 1981 on between 7 and 30 postvaccination complications observed in Latin America after the administration of different SMB vaccines used in the treatment of 300,000 patients each year.

c. Duck Embryo Vaccine (DEV). DEV was considered safe enough to be developed on a large scale for use in preexposure immunization, although the official rate of neuroparalytic complications was about 1:32,000 recipients (Hattwick, 1974; Kuwert *et al.*, 1978a). In spite of successive improvements in concentration, inactivation, and purification techniques, recent official figures from the United States have been pessimistic: "With DEV, anaphylaxis developed in slightly $<1\%$ of recipients. Neuroparalytic accidents, including transversomyelitis, cranial or peripheral neuropathy and encephalopathy, are estimated to occur in about 1 in 24,000 vaccine recipients" (Meyer, 1980).

Today, in spite of all the technical improvements made to these different traditional vaccines, the WHO has globally recorded one accident in 19,525 people treated (WHO, 1982). These figures are underestimated due to the poor quality of the surveillance system. However, even today 36 times more doses of traditional vaccines than of HDCS vaccine are administered (WHO, 1982), and it is evident that the risks of a severe neurological accident or even death following rabies treatment remain at a very high level.

2. *General Safety and Tolerance*

Vaccines prepared from cell-grown virus were developed to eliminate the risk of encephalitic accidents. Early studies clearly showed that HDCS vaccine provided total safety. In spite of repeated injections, it had good tolerance, provoking only the local and general side effects common to all modern inactivated vaccines. Table IV shows the percentages of reactions reported in France for hundreds of vaccinations in volunteers (Dureux, 1974; Armengaud *et al.*, 1975). In order to ensure that the various immunization procedures were perfectly tolerated, different injection routes were

TABLE IV
HDCS Rabies Vaccine Side Effects

Reaction	Number of cases (%)	
	From Dureux (1974)	From Armengaud *et al.* (1975)
Local		
Erythema	4.9	11.5
Induration	7.3	3.5
Pain	7.4	14.5
Itching	0	3.0
General		
Fever	3.7	1.5
Malaise	3.7	0.7
Shivering	0	0
Allergic		
Rash	0	0
Urticaria	0	0

employed, and different intervals between injections respected throughout the trials. No serious side effects were seen after primovaccination, whatever system was adopted (Aoki *et al.*, 1975; Ajjan *et al.*, 1978). In some trials (Kuwert, 1976), the number of successive inoculations or postexposure treatment was increased up to 9, and still no abnormal reactions were observed. Booster injections induced no particular adverse reactions.

In some countries, follow-up surveys have provided a broader overview of possible side effects. In the United States a survey of State Health Departments (CDC, 1980b) over a 3-month period corresponded to 25,200 doses of Mérieux HDCS vaccine given to 2500 patients, the majority of whom received postexposure treatments. The survey showed that 4 patients (1:625 treated) had systemic allergic reactions ranging from hives to anaphylactic shock. Two cases were complicated by the simultaneous administration of human rabies globulin.

The most complete follow-up study of reactions was conducted in Britain between 1976 and 1983 and covered more than 40,000 doses used for both pre- and postexposure immunization (Gardner, 1983). The author reported no serious reactions resulting in death or permanent neurological damage. Two neurological complications were observed: one transient, the second causing residual wasting of the deltoid muscle. Local, usually minor, reactions were clearly observed in 15% of cases (370 out of 2404 vaccinees): 7% with pain, 6% with erythema, 5% with swelling, 3% with aching and stiffness, 0.3% with local sensory changes, 0.1% with stinging, and 0.1% with lymphadenopathy. Systemic reactions were also observed in 7% of cases: 2% described as influenza-like, 1.5% with headache, 1% with fever,

1% with fatigue, 1% with malaise; 1% of the vaccinees also presented allergic reactions, some of which were serious enough to advise against further immunization with HDCS vaccine (Gardner, 1983).

Today after 10 years of general use and more than 2.4 million doses administered to 533,000 patients throughout the world, it is possible to make general evaluation of the vaccine (Kuwert *et al.*, 1984). Few severe cases have been reported following use of HDCS vaccine: 1 Guillain-Barré syndrome (Boe and Nyland, 1980) and 1 case of transient neuroparalytic illness where the relationship between vaccination and the neurological accident was not clearly established (Bernard *et al.*, 1982b): "Even if 2 cases are accepted as due to vaccination by HDCS, the neurocomplication rate would be only $\leq 1{:}260{,}000$, thus approaching the corresponding values of the most safe vaccines" (Kuwert *et al.*, 1984).

3. *Tolerance in Children*

The treatment of children with traditional rabies vaccines was a major problem in the past. A classical reaction was to reduce the dose in children (Lépine *et al.*, 1973) to diminish pain during injection. This, however, did not reduce the severity of complications which were sometimes reported and clinically described (Vejjajiva, 1967; Timm and Wolter, 1971; Lery *et al.*, 1975; Kuwert *et al.*, 1981), but no specific pediatric statistics are available.

The development of HDCS vaccine was regarded optimistically by pediatricians, and in the first study, carried out in Germany, Kuwert *et al.* (1976) reported that 17 children aged between 4 and 12 years perfectly tolerated six doses of Mérieux HDCS vaccine. Two years later, Kuwert *et al.* (1977a) studied a large group of 950 vaccinees including 185 children. They reported an absence of any major postvaccinal reactions even when the vaccine was given to children younger than 2 years of age, at the dose and the schedule used for adults. However, it was children who displayed the most marked endotoxic reactions to a pyrogenic lot. Thus two additional tests—pyrogenic determination test in the rabbit, and the *Limulus* amoebocyte lysate clotting test—were proposed for inclusion in control procedures to ensure better tolerance to HDCS vaccine (Kuwert *et al.*, 1978a).

A recent study (Ajjan *et al.*, 1983) reported the follow-up of 289 children under 15 years of age who received HDCS postexposure treatment. There were a few mild reactions: 11 children (3.8%) developed a fever of 38–38.5°C after the third or fourth injection; local induration was observed in 14 children (4.8%); 19 (6.5%) complained of slight local pain when touched, and 29 (10%) had axillary or posterior cervical adenopathy with no sign of inflammation. None of these reactions required discontinuation of the antirabies treatment. These results confirm the safety of the HDCS treatment as already reported by Klietmann *et al.* (1978), Plotkin and Wiktor (1979),

and Thongcharoen *et al.* (1982b). The safe and well accepted postexposure treatment of children is today routinely applied; for instance, out of 5654 subjects treated in the United States in 1980 and 1981, 8% were under 5 and 34% under 15 years of age (Helmick, 1983).

4. Tolerance in the Pregnant Woman

Very few papers have been published regarding cases of rabies or their treatment in pregnant women using Semple vaccine (Vibulbandhitkij, 1980), or DEV (Cates, 1974; Spence *et al.*, 1975). Even with HDCS vaccine, few cases of vaccination have been described (Klietmann *et al.*, 1978; Varner *et al.*, 1982). Very recently, the Antirabies Centers in France (Ajjan, 1983) initiated a retrospective study of a dozen cases of postexposure treatment with SMB and HDCS vaccines. In all cases, the pregnancy proceeded normally and the babies were unaffected. The authors intend to broaden surveillance in this respect.

F. Preexposure Vaccination

1. Early Investigations

After the first publication describing rabies HDCS vaccine (Wiktor *et al.*, 1964), and although the WHO Rabies Expert Committee (WHO, 1966) strongly recommended experimental trials of the new vaccine, it took several years to organize the first clinical investigations. After extensive experiments on laboratory animals (Sikes *et al.*, 1971), Wiktor, Koprowski, and Plotkin decided to carry out the first inoculations of human volunteers in November 1971 (Wiktor *et al.*, 1973b). This successful experiment was undertaken using a split product (Plotkin, 1980).

Clinical investigations of the Mérieux HDCS whole-virion vaccine were conducted in Iran and France according to the detailed field protocol for the assessment of HDCS vaccine immunogenicity (WHO, 1973). The first results of serological titrations after vaccination were published by Bahmanyar in 1974. Groups of subjects, all previously nonimmunized against rabies, were vaccinated intramuscularly with either 1 or 3 doses at days 0, 3, 7, or 4 doses at days 0, 1, 2, 3 or days 0, 3, 7, and 21. A booster dose was administered on day 91. Serum samples were collected at days 0, 3, 7, 21, 35, 91, and 104 and showed that a single dose rapidly induced detectable antibody, but its level 21 and 35 days after the single dose was not judged sufficient "to offer dependable protection against the invasion of street virus" (Bahmanyar, 1974). However, a four-dose schedule induced a strong antibody response.

From 1973 to mid-1974, 694 subjects received preexposure vaccination in eight French rabies centers; 107 accepted a complete serological follow-up

and all of them presented seroconversion after two injections (Dureux, 1974). In September 1974, the French Ministry of Health granted a license for the preventive use of the new HDCS vaccine. Different schedules were compared (Fig. 4) in an attempt to define the simplest and most acceptable preexposure preventive regimen; two doses injected intramuscularly at days 0 and 28 (Soulebot, 1974), three doses injected subcutaneously at days 0, 28, and 56 (Dureux and Canton, 1975; Armengaud *et al.*, 1975), or three doses also injected at days 0, 28, and 56 with a comparison of the subcutaneous and intramuscular routes (Ajjan *et al.*, 1978).

2. *Primovaccination*

Following these early investigations, experimental preventive vaccinations were conducted by different European research teams and led to the proposal of other preexposure vaccination regimens.

In Germany (Kuwert *et al.*, 1976), 227 subjects received subcutaneous injections of HDCS vaccine for preexposure immunization. Careful serological monitoring was made in two groups; 13 people who had received three doses at days 0, 28, and 56, and 46 people who had received three doses at days

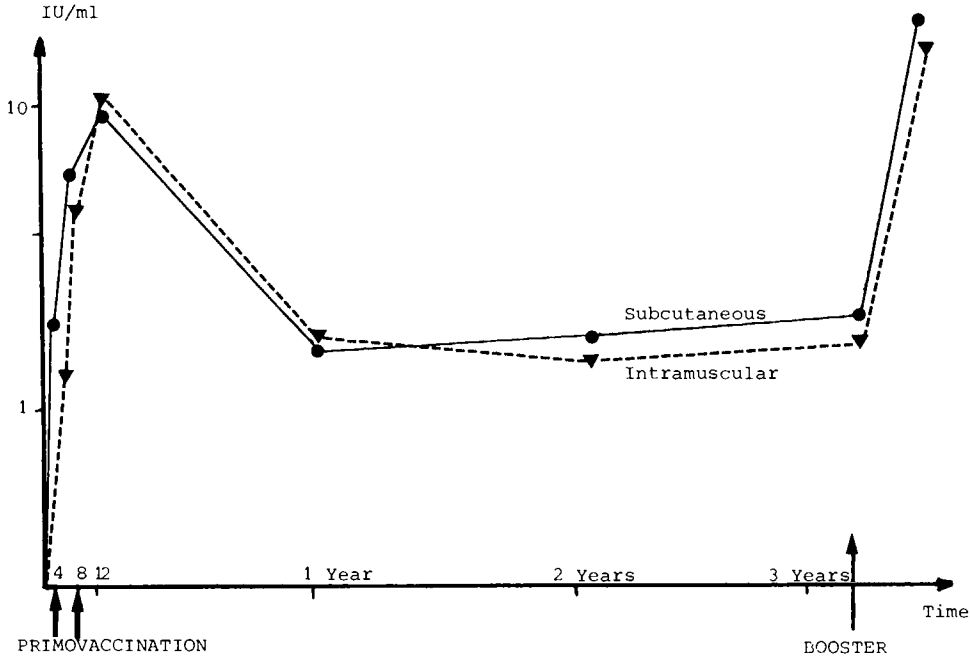


Fig. 4. Seroneutralizing antibodies (in IU/ml) following preexposure HDCS rabies vaccination and booster injection.

0, 7, and 14. Good antibody titers were obtained after only one dose, but to ensure the best protection, a prophylactic schedule of three doses at 4-week intervals was recommended for the general population, and even four doses at days 0, 3, 7, and 21 for high-risk groups (WHO/IABS, 1978; Kuwert *et al.*, 1981).

In 1974, the British Medical Research Council initiated a series of studies of HDCS vaccine, comparing results obtained with the intramuscular and intradermal routes. The first study (Aoki *et al.*, 1975) showed that 29 out of 31 volunteers had a sufficient titer after a single dose, and all of them after two doses. The follow-up study carried out 2 years later (Nicholson *et al.*, 1978) recommended a primary series of 2 × 1 ml injections given intramuscularly or subcutaneously, at an interval of 4 weeks.

Since 1976, HDCS vaccine has been introduced into many European countries for preexposure vaccination: three 1-ml doses given deep-subcutaneously at a 1-month interval in Belgium (Costy-Berger, 1978), two doses at days 0 and 30 in Switzerland (Keller *et al.*, 1981) and Italy (Trivello *et al.*, 1982).

3. *Booster Inoculations*

Early serological investigations showed that the neutralizing antibodies diminished consistently after an early peak 1 month (Bahmanyar, 1974) or 3 months (Ajjan *et al.*, 1978) after preventive HDCS vaccination. A booster injection was thus necessary to offer continuous and long-lasting protection.

Ajjan *et al.* (1978) measured the evolution of neutralizing antibodies over a 1-year period in 62 subjects primovaccinated with two doses: all the subjects, whatever the injection route employed, presented antibody levels superior to 0.5 IU, 48 weeks after primoimmunization. Kuwert demonstrated the persistence of neutralizing antibodies in all subjects monitored for 200–248 days (Kuwert *et al.*, 1976) and then for about 2 years (Kuwert *et al.*, 1978a).

In France, it was considered safe to recommend a first booster after 1 year and the second 3 years later (Dureux and Canton, 1975; Ajjan *et al.*, 1978). In Germany, a first booster after 18 months to 2 years was recommended (Kuwert *et al.*, 1976) with subsequent boosters every 2 or 3 years (Kuwert *et al.*, 1978a). In the United Kingdom, Nicholson *et al.* (1978) recommended a first booster 6 months after primovaccination for high-risk populations and at 1 year for subjects without special risk, "the interval between subsequent booster doses could be 2 or possibly 3 years" (Nicholson *et al.*, 1978). In Switzerland, it was reported that antibody titers had decreased to insufficient values a year after primovaccination (Keller

et al., 1981), thus indicating the need for an early booster for high-risk groups.

In all these different studies, the effect of the first booster was always very marked in all vaccinees. Today, with longer term evaluations of the duration of immunity available (see Section III, H) it is possible to recommend that the second booster be given after a 3-year interval (Fig. 4).

4. Preexposure Vaccination: Official Recommendations

After the publication of the results of clinical and follow-up studies of immunity in healthy people preventively immunized by primovaccination and often boosters, the authorities in some countries made their official recommendations regarding preexposure vaccination against rabies. Table V summarizes some of these recommendations and indicates the differences between them.

In France, the national experts and producers early advocated a simple schedule of two 1-ml doses at days 0 and 28, given subcutaneously or intramuscularly; the first booster was recommended 1 year after primoimmunization. In Germany, official recommendations were published in 1977 (Kuwert *et al.*, 1981) giving the choice of three or four 1-ml doses injected either at intervals of 4 weeks at days 0, 28, and 56, or rapidly at days 0, 3, 7, and 21. In the United Kingdom, the Mérieux HDCS vaccine was licensed in 1976 with the recommendation of a preexposure schedule of a primary series of two doses of vaccine: "Each dose consists of 1 ml vaccine given intramuscularly with a 4 week interval; a 3rd dose of 1 ml is given after 12 months. Reinforcing doses are given every 2–3 years depending on the risk of continuing exposure" (Gardner, 1983).

In the United States, follow-up studies of primoimmunization and booster

TABLE V
Official Recommendations for Preexposure Rabies Vaccination[a]

Source	Volume	Route	Schedules	Booster
France, United Kingdom	1 ml	sc or im	Days 0, 28	1 year, then every 3 years
Germany	1 ml	im	days 0, 28, 56, or 0, 3, 7, 21	
United States	1 ml	im	Days 0, 7, 28	As required or every 2 years
WHO, 1980[b]	1 ml		Days 0, 7, 28, or 0, 28, 56	Boosters should be administered at intervals of 1–3 years

[a]From Roumiantzeff *et al.* (1983).
[b]The preference is for three injections of a potent rabies vaccine (at least 2.5 IU).

effects were less clear, as they were limited to only a few experimental trials of the HDCV split-product vaccine (Plotkin, 1980) until licensing of the Mérieux HDCS vaccine in June 1980 (Meyer, 1980). The Immunization Practices Advisory Committee then published very clear recommendations (CDC, 1980a) for the HDCS vaccine, which "is the vaccine of choice whenever available. Three 1 ml injections of HDCV should be given intramuscularly (for example, in the deltoid area) one at each of days 0, 7 and 21 or 28." But the recommendation for a first booster varied with the level of rabid risk exposure from 6 months to 2 years after the three dose primoimmunization. At that time, it was recommended that the immune status of each subject at risk be determined by the rabies antibody titer (CDC, 1980a). A year later, the Immunization Practices Advisory Committee "reviewed data on seroconversion in persons properly vaccinated with HDCS vaccine. The data showed that 100% of vaccinees (510/510) had protective antibody levels following preexposure treatment. In view of these findings. . . the ACIP now sees no reason to continue routine serologic testing of persons who receive the recommended preexposure treatment. . . 3 intramuscular, 1.0 ml doses at days 0, 7 and 21 or 28" (CDC, 1981b).

The differences between these national authorities are reflected in the latest recommendations from the Seventh WHO Rabies Expert Committee in 1980: "A preexposure rabies vaccination preferably consists of 3 injections of a potent rabies vaccine given at days 0, 7 and 28, or 0, 28 and 56. A further booster should be administered at intervals of 1 to 3 years" (WHO, 1980). We decided to run a trial using a single batch of HDCS vaccine to compare the two preexposure schedules proposed by the WHO experts. The first schedule, as adopted by the United States Committee, with injections at days 0, 7, and 28, was undoubtedly better. An immune state was more rapidly obtained and there was a significant difference in antibody levels at 28 days. The highly significant advantage a month after the third injection is even more important (Roumiantzeff *et al.*, 1983).

5. *Comparison between HDCS and Traditional Vaccines*

For preexposure immunization, the comparison of results can be restricted to DEV and SMB vaccines. These two traditional vaccines were or are recommended for preexposure vaccination in different countries. DEV was the first rabies vaccine to be considered safe enough for preexposure use, particularly in the United States (Hattwick, 1974; CDC, 1976). SMB vaccine was recommended in some countries including France, and is still used in rabies centers for prophylactic immunization, particularly in Latin America. The comparative results of safety and side-effect tests are reviewed in Section III,E, so here we would like to compare their activity only.

a. Comparison between HDCS and DEV. It has often been reported that DEV has limited potency. In schedules of either two doses 1 month apart with a booster 6–7 months later, or in the more rapid program of three doses at 1-week intervals and a booster 3 months later, only 80–90% of vaccinees could expect neutralizing antibodies (Corey and Hattwick, 1975; CDC, 1976). Even an accelerated protocol of four to five doses of purified DEV over a 2-week period (Nelson *et al.*, 1977) gave neutralizing antibodies in only 84.8% vaccinated subjects. It was thus agreed that all those receiving a DEV postexposure vaccination should have their serum tested (CDC, 1976, 1980a).

Early serological investigations of the new HDCS vaccine quickly demonstrated that the response obtained with one or two injections was markedly better than that achieved with DEV (Wiktor, 1974; Kaplan, 1974). Bahmanyar's study (1974), evaluating serological profiles, concluded that "to the best of our knowledge, no other rabies vaccine has yet been demonstrated to be as immunogenic in man as the HDCS vaccine used in the present trial." Few truly comparative preexposure trials were conducted, as the leading investigators soon moved over from DEV to HDCS vaccine.

Schell *et al.* (1980) produced a highly purified and concentrated DEV with a minimal potency of 2.5 IU, and they claimed that a DEV with such a high antigenic value could compete with HDCS vaccine. Glück *et al.* (1984) recently proposed a new DEV with 10 IU per dose.

A double-blind study was instigated in the United States to demonstrate that 2, 3, "or 4 doses of HDCS split-product vaccine developed antibody titers and that the mean neutralizing titer was higher than with 4 doses of DEV" (Hafkin *et al.*, 1978). In 1980, HDCS vaccine was officially advocated as the vaccine of choice for preexposure use (CDC, 1980a), and then a year later it was agreed that mandatory serological controls were no longer necessary (CDC, 1981b). The production of DEV was definitively abandoned in the United States in 1981 (CDC, 1981a).

b. Comparison between HDCS and SMB Vaccines. Comparisons between HDCS and SMB vaccines are, to our knowledge, very limited.

In France, during early experiments with the HDCS vaccine, a very limited comparison showed that a similar antibody level was obtained after only two HDCS vaccinations and after 12 injections of SMB (Dureux, 1974). In Belgium, Costy-Berger (1978), working with a small group of volunteers, found that three HDCS vaccine doses elicited an antibody response that was five times superior to that obtained with the classic SMB vaccine schedule. In Latin America, a preexposure SMB vaccine schedule, proposed by Fuenzalida (1972), recommended three 2-ml doses injected on alternate days for

regular use in high-risk groups. Diaz (1982) reported 96% seroconversion; he compared these results favorably with published reports using HDCS vaccine and pointed out that SMB vaccine was the only vaccine for human use produced in Latin America.

HDCS vaccine today is used principally for postexposure treatment in less wealthy countries where the risk of rabies is high. In developed countries, preventive vaccination is on the increase, and HDCS is the product of choice for simple and sure prophylaxis. However, the spread of preventive vaccination worldwide will depend on the development of a more economical and equally efficient vaccine.

G. Postexposure Treatment

Louis Pasteur originally proposed the idea of an immunological treatment for rabies, consisting of active immunization after potential infection. By taking advantage of the slow spread of virus in the body, it is possible to create a solid immune state after exposure to rabies and before the onset of the disease, thus leading to total protection. The value of postexposure treatment was difficult to prove and widely discussed until the appearance of modern cell culture vaccines (Turner, 1977). This classical therapeutic procedure is now recommended all over the world by WHO experts, whatever the potential risk (WHO, 1980). The situation is very complex, however, because of the great differences in activity seen between the various types of rabies vaccine, and because this active immunization can be used alone or combined with serum or immunoglobulin therapy (WHO, 1980). It is thus necessary to distinguish the different facets of this complex situation.

1. Early Investigation

a. Serological Investigations. The high antigenicity found in the first experimental batches of HDCS vaccine (Koprowski, 1966; Sikes *et al.*, 1971; Wiktor, 1974) opened the way for preliminary human trials. After the demonstration of good tolerance, these first investigations were limited to serological tests for the evaluation of both pre- and postexposure immunizations.

The preliminary study carried out by Bahmanyar (1974) compared five vaccination schedules using an experimental batch prepared at the request of the WHO by the Institut Mérieux. Four groups received the vaccine alone, and one group received a combination of antirabies serum and vaccine. The antibody profiles were analyzed in the context of a possible future postexposure vaccination, and Bahmanyar concluded that "prompt and dependable immunity [was] induced by only 4 or 5 doses of this vaccine."

Early results of safety and serological activity studies in France and Germany (Soulebot, 1974; Armengaud *et al.*, 1975; Kuwert *et al.*, 1976), providing evidence of the high potency of the new HDCS vaccine, enabled those responsible for Rabies Treatment Centers to use the new vaccine in some postexposure treatments. In France, 317 people who had been in contact with rabid animals received six 1-ml subcutaneous injections at days 0, 3, 7, 14, 30, and 90 (Dureux, 1974). Very rapidly, the new vaccine was distributed to eight Rabies Centers for experimental use under controlled conditions; the French license for postexposure treatment was granted in March 1977 following publication of the field study by Bahmanyar *et al.* (1976). In Germany, the high level of neutralizing antibodies which had been observed in vaccinees since 1973 encouraged Kuwert *et al.* (1977b) to treat 16 patients who had suffered bites, scratches, cuts, or abrasions caused by proven rabid animals, and 8 patients who had come into close contact with the saliva of proven rabid animals. The consistently satisfactory results achieved with these patients confirmed the value of the postexposure schedule as proposed by Kuwert *et al.*, called the "Essen Protocol"; this had already been recommended by the WHO Rabies Expert Group (WHO, 1973). This schedule consisted of six 1-ml intramuscular injections of HDCS whole virion vaccine, at days 0, 3, 7, 14, 30, and 90. Kuwert *et al.* (1977a) published a further report concerning the treatment of 108 individuals after contact with proven rabid (90 people) or suspected rabid (18 people) animals. The HDCS vaccine was licensed and officially recommended in Germany for human postexposure use in June 1977.

b. Early Evidence of Efficacy in the Field. The Pasteur Institute Rabies Reference Center in Teheran was selected by the WHO scientific group because of its capacity to treat high-risk patients severely bitten by rabid animals (Baltazard *et al.*, 1955; Bahmanyar, 1966).

Remarkable results providing a clear guarantee of the value of postexposure treatment using HDCS vaccine were published by Bahmanyar *et al.* (1976; Bahmanyar, 1978). These authors were "convinced of the safety of the HDCS vaccine and promptness of the antigenic response in humans, and therefore, with the consent of the responsible authorities, used HDCV in conjunction with antiserum to treat, after exposure, 45 persons severely bitten by 2 wolves and 6 dogs—animals confirmed by laboratory procedures to be rabid." This study provided a wealth of detail concerning the biting animals, the exposed subjects, and the nature and number of wounds per victim, ranging from a single penetrating wound to more than 25 multiple severe lacerations about the face and head. The data were presented for 8 groups corresponding to each biting animal. The treatment combined serotherapy and vaccination and was implemented as soon as possible after

infection by the rabies virus and programmed according to WHO recommendations (WHO, 1973). Two batches of Institut Mérieux vaccine were used in the treatment protocol, which in some cases was implemented up to 14 days after rabid infection. The average antibody rates achieved were excellent in all 45 individuals and were usually considered to reach an efficient level as from day 7 or day 14. But the most impressive results concerned the complete protection obtained in all these 45 people. Historical patterns in Iran indicated that a mortality rate of at least 35% could be expected. The results were even more surprising for the two groups whose treatment was implemented long after the bite. All previous results had indicated that, under such conditions, classical treatments always ended in failure.

The authors kept in contact with all the patients who received this postexposure treatment (Fayaz *et al.,* 1981) except for a 90-year-old man who died of a heart attack 5 months after treatment. All had remained healthy for the 4 years after exposure, and 27 of them agreed to give blood samples and receive a booster injection.

2. *Generalization of Use of HDCS Vaccine for Postexposure Treatment: Official Recommendations*

After the early publications in Europe and reports on the successful protection obtained by Bahmanyar *et al.* (1976), the use of HDCS vaccine for postexposure treatment developed very rapidly; the only limitations to its use were the availability of the product and the cost of a course of treatment. HDCS vaccine was used increasingly in Europe for treatment after severe bites, and reports were soon published in the United Kingdom (Gardner, 1977), in Sweden (Grandien, 1977), and in Switzerland (Mean *et al.,* 1978).

At the WHO/IABS Symposium held in Marburg in 1977, one of the Working Groups recommended a postexposure six-dose schedule using HDCS vaccine at a minimum antigenic value of 2.5 IU per dose, which "should guarantee a rate of seroconversion of almost 100% within 21 days. The RFFIT should preferably be used for rapid determination and monitoring of conversion. . . . [It was] suggested that the serum be tested 4 weeks after the last inoculation and at that time a minimum value of 0.5 IU per ml be attained."

The WHO Rabies Expert Committee (WHO, 1980) later slightly modified this recommendation by adding that "the 90 day dose is not used in some countries." This was probably intended to cover the official position in the United States. In fact, in June 1980, HDCS whole virion vaccine had been licensed in the United States (Meyer, 1980). The ACIP Committee had stated (CDC, 1980a) that "HDCS is the vaccine of choice whenever available and

should be administered in conjunction with RIG." However, the WHO-recommended vaccination schedule (WHO/IABS, 1978) was not retained; studies conducted by the CDC led the ACIP to recommend that "5 × 1 ml doses of HDCV should be given intramuscularly. The first dose should be given as soon as possible after the exposure; an additional dose should be given on each of days 3, 7, 14, and 28 after the first dose." At that time, the validity of postexposure treatment was assessed by the testing of rabies antibody in a serum specimen collected 2–3 weeks after the last dose. In 1981, the procedure was simplified (CDC, 1981b); reviewed data had shown that 99.9% of persons (1299 out of 1300) had protective antibody levels following postexposure treatment. This obviated the need to continue routine serological testing of those receiving the recommended regimen.

Table VI summarizes the official recommendations relevant at present, showing the great similarities in recommended postexposure treatment schedules.

From 1977, the use of HDCS vaccine spread throughout the world. For example, a license was granted in 1978 for Thailand and the vaccine soon was used on a large scale (Thongcharoen *et al.*, 1982a). In Canada, the reactions of some patients in a group of 42 under treatment with traditional DEV vaccine made it necessary to complete the treatment course with samples of HDCS obtained from Europe (Dempster *et al.*, 1979), thus paving the way for the official introduction of the new vaccine (Health and Welfare, Canada, 1979). By 1983, more than 100 countries were using the vaccine.

3. *Postexposure Treatment in Children*

Some reports were published concerning postexposure treatment in children using traditional vaccines, particularly in high-risk regions, despite the danger of neurological accidents (Vejjajiva, 1967; Kuwert *et al.*, 1981). The high rate of severe bites in children was discussed; for instance, Triverdi

TABLE VI
Official Recommendations for Postexposure Rabies Vaccination[a]

Source	Volume	No.	Schedules
France, Germany, United Kingdom	1 ml	6	Days 0, 3, 7, 14, 30, and 90
United States	1 ml	5	Days 0, 3, 7, 14, and 28
WHO, 1980[b]	—	6	Days 0, 3, 7, 14, 30 and 90[c]

[a]From Roumiantzeff *et al.* (1983).
[b]Cell culture vaccine (concentrated, minimum potency 2.5 IU).
[c]90-day dose is not used in some countries.

(1981) reported that in India children under the age of 15 years constituted 65% of cases in his Semple vaccine study.

Early experimental studies of the new HDCS vaccine concerned only adult volunteers, and few follow-up reports included a subgroup of children. Mean and Tanner (1978) studied the treatment of 359 patients, including 89 children less than 15 years old; 47 of them received vaccine and rabies immunoglobulin. Subjects under 15 years of age presented a better early immune response, a higher percentage of neutralizing antibodies at day 14, and a less marked antibody inhibition effect of the specific immunoglobulin.

Plotkin and Wiktor (1979) reported three cases of children treated with the split-product vaccine who acquired a significant antibody titer after the fourth and fifth injections. When the Mérieux rabies vaccine was licensed in the United States, Plotkin (1981) reiterated his advice to pediatricians facing cases of rabies exposure in children; the full vaccine schedule was well tolerated, and there was no reason to reduce the dosage volumes in infants or young children.

Thongcharoen *et al.* (1982b) in Thailand, reported on the vaccination of 50 children under 13 years of age; the youngest was 12 months old, and 27 were less than 6 years old. None of the treated children developed the disease, and this in a country where 45% of the 200–300 rabies deaths recorded each year are seen in untreated children.

Ajjan *et al.* (1983) reported on the clinical evaluation in France of 289 vaccination children, 29 of whom underwent serological investigations at day 30 (Table VII). The group included 172 boys and 117 girls; 55% had been bitten by dogs and 27% by cats. The sex and age group distributions (about 6% less than 3 years old, 57% between 3 and 10 years) roughly represented the risk level of bites in children (Chun *et al.*, 1982). Many of the cases corresponded to the WHO classification of major bites to limbs and sometimes to the head and neck; in 26% of cases, animal rabies was confirmed by laboratory tests. All the children received the postexposure schedule, with a full 1-ml dose at each injection on days 0, 3, 7, 14, 30, and 90. Only 14 children received antirabies serum or immunoglobulin. Blood specimens for serological testing were obtained from 23 children on day 30 just before injection of the fifth dose, thus indicating their immune status 14 days after the fourth dose. All these children presented a very high antibody titer ranging from 10 to 385 IU/ml (Table VII). It is perhaps interesting to note that, even in an area of France where the danger of rabies is recognized and antirabies treatment well organized, 20% of the children, many of them bitten by proven rabid animals, were treated after a dangerous delay of more than 7 days.

These different studies demonstrate that the HDCS vaccine, well toler-

TABLE VII
Antibody Responses in Children after the Fourth Dose of HDCS Rabies Vaccine in Postexposure Treatment

No. of children	Animal source	Age range (years)	Severity of exposure[a]			Lapse from contact to treatment (days)	Proven rabid animal	Titer after 4 doses (IU/ml)[b]
			1	2	3			
6	Cat	3–12	—	4	2	2–8	2	83, 25, 40, 22, 29, 125
7	Dog	7–12	—	7	—	1–5	7	48, 86, 10, 37, 10, 5, 36
4	Sheep	3–12	—	4	—	12–25	4	31, 37, 385, 43
5	Fox	9–13	—	5	—	1–3	5	38, 41, 151, 45, 82
1	Wild mouse	10	—	—	1	1	?	37
23								

[a] WHO classification.
[b] The geometric mean is 55 IU/ml.

ated in children, should be used regularly for the postexposure treatment of rabies.

4. *Combination of Antirabies Serum or Human Immunoglobulin with Vaccine*

The efficacy of passive immunity was clearly demonstrated by Baltazard *et al.* (1955). The addition of 1 or 2 doses of a potent animal serum reduced the fatality rates observed in a group receiving 21 doses of nervous tissue vaccine alone. When the same research group in Teheran initiated investigations of the new HDCS vaccine, one of the five groups of volunteers received antirabies serum at 50 IU/kg, and 1 ml of vaccine on days 0, 3, 7, and 21. Interference was observed, and the question was raised "whether the administration of homologous or heterologous serum will still be profitable in severe exposure when such a highly immunogenic vaccine will become available for general use" (Bahmanyar, 1974). When the first field trial was launched, involving 45 severely bitten individuals (Bahmanyar *et al.*, 1976), the consent of the authorities was rapidly obtained for the use of HDCS vaccine combined with antiserum.

The development of human antirabies immunoglobulin (HRIG) improved these conditions. A single injection of an optimal dose of 20 IU/kg on the first day of a complete six-dose vaccination schedule provoked an acceptable immunosuppression effect (Mean and Turner, 1978; Klietmann *et al.*, 1981) and no significant interference (Kuwert *et al.*, 1978b; Nicholson and Turner, 1978). The introduction of the highly potent HDCS vaccine has certainly reduced the use of passive immunity, though the WHO always recommends the addition of serum to vaccine for severe Class III cases of exposure (WHO, 1980). A wider use of HRIG has been recommended officially in the United Kingdom (Gardner, 1983) and particularly in the United States: "Postexposure antirabies immunization should always include both passively administered antibody (preferably RIG) and vaccine (preferably HDCV) with one exception: persons who have been previously immunized" (CDC, 1980a). This recommendation was reinforced (CDC, 1982a) when one case of possible vaccine failure was reported (Devriendt *et al.*, 1982). HRIG "was omitted from this patient's initial postexposure prophylactic regimen and may be related to the failure of the treatment." It should perhaps be noted that this American patient did not receive proper local wound treatment after the bite, and that this case constitutes the only reported failure after complete postexposure treatment (Kuwert *et al.*, 1984). The recommendation regarding a combination of vaccine and HRIG is well respected in the United States, and a recent survey has shown that, in 1981, 86% of the 5654 treatments given included this combination (Helmick, 1983).

5. *Comparison of HDCS and Traditional Vaccines*

Experimental vaccinations in animals showed the high immunogenicity and surprising protective power of HDCS vaccine against rabies challenge in the monkey, in contrast with DEV and SMB vaccines (Sikes *et al.*, 1971; Wiktor, 1974).

Some of the early serological investigations of HDCS vaccine included control groups who were given traditional vaccines. Cabasso *et al.* (1974) consistently observed a more rapid and effective seroconversion with two protocols using 4 doses of HDCS split vaccine rather than 14 DEV injections and 2 boosters. Soulebot and Mackowiak (1975) obtained a higher level of seroneutralizing antibodies with 5 doses of HDCS vaccine than with more than 10 or even 15 doses of nervous tissue vaccine. Shah *et al.* (1976) demonstrated that 2–4 doses of HDCS vaccine gave a rapid antibody response equivalent to that obtained after 14 doses of Semple vaccine. Kuwert *et al.* (1976) demonstrated that HDCS vaccine was of a much higher antigenicity, and presumably immunogenicity, than vaccines previously used in Germany, such as Hempt or DEV. Several other groups confirmed this superiority (Grandien, 1977; Klietmann and Koslowski, 1978; Thongcharoen *et al.*, 1982a; Larghi *et al.*, 1983). The surprising booster effect obtained with a single dose of HDCS was also reported (Kuwert *et al.*, 1976).

The high quality of the immune response was demonstrated by a rapid increase in IgG, as opposed to the prolonged IgM response obtained after Semple or DEV treatment and considered rather ineffective (Turner, 1978).

After these studies and the Iranian field trial (Bahmanyar *et al.*, 1976) it was generally acknowledged that HDCS was the best vaccine available for postexposure treatment, and many countries have gradually eliminated the use of other vaccines.

H. Problems Regarding HDCS Vaccine Use

The basic theory of immunization is that no case of rabies has occurred in persons known to have rabies antibodies in their serum at the time of exposure (Hattwick, 1974). For postexposure treatment, active immunization immediately after possible infection must rapidly provide sufficient neutralizing antibodies before viral invasion and, of course, before the onset of the disease (Turner, 1977). Certain theoretical and practical aspects of HDCS use have been elucidated through our increasing experience; however, some major problems are still to be resolved.

1. *Immunization Programs*

The risks of neurological damage or death have limited the use of traditional vaccines to postexposure therapy. The paucity of antigens in these vaccines has made it necessary to use a prolonged series of daily injections

extending over 2 or 3 weeks (Turner, 1977), only limited by the tolerance and acceptance of these injections.

This situation changed with the new concentrated HDCS vaccine. For preventive use, two or three appropriately spaced doses ensure a rapid induction of virus neutralizing antibody. For postexposure treatment, a program of five to six doses given over a 90-day period ensures reliable protection. Some investigators tried to find methods for simplified or accelerated immunization, first in preexposure use (Turner *et al.*, 1976; Nicholson *et al.*, 1983) and later for postexposure treatment (Warrell *et al.*, 1983). It has been suggested that such simplified schedules, involving fewer doses in a shorter program, should be considered with care.

2. *Markers of Immunity*

Prompt and suitable immunization is necessary when there is a high risk of rabies virus contamination. The best marker is the early neutralizing IgG class antibody (Kuwert *et al.*, 1978b) detected either at day 7 (Bahmanyar *et al.*, 1976; Kuwert *et al.*, 1978c) or at day 14 (Mean and Tanner, 1978). Technical problems, however, have limited reproducible detection earlier in the treatment course (Kuwert *et al.*, 1978c). New serological techniques, such as ELISA, may increase the sensitivity of this detection, but the protective value of these antibodies is still to be proved.

Other accepted markers of rapid immunization are cell-mediated immunity and interferon induction. Nicholson *et al.* (1979a) found a lymphocyte stimulation, probably T specific, in 8 out of 10 HDCS vaccinees 30 days after the first four doses of a classic postexposure regimen. Interferon induction in man following vaccination has rarely been reported (Nicholson *et al.*, 1979b; Fornosi *et al.*, 1983). Standardized techniques and protocols are needed (WHO/IABS, 1978; WHO, 1980) to understand "which member of the trinity—antibody, CMI and interferon—is most important in preventing rabies" (Turner, 1983).

3. *Combination of Passive and Active Immunization*

The combination of serum or HRIG with vaccine is certainly an important factor in postexposure treatment and there is no theoretical objection to using this combination. When animal serum is used, however, the main objection is the permanent risk of sensitization to an heterologous serum. With HRIG, the main restriction is the limit of a 20 IU/kg dose given once, at the same time as the first vaccine injection.

4. *Duration of Immunity*

One of the most important practical problems in rabies prophylaxis is the duration of immunity and the guarantee of permanent protection. Traditional vaccines were used mainly in emergencies for postexposure treat-

ment, and doctors accepted a limited level of protection. The highly immunogenic HDCS vaccine has given users a sense of security, which has increased with the generalization of preexposure prevention. It is thus essential to emphasize the limits of its protection, and the conditions of usage necessary to obtain long-term security. Durable immunity after postexposure treatment was demonstrated by Fayaz *et al.* (1981) in their follow-up of Bahmanyar's major study. No cases of rabies or any side effects were observed in the 45 vaccinees. Of these, 27 agreed to provide a serum sample 4 years after the initial treatment, and the resulting titers, from 2 to 91 IU/ml with a mean of 25.5 IU/ml, were surprisingly high. A single booster given 4 years after the initial treatment increased neutralizing antibody levels to between 36 and 650 IU/ml, measured 10 days after the booster injection.

Immune status after preexposure vaccination has often been evaluated in subjects at risk and requiring permanent protection. The rhythm of routine boosters depends on the duration of immunity bestowed by primoimmunization. Investigations undertaken 1 or 2 years after primoimmunization came to the conclusion that booster injections could be recommended 1 year after primoimmunization in France and the United Kingdom, and 2 years after in Germany and the United States; intervals clearly related to the number of primary doses. Such schedules should be seen in the context of the prevailing risk. Laboratory personnel, a very high-risk group, require a vaccination (CDC, 1980a) or rabies antibody check (Gardner, 1983) every 6 months to ensure permanent protection.

Two years after vaccination with two subcutaneous doses, a group of 20 veterinary students showed a mean titer of 1.25 IU/ml; two subjects had a borderline titer of 0.5 IU. After 2 years, a 1-ml subcutaneous booster raised the mean antibody level to 28.5 IU/ml; 3 years later, the mean titer was 2.20 IU and all the students presented a protective antibody level superior to 0.5 IU/ml (Ajjan *et al.*, 1983). A group of laboratory personnel was vaccinated with two subcutaneous doses and monitored after a single booster given 1 or 2 years later. Their antibody levels slowly decreased over the 4 years following the booster. Only 1 subject out of the 49 tested after 4 years had a titer inferior to 1 IU/ml. It is interesting to note that subjects who received a booster after 2 years presented a significantly higher geometric mean than the group revaccinated after only 1 year (Drucker *et al.*, 1984).

5. *Choice of Injection Route; Intradermal Vaccination*

The intradermal administration of rabies vaccine has been the subject of much discussion. The quality of intradermal immunization was already recognized with traditional vaccines and recommended for booster doses of

nervous tissue vaccine (Gamet and Atanasiu, 1971) for SMB (Lépine *et al.*, 1973) or for DEV vaccine (Schnurrenberger *et al.*, 1965).

The high potency of HDCS vaccine, and also the financial considerations due to its cost, have led some researchers to look for more economical regimens for pre- and postexposure immunization. During the early development of HDCS vaccine in France, Soulebot (1974) showed that a 0.1-ml intradermal injection of HDCS vaccine gave a significant level of antibody in the majority of volunteers after 7 days, and a second injection gave a protective level of antibodies in all vaccinees. Later investigations confirmed these results (Ajjan *et al.*, 1978).

In the United Kingdom, Aoki *et al.* (1975) made the first comparison of an intramuscular prophylactic regimen with a more economical regimen using two 0.1-ml doses injected intradermally. Several investigations have continued these comparisons (Turner *et al.*, 1976; Nicholson *et al.*, 1978, 1979a, 1981; Warrell *et al.*, 1983); the intradermal method has sometimes been applied routinely (Furlong and Lea, 1981) although 1.0-ml intramuscular vaccinations give significantly better results that the 0.1-ml intradermal ones.

In Germany, Cox and Schneider (1976) concluded that the intradermal route should be restricted to booster inoculations. In other trials, attempts were made to reduce the dose, by using either the intradermal route (Klietmann *et al.*, 1981, 1983) or the addition of an aluminum adjuvant (Kuwert *et al.*, 1978d; Klietmann *et al.*, 1983).

In the United States, Bernard *et al.* (1982a) undertook a study to evaluate low-dose primovaccinations and boosters, given either intradermally or subcutaneously. While the recommended 1.0-ml intramuscular regimen produced higher titers, the 0.1-ml interdermal regimen gave adequate titers in all subjects without particularly adverse reactions, and also provided a consistently good booster effect in subjects previously immunized with DEV. The ACIP Committee examined data regarding 1500 people who had received Mérieux HDCS vaccine intradermally; all of them had developed adequate antibody. The Committee concluded that three 0.1-ml intradermal doses given at days 0, 7, and 21 or 28 would be an acceptable alternative regimen "with appropriate packaging and labelling changes." The Committee suggested that routine serological testing to confirm a satisfactory antibody response was not necessary, whether or not the recommended intramuscular or intradermal regimens had been used (CDC, 1982b).

Table VIII summarizes the main trials published since 1975 showing the great variety of proposed protocols and the lack of standarization.

Many of these investigations emphasized the technical difficulties in giving effective intradermal injections. Skilled medical personnel in specialized Rabies Centers are essential (Turner *et al.*, 1976; Warrell *et al.*, 1983) be-

TABLE VIII
Main Trials for Intradermal Preexposure Rabies Vaccination[a]

Volume	No. of sites	No. of injections	Days	Reference
0.1 ml	1	2	0, 28	Aoki *et al.* (1975)
0.1 ml	1	1	0	Turner *et al.* (1976)
0.1 ml	1	4	0, 1, 2, 3, or 0, 3, 7, 14	Cox and Schneider (1976)
0.1 ml	1	3	0, 28, 56	Nicholson *et al.* (1978)
0.1 ml	1	2	0, 28	Furlong and Lea (1981)
0.1 ml	8	1	0	Nicholson *et al.* (1981)
0.1 ml	1	2	0, 30	Ajjan *et al.* (1980)
0.05 ml	2	2	0, 30	Ajjan *et al.* (1980)
0.1 ml	1	3	0, 7, 28	Bernard *et al.* (1982a)

[a]From Roumiantzeff *et al.* (1983).

cause the level of immunity obtained is directly dependent on successful intradermal administration. However, the problem is that a generalization of these techniques is also badly needed in remote regions of developing countries.

The use of a jet injector (Bernard *et al.*, 1982a) does not improve the quality of the vaccination, but this idea should be reconsidered using more efficient injectors. A single-dose syringe of freeze-dried HDCS vaccine, which after reconstitution is ready for intradermal injection, could help toward a solution of this technical problem and respond to the United States ACIP Committee's request to manufacturers for improvements in the packaging and labeling of vaccine for intradermal use (CDC, 1982b). The multisite injection technique (Turner *et al.*, 1976; Warrell *et al.*, 1983) may also provide an efficient solution, not only by increasing the security of the injection, but also by improving the immunological response.

These intradermal preexposure vaccinations paved the way to experimental postexposure treatments associating the effects of multisite and intradermal injections. Timms (1981) reported on the first cases of intradermal treatment in Kenya. Warrell *et al.*, (1983) reported on the serological investigations of different postexposure HDCS regimens. The highest antibody titers were obtained with the intramuscular reference regimen and the eight multisite intradermal regimen, which requires only a quarter of the dose. Subsequently, Harverson (1983) stated that since 1979 he had administered 328 postexposure intradermal treatments in Thailand.

6. *Postexposure Treatment of Previously Vaccinated Subjects*

It is important to have a clearly defined strategy for the efficient treatment of previously vaccinated subjects. This situation is more and more common, because an increasing number of high-risk subjects are preventively immunized.

When DEV was the only vaccine available for pre- and postexposure, a simplified treatment was recommended for a previously vaccinated individual; as soon as possible after exposure, five daily doses of DEV should be given, followed by a booster given 20 days after the fifth dose, but only if the rabies antibody level was known (WHO/IABS, 1978; CDC, 1976). This recommendation still applies today for those previously vaccinated with traditional vaccines; only a fully documented immune status can permit the use of simplified HDCS postexposure treatment (WHO/IABS, 1978).

In all other cases, particularly in countries where the disease is endemic or in the case of a severe bite, the risk is too high and a complete HDCS six-dose treatment must be rapidly applied, even with the addition of HRIG. The situation differs for those persons previously immunized with HDCS vaccine, as the consistently high level of immunity following preexposure vaccination has encouraged some groups to advocate a single booster in the case of exposure (Cox and Schneider, 1976; Kuwert *et al.,* 1978a).

However, revaccination is one of the major problems facing the medical profession which "requires assessment of all risk factors and immunological conditions." Therefore, special Advisory Services should be established to deal with each individual case (WHO/IABS, 1978) in these two situations: (1) in a previously vaccinated individual with a proven antibody level, a single injection of HDCS vaccine may be sufficient to provoke a marked anamnestic reaction (WHO/IABS, 1978); (2) For a previously vaccinated individual whose level of neutralizing antibody has not been determined, the use was recommended of potent vaccines on days 0, 10, 20, and 90 after reexposure.

Then WHO (1980) clarified its position even further. Those individuals who have previously shown an antibody response following preexposure or postexposure vaccination should receive a single booster dose except in cases of severe bites, in which case additional boosters could be considered at days 0, 3, and 7. However, for those whose neutralizing antibody titer has not been determined, three doses of a potent vaccine should be given on days 0, 3, and 7.

The omission of HRIG from this reduced course of postexposure treatment was originally simply advised (WHO/IABS, 1978), but today it is a clear recommendation (WHO, 1980; Gardner, 1983).

The United States experts (CDC, 1980a) tried to simplify the recommended treatment even further to two 1-ml doses given intramuscularly, one immediately after exposure and the second 3 days later. HRIG should not be given to an individual with demonstrated rabies antibodies, but for those of unknown immune status a full primary postexposure treatment with HRIG was suggested.

With the exception of laboratory technicians who are regularly checked, it is almost impossible to maintain reliable antibody measurements of the general population. Moreover, such serological follow-up would not be economical, bearing in mind that antibody testing may be more expensive than vaccination. The ACIP Committee had the merit of considering both the practical and the economic points and, because of the excellent results of pre- and postexposure HDCS vaccination, recommended discontinuation of routine serologic testing "except for persons vaccinated with DEV or those whose immune response might be diminished by drug therapy or for other reasons" (CDC, 1981b).

The tragic death of a young American in Kenya (CDC, 1983a) should serve as a lesson for the future. Preexposure vaccination is an excellent prevention which bestows a primary level of protection and will solidly prepare and simplify postexposure treatment which remains essential in the case of a new rabies contact (CDC, 1983b).

I. Different Rabies Vaccines Prepared on Human Diploid Cells

It is necessary to describe briefly and distinguish between the different vaccines presently prepared on human diploid cells.

Immediately following the first adaptation of rabies virus to a human diploid cell strain (Wiktor *et al.*, 1964), experimental vaccines were prepared by Wyeth Laboratories, which in April 1971 received from the Wistar Institute the PM rabies strain adapted to WI-38 human diploid cells (Wiktor *et al.*, 1978a). The Wistar technique initially produced whole virions (Wiktor *et al.*, 1969; Koprowski, 1973). Working in the Wyeth laboratory, Tint *et al.* (1974) introduced an inactivation process which had already been described by Neurath *et al.* (1972). The rabies virus suspension is treated with 0.1% tri-*n*-butyl phosphate (TNBP) in the presence of 0.1% Tween 80. TNBP dissociates the lipids and most of the glycoproteins to release subviral components (Neurath *et al.*, 1972; Tint, 1981) which retain the main immunological properties (Cabasso *et al.*, 1974).

It is interesting to note that this vaccine was first used on man in 16 volunteers from the Wistar Institute in November 1971. These first trials were later reported by some of the volunteers (Wiktor *et al.*, 1973b, 1978a; Kaplan, 1974; Plotkin, 1980).

The exact nature and composition of the different human diploid cell strain vaccines must be clearly defined. We support the statement of the clinician who, from the beginning, worked with both the Wyeth and Mérieux vaccines: "There is a tendency, which is not completely justifiable, to combine all data on the clinical use of HDCV regardless of the method of manufacture. However, since there are both whole virion (WV) and split product (SP) human cell culture vaccines, an attempt will be made in the following discussion to indicate clearly which is which" (Plotkin, 1980).

The following investigations reported good results in different preexposure schedules (Plotkin *et al.*, 1976), definitively better than the duck embryo vaccine (Hafkin *et al.*, 1978) and with a safe booster reponse 1 year later (Rosanoff and Tint, 1979). New data have been published concerning postexposure trials in the United States (Anderson *et al.*, 1980a,b) with and without the use of HRIG (Mertz *et al.*, 1982) and possible postexposure treatment modifications (Anderson *et al.*, 1981a,b). The Wyeth HDCS vaccine (SP) was licensed in the United States in 1982.

The HDCS PM virus vaccine strain was transferred from the Wistar Institute to Behringwerke AG in Germany as early as February 1969 (Wiktor *et al.*, 1978a). However, experimental vaccine development was reported only later (Hilfenhaus *et al.*, 1976) after a license agreement was obtained from Institut Mérieux. A purified HDCS vaccine (whole virion) was developed (Majer *et al.*, 1977a,b, 1978) using WI-38, then MRC-5 diploid cells (Majer *et al.*, 1978) and with centrifugation in a sucrose gradient (Hilfenhaus *et al.*, 1976). Preexposure (Cox *et al.*, 1978) and postexposure use (Kuwert *et al.*, 1978a; Klietmann *et al.*, 1978) were reported with good tolerance and satisfactory serological results.

The CL60 strain of fixed rabies virus used in Canada for the production of hamster kidney cell vaccine (Fenje, 1960) was adapted to MRC-5 human diploid cells (Fenje *et al.*, 1981). Concentrated vaccine was tried in rabbits (Fenje *et al.*, 1981). Clinical pre- and postexposure investigations are in progress using concentrated and purified vaccine (Johnson *et al.*, 1984).

IV. OTHER CELL CULTURE VACCINES

The dramatic side effects and low potency observed with traditional vaccines rapidly oriented research toward the growth of rabies virus in cell culture. Before 1955, the publication date of SMB vaccine (Fuenzalida and Palacios, 1955) and DEV (Peck *et al.*, 1955, 1956), other cell culture methods had already been explored (Sanders *et al.*, 1953). But it is certainly the work by Kissling (1958) and Kissling and Reese (1963) which opened the way to the development of inactivated vaccines of significant NIH test value, by the concentration of rabies virus produced in hamster kidney tissue cul-

ture. Several vaccines were experimented with, but, as suggested by Clark *et al.* (1975), it is perhaps wiser to consider here only those that were used in human subjects.

Since 1965, the date of the first international standardization symposium on rabies, it has been interesting to watch the competition between different candidate vaccines and the increasing predominance of HDCS vaccine. Such competition was possible because the WHO Rabies Expert Committee accepted a large variety of cell systems (Lang, 1974; Perkins, 1974) and a clear list of vaccine strains (WHO, 1980). More recently, a new approach has enabled the development of virus rabies culture on an heteroploid cell line.

A. Vaccines in Use Prepared from Primary Animal Cells

1. Rabies Vaccines Prepared from Hamster Kidney Cells

Following the lines of Kissling's work, but without direct reference to it, Fenje (1960) proposed an inactivated vaccine prepared from fixed SAD rabies virus, adapted to and produced on primary hamster kidney cells. The presence of horse serum limited its human use, but an interesting modification was achieved by substituting a medium without serum, and further, its potency was reinforced by an aluminum phosphate adjuvant (Fenje and Pinteric, 1966). This vaccine was used as an experimental booster (Christensen and Muller, 1974) and in preexposure treatment (Fenje, 1974; Garner *et al.*, 1976). In 1968, it was licensed in Canada for booster and preexposure vaccination (WHO, 1973; Furesz *et al.*, 1977).

The Vnukovo 32 strain was adapted in the USSR by different animal cell passages from the SAD strain grown in hamster kidney cells (Selimov *et al.*, 1974), and it served to produce an ultraviolet-inactivated vaccine prepared from Syrian hamster kidney cells. Good results after postexposure treatment using 1–25 daily doses were reported with the vaccine alone or in combination with rabies γ-globulin (Selimov *et al.*, 1974). Later, this vaccine was improved in the USSR by concentration and purification. The resulting Vnukovo 32-107 vaccine has been widely used in the USSR since 1978 (Barth, 1978); official reports indicate that more than 100,000 people have been treated with 9–17 doses after exposure (Selimov *et al.*, 1978). Today, this vaccine is also used throughout Eastern Europe (Selimov *et al.*, 1981; Sinnecker, 1981).

In China, a local strain of fixed virus was adapted to primary hamster kidney cells, thus allowing the development of plain and aluminum-adjuvanted vaccines, which have given encouraging pre- and postexposure vaccination trial results (Fangtao *et al.*, 1983).

2. *Rabies Vaccine Prepared from Chick Embryo Cells*

The HEP and LEP Flury strains were adapted to chick embryo cells for the production of live vaccines. The HEP vaccine was used experimentally in humans (Ruegsegger and Sharpless, 1962), but these live vaccines have not been accepted for general human use (Wiktor, 1973; WHO, 1973).

Kondo (1965) adapted the Flury HEP strain to chick embryo cells, grown in serum-free medium 199. Kondo *et al.* (1974) were able to produce a β-propiolactone-inactivated vaccine for preliminary human experimental use. A concentrated vaccine was developed and assayed in very limited pre- and postexposure vaccination trials (Kondo, 1978). A lyophilized vaccine is now manufactured in Japan and used for pre- and postexposure vaccination (Yamada *et al.*, 1979).

A similar vaccine adapting the Flury LEP strain to primary chick embryo cells is presently under study.

3. *Rabies Vaccine Prepared from Fetal Calf Kidney Cells*

In 1972, the Pasteur strain was adapted to different cell systems. Atanasiu *et al.* (1974a) selected fetal calf kidney cells to grow the Pasteur strain because of the high level of protective antigens and the possibility of large batch production (Atanasiu *et al.*, 1974b). The resulting product available for human use is a β-propiolactone-inactivated vaccine, prepared on bovine fetal kidney cells, concentrated, and purified by zonal centrifugation (Antanasiu *et al.*, 1977, 1978). Some experimental studies have shown that this vaccine bears comparison with SMB or HDCS vaccines in preexposure treatment (Guesry *et al.*, 1981) and is up to the standard of nervous tissue vaccine (Chadli *et al.*, 1984) or SMB vaccine (Loucq *et al.*, 1984) for postexposure treatment.

4. *Rabies Vaccine Prepared from Primary Dog Kidney Cells*

Van Wezel and Van Steenis (1978) selected primary dog kidney cells with a view to the possible adaptation of the cell culture to a microcarrier for the potential large-scale production of rabies vaccine using the PM strain adapted to human diploid cells. The vaccine is concentrated by ultrafiltration, purified by gel filtration, and then inactivated by β-propiolactone; it has been shown that adsorption on aluminum phosphate adjuvant enhances the antibody production in experimental animals (Van Wezel and Van Steenis, 1978). Good results have been reported in preexposure and postexposure trials conducted in the Netherlands; results comparable to those obtained with HDCS vaccine (Van Steenis *et al.*, 1981, 1984).

B. Vaccines under Development

1. Vaccine Prepared from Rhesus Diploid Cells

The Kissling rabies virus strain (Mitchell *et al.*, 1971) was adapted to diploid fetal rhesus lung cells (Berlin *et al.*, 1982). A β-propiolactone inactivated vaccine, adsorbed and concentrated on aluminum phosphate, and with an NIH potency of at least 2.5 IU, is presently under trial in humans. Early results of preexposure vaccination trials (Berlin *et al.*, 1982) and of investigations simulating postexposure protocols (Berlin *et al.*, 1983) have been published.

2. Rabies Vaccine Prepared from Chick Embryo Cells

A new vaccine, designated PCEC (purified chick embryo cell) (Barth *et al.*, 1983a,b) to differentiate it from the Japanese vaccine (Kondo, 1965), is presently under study. Barth *et al.* (1983a,b) selected the Flury LEP strain they had already used experimentally and which had been particularly effective when adapted to WI-38 human diploid cells (Barth *et al.*, 1974). Instead of the Japanese Flury HEP strain, the Flury LEP C25 strain is being used; both these strains are accepted by the WHO Rabies Expert Committee (WHO, 1980). The same β-propiolactone inactivation is used, but purification and concentration by zonal centrifugation in a sucrose gradient replaces polyethylene glycol precipitation followed by high-speed centrifugation. The antigen concentration is standardized at 2.5 IU per dose. Preliminary results for booster and preexposure (Barth *et al.*, 1983a) and postexposure treatment in combination with HRIG (Vodopidja *et al.*, 1984) have recently been reported.

3. Rabies Vaccine Produced on VERO Cells

The HDCS rabies vaccine, with its high safety and potency, has proved its reliability and efficacy. However, the relatively low titer of virus produced by HDCS makes it an expensive vaccine.

The opportunity offered by the microcarrier technique (Van Wezel, 1967) for the mass culture of anchorage-dependent cells, has stimulated the reassessment of inactivated poliomyelitis vaccine and the development of the VERO cell line, a simian nontumorigenic heteroploid cell line. A cell-cultured purified rabies vaccine is now feasible on an industrial scale, using VERO cells.

a. Manufacturing. A cell-seed system with primary and working-cell banks (Montagnon *et al.*, 1981) is prepared using the VERO cell line from the American Type Culture Collection (ATCC). This line was developed in Japan in 1962 from a *Cercopithecus aethiops* monkey kidney cell (Yasamura

and Kawahita, 1963). The cell growth flow sheet is shown diagrammatically in Fig 6. Large-scale management of the culture in tanks of several hundred liter capacity is ensured by devices which automatically regulate temperature, pH, pO_2, pressure, and stirring speed (Fig. 5). The microcarrier support used is Cytodex-1 from Pharmacia (Fig. 7).

The virus strain is the same as that used for the production of HDCS vaccine, i.e., PM 1503-3M. The cells are inoculated during their growth phase, and their infectivity is checked daily by immunofluorescent examination after labeling of the cells on beads with an antibody fluorescein-isothiocyanate conjugate (Fig. 8). As the rabies virus is not cytolytic, several harvests are possible. The infectious virus titers achieved are regularly $10^{6.6}$ $TCID_{50}$ per milliliter or higher. In order to achieve good inactivation, each harvest must be concentrated several times; concentration factors of up to a hundred are easily obtained by the use of ultrafiltration membranes with a retention efficacy of 10,000 or 100,000 molecular weight. β-Propiolactone is used for inactivation (1:4000 at 4°C) and the residue is hydrolyzed by gently warming the solution for 2 hr at 37°C. Although the whole process takes 24 hr, inactivation curves indicate that at 12 hr the viral suspension is already inactivated. The vaccine is purified by zonal centrifugation using a sucrose gradient (Tayot and Montagnon, 1973) to remove any nonanti-

Fig. 5. Cytogenerator for mass culture of VERO cell.

cell number	1×10^9	5×10^9	25×10^9	150×10^9	500×10^9
surface area m^2	0.60	3.0	15.0	90.0	300.0
equivalent 1 L Roux bottle	30	150	750	4500	15000

note : a one gram per liter concentration of Cytodex-1 (Pharmacia) was considered for this example

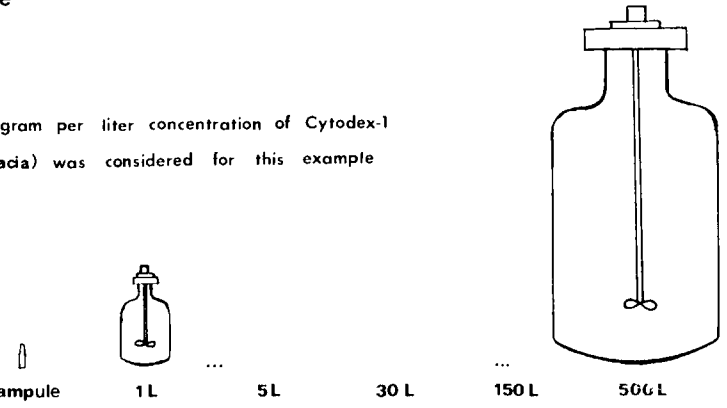


Fig. 6. Flowsheet for scaling up VERO cell production in cytogenerator.

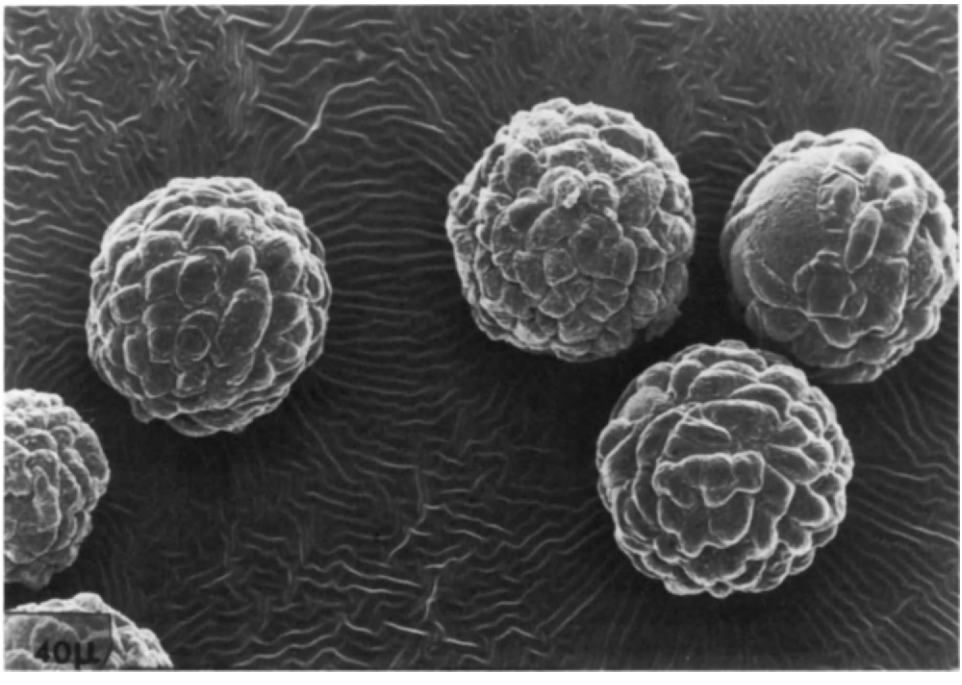


Fig. 7. Scanning electron micrograph of VERO cells grown on microcarrier after 72 hr.

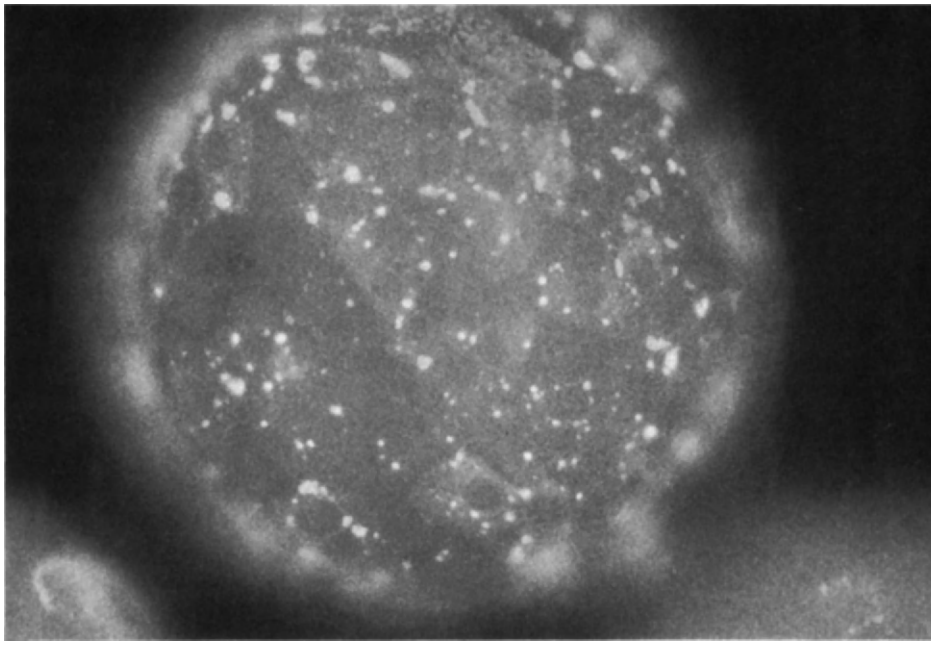


Fig 8. VERO cells on microcarrier with fluorescent intracytoplasmic viral inclusions 5 days after infection.

genic material and thus obtain a well-tolerated vaccine. The final product is stabilized with human albumin. The vaccine is supplied in the form of a stable lyophilized suspension; each injection dose is 0.5 ml and contains at least 2.5 IU of rabies antigen.

b. Controls. Besides the classical tests already described, two other controls are included for vaccine produced from an heteroploid cell line: a tumorigenicity test and a test for residual cellular DNA. The tumorigenicity test is performed on newborn rats according to the procedure described by Van Steenis and Van Wezel (1982) using antithymocyte sera and inoculation with 10^6 VERO cells. For positive controls, other rats receive HeLa, KB, and HEP 2 cells under the same conditions. The amount of cellular DNA which can be detected after purification is very small, and techniques such as spectrofluorimetry are insufficiently accurate. Molecular hybridation, a more sophisticated technique using a radioactive element, is presently under investigation.

c. Results. Montagnon *et al.* (1984) have demonstrated the absence of tumoral nodules and metastases in 100 vaccinated laboratory animals and

TABLE IX
Potency and Stability of VERO Cell Vaccine[a]

Vaccine batch	Initial titer	6 Months at 4°C	9 Months at 4°C	12 Months at 4°C	18 Months at 4°C	1 Month at 37°C
I	12.9	—	—	—	—	6.5
II	10.3	4.9	13.9	10.8	8.3	6.5
III	2.9	4.7	10.5	6.3	7.3	4.7
IV	6.5	—	6.1	8.6	14.0	4.2
V	3.2	—	5.3	6.9[b]	7.0	5.8
VI	14.6	15.1	—	—	—	20.9
VII	3.2	—	—	—	—	9.7
VIII	4.6	8.6	—	—	—	7.7
IX	3.0	—	—	—	—	—

[a]Vaccine was stored at 4°C. The IU per dose was estimated by the NIH test. For comparison, results obtained on a sample kept 1 month at 37°C are given.
[b]Result at 16 months.

evaluated the residual cellular DNA of the vaccine at less than 50 pg per dose, a level equivalent to that seen in poliomyelitis vaccine. The potency of the new rabies vaccine produced on VERO cells is at least the 2.5 IU per dose required by the NIH test. The stability of the vaccine is quite similar to that found in HDCS vaccine. Table IX summarizes the technical results available at present. Preliminary results regarding booster and preexposure have been reported (Drucker *et al.*, 1984) showing a perfect tolerance. Preexposure and postexposure trials are under way in different parts of the world.

V. CONCLUSIONS

This chapter has described the remarkable advances achieved in rabies vaccine technology since 1960 thanks to the improved application of rabies virus culture on animal and human diploid cells. For HDCS vaccine in particular, we have seen remarkable developments in its application to prevention and above all postexposure treatment—developments possible because of the vaccine's total safety and very high activity. However, in poorer countries where the rabies risk is at its maximum, generalization of the use of HDCS vaccine has been limited by its availability and cost. Most of this vaccine is thus destined for emergency postexposure treatment. New technologies, particularly mass production of virus using the microcarrier system, may in the near future enable the production of purified vaccine at a much reduced price.

A vaccine presenting these qualitities would be an excellent candidate for the worldwide development of a true prophylaxis for rabies in humans, in association with a campaign for the eradication of canine rabies. It would

then be possible to envisage vaccine combinations enabling a wider application of rabies prevention.

REFERENCES

Acha, P. N., and Arambulo, P. V. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Ajjan, N., (with the collaboration of the Antirabies Centers in France) (1983). 3rd International Conference on the Impact of Viral Diseases on the Development of Middle East and African Countries, Kuwait.

Ajjan, N., Soulebot, J. P., Stellmann, C., Biron, G., Charbonnier, C., Triau, R., and Mérieux, C. (1978). *Dev. Biol. Stand.* **40**, 89–100.

Ajjan N., Soulebot, J. P., Triau, R., and Biron, G. (1980). *J. Am. Med. Assoc.* **244**, 2528–2531.

Ajjan, N., Straddy, A., Xueref, C., and Roumiantzeff, M. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.), Springer Verlag, Berlin.

Anderson, G. R., Schnurrenberger, P. R., Masterson, R. A., and Wentworth, F. H. (1960). *Am. J. Hyg.* **71**, 158–167.

Anderson, L. J., Winkler, W. G., Hafkin, B., Keenlyside, R. A., D'Angelo, L. J., and Deitch, M. W. (1980a). *J. Am. Med. Assoc.* **244**, 781–784.

Anderson, L. J., Sikes, R. K., Langkop, C. W., Mann, J. M., Smith, J. S., Winkler, W. G., and Deitch, M. W. (1980b). *J. Infect. Dis.* **142**, 133–138.

Anderson, L. J., Baer, G. M., Smith, J. S., Winkler, W. G., and Holman, R. C. (1981a). *Am. J. Epidemiol.* **113**, 270–275.

Anderson, L. J., Winkler, W. G., Smith, J. S., Costin, C., and Swartz, T. A. (1981b). *Proc. WHO Consultations Essen,* pp. 300–306. International Green Cross, Geneva.

Aoki, F. Y., Tyrrell, D. A. J., Hill, L. E., and Turner, G. A. (1975). *Lancet* **1**, 660–662.

Appelbaum, E., Greenberg, M., and Nelson, J. (1953). *J. Am. Med. Assoc.* **151**, 188–191.

Arko, R. J., Wiktor, T. J., and Sikes, R. K. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23**, 292–294.

Armengaud, M., Auvergnat, J. C., Picot, N., Saurat, P., and Ganière, J. P. (1975). *Rev. Med. Toulouse* **11**, 531–535.

Atanasiu, P. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23**, 314–318.

Atanasiu, P., Bahmanyar, M., Baltazard, M., Fox, J. P., Habel, K., Kaplan, M. M., Kissling, R. E., Komarov, A., Koprowski, H., Lepine, P., Perez Gallardo, F., and Schaeffer, M. (1957). *Bull. W.H.O.* **17**, 911–932.

Atanasiu, P., Ribeiro, M., and Tsiang, H. (1972). *Ann. Inst. Pasteur Paris* **123**, 427–441.

Atanasiu, P., Gamet, A., Velch, A., and Tsiang, H. (1974a). *Proc. Symp. Immunol.* **21**, 207–212.

Atanasiu, P., Tsiang, H., and Gamet, A. (1974b). *Ann. Microbiol. (Paris)* **125B**, 419–432.

Atanasiu, P., Tsiang, H., Lavergne, M., and Chermann, J. C. (1977). *Ann. Microbiol. (Paris)* **128B**, 297–302.

Atanasiu, P., Tsiang, H., Reculard, P., Aguilon, F., Lavergne, M., and Adamovicz, P. (1978). *Dev. Biol. Stand.* **40**, 35–44.

Baer, G. M. (1978). *Am. J. Clin. Pathol.* **1**, 185–187.

Baer, G. M. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Baer, G. M. (1984). *In* "Control of Virus Diseases" (E. Kurstak, and R. G. Marusyk, eds.), pp. 79–92.

Bahmanyar, M. (1966). *Symp. Immunobiol. Stand.* **1**, 307–312.
Bahmanyar, M. (1974). *Symp. Immunobiol. Stand.* **21**, 231–239.
Bahmanyar, M. (1978). *Dev. Biol. Stand.* **40**, 163–165.
Bahmanyar, M., Fayaz, A., Nour-Salehi, S., Mohammadi, M., and Koprowski, H. (1976). *J. Am. Med. Assoc.* **24**, 2751–2754.
Baltazard, M., Bahmanyar, M., Ghodssi, M., Sabeti, A., Gajdusek, C., and Rouzbehi, E. (1955). *Bull. W.H.O.* **13**, 747–772.
Barth, R. (1978). *Dev. Biol. Stand.* **40**, 275.
Barth, R., and Jaeger, O. (1980). *Zentralbl. Bakteriol. Parusitenk., Infektionskr. Hyg. Abt. 1, Orig. Reihe B.* **169**, 488–494.
Barth, R., Bijok, U., Gruschkau, H., Sherde, J., and Vodopidja, J. (1983). *Lancet* **1**, 700.
Barth, R., Bijok, U., Gruschkau, H., Jaeger, D., and Weinmann, E. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Barth, R., Jaeger, O., and Weinmann, E. (1974). *Symp. Immunobiol. Stand.* **21**, 253–256.
Bell, J. F., Wright, J. T., and Habel, K. (1949). *Proc. Exp. Biol. Med.* **70**, 457–461.
Berlin, B. S., Mitchell, J. R., Burgoyne, G. H., Oleson, D., Brown, W. E., Goswick, C., and McCullough, N. B. (1982). *J. Am. Med. Assoc.* **247**, 1726–1728.
Berlin, B. S., Mitchell, J. R., Burgoyne, G. H., Brown, W. E., and Goswick, C. (1983). *J. Am. Med. Assoc.* **249**, 2663–2665.
Bernard, K. W., Roberts, M. A., Sumner, J., Winkler, W. G., Mallonee, J., Baer, G. M., and Chaney, R. (1982a). *J. Am. Med. Assoc.* **247**, 1138–1142.
Bernard, K. W., Smith, P. W., Kader, F. J., and Moran, M. J. (1982b). *J. Am. Med. Assoc.* **248**, 3136–3138.
Boe, E., and Nyland, H. (1980). *Scand. J. Infect. Dis.* **12**, 231–232.
Burridge, M. J., Baer, G. M., Sumner, J., and Sussman, O. (1982). *J. Am. Med. Assoc.* **248**, 1611–1614.
Cabasso, V. J., Dobkin, M. B., Roby, R. E., and Hammar, A. H. (1974). *Appl. Microbiol.* **27**, 553–561.
Cates, W. (1974). *Am. J. Obstet. Gynecol.* **44**, 893–895.
Centers for Disease Control. (1976). *Morbidity and Mortality Weekly Report* **25**, 403–406.
Centers for Disease Control. (1980a). *Morbidity and Mortality Weekly Report* **29**, 265–280.
Centers for Disease Control. (1980b). *Morbidity and Mortality Weekly Report* **29**, 609–610.
Centers for Disease Control. (1981a). *Morbidity and Mortality Weekly Report* **30**, 407–408.
Centers for Disease Control. (1981b). *Morbidity and Mortality Weekly Report* **30**, 535–536.
Centers for Disease Control. (1982a). *Morbidity and Mortality Weekly Report* **31**, 135.
Centers for Disease Control. (1982b). *Morbidity and Mortality Weekly Report* **31**, 279–285.
Centers for Disease Control. (1983a). *Morbidity and Mortality Weekly Report* **32**, 494–495.
Centers for Disease Control. (1983b). *Morbidity and Mortality Weekly Report* **32**, 601–603.
Chadli, A., Sureau, P., and Arrouji, A. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Chippaux, A., Chaniot, S., Piat, A., and Netter, R. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Christensen, P. E., and Muller, J. (1974). *Symp. Immunobiol. Stand* **21**, 180–181.
Chun, Y. T., Berkelhamer, J. E., and Herold, T. E. (1982). *Pediatrics* **89**, 119–120.
Clark, H. F., Wiktor, T. J., and Koprowski, H. (1975). *In* "The Natural History of Rabies" (G. M. Baer, ed.), Vol. 2, pp. 341–365. Academic Press, New York.
Cohen, D., Tierkel, E. S., Sikes, R. K., and Chen, S. M. (1964). *Bull. W.H.O.* **31**, 426–429.
Corey, L., and Hattwick, M. A. W. (1975). *J. Am. Med. Assoc.* **232**, 272–276.
Costy-Berger, F. (1978). *Dev. Biol. Stand.* **40**, 101–104bis.
Cox, J. H., and Schneider, L. G. (1976). *J. Clin. Microbiol.* **3**, 96–101.
Cox, J. H., Dietzschold, B., and Schneider, L. G. (1977). *Infect. Immun.* **16**, 754–759.
Cox, J. H., Klietmann, W., and Schneider, L. G. (1978). *Dev. Biol. Stand.* **40**, 105–108.

Crick, J. (1983). In "Rabies a Growing Threat" (J. R. Pattison, ed.), pp. 18–26. Van Nostrand-Reinhold, New York.

Dean, D. J., Evans, W. M., and Thompson, W. R. (1964). *Am. J. Vet. Res.* **25,** 756–763.

Dempster, G., Stead, S., Zbitnew, A., Rhodes, A. J., and Zalan, E. (1979). *Can. Med. Assoc. J.* **120,** 1069–1074.

Devriendt, J., Staroukine, M., Costy, F., and Vanderhaeghen, J. J. (1982). *J. Am. Med. Assoc.* **248,** 2304–2306.

Diaz, A. M. O. (1982). *Am. J. Epidemiol.* **115,** 274–277.

Dierks, R. E., and Gough, P. M. (1973). *In* "Laboratory Techniques in Rabies, 3rd ed. *W.H.O. Monogr. Ser.* **n23,** 147–150.

Drucker, J., Ajjan, N., Roumiantzeff, M., Triau, R., and Straddy, A. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Drucker, J., Roumiantzeff, M., Bussy, L., and Defayolle, M. (1984). Submitted for publication.

Dureux, J. B. (1974). *Document Institut Mérieux,* Lyon and Nancy, France.

Dureux, J. B., and Canton, P. (1975). *Rev. Med. (Paris)* **16,** 705–720.

Ellenbogen, C., and Slugg, P. (1973). *J. Infect. Dis.* **127,** 433–436.

Fangtao, L., Fanzhen, Z., Longmu, L., Xiaozhen, L., Rongfang, Z., Yongxin, Y., and Naimin, C. (1983). *J. Infect. Dis.* **147,** 467–473.

Fayaz, A., Simani, S., Nour-Salehi, S., and Bahmanyar, M. (1981). *J. Am. Med. Assoc.* **246,** 2334–2335.

Fenje, P. (1960). *Can. J. Microbiol.* **6,** 605–609.

Fenje, P. (1974). *Symp. Immunobiol. Stand.* **21,** 148–156.

Fenje, P., and Pinteric, L. (1966). *Symp. Immunobiol. Stand.* **1,** 381–390.

Fenje, P., Cho, H. C., Johnson, S. E., and Carlson, J. A. K. (1981). *Proc. WHO Consultations Essen,* pp. 73–77. International Green Cross, Geneva.

Fermi, C. (1908). *Z. Hyg. Infekt. Kr.* **58,** 233–276.

Foundation Mérieux (1968). "Table Ronde sur les cellules diploïdes humaines," Fond. Mérieux, ed., Lyon, France.

Fornosi, F., Talas, M., and Somlo, G. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Fuenzalida, E. (1972). *Bull. W.H.O.* **46,** 561–563.

Fuenzalida, E. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23,** 216–220.

Fuenzalida, E., and Palacios, R. (1955). *Bol. Inst. Bacteriol. Chile* **8,** 3–10.

Fuenzalida, E., Palacios, R., and Borgoño, J. M. (1964). *Bull. W.H.O.* **30,** 431–436.

Furlong, J., and Lea, G. (1981). *Lancet* **1,** 1311.

Furesz, J., Boucher, D. W., and Contreras, G. (1977). *In* "Comparative Diagnosis of Viral Diseases" (E. Kurstak and C. Kurstak, eds.), Vol. 2, pp. 209–247. Academic Press, New York.

Gamet, A., and Atanasiu, P. (1971). *Presse Med.* **79,** 1113–1114.

Gardner, S. (1977). *Health Trends* **9,** 35–38.

Gardner, S. D. (1983). *In* "Rabies A Growing Threat" (J. R. Pattison, ed.), pp. 39–49. Van Nostrand-Reinhold, New York.

Garner, W. R., Jones, D. O., and Pratt, E. (1976). *J. Am. Med. Assoc.* **235,** 1131–1132.

Glück, R., Keller, H., Mischler, R., Wegmann, A., and Germanier, R. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Grandien, M. (1977). *J. Clin. Microbiol.* **5,** 263–267.

Greenwood, M. (1945). *Bull. W.H.O.* **12,** 301–304.

Guesry, P., Sureau, P., Levy, L., Cerisier, Y., Gamet, A., and Atanasiu, P. (1981). *Proc. WHO Consultations Essen,* pp. 314–323. International Green Cross, Geneva.

Gusic, B. (1970). *Proc. Symp. Human Diploid Cells,* pp. 205–216. Yugoslav Academy of Sciences, Zagreb, Yugoslavia.
Hafkin, B., Hattwick, M. A. W., Smith, J. S., Alls, M. E., Yager, P. A., Corey, L., Hoke, C. H., and Baer, G. M. (1978). *Am. J. Epidemiol.* **107**, 439–443.
Harverson, G. (1983). *Lancet* **2**, 566.
Hattwick, M. A. W. (1974). *Public Health Reviews* **3**, 229–274.
Hayflick, K., and Moorhead, P. S. (1961). *Exp. Cell. Res.* **25**, 585–621.
Health and Welfare, Canada (1979). *Canadian Disease Weekly Report.*
Held, J. R., and Lopez Adaros, H. (1972). *Bull. W.H.O.* **46**, 321–327.
Helmick, C. G. (1983). *J. Am. Med. Assoc.* **250**, 1990–1996.
Hempt, A. (1925). *Ann. Inst. Pasteur Paris* **39**, 632–640.
Hilfenhaus, J., Köhler, R., Barth, R., Majer, M., and Mauler, R. (1976). *J. Biol. Standard.* **4**, 263–271.
Instituto Nacional de Salud, Bogota, Colombia (1981). *Serie de Notas Tecnicas* **4**, 1–18.
Jacobs, J. P., Jones, C. M., and Baille, J. P. (1970). *Nature (London)* **227**, 168–170.
Jervis, G. A. (1954). *Bull. W.H.O.* **10**, 837–844.
Johnson, S. E., Pearson, E. W., and Ing, W. K. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Kaplan, M. (1974). *Symp. Immunobiol. Stand.* **21**, 226–230.
Keller, H., Simona, R., Spengler, G. A., and Steck, F. (1981). *Proc. WHO Consultations Essen,* pp. 90–95. International Green Cross, Geneva.
Kissling, R. E. (1958). *Proc. Soc. Exp. Biol. Med.* **98**, 223–225.
Kissling, R. E., and Reese, D. R. (1963). *J. Immunol.* **91**, 362–368.
Klietmann, W., Koslowski, L. (1978). *Wehrmed. Monataschr. Heft* **8**, 239–245.
Klietmann, W., Domres, B., and Cox, J. H. (1978). *Dev. Biol. Stand.* **40**, 109–113.
Klietmann, W., Schottle, A., Klietmann, B., and Cox, J. H. (1981). *Proc. WHO Consultations Essen,* pp. 330–337. International Green Cross, Geneva.
Klietmann, W., Klietmann, B., Cox, J. H., Biron, G., and Charbonnier, C. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Kondo, A. (1965). *Virology* **27**, 199–204.
Kondo, A. (1978). *Dev. Biol. Stand.* **40**, 147–153.
Kondo, A., Takashima, Y., and Suzuki, M. (1974). *Symp. Immunobiol. Stand.* **21**, 182–189.
Koprowski, H. (1966). *Symp. Immunobiol. Stand.* **1**, 357–366.
Koprowski, H. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23**, pp. 256–260.
Koprowski, H., Black, J., and Nelson, D. J. (1954). *J. Immunol.* **72**, 94.
Kuwert, E. K. (1967). *Arch. Hyg. Bakteriol.* **151**, 130–145.
Kuwert, E. K. (1981). *Proc. WHO Consultations Essen,* pp. 11–12. International Green Cross, Geneva.
Kuwert, E. K., Marcus, I., and Hoher, P. G. (1976). *J. Biol. Stand.* **4**, 249–262.
Kuwert, E. K., Marcus, I., Werner, J., Hoher, P. G., Thraenhart, O., Hierholzer, E., Iwand, A., Helm, E. B., Wiktor, T. J., and Koprowski, H. (1977a). *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 1 Orig. Reike A* **239**, 437–458.
Kuwert, E. K., Marcus, I., Hoher, P. G., Werner, J., Iwand, A., and Helm, E. B. (1977b). *Med. Klin. (Munich)* **72**, 797–805.
Kuwert, E. K., Marcus, I., Werner, D., Iwand, A., and Thraenhart, O. (1978a). *Dev. Biol. Stand.* **40**, 79–88.
Kuwert, E. K., Werner, J., Marcus, I., and Cabasso, V. J. (1978b). *Dev. Biol. Stand.* **40**, 129–136.
Kuwert, E. K., Werner, J., Marcus, I., and Cabasso, V. J. (1978c). *J. Biol. Stand.* **6**, 211–219.
Kuwert, E. K., Menzel, H., Marcus, I., and Maser, M. (1978d). *Dev. Biol. Stand.* **40**, 29–34.

Kuwert, E. K., Werner, J., Marcus, I., and Scheiermann (1981). *Proc. WHO Consultations Essen*, pp. 31–51. International Green Cross, Geneva.

Kuwert, E. K., Triau, R., and Thraenhart, O. (1984). *In* "Rabies in the Tropics" (Tunis Congress).

Lang, R. (1974). *Symp. Immunobiol. Stand.* **21,** 347–370.

Larghi, O. P., and Fuenzalida, E. (1972). *Ann. Inst. Pasteur Paris* **123,** 421–426.

Larghi, O. P., Varela-Diaz, V. M., Soto, E., Imas B., Cuba-Caparo, A., and Fuenzalida, E. (1976). *Ann. Microbiol. (Paris)* **127B,** 567–572.

Larghi, O. P., Diaz, A. M. O., Arrossi, J. C., Resigno, G. G. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Lavender, J. F. (1970). *Appl. Microbiol.* **19,** 923–927.

Lavender, J. F., and Van Franck, R. M. (1971). *Appl. Microbiol.* **22,** 358–365.

Lépine, P., and Gamet, A. (1974). *Bull. Acad. Natl. Med. (Paris)* **158**(3), 191–194.

Lépine, P., Atanasiu, P., Gamet, A., and Vialat, C. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23,** 204–212.

Lery, L., Gaillard, L., and Delphin, D. (1975). *Arch. Fr. Pediatr.* **32,** 563–567.

Loucq, C., Albert, J. P., Michel, P., Harry, T. O., Rollin, P., and Sureau, P. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

MacFarlane, J. O., and Culbertson, C. G. (1954). *Can. J. Public Health* **45,** 28.

Majer, M., Herrmann, A., Hilfenhaus, J., Mauler, R., Lehmann, H. G., Hennessen, W., and Kuwert, E. K. (1977a). *J. Biol. Stand.* **5,** 249–256.

Majer, M., Herrmann, A., Hilfenhaus, J., Reichert, E., Mauler, R., and Hennessen, W. (1977b). *Dev. Biol. Stand.* **36,** 285–289.

Majer, M., Hilfenhaus, J., Gruschkau, H., Mauler, R., and Hennessen, W. (1978). *Dev. Biol. Stand.* **40,** 25–28.

Mathew, T., Raichowdhuri, A. N., Bhola, S. R., and Topa, P. K. (1981). *Int. Congr. Virol. 5th* 08 Poster, p. 45.

Mean, F., and Tanner, F. (1978). *Med. Hyg.* **36,** 3818–3837.

Mean, F., Steck, F., and Tanner, F. (1978). *Dev. Biol. Stand.* **40,** 159–161.

Menon, M. N., Mathew, Z., Mathew, T., and Adlakha, S. C. (1976). *Bull. Off. Int. Epizoot.* **86,** 341–349.

Mertz, G. J., Nelson, K. E., Vithayasai, V., Makornkawkeyoon, S., Rosanoff, E. I., Tint, H., and Wiktor, T. J. (1982). *J. Infect. Dis.* **145,** 720–727.

Meyer, H. M. (1980). *J. Infect. Dis.* **142,** 287–289.

Mitchell, J. R., Everest, R. E., and Anderson, G. R. (1971). *Appl. Microbiol.* **22,** 600–603.

Montagnon, B. J., Fanget, B., and Nicolas, A. J. (1981). *Dev. Biol. Stand.* **47,** 55–64.

Montagnon, B. J., Fournier, P., and Vincent-Falquet, J. C. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Nelson, K. E., Vithayasai, V., Makornkawkayoon, S., Batt, M. D., Panas-Amporn, K., and Anderson, J. T. (1977). *J. Am. Med. Assoc.* **238,** 218–220.

Netter, R., and Perkins, F. T. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23,** 343–353.

Neurath, A. R., Vernon, S. K., Dobkin, M. B., and Rubin, B. A. (1972). *J. Gen. Virol.* **14,** 33–48.

Nicholson, K. G., and Turner, G. S. (1978). *Dev. Biol. Stand.* **40,** 115–120.

Nicholson, K. G., Turner, G. S., and Aoki, F. Y. (1978). *J. Infect. Dis.* **137,** 783–788.

Nicholson, K. G., Cole, P. J., Turner, G. S., and Harrison, P. (1979a). *J. Infect Dis.* **140,** 176–182.

Nicholson, K. G., Kuwert, E. K., Werner, J., and Harrison, P. (1979b). *Arch. Virol.* **61,** 35–39.

Nicholson, K. G., Prestage, H., Cole, P. J., Turner, G. S., and Bauer, S. P. (1981). *Lancet,* **2,** 915–918.

Nicholson, K. G., Burney, M. I., Ali, S., and Perkins, F. T. (1983). *Lancet* **1**, 916–918.
Nicolas, A. J., Patet, J., Vincent-Falquet, J. C., Branche, R., Delaiti, P., Montagnon, B., Peyron, L., and Soulebot, J. P. (1978). *Dev. Biol. Stand.* **40**, 17–24.
Ofsanpan (1976). *Monthly Report* **8.**
Pait, C. F., and Pearson, H. E. (1949). *Can. J. Public Health* **39**, 875–877.
Pasteur, L. (1885). *C. R. Hebd. Seances. Acad. Sci.* **101**, 765–774.
Peck, F. B., Powell, H. M., and Culberston, C. G. (1955). *J. Lab. Clin. Med.* **45**, 679–683.
Peck, F. B., Powell, H. M., and Culberston, C. G. (1956). *J. Am. Med. Assoc.* **162**, 1373–1376.
Perkins, F. T. (1974). *Symp. Immunobiol. Stand.* **21**, 371–375.
Peterman, H. G., Lang, R., and Branche, R. (1967). *C. R. Hebd. Seances Acad. Sci.* **265**, 2143–2144.
Plotkin, S. A. (1980). *Rev. Infect. Dis.* **2**, 443–447.
Plotkin, S. A. (1981). *Pediatrics* **68**, 131–132.
Plotkin, S. A., and Wiktor, T. J. (1979). *Pediatrics* **63**, 219–221.
Plotkin, S. A., Wiktor, T. J., Koprowski, H., Rosanoff, E. I., and Tint, H. (1976). *Am. J. Epidemiol.* **103**, 75–80.
Putzi, R., and Luthy, R. (1983). *Schweiz. Rundsch. Med. Prax.* **72**, 1583–1586.
Rosanoff, E. I. (1979). *J. Biol. Stand.* **7**, 1–7.
Rosanoff, E. I., and Tint, H. (1979). *Am. J. Epidemiol.* **110**, 322–327.
Roumiantzeff, M., Charbonnier, C., and Ajjan, N. (1983). *In* "Rabies a Growing Threat" (J. R. Pattison, ed.), pp. 33–38, Van Nostrand-Reinhold, New York.
Roumiantzeff, M., Armand, J., Thoinet, M., and Tayot, J. L. (1984). *In* "New Approaches to Vaccine Development" (G. Torrigiani, ed.). WHO, Geneva.
Rubin, R. H., Hattwick, M. A. W., Jones, S., Gregg, M. B., and Schwartz, V. D. (1973). *Ann. Intern. Med.* **78**, 643–649.
Ruegsegger, J. M., and Sharpless, G. R. (1962). *Arch. Intern. Med.* **110**, 754–757.
Sanders, M., Kiem, I., and Lagunoff, D. (1953). *AMA Arch. Pathol.* **56**, 148–225.
Schell, K. R., Fürst, M., Haberstich, H. U., Mischler, R., Hoskins, J. M., and Wegmann, A. (1980). *J. Biol. Stand.* **8**, 97–106.
Schild, G. C., and Ferguson, M. (1982). *J. Gen. Virol.* **59**, 197–201.
Schnurrenberger, P. R., Bashe, W. J., and Helwig, J. H. (1965). *Am. J. Epidemiol.* **81**, 146–149.
Sehgal, S., and Gupta, O. M. H. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Seligmann, B. (1973a). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23**, 192–198.
Seligmann, B. (1973b). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23**, 279–286.
Selimov, M., *et al.* (1974). *Symp. Immunobiol. Stand.* **21**, 179.
Selimov, M. A., Klyueva, E. V., Aksenova, T., Lebedeva, I. R., and Gribencha, L. (1978). *Dev. Biol. Stand.* **40**, 141–146.
Selimov, M., Klyueva, E. V., Aksenova, T., Elbert, L., Gribencha, L., Antonova, L., and Shpirnova, N. (1981). *Proc. WHO Consultations Essen,* p. 64. International Green Cross, Geneva.
Sellers, T. F. (1948). *Am. J. Trop. Med.* **28**, 453–456.
Semple, D. (1911). *Sci. Mem. Med. Sanit. Dep. India,* **n44.**
Shah, U., and Jaswal, G. S. (1976). *J. Infect. Dis.* **134**, 25–29.
Shah, U., Jaswal, G. S., Mansharamani, H. J., Plotkin, S. A., and Wiktor, T. J. (1976). *Br. Med. J.* **1**, 997.
Sikes, R. K., and Larghi, O. P. (1967). *J. Immunol.* **99**, 545–553.

Sikes, R. K., Cleary, W. F., Koprowski, H., Wiktor, T. J., and Kaplan, M. M. (1971). *Bull. W.H.O.* **45**, 1–11.

Sinnecker, H. (1981). *Proc. WHO Consultations Essen*, p. 313. International Green Cross, Geneva.

Smith, J. S., Yager, P. A., and Baer, G. M. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23**, 354–358.

Soulebot, J. P. (1974). *In* "Prophylaxie de la Rage Humaine" (J. B. Dureux, ed.), pp. 149–155. Expansion Scientifique, Paris.

Soulebot, J. P., and Mackowiak, C. (1975). *Symbioses* **7**, 27–43.

Spence, M. R., Davidson, D. E., Dill, G. S., Boonthai, P., and Sagartz, J. W. (1975). *Obstet. Gynecol.* **123**, 655–656.

Svet-Moldavskij, G. J., Andjaparidze, O. G., Unanov, S. A., *et al.* (1965). *Bull. W.H.O.* **32**, 47–58.

Sweet, B. H. (1966). *Symp. Immunobiol. Stand.* **1**, 391–396.

Tayot, J. L., and Montagnon, B. J. (1973). *In* "Symposium Européen de la Centrifugation Zonale," pp. 195–204. Cite Nouvelle, Paris.

Thongcharoen, P., Wasi, C., Chavanich, L., and Sirikawin, S. (1982a). *In* "Viral Diseases in South East Asia and the Western Pacific" (J. S. MacKenzie, ed.) pp. 606–611. Academic Press, New York.

Thongcharoen, P., Wasi, C., and Chavanich, L. (1982b). *Lancet* **2**, 436–437.

Thraenhart, O., and Kuwert, E. K. (1979). *Zentralbl. Bakteriol. Abts. Parasitenk. Infektionskr. Hyg. Orig., Reike A* **245**, 429–441.

Tierkel, E. S., and Sikes, R. K. (1967). *J. Am. Med. Assoc.* **201**, 911–914.

Timm, H., and Wolter, M. (1971). *J. Am. Med. Assoc.* **218**, 738.

Timms, G. L. (1981). Oral presentation in *"Séminaire International sur les Vaccinations en Afrique, 2ième*, Dakar, Senegal.

Tint, H. (1981). *Proc. WHO Consultations Essen*, pp. 87–89. International Green Cross, Geneva.

Tint, H., Dobkin, M. B., and Rubin, B. A. (1974). *Symp. Immunobiol. Stand.* **21**, 132–144.

Trejos, A., Lewis, V., Fuenzalida, E., and Larghi, O. P. (1974). *Ann. Immunol. (Paris)* **125C**, 917–924.

Trivello, R., Gasparini, V., Marcati, F., Moschen, M. E., Renzulli, G., Rocco, S., Canova, F., and Rausa, G. (1982). *Ann. Sclavo* **24**, 151–161.

Triverdi, C. R. (1981). *J. Indian Med. Assoc.* **76**, 134–136.

Turner, G. S. (1977). *In* "Rabies, the Fact" (C. Kaplan, ed.), pp. 104–113. Oxford Univ. Press, London and New York.

Turner, G. S. (1978). *J. Gen. Virol.* **40**, 595–604.

Turner, G. S. (1983). *In* "Rabies a Growing Threat" (J. R. Pattison, ed.), pp. 27–32. Van Nostrand-Reinhold, New York.

Turner, G. S., Aoki, F. Y., Nicholson, K. G., Tyrrell, D. A. J., and Hill, L. E. (1976). *Lancet* **1**, 1379–1381.

Turner, G. S., Nicholson, K. G., Tyrrell, D. A. J., and Aoki, F. Y. (1982). *J. Hyg.* **89**, 101–110.

Van Der Marel, P., and Van Wezel, A. L. (1981). *Dev. Biol. Stand.* **50**, 267–275.

Van Steenis, G., and Van Wezel, A. L. (1982). *Dev. Biol. Stand.* **50**, 37–46.

Van Steenis, G., Van Wezel, A. L., Van Der Marel, P. J., and Hannik, C. A. (1981). *Proc. WHO Consultations Essen*, pp. 78–86. International Green Cross, Geneva.

Van Steenis, G., Hannik, C. A., Van Der Marel, P., Osterhaus, A. D. M. E., Van Wezel, A. L., De Groot, I. G. M., and Ruitenberg, E. J. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.

Van Wezel, A. L. (1967). *Nature (London)* **216**, 64–65.

Van Wezel, A. L., and Van Steenis, G. (1978). *Dev. Biol. Stand.* **40,** 69–75.
Van Wezel, A. L., Van Steenis, G., Hannik, C. A., and Cohen, H. (1978). *Dev. Biol. Stand.* **41,** 159–168.
Varela-Diaz, V. M., Imas, B., Soto, E., Larghi, O. P., Cuba-Caparo, A., and Fuenzalida, E. (1974). *Ann. Immunol. (Paris)* **125C,** 925–938.
Varner, M. W., McGuinness, G. A., and Galask, R. P. (1982). *Am. J. Obstet. Gynecol.* **143,** 717–718.
Vejjajiva, A. (1967). *J. Med. Assoc. Thailand* **50,** 806–811.
Vibulbandhitkij, S. (1980). *In* "Rabies"(P. Thongcharoen), pp. 235–251 (in Thai). Aksonnsmai Press, Bangkok.
Vincent-Falquet, J. C., Callard, R., Souvras, M., Xueref, C., Gibelin, N., and Peyron, L. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Vodopija, K., Smerdel, S., and Bijok, U. (1984). *In* "Rabies in the Tropics" (Tunis Congress) (E. Kuwert, ed.). Springer Verlag, Berlin.
Warrell, M. J., Warrell, D. A., Suntharasamai, P., Viravan, C., Sinhaseni, A., Udomsakdi, D., Phanfung, R., Xueref, C., Vincent-Falquet, J. C., Nicholson, K. G., Bunnag, D., and Harinasuta, T. (1983). *Lancet* **2,** 301–304.
Wasi, C., Chaiprasithikul, P., and Thongcharoen, P. (1983). *Lancet* **1,** 1272.
Wattanasri, S., Boonthai, P., and Thongcharoen, P. (1982). *Lancet* **2,** 870.
Webster, L. T., and Dawson, J. R. (1935). *Proc. Soc. Exp. Biol. Med.* **32,** 570.
WHO (1966). Rabies Expert Committee, 5th Report, *W.H.O. Tech. Rep. Ser.* **n321.**
WHO (1973). Rabies Expert Committee, 6th Report, *W.H.O. Tech. Rep. Ser.* **n523.**
WHO (1980). Rabies Expert Committee, 7th Report, *Report of Consultations WHO Rabies* **80.188.**
WHO (1981). "Requirements for Biological Substances." *W.H.O. Tech. Rep. Ser.* **n658.**
WHO (1982). World Survey of Rabies XX for Years 1980–1981. *WHO/Rabies* **82.193.**
WHO/IABS, Working Group 1 (1978). *Dev. Biol. Stand.* **40,** 267–271.
Wiktor, T. J. (1966). *Symp. Immunobiol. Stand.* **1,** 65–80.
Wiktor, T. J. (1973). *In* "Laboratory Techniques in Rabies," 3rd ed. *W.H.O. Monogr. Ser.* **n23,** 101–123.
Wiktor, T. J. (1973). *In* "Laboratory Technique in Rabies," pp. 101–123. WHO, Geneva.
Wiktor, T. J. (1974). *Symp. Immunobiol. Stand.* **21,** 102–118.
Wiktor, T. J., and Koprowski, H. (1965). *Proc. Soc. Exp. Biol. Med.* **118,** 1069–1073.
Wiktor, T. J., and Koprowski, H. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3938–3942.
Wiktor, T. J., Fernandes, M. V., and Koprowski, H. (1964). *J. Immunol.* **93,** 353–366.
Wiktor, T. J., Sokol, F., Kuwert, E., and Koprowski, H. (1969). *Proc. Soc. Exp. Biol. Med.* **131,** 799–805.
Wiktor, T. J., Aasleasted, H. G., and Kaplan, M. M. (1972). *Appl. Microbiol.* **23,** 914–918.
Wiktor, T. J., György, E., Schlumberger, H. D., Sokol, F., and Koprowski, H. (1973a). *J. Immunol.* **110,** 269–276.
Wiktor, T. J., Plotkin, S. A., and Grella, D. W. (1973b). *J. Am. Med. Assoc.* **224,** 1170–1171.
Wiktor, T. J., Plotkin, S. A., and Koprowski, H. (1978a). *Dev. Biol. Stand.* **40,** 3–9
Wiktor, T. J., Boegel, K., Cox, J. H., Diaz, A. M., Fitzgerald, E., Netter, R., Turner, G., Schneider, L. G., and Van Steenis, G. (1978b). *Dev. Biol. Stand.* **40,** 187–193.
Yamada, A., Sakamoto, K., and Nonaka, J. (1979). *Basic and Clinical Studies* **13,** 34–38. In Japanese.
Yasamura, Y., and Kawakita, Y. (1963). *Nippon Rinsho* **21,** 1201–1215.

19

Epidemiology and Diagnosis of Viral Hemorrhagic Fevers

G. VAN DER GROEN

Institute of Tropical Medicine, Antwerp, Belgium

I. INTRODUCTION

The clinical manifestations of viral hemorrhagic fevers are not distinctive enough to enable the clinician to make a specific diagnosis without the aid of laboratory tests. That is the reason why rapid laboratory diagnosis tests for this group of viruses is important. Although differences among the various diseases can be demonstrated in a relatively large number of cases, there is enough variability when considering a few patients to render those differences practically useless.

An important aspect common to almost all viral hemorrhagic fevers is

ISBN 0-12-429601-7

that the incidence of hemorrhage is relatively low and occurs almost always after the onset of fever. These fevers generally have an acute onset accompanied with chills, headache, myalgia, arthralgia, nausea, vomiting, and conjunctivitis. Such symptoms are nonspecific and simulate many other viral, protozoal, and bacterial diseases; this makes it particularly difficult for the physician to decide whether patients must be kept in isolation when a life-threatening hemorrhagic fever is suspected.

This chapter does not give a complete survey of the knowledge of hemorrhagic fevers but focuses attention on important new developments with far-reaching consequences in the viral hemorrhagic fever world (see Table I).

II. EPIDEMIOLOGY

A. Ebola Hemorrhagic Fever

1. History

Ebola virus was first isolated from two separate outbreaks in northern Zaire and southern Sudan in the year 1976 and named for a small river near the epidemic area in northern Zaire. Another case occurred in Tandala, Zaire in 1977, and an additional outbreak involving 34 cases occurred in 1979 in southern Sudan in exactly the same place. The mortality rate in the Zaire outbreak was 88% as compared to 53% in Sudan.

2. Epidemiology

The Ebola virus (EBO) is an RNA virus. From electron microscopy, EBO particles are pleomorphic, appearing as long filamentous forms (sometimes with extensive branching) or as U-shaped, 6-shaped, or circular forms. The particles vary greatly in length, but have a uniform diameter of approximately 80 nm. Unit length associated with peak infectivity for EBO is 970 nm.

Although the Zaire and Sudan EBO isolates are closely related, biological, immunological, and chemical data indicate that two distinct biotypes exist in nature and that the differences reside in VP1 and VP3 viral proteins and also in oligonucleotide patterns. Zaire strains are much easier to isolated in cell culture and are much more pathogenic for suckling mice. Viral protein and oligonucleotide patterns were remarkably similar within the group, suggesting an unusually high genetic stability among viruses within Zaire and Sudan over 2- and 3-year periods, respectively. Antigenic differences between the two strains are not symmetrical. Ebola Sudan antisera react less well with Ebola Zaire antigens than do Ebola Zaire antisera with Ebola Sudan antigens. This illustrates the importance of using both Zaire and Sudan antigens when serologic screening tests are performed.

TABLE I
Hemorrhagic Fever Viruses

Virus	Disease	Occurrence
Togaviruses		
Alphavirus	Chikungunya	Asia,[a] Africa
Flavivirus	Yellow fever	Africa, Central America, Caribbean
	Dengue	Asia, Caribbean, Africa
	Kyasanur Forest disease	India (Mysore State)
	Omsk hemorrhagic fever	USSR
Bunyaviruses	Rift Valley fever	Africa, Arabia
	Congo-Crimean hemorrhagic fever	Asia, USSR, Bulgaria, Pakistan, Iraq, Africa
	Hemorrhagic fever with renal syndrome	USSR, Czechoslovakia, Yugoslavia, Japan, Korea, United States
Arenaviruses	Lassa	Africa
	Junin	Argentina
	Machupo	Bolivia
Filoviruses[b]	Marburg	Africa [imported to Europe (Marburg) through monkeys] (Kenya, South Africa)
	Ebola	Africa (Zaire, Sudan)

[a]Geographical distribution confirmed by virus isolation.
[b]New proposed name (Kiley *et al.*, 1982).

Despite enormous efforts the ultimate source of EBO in nature is unknown. For example, more than 1000 animals, mostly mammals, representing 93 different species, were sampled in Zaire in 1979. No EBO isolates were obtained. However, immunofluorescent techniques have shown the presence of antibodies against EBO in sera from monkeys (Kenya) (B. K. Johnson *et al.*, 1982), guinea pigs (Zaire) (Stansfield *et al.*, 1982), and rabbits (Central African Republic) (Institut Pasteur, 1981). In the laboratory, monkey, mouse, guinea pig, and hamster have been experimentally infected.

When results obtained by indirect immunofluorescent antibody (IFA) testing can be confirmed by other methods, we have to accept that the virus or a serologically related virus is more widespread than the Ebola epidemic areas have indicated.

Antibodies against EBO or a closely related agent in humans were detected in Senegal (Institut Pasteur, 1981), Liberia (Knobloch *et al.*, 1982), Cameroon (Bergmann, 1981, 1982), Gabon (Georges, 1979), Central African Republic (Georges, 1979), Sudan, northern and eastern Zaire, Uganda, Northern Rhodesia (van der Groen and Pattyn, 1979), Kenya (B. K. John-

son *et al.*, 1982), and the Republic of Congo (van der Groen and Trexler, 1982). In some villages of a pygmy population in southeastern Cameroon, a prevalence as high as 17% was found (Bergmann, 1982). There is evidence that EBO or a related agent had been circulating in Zaire in 1972, 4 years before the first outbreak was noticed (van der Groen and Pattyn, 1979). The absence of epidemics in areas where circulating antibodies against the virus were detected is perhaps an indication that EBO or a related virus circulates more frequently in a subclinical or mild form.

In human cases transmission appears to occur only by close personal contact with a patient or by exposure to infected body fluids or organs. In Zaire, 30% of the cases were iatrogenic. Transmissibility was low, and the disease could not sustain itself even in a fully susceptible community living under primitive conditions. Import of this virus in Western countries would have no epidemic potential in the community. However, nursing personnel should avoid direct contact with the patient and his excreta.

3. Clinical

Early in the disease symptomatology is nonspecific, making diagnosis very difficult until additional similar cases appear and the severe hemorrhagic syndrome becomes apparent. Bleeding occurs in 70% of all cases, mainly from the gastrointestinal tract. The incubation period was usually 6–8 days.

4. Treatment

Experience in the use of convalescent EBO antibody for therapy is very limited and has been successfully reported on only one occasion, and this only in combination with human leukocyte interferon (Emond *et al.*, 1977). Subsequent attempts to treat experimentally infected monkeys with human convalescent antibody alone failed (E.T.W. Bowen, personal communication, 1981). Heat-inactivated and formalin-inactivated EBO Zaire given intramuscularly protected guinea pigs when challenged intraperitoneally with infectious EBO Zaire (Lupton *et al.*, 1980). This is perhaps the first step toward an inactivated vaccine.

For further reading on Ebola, see review article of Kiley *et al.* (1982).

B. Marburg Hemorrhagic Fever

1. History

Marburg hemorrhagic fever was discovered in 1967 when the disease was transmitted to German and Yugoslavian scientists who had come in contact with blood or organs from monkeys imported from Uganda. Subsequently, one primary and two contact cases were reported in 1975 from South Africa. However, Spence and Gear (1982) detected inclusions indistinguishable from those of the Marburg-Ebola virus group in wax-embedded liver

sections from a white female who died during 1973 of an undiagnosed hemorrhagic fever in Durban. In January 1980, two cases occurred in Western Kenya (Smith *et al.*, 1982). The overall mortality rate was 24%.

2. *Epidemiology*

Marburg (MBG) is an RNA virus and morphologically identical with EBO. However, unit length associated with peak infectivity is 790 nm. The virus is structurally related to the EBO virus; however, the differences in oligonucleotide maps and viral proteins is large enough to differentiate it clearly from EBO. There is a close correspondence with the VP2 nucleocapsid proteins of EBO. Both viruses are different from any other animal virus known and are members of a new virus family with the proposed name Filoviridae.

Both viruses are handled as class 4 agents, and laboratory work is performed in P4 laboratories (van der Groen and Trexler, 1982). This is one of the reasons why we had to wait so long before these viruses were fully characterized.

The monkeys implicated as the source of the 1967 MBG outbreak in Germany and Yugoslavia were vervets (*Cercopithecus aethiops*) recently imported from Uganda. These monkeys did not appear to be ill, although the species exhibits a severe form of disease when infected experimentally. All subsequent efforts failed to isolate MBG virus from wild vervet monkeys. The infection was transmitted to man only by direct contact with infected organs or blood. One secondary case was in a woman believed to have been infected during sexual intercourse 12 weeks after clinical recovery of her husband. The first case of MBG disease in Germany was nursed at home, and the other cases had many contacts in the community during 93 days. None of their contacts became infected. It is clear that the transmissibility of MBG disease is low and does not present an epidemic danger in a country with a reasonable, well-organized health care system.

The natural reservoir is unknown. However in Tandala, Zaire, two wild guinea pigs had radioimmunoassay-confirmed antibody to MBG virus at reciprocal titers of 32 and 128 (Stansfield *et al.*, 1982). The possible role of guinea pigs in the spread of MBG disease must be studied further.

Based on indirect fluorescent antibody testing, MBG antibodies were detected less frequently than EBO. Serologic evidence was found in Liberia (Knobloch *et al.*, 1982), Central African Empire (Saluzzo *et al.*, 1981), and Kenya (Smith *et al.*, 1982).

3. *Clinical Features and Treatment*

Extensive clinical descriptions of the European (Siegert and Schlenczka, 1971; Martini, 1971), South African, and Kenyan (Smith *et al.*, 1982) MBG disease were given. A specific hazard appears to exist in relation to genital

carriage during convalescence. Virus was isolated on two occasions from the semen of the patient up to 3 months after the time of his infection. For further reading on MBG disease, refer to the review of Kiley *et al.* (1982).

C. Lassa Fever

1. History

Lassa fever is perhaps the most publicized of all the viral hemorrhagic fevers. The first known victim was an American nurse who was infected at a small mission station in Lassa township in Nigeria. This was the start of a dramatic episode, in which two other nurses died, one of whom was evacuated to the United States by a commercial airliner without strict containment conditions during transport. The arrival of this strange disease from Africa with a case fatality rate of 25% among the first cases in the civilized world, and the additional infection of two laboratory workers working on the specimens, initiated the bad reputation of this virus, which is out of proportion to its clinical importance in the New World. Since then, Lassa fever episodes have occurred at regular intervals both in communities and hospitals in Nigeria, Liberia, and Sierra Leone. An excellent review of these outbreaks was given by Galbraith and colleagues (1978). Cases of Lassa fever occur regularly in Sierra Leone even today. The mortality rate in that area should be considerably lower (<2%) than that indicated by the earlier cases (P. Webb, personal communication, 1982).

2. Epidemiology

Lassa virus belongs to the group of the arenaviruses. It has been isolated repeatedly from the multimammate rat *Mastomys natalensis* in Sierra Leone and Nigeria. The rodent reservoir, *Mastomys natalensis,* becomes persistently infected and sheds the virus in urine for prolonged periods. The virus is transmitted by ingesting food contaminated by rodent urine or feces (Walker *et al.*, 1982). Lassa virus antibodies associated with human illness have been demonstrated in numerous West African countries including Ivory Coast, Ghana, Senegal, Guinea, Gambia, Upper Volta, Mali, Liberia, and the Central African Empire (Monath and Casals, 1975). In 1977, a second arenavirus was isolated from *Mastomys natalensis* in Mozambique. Limited human serologic studies have shown the presence of human antibody to the virus. The interest in the Mozambique virus was stimulated by the finding that it caused asymptomatic infection in rhesus monkeys, which were thereafter clinically resistant to otherwise lethal Lassa virus challenge (Kiley *et al.*, 1979). In 1981, six strains of Mozambique virus, identified by means of specific monoclonal antibodies to the agent, were recovered from

visceral tissues of *Mastomys natalensis* in Zimbabwe (K. M. Johnson, 1981b). Immunofluorescent antibodies to Mozambique virus were found in *Mastomys natalensis* and *Aethomys chrysophilus*. In 1982, eight Lassa-related virus strains were isolated from *Praomys* sp. in the Central African Republic (CAR) (Gonzalez *et al.*, 1982). The use of a battery of monoclonal antibodies has shown that the CAR isolates from *Praomys* sp. are identical to each other and different from Lassa, Mozambique, and lymphocytic choriomeningitis. Human and animal sera to Lassa, Mozambique, or CAR virus appear to cross-react to some degree with heterologous antigens, but the patterns do not give clear evidence of how closely the viruses are related. In terms of host–parasite relationships a variety of questions must be answered. Is *Aethomys chrysophilus* really involved in natural maintenance of Mozambique virus? Where in Africa is the geographic ecological boundary between Lassa, Mozambique, and CAR virus? Is the Mozambique and CAR type of virus capable of producing serious acute disease in man? Will additional Lassa virus be isolated if one examines other African rodent species? What is the real method of transmission in an endemic area? An extensive study by P. A. Webb and colleagues (personal communication, 1982) showed that, in the population of a small village in eastern Sierra Leone with endemic Lassa fever, there was no correlation between seroconversion and the presence or absence of *Mastomys* in a given house, nor was there correlation between the presence of virus or antibody-positive *Mastomys* and seroconversion.

A long-term study of Lassa fever in an endemic setting in the Eastern Province of Sierra Leone, performed by Webb *et al.* (1982), revealed that, even using unsophisticated isolation techniques and minimal protective clothing, the risk of acquiring Lassa fever in a hospital setting is small. We know that hospital practice in parts of tropical Africa may be particularly hazardous because of certain factors that do not apply in other countries: patients must often be nursed in open wards where cleanliness is inadequate and decontamination impossible, and infection may occur through the necessary economy of reusing nonsterile equipment such as needles. Therefore, it is time to ask whether public alarm and expensive quarantine and surveillance procedures are necessary in order to avoid importation of Lassa fever into other continents with higher hygienic standards by travellers returning from parts of Africa where the disease is indigenous.

Ten Lassa cases imported to different places (United States, Great Britain, Canada) are well documented (Banatvala, 1982; Galbraith *et al.*, 1978; Best, 1976). As far as I am aware, not a single case of transmission of Lassa fever from person to person has occurred among more than 2000 persons who had contact with these imported cases. In cases of Lassa fever imported to countries outside Africa, application of Emond's criteria (Emond *et al.*,

1978) will facilitate the decision of the clinician. These criteria are based mainly on epidemiological information. The possibility of Lassa fever should be considered whenever a patient comes from a rural area where Lassa is endemic. A detailed travel history is very relevant.

Perhaps the recommendations for surveillance and quarantine procedures relevant to importation of Lassa fever cases should be revised (Cooper *et al.*, 1982; Emond *et al.*, 1978). Lassa remains a danger for nursing and laboratory personnel. Virological work with this virus should by preference be performed in maximum containment facilities.

3. *Clinical Features*

Lassa virus causes a disease ranging from subclinical to fatal infection. The incubation period ranges from 3 to 16 days, and the disease begins insidiously with nonspecific symptoms. It is marked by a prolonged viremia and the simultaneous presence of both virus and specific antibodies in the blood during the second week of illness. Field studies in Kenema, Sierra Leone have shown that no single symptom or sign was significantly correlated with laboratory-diagnosed Lassa fever (J. B. McCormick *et al.*, personal communication). Combinations of findings such as pharyngitis, conjunctivitis, and bleeding, however, were definitely predictive in the minority of patients. Lassa may be a common cause of febrile illness and asymptomatic infection. The absence of any specific symptom in the early stage of the disease makes it difficult for the clinician to make decisions concerning isolation and treatment procedures.

4. *Treatment*

The passive administration of Lassa immune plasma may suppress viremia and favorably alter the clinical outcome of the disease. However, this has not always been found to be true. The final outcome of this treatment is dependent on many parameters. These include the time after infection when the plasma was given; the strength of the viremia [$>10^5$ TCD_{50}/ml virus in the blood indicates fatal hemorrhagic disease (J. B. McCormick, personal communication)]; and the quality of the immune plasma. A breakthrough in the treatment of Lassa with immune plasma was achieved by Jahrling and his co-workers (1982), showing the extreme importance of the presence of neutralizing antibody in order to have a favorable immune plasma treatment. First, they showed that, in guinea pigs surviving from virulent Lassa challenge, neutralizing antibodies were developed late in convalescence, whereas in susceptible animals no neutralizing antibodies were detected. In both cases fluorescent antibody response did occur early after infection, clearly showing the distinct nature of both types of antibody re-

sponses. In later work they showed that susceptible guinea pigs after challenge with virulent Lassa survived when they were treated with immune plasma rich in neutralizing antibodies and died when treated with immune plasma rich in fluorescent antibodies but lacking neutralizing antibodies. The same results could be obtained in the monkey model.

These are encouraging results and explain perhaps the lack of success obtained with immune plasma in the past, due to incomplete characterization of the immune plasma.

Ribavirin, an antiviral drug, had a beneficial effect when tested in monkeys infected with Lassa virus (Jahrling *et al.*, 1980). Evaluation of field trials with ribavirin on Lassa fever patients in an endemic area in Sierra Leone is now in progress (J. B. McCormick, personal communication).

D. Junin Virus of Argentinian Hemorrhagic Fever

Junin virus was first isolated in 1958. It still causes annual outbreaks in an area of intensive agriculture known as the wet pampa in Argentina. The mortality rate varies from 3 to 15%. The disease is seasonal, coinciding with the maize harvest, when rodent populations reach their peak. People harvesting maize are most commonly affected. The main reservoir hosts of Junin are *Calomys* species.

The virus is present in urine and saliva. The mode of transmission of Junin virus is either by air from dust particles contaminated by rodent excreta or by eating contaminated foodstuffs.

Argentinian hemorrhagic fever was until now the only hemorrhagic fever which could be treated with immune plasma, with rather encouraging results. Provided the immune plasma was given within 8 days of the onset of symptoms, clinical improvement was recorded. Maiztegui and co-workers demonstrated the formation of neutralizing antibodies in human sera early after infection (Maiztegui, 1982). Jahrling and his group demonstrated the much earlier formation of neutralizing antibodies in Junin-infected monkeys in comparison with Lassa-infected monkeys.

Assuming that the presence of neutralizing antibodies in the immune plasma for treatment of human Junin cases is equally important as it was for treatment of Lassa-infected guinea pigs, we understand why immune plasma treatment of Argentinian hemorrhagic fever cases was so successful. Maiztegui and colleagues plan to determine the critical titer of neutralizing antibodies required in immune plasma in order to be effective.

One complication of immune plasma treatment has been the development of late neurological symptoms, but this phenomenon is rare.

A live attentuated vaccine against Junin virus is under study (Barrera Oro and Eddy, 1982).

E. Machupo Virus of Bolivian Hemorrhagic Fever

Bolivian hemorrhagic fever was first recognized in 1959 in northeastern Bolivia. The disease has continued to occur more or less annually. The mortality rate has varied from 5 to 30%. The disease is closely similar to Argentinian hemorrhagic fever. The rodent reservoir of Machupo virus is *Calomys callosus*. Transmission to man is probably by food contaminated with rodent excreta. Spread of the disease is best achieved by rodent control and by improved housing. For further reading on arenaviruses causing hemorrhagic fevers, see Simpson and Metselaar (1982); Murphy (1977); Jahrling (1980); International Symposium on Arenaviral Infections (1975).

F. Hemorrhagic Fever with Renal Syndrome

1. *History*

An epidemic of hemorrhagic fever with an associated renal syndrome occurred among United Nations troops during the Korean war. Since that time, this disease has become known as Korean hemorrhagic fever (KHF).

Hemorrhagic fevers with a syndrome very similar to KHF have been reported throughout Euroasia: e.g., hemorrhagic fever with renal syndrome in the USSR, or nephrosonephritis; Songo fever or epidemic hemorrhagic fever in China, with more than 20,000 cases reported annually since 1931; nephropathia epidemica (NE) in Scandinavia, with several hundred cases reported annually since 1934; epidemic nephritis in Eastern Europe since 1934; and epidemic hemorrhagic fever in Japan since 1960. The World Health Organization has recommended the name hemorrhagic fever with renal syndrome (HFRS) for the above-mentioned disease (WHO, 1982b).

The isolation by Lee and Lee in 1976 of the virus causing KHF, now called Hantaan virus (named after the Hantaan river), from the lungs of the natural reservoir *Apodemus agrarius coreae,* was the start of a new era in the study of hemorrhagic fevers with renal syndrome throughout the world.

2. *Epidemiology*

The virus was initially isolated by serial passage of lung tissue to uninfected *A. agrarius* mice. A breakthrough was the detection by H. W. Lee of KHF antigen in thin sections of frozen lung tissue of recipient mice, and of KHF antibodies in the sera, by means of an indirect immunofluorescence test. Subsequently the virus was adapted to grow in Fisher and Wistar laboratory rats, in nude mice, and in Vero E6 and A549 (human lung carcinoma) continuous cell lines. The facilitation of *in vitro* propagation of the virus contributed to a real explosion of knowledge about this virus and

related ones. Virions resembling those of known Bunyaviridae have been found in KHF-infected cells by electron microscopy. Very recently, J. Dalrymple has demonstrated the RNA genome of the Bunyavirus group in KHF virus-infected cells.

Besides the isolation from the natural reservoir, *Apodemus agrarius*, Hantaan virus was very recently isolated from 23 *Rattus norvegicus* trapped in Seoul, Korea (Lee and Johnson, 1982). Wistar rats were much more sensitive than *Apodemus* mice in detecting this wild rat virus. Detection of Hantaan virus in urban rats significantly expands the epidemiology for the transmission of the virus to humans.

Clinically apparent infections with Hantaan virus occurred in nine persons at Korea University (Seoul) between 1971 and 1979. All were directly related to trapping of wild rodents or work with naturally or experimentally infected wild and laboratory rodents in a designated animal suite. From five patients Hantaan virus was isolated by propagation the virus in *Apodemus* mice. Five of nine infections occurred within 4 months of the initiation of experimental studies of infections in Wistar rats with Hantaan virus, a finding suggesting that this rodent may be a highly infectious host for Hantaan virus (Lee and Johnson, 1982). Viral survival in infectious rodent urine-borne aerosols at low relative humidity appears to be one of the most important modes of transmission. In addition, out of 23 Wistar rats used for experimental work in four different Japanese laboratories, two identical virus strains (SR-11) were isolated which were indistinguishable antigenically from Hantaan virus. This was the first report of direct isolation of Hantaan virus by inoculation into Vero E6 cell cultures (Kitamura *et al.*, 1982).

It is clear that for Hantaan virus three epidemiological types can be distinguished: rural, urban, and experimental animal types. Besides Hantaan virus there is overwhelming epidemiological evidence for the existence of at least two other antigenically related viruses that are distinguishable serologically from Hantaan. The presence of these viruses was detected by performing the immunofluorescent antibody (IFA) technique on frozen lung sections using sera containing antibodies for hemorrhagic fever with renal syndrome.

In 1980, Brummer-Korvenkontio *et al.* reported recovery from the pulmonary tissues of *Clethrionomys glareolus* of an agent similar to Hantaan virus, which appears to be etiologically linked to a clinical syndrome termed nephropathia epidemica (NE) in Finland. Clinically nephropathia epidemica is identical in Norway, Sweden, and Finland but is milder than the East-Asian HFRS caused by Hantaan virus. Mortality is 0.5% compared to 5–10% for KHF (Lähdevirta *et al.*, 1982). The antisera of Scandinavian patients exhibited a pattern of high reactivity toward the NE antigen and

showed slightly reduced reactivity (2–4 times) to Hantaan virus, clearly showing the relationship between Scandinavian and East-Asian HFRS.

A new hemorrhagic fever with renal syndrome-related virus was detected in lung sections from *Microtus* species, captured wild in Prospect Hill, Frederick, Maryland. This antigen reacted with seropositive *Microtus* species, with convalescent sera from a patient with KHF, and with serum from Fisher rats experimentally infected with Hantaan virus. Seronegative *M. pennsylvanicus* bred in captivity have developed antibody and antigen in lung after intramuscular inoculation with suspensions of antigen-bearing lung tissue from two wild-caught seropositive voles. The role of this virus in human infection must still be elucidated.

Antigens related to Hantaan virus have been detected in urban, laboratory, and wild rodents with a wide geographic distribution. A summary is given in Table II.

Tkachenko and colleagues (Tkachenko *et al.*, 1981; Tkachenko, 1982) showed the presence of HFRS antigen in 14 different wild rodents in 13 European and 3 Far Eastern regions of the USSR using antigen obtained by enzyme-linked immunosorbent assay. Sera from USSR HFRS patients of the endemic area were used in the test. HFRS viral strain Ufa–3952 has been recovered from the pulmonary tissue of *Clethrionomys glareolus* trapped in Bashkiria (European focus of HFRS) and is now passed in Wistar rats. HFRS viral strain 81–660 Ussury has been recovered from the pulmonary tissue of *Apodemus agrarius* trapped in Ussury region (Far Eastern focus of HFRS) and is passed in *A. agrarius*. Natural foci of HFRS in USSR may be characterized as polyhostal. In some sera collected from patients with HFRS in the acute period, HFRS viral antigen was detected by ELISA, but in low titers.

Sera from the European focus of HFRS in USSR react eight times as strongly with the antigens from the same area as with antigen isolated in Far Eastern USSR, whereas convalescent sera from the Far Eastern USSR react 64–128 times as strongly with Far Eastern antigen than with those collected in the European USSR. These findings point to the existence in the USSR of two serotypes of the HFRS agent.

On the evidence of serological tests for Hantaan and nephropathia epidemica virus (NE), both viruses or viruses related to them exist worldwide. Serological tests were based on indirect immunofluorescent antibody testing using Hantaan virus in rodent lung and cell culture and NE virus in lung sections of *Clethrionomys glareolus;* with a recently developed blocking and neutralization test for Hantaan virus, distinct patterns among IFA-positive sera could be obtained. The presence of blocking or neutralizing antibody was interpreted as indicating a serum more specifically related to the Hantaan antigen than to the other antigens mentioned. These findings are summarized in Table III. Four distinct patterns can be observed. Convalescent phase sera from patients with HFRS in Korea, Japan, China, Far Eastern

TABLE II
Hemorrhagic Fever with Renal Syndrome (HFRS) Antigen Detection in Lung Sections of Animals by Indirect Immunofluorescent Antibody Technique

Species	Origin	Serum used in the test[a]			
		A	B	C	D
Wildlife					
Microtus pennsylvanicus	United States (Prospect Hill, Maryland)	+	+	+	
Microtus montebelli[b]	Japan	+			
Apodemus speciosus[b]	Sweden, USSR[c]		+		+[c]
Clethrionomys glareolus[d]	Sweden, USSR[c]		+		+[c]
Clethrionomys rufocanus[d]	Korea	+			
Apodemus agrarius coreae	USSR				+
Apodemus agrarius	USSR				+
Apodemus silvaticus	USSR				+
Apodemus flavicollis	USSR				+
Apodemus peninsulae	USSR				+
Sorex araneus	USSR				+
Rattus norvegicus	USSR				+
Microtus fortis	USSR				+
Microtus arvalis	USSR				+
Micromys minutus	USSR				+
Mus musculus	USSR				+
Clethrionomys rutilus	USSR				+
Eutamias sibiricus	USSR				+
Urban					
Rattus rattus	Korea, Japan	+			
Rattus norvegicus	Korea, Japan				
Seaport area					
Rattus norvegicus	United States (Houston, Texas)	+			
Laboratory rats Wistar rat	Japan, Korea	+			

[a] A: Convalescent serum from KHF patient; B: convalescent serum from NE patient; C: serum of the animal; D: convalescent serum of patient with HFRS in USSR.

[b] Umenai *et al.* (1981).

[c] Tkachenko *et al.* (1981). Used antigen obtained by ELISA technique on lung tissue homogenates.

[d] Brummer-Korvenkontio *et al.* (1982).

USSR, and the United States reacted well with Hantaan virus and feebly or not at all with NE; they contained blocking or neutralizing antibodies. We now know that HFRS in these countries is caused by the same virus or antigenically related viruses which are distinguishable serologically from the virus that causes HFRS in European USSR, Scandinavia, and Yugoslavia,

TABLE III
Indirect Immunofluorescent Antibody Titers to Korean Hemorrhagic Fever (KHF) Virus, Nephropathia Epidemica (NE) Virus, and Prospect Hill (PH) Virus, and KHF Blocking and Neutralizing Antibody[a]

Origin of serum	IFA			KHF Virus blocking antibody	KHF virus neutralizing antibody
	KHF	NE	PH		
Korea, South	+	−/+	NT	+	+
China	+	−	NT	+	+
Japan	+	−	NT	+	+
Far Eastern USSR	+	−	NT	+	+
United States	+	−	−	+	NT
United States (*Rattus norvegicus*) nonport areas	+	−	−	NT	NT
United States (*R. norvegiucs*) port areas	+	NT	NT	NT	+
European USSR	+	+	NT	+	+
Sweden	+	+	NT	+	+
Yugoslavia	+	+	NT	+	+
European USSR	+	2 to 4+	NT	−	−
Sweden	+	2 to 4+	NT	−	−
Yugoslavia	+	2 to 4+	NT	−	−
United States (*Microtus pennsylvanicus*) nonport areas	+	−	+	−	NT

[a]Using sera from patients with HFRS or animals with HFRS antigens in the lungs. +, Reacts well with KHF antigen in IFA; −, no reaction; ±, weak reaction; 2 to 4, titers 2 to 4 times higher; NT, not tested.

where the sera exhibited a pattern of high reactivity to NE and slightly reduced reactivity to Hantaan virus by IFA, but failed to block or neutralize Hantaan virus. A third pattern was found in Europe, where sera react equally well with both KHF and NE antigen and have blocking or neutralizing antibodies. These data suggest that both Hantaan and NE may coexist or that other serotypes of HFRS in Europe exist. A fourth pattern was found in the United States in sera from wild rodents, exhibiting a pattern of high reactivity to Hantaan and PH antigen, but failing to block KHF. So at least two distinct serotypes of HFRS are circulating in the United States. In addition, antibody to Hantaan virus has been found occasionally in human sera from Alaska, Bolivia, India, Iran, Central African Republic, and Gabon. Antibodies to Hantaan virus were detected in human sera from

people working in animal care rooms in Belgium. Also Wistar rats used for experimental work contained Hantaan antibodies. In 1983, in the author's laboratory, antibody to Hantaan virus was found in sera from wild *Clethrionomys* species trapped in the northeastern part of Belgium (Hasselt) (3 positive sera out of a total of 686 sera, had titers of 1/128, 1/256, and 1/64, respectively, when tested by IFA on Vero E6 infected with Hantaan 76–118 strain).

Antibodies to Hantaan virus have been detected in the sera from all rodent species mentioned in Table II except those described by Tkachenko, which were tested against European and Far Eastern HFRS antigens of the USSR. However, from Table III we can deduce that those sera will also react with the Hantaan antigen. The term hemorrhagic fever with renal syndrome is perhaps misleading, since bleeding occurs only in one-fifth of the cases. All patients have proteinuria and azotemia, and some show petechiae, hemoconcentration, hypotension, and renal failure. Gajdusek *et al.* (1982) has suggested the name "muroid virus nephropathies" to replace HFRS, because the majority of patients have some degree of renal involvement whereas many have no hemorrhages.

The worldwide spread of this group of viruses should make clinicians more aware about the possibility that these viruses can be the etiologic agent for patients with fever of unknown origin associated with albuminuria and renal dysfunction. Surveys of urban rats worldwide are urgently needed, and port cities represent the most logical place to start.

If not explicitly stated, the information mentioned in this short review on HFRS was based on an excellent review article in the *Lancet* (see Muroid Virus Nephropathies, 1982), and the review article of Lee (1982).

G. Crimean Congo Hemorrhagic Fever

This fever was first recognized in the Crimea in 1944. A similar virus was isolated in Zaire in 1956, but the viruses were shown to be identical only much later by work done by Casals in 1969. The disease in man, as confirmed by virus isolation and antibody development, has been reported in Uganda and Zaire (1967), Bulgaria (1969), Pakistan (1970), Dubai (1979), and Iraq (1981) (Al Tikriti *et al.*, 1981a,b). Seroepidemiological surveys revealed the possible occurrence of inapparent infections in man and animals in Bulgaria, Iran, Egypt, Turkey, Afghanistan, India, Greece, Hungary, Yugoslavia, and Nigeria.

Crimean Congo hemorrhagic rever (CCHF) is caused by a virus belonging to the Bunyaviridae. The virus is transmitted by ticks and has repeatedly been isolated from them, most often the *Hyalomma* genus. Isolations from cattle and goat and the high prevalence in domestic mammals (cattle, sheep,

goat, camel) suggest a possible role of domestic animals in the spread of the virus.

The incubation period of CCHF is relatively short (3–6 days). South Africa recently reported its first documented indigenous case of CCHF with hemorrhagic manifestations and death. The patient was bitten by a tick of the genus *Hyalomma* in the Transvaal province of South Africa. Pools of ticks collected near the area where the boy died contained CCHF virus (Centers for Disease Control, 1981a). Antibodies to CCHF virus were detected by IFA and reversed passive hemagglutination inhibition tests in sera from family members. Antibodies were also detected in cattle, sheep, and antelope. Serological evidence for CCHF was also found in sera of hares which had been collected between 1977 and 1979 at various sites in South Africa, confirming the presence of the virus in all four provinces (Swaenepoel *et al.*, 1982).

Treatment is supportive. A vaccine has been developed in the USSR for those at high risk.

For further reading see the excellent reviews by Hoogstraal (1979), Casals (1978), and Simpson (1978).

H. Rift Valley Fever

Rift Valley fever (RVF) is an acute febrile arboviral disease. The virus is now classified as being of the genus *Phlebovirus* of the Bunyaviridae family. It was primarly a disease of sheep, cattle, other domestic animals, and man, first recognized in Kenya in 1931. Until 1977, the disease was geographically confined to Sub-Saharan Africa, with epizootics in Kenya, South Africa, southern Zimbabwe, Sudan, Uganda, United Republic of Tanzania, and Zambia. In Mali, Nigeria, Congo, Botswana, and Mozambique no epizootics have been reported, but the RVF virus was demonstrated by virus isolation or by the presence of antibody in human and animal subjects. RVF was believed to be benign for man. However, in 1975 the first fatal cases of RVF in man were reported in South Africa. In 1977, the disease was for the first time identified in Egypt, and it reappeared with reduced intensity in the following years. It caused enormous losses of sheep and cattle in Egypt, and human infection was now also very extensive. During the 3-month epidemic, 18,000 acute cases and nearly 600 deaths occurred. The disease in man was typically an acute febrile illness with severe ocular, encephalitic, or fatal hemorrhagic complications, and hepatitis occurred frequently. Mortality in sheep and cattle may be 10–30%; abortion in pregnant animals and mortality among the newborn was near to 100%.

RVF is vector borne, and at least 26 species of arthropods, mostly mosquitoes but also *Culicoides* midges, can be infected. Transmission of RVF

virus by aerosol from blood and other infected animal body fluids is implicated in the frequent infections of virologists, veterinarians, and abattoir workers.

The events in Egypt have changed completely the perception of RVF and made the virus a potential hazard for other candidate receptive countries in the Mediterranean and South West Asia Regions. Predictions regarding further spread outside Egypt cannot be given because the precise mechanism of the natural maintenance cycle of RVF as well as the way it was introduced into Egypt is not known.

It is not excluded that the South African and Egyptian RVF strains possessed enhanced virulence in comparison with strains circulating before 1975.

Treatment/Prevention

Observations in laboratory animals suggest that interferon inducers, antiviral drugs, or immune serum could be useful. Formalin-inactivated and attenuated types of vaccine are already available. The formalin-inactivated cell culture vaccine is expensive, and the limited supply allows only vaccination of exposed laboratory workers and veterinarians.

An inexpensive attenuated vaccine which can easily be produced in large quantities was successfully used for veterinary use. However, it caused a small number of sheep to abort, and it has to be studied further to determine the frequency of this side effect. It is therefore not recommended for use in areas that now are free of RVF because, if virulence change should occur, the cycle of transmission might be initiated.

For further information concerning RVF, refer to three excellent recent review articles: Shope *et al.* (1982); La Fièvre de la Vallée du Rift, 1981; Kark *et al.*, (1982).

I. Dengue Hemorrhagic Fever/Dengue Shock Syndrome

Dengue has been known for more than a century in the tropical areas of southeast Asia and the Western Pacific region. Since 1953 more patients were seen with infections complicated by hemorrhagic fever and shock syndromes (DHF/DSS). DHF/DSS was reported in the Philippines, Vietnam, China, Thailand, Burma, Malaysia, Singapore, Indonesia, India, and Sri Lanka. This disease is among the 10 leading causes of hospitalization and death in children in at least eight Asian countries. Over half a million persons have been hospitalized with this syndrome in the past 20 years. Dengue has also been prevalent in tropical areas of Africa, the Mediterranean Region of Europe, the Caribbean and the Americas. In 1980, DEN 1 was

transmitted within the borders of the United States for the first time in nearly 40 years.

Dengue is caused by four serotypes of DEN virus, belonging to the flavivirus group. Infection (primary) with one DEN type elicits 3–6 weeks of protection against infection with another type (secondary infection). After this period, exposure to a second DEN type results in infection and disease. Dengue virus infection is transmitted to man through mosquito bites. *Aedes aegypti* is the most efficient of the mosquito vectors. Man is the reservoir of the virus, and studies in Malaysia have shown that the monkey is a natural reservoir.

Clinically, classical dengue, dengue hemorrhagic fever without and with shock, can occur. One of the intriguing questions of dengue is how a benign disease (classical dengue) is transformed to a severe disease often accompanied by shock and hemorrhage.

Two main pathophysiological changes occur in DHF/DSS. One is an increased vascular permeability; the second is a disorder in hemostasis which involves vascular changes, thrombocytopenia, and coagulopathy. A constant finding in DHF/DSS is the association with "secondary" antibody response. Very interesting is the observation that DHF/DSS occurs in infants who acquired maternal dengue antibody and subsequently experienced a dengue infection. One factor which may contribute to that is the enhancement of virus multiplication in monocytes by "enhancement antibodies." It was established that at nonneutralizing concentrations infectious DEN antibody immune complexes were formed which attached to monocytes via the FC reception, stimulating the phagocytosis. By a still unknown mechanism the virus successfully replicates in the monocyte instead of being destroyed. This may trigger the production by monocytes of chemical mediators that interact with blood clotting, complement, and vascular permeability systems. If this hypothesis is confirmed, signs and symptoms of DHF/DSS could be related in a simple way to effector activities of the activated cell system.

The "enhancement" effect of sera correlates with the hemagglutination-inhibition titers of these sera and is extended to nondengue flavivirus serum DEN 2. Monoclonal antibodies were developed which possess strong enhancing activity having hemagglutination inhibition but weak or no neutralizing activities. Antibodies directed toward type-specific determinants neutralize, whereas antibodies against group-specific determinants enhance.

DEN epidemiology remains still a fascinating puzzle. One question is, Why does DHF/DSS occur only with some, but not with all, secondary dengue infections?

The study of DEN immunopathology can be extremely relevant to help

to understand the hemorrhagic phenomenon of the other viral hemorrhagic fevers.

Theoretically, vaccine requires four separate components for the four types of DEN. In Bangkok the development of attenuated DEN strains was started. The method selected was that of serial passage of virus in primary tissue culture. Types 1, 2, and 4 have been passaged in primary dog kidney cell cultures, and DEN 3 has been passaged in African green monkey cells. In the United States DEN 1 and 4 have reached the stage of vaccine production and safety testing. DEN 2 live attenuated vaccine has been produced, and volunteers have been vaccinated successfully. However, in persons not immune to flaviviruses antibody response was low (WHO, 1982a).

Can DHF/DSS be controlled by using a monovalent vaccine? Studies on DHF/DSS in Bangkok have shown a consistent predominance of secondary DEN 2 infection over a 20-year period. These results suggest that DSS might be controlled using a DEN 2 vaccine (Halstead, 1982a).

For further reading, refer to WHO (1980), Halstead (1981, 1982a), and CDC (1981b).

III. DIAGNOSIS

The clinical manifestations of viral hemorrhagic fevers are not distinctive enough to enable the clinician to make a specific diagnosis without the aid of laboratory tests.

A rapid diagnosis of viruses causing hemorrhagic fevers is needed since these agents are capable of afflicting heavy losses to human life and sometimes to livestock. The most rapid way is direct detection of viral hemorrhagic fever (VHF) antigen in material from the patient or infected animal. This can be done by the methods described in Section III, A–D below.

A. Electron Microscopy

In a few situations, early diagnosis was achieved by direct electron microscopic examination of autopsy material (LAS, MBG, EBO) (Winn *et al.*, 1975; Johnson *et al.*, 1977) and acute blood specimens [EBO (Emond *et al.*, 1977; Smith *et al.*, 1982) and RVF (Ellis, 1979)]. Electron microscopy remains a powerful tool because morphology can help to distinguish among members of the heterogeneous group of hemorrhagic fever viruses. Research should be done to study inactivation procedures of biological samples coming from the patient in order to study the morphology without

danger for the investigator, as for example, β-propionolactone inactivation of human sera (Lloyd *et al.*, 1982).

B. Indirect Immunofluorescent Antibody Technique

A second direct attack is to reveal the presence of VHF antigen in intact cells from the patient or animal using the indirect immunofluorescent antibody (IFA) technique. This technique was successfully applied for DEN antigen in peripheral lymphocytes and mononuclear leukocytes in the skin (Halstead, 1981), for Junin in urine sediment cells (Ruggiero *et al.*, 1981), for Lassa in acetone-fixed epithelial cells collected from eye scrapings, and for KHF antigens in frozen lung tissue sections of infected animals.

The diagnosis still represents many difficulties because few laboratories are equipped to conduct the necessary investigations in safety. Trypsin digestion of formalin-fixed tissue or cells before fluorescence microscopy allowed a specific diagnosis of MBG, EBO, RVF, Congo, LAS, and Hantaan virus (Kurata *et al.*, 1982; van der Groen, 1982). Two hours' fixation of Vero cells infected with EBO, LAS, or MBG in buffered 3.5% formaldehyde solution is enough completely to inactivate the antigens (van der Groen, 1982). This technique appears to be especially suitable for investigation of class 4 agents without danger for the investigator and for retrospective investigation of stored tissues. MBG antigen was successfully detected with this procedure in formaldehyde-fixed biopsy kidney tissue (Smith *et al.*, 1982).

C. Antigen Capturing in Homogenates of Infected Organs

The test uses recipients coated with specific antiviral antibodies. Suspensions or body fluids were added, and virus antigen was allowed to absorb. The trapped antigen is detected by addition of virus-specific IgG coated with an enzyme (double-layer ELISA) or with a radioactive label [solid-phase radioimmunoassay (SPRI)]. This technique was successfully applied by Tkachenko *et al.* (1981) for HFRS antigen determinations in sera from HFRS patients and from organ homogenates of infected animal reservoirs. This technique is now in development for EBO, MBG, LAS, and RVF. The antigen in homogenates of organs was directly detected by microimmunodiffusion test (RVF) (Swaenepoel, 1981).

The availability of a battery of highly specific monoclonal antibodies will make it possible in future to specify directly the VHF antigen with the above-mentioned techniques. Monoclonals against different strains of Lassa (Buchmeier *et al.*, 1981; Gonzalez, 1983), EBO (McCormick *et al.*, 1983) and DEN 1, 2, 3, and 4 (Henchal *et al.*, 1982) have been produced.

Since it is not always possible to detect viral hemorrhagic fever (VHF) antigens directly in samples from the patient, it is necessary to inoculate suspected specimens into susceptible host systems for virus isolation. The most commonly used host systems for primary isolations of VHF viruses are summarized in Table IV. Baby mice and Vero cells are good substrates with a broad spectrum for different hemorrhagic fever viruses (HFV). The time taken to reach a specific diagnosis can be shortened by the daily examination of inoculated systems without wasting undue time waiting for the appearance of signs of virus infectivity. Intracellular hemorrhagic fever antigens can be detected by IFA, and extracellular antigens in the supernatant by EM, from a few hours postinoculation to a few days postinoculation in experimentally infected CV1 cells. These cells were superior to other cell lines tested (Vero, BHK-21, *Aedes albopictus*) and deserve perhaps more attention for primary isolation assays of HFV (El Mekki and van der Groen, 1981).

Antigen in the supernatant of infected cell cultures was successfully detected with (1) double-layer ELISA technique as previously described for KHF (this technique is now in development for EBO, MBG, and Lassa); (2) reverse passive hemagglutination test (RPHT), using erythrocytes coated with virus-specific antibodies: LAS (Goldwasser *et al.*, 1980), RVF, Congo (Swaenepoel, 1982); (3) immune electron microscopy of formaldehyde- or glutaraldehyde-inactivated supernatants.

TABLE IV
Host Systems Used for Primary Isolations of Hemorrhagic Fever Viruses

Virus	Animal	Cell culture
EBO Zaire	Baby mice, guinea pig, rhesus monkeys	Vero
EBO Sudan	Guinea pig, rhesus monkeys	Vero
Marburg	Guinea pig, vervet/rhesus monkeys	Vero/LLCMK2
Hantaan	Wistar rats, *Apodemus coreae*	E6
Lassa: Josiah Mozambique and CAR	Guinea pig	Vero
Congo/Crimean	Baby mice	CER/BHK-21
RVF	Baby mice	BHK-21/Vero Primary sheep/bovine cell line
DEN	Baby mice, inoculation of mosquitos	*Aedes pseudoscutularis*, *Aedes albopictus*, Vero/BHK-21

Also here research is necessary in order to find the suitable technique to inactivate the supernatant and to keep antigenecity intact. Gamma irradiation successfully inactivates RVF, Congo, MBG, EBO, LAS (Elliot *et al.*, 1982), YF, DEN, CHIK, and Hantaan (van der Groen, 1983) and the antigens can still be used in immunological reactions.

Intracellular hemorrhagic fever antigen was demonstrated directly in organs of inoculated animals or inoculated cell cultures (1) by indirect immunofluorescence antibody testing (for all VHF) in acetone-fixed infected cells or frozen sections of infected organs; sensitivity of DEN antigen detection in inoculated suckling mouse brain by indirect FA technique, was markedly increased by using the biotin-avidin system (unlabeled antiviral antibody, biotinyl-anti-IgG, and fluorescein-conjugated avidin D) (Centers for Disease Control, 1981b); (2) by indirect immunoperoxidase antibody testing in mosquitoes for DEN (Igarashi *et al.*, 1981), and for DEN, Hantaan, YF, and CHIK in cell culture (van der Groen, 1983, unpublished); the immunoperoxidase test allows reading with an ordinary light microscope and is perhaps more suitable for work in the field; and (3) by immunoenzymatic quantification of hemorrhagic fever antigens directly in the wells of a plate with fixed inoculated cell monolayer by performing ELISA directly in the wells. This technique is especially suitable for titration or quantitation in cell culture of antigens which give no cytopathic effect—e.g., KHF in E6 cells. This technique is in progress (Tsai, 1982, personal communication).

D. Serology

The hemagglutination-inhibition test has been the serological test most widely used for hemorrhagic fever antibody determinations, except for EBO, MBG, and LAS. However, for DEN, RVF, and Congo, marked cross-reactions with other viruses in the same group did occur. Complement fixation was also used except for EBO, also with the disadvantage of not being specific.

More recently, the determination of hemorrhagic fever viral antibodies was performed on γ-irradiated inactivated acetone-fixed antigen slides using the indirect immunofluorescent antibody test (Johnson *et al.*, 1981a; McCormick *et al.*, 1982; Wulff *et al.*, 1978). The antigen consisted of Vero cells infected with HFV, and A549 or E6 cells infected with Hantaan (French *et al.*, 1981; McCormick, 1982). Much seroepidemiological information was obtained with this test, most of which was not published until now, because reproducible results could not always be obtained by different laboratories (Smith *et al.*, 1982), especially when African sera were screened. In African sera a lot of aspecific fluorescence occurs at dilutions lower than 1:16.

Titers of the same sera showed considerable variations in the IFA test

depending on the antigen concentration present in the rodent lung sections when antibodies against HFRS were tested (Tkachenko *et al.*, 1981). There is an absolute need for standardization of IFA test and confirmation of these results by means of other sensitive tests, suitable for mass-screening procedures. For LAS and EBO, sensitive radioimmunoassay and enzyme filtration assays were developed when exactly the same antigen (intact infected cells) can be used in both tests (Richman *et al.*, 1982). The Spearman rank correlation coefficient of IFA and RIA methods for EBO was 0.74, $p < 0.01$ (Stansfield *et al.*, 1982), showing a reasonable correlation.

In order to obtain reproducible results, it is necessary to work with a common available inactivated antigen and standard concentration of the antigen. This was nicely demonstrated for HFRS antibody determination with the SPRIA and double-layer ELISA test using a constant amount of antigen (Tkachenko *et al.*, 1981). The latter technique is now also in development for LAS and RVF antibody determination (Nicholson and Peters, 1982, personal communication).

Other tests where the antigen can be carefully controlled is the reversed passive agglutination-inhibition test, successfully applied for LAS, Congo, and RFV antibody determinations (Goldwasser *et al.*, 1980; Swaenepoel *et al.*, 1982). The simplicity of the test and stability of the reagents make this test perhaps suitable for serum epidemiological work in the field.

The immunofiltration assay for LAS antibody determination as described by Richman *et al.* (1982) using peroxidase or radioactive labeled protein A as universal conjugate should allow antibody determination in the sera of different animal species. This method needs wider application for the other antigens.

ELISA test using 3.5% buffered formaldehyde-inactivated (24 hr at 37°C, 1 week at 4°C) extracellular LAS, MBG, and EBO antigen was developed in the author's laboratory. An ELISA result was positive when the difference between the absorption of serum in the presence of antigen and the absorption of serum in the presence of control antigen (formaldehyde-treated supernatant of noninfected cells) was significant by t test. Each serum dilution was tested at least on two wells containing antigen and two wells containing control antigen.

Usually a neutralization test should be used to confirm serodiagnostic results. Neutralization tests using plaque reduction were described for DEN (WHO, 1980), RVF (Kark *et al.*, 1982), LAS (Jahrling *et al.*, 1982), Congo (Casals, 1978), and Hantaan (Le Duc *et al.*, 1982).

For MBG and EBO, however, neutralization tests by plaque reduction assay are very difficult to perform in a reproducible manner (Moe *et al.*, 1981) because the plaquing technique itself is not well standardized. Modified plaque assays are now in progress (L. H. Elliott, 1982, personal communication).

IV. CONCLUSIONS

The correlation between the presence of neutralizing antibodies in immune plasma and successful treatment of Junin patients and animals experimentally infected with Lassa should be studied further and eventually extended to the other VHF for which no vaccine is available.

Further knowledge of the exact mechanism by which hemorrhagic phenomena occur in dengue shock syndrome can be the clue to understanding the hemorrhagic phenomenon of the other HFV. The role of enhancement antibodies in the other VHF should be studied.

The risks of community spread of VHF with high mortality rates, such as EBO, MBG, and LAS, by importation into countries outside Africa have been exaggerated. It is hospital and laboratory spread that requires attention. Nosocomial transmission can probably be prevented by preventing close contact with infected tissue and samples. Simple barrier nursing techniques and limitation of laboratory investigation except with adequate containment should be established in areas of virus activity.

The worldwide spread of hemorrhagic fever with renal syndrome viruses should make clinicians more aware of the possibility that these viruses can ber the etiologic agent for patients with fever of unknown origin associated with albuminuria and renal dysfunction.

Further efforts should be made to develop techniques for inactivation of biological samples containing class 4 (highly pathogenic) VHF antigens with preservation of their antigenic structure so that rapid diagnosis of immunospecific tests is still possible and diagnosis can be performed without danger for the investigator.

There is need for standardization of serologic techniques used for VHF seroepidemiological studies.

The role of guinea pigs as an incidental host for Marburg and Ebola viruses, the HFV with the highest mortality rate for man, should be further studied. The striking electron micrographic resemblance of Ebola and Marburg virus to some plant rhabdoviruses, and the fact that guinea pigs are strict herbivores, may make investigative efforts in plant virology worthwhile.

REFERENCES

Al Tikriti, S. K., Hassan, F. K., Moslih, I. M., Jurji, F., Mahmud, M. I. A., and Tantawi, H. H. (1981a). *J. Trop. Med. Hyg.* **84**, 117–120.

Al Tikriti, S. K., Al-Ani, F., Fumi, F. J., *et al.* (1981b). *Bull W. H. O.* **59**, 85–90.

Banatvala, J. E. (1982). *Br. Med. J.* **285**, 1653.

Barrera Oro, J. G., and Eddy, G. A. (1982). *Abstracts of 4th International Conference on Comparative Virology*, p. 203. Banff, Canada.

Bergmann, J. F. (1981). Thèse pour le Doctorat en Médecine, Université René Descartes, Faculté de Médecine, Cochin, Port-Royal.

Bergmann, J. F. (1982). *Med. Maladies Infectieuses* **12**, 638–642.

Best, E. W. R. (1976). *Can. J. Public Health* **67**, 361–366.

Brummer-Korvenkontio, M., Vaheri, A., Hovi, T. von Bonsdorff, C., Vuorimies, J., Manni, T., Pentineu, K., Oker-Blom, N., and Lähdevirta, J. (1982). *Scand. J. Infect. Dis.* **14**, 315–316.

Buchmeir, M. J., Lewicki, H. A., Tomori, O., and Oldstone, M. B. (1981) *Virology* 73–85.

Casals, J. (1978). *In* "Ebola Virus Haemorrhagic Fever" (S. R. Pattyn, ed.), pp. 301–318. Elsevier/North-Holland Biomed. Press, Amsterdam.

Centers for Disease Control (1981a). *Morbidity and Mortality Weekly Report* **30**, 349–350.

Centers for Disease Control (1981b). Dengue Surveillance Reference and Research Report, CDC Puerto Rico and Colorado.

Cooper, C. B., Gransden, W. R., Webster, M., King, M., O'Mahony, M., Young, S., and Banatvala, J. E. (1982). *Br. Med. J.* **285**, 1003–1005.

Elliott, L. H., McCormick, J. B., and Johnson, K. M. (1982). *J. Clin. Microbiol.* **16**, 704–708.

Ellis, D. E. (1979). *J. Gen. Virol.* **42**, 329–337.

El Mekki, A. A., and van der Groen, G. (1981). *J. Virol. Methods* **3**, 61–69.

El Mekki, A., van der Groen, G., Pattyn, S. R. (1983). Abstracts of the Conference on the World Development, Kuwait.

Emond, R. T. D., *et al.* (1977). *Br. Med. J.* **2**, 541.

Emond, R. T. D., Smith, H., and Welsby, P. D. (1978). *Br. Med. J.* **1**, 966–967.

French, G. R., Foulke, R. S., Brand, D. A., Eddy, G. A., Lee, H. W., and Lee, P. W. (1981) *Science* **211**, 1046–1048.

Gajdusek, D. C., Goldgaber, D., Millard, E. and Ono, S. (1982) "Bibliography of hemorrhagic fever with renal syndrome (muroid virus nephropathies)." National Institutes of Health, Bethesda, Maryland.

Galbraith, N. S., Berrie, J. R. H., Forbes, P., and Young, S. (1978). *R. Soc. Health J.* **98**, 152–160.

Georges, A. J. (1979). *Annu. Rep. Inst. Pasteur Bangui.*

Goldwasser, R. A., Elliott, L. H., and Johnson, K. M. (1980). *J. Clin. Microbiol.* **11**, 593–599.

Gonzalez, J. P., McCormick, J. B., Saluzzo, J. F., Herve, J. P., Johnson, K. M., and Georges, A. J. (1982). *Intervirology.*

Halstead, S. B. (1981). *Am. J. Epidemiol.* **114**, 632–648.

Halstead, S. B. (1982a). *Semin. Hematol.* **19**, 116–131.

Halstead, S. B. (1982b). *Abstracts of 4th International Conference on Comparative Virology,* Banff, Canada, p. 95.

Henchal, E. A., Gentry, M. K., McCown, J. M., Brandt, W. E. (1982). *Am. J. Trop. Med. Hyg.* **31**, 830–836.

Hoogstraal, H. (1979). *J. Med. Entomol.* **15**, 307–417.

Institut Pasteur (1981). *Inst. Pasteur Dakar, Senegal Annu. Rep.*

International Symposium on Arenaviral Infections of Public Health Importance (1975). **52**, 381–766.

Jahrling, P. B. (1980). *In* "Manual of Clinical Microbiology" pp. 884–890. Am. Soc. Microbiol., Washington, D. C.

Jahrling, P. B. (1982). *Abstracts of 4th International Conference on Comparative Virology,* Banff, Canada, p. 101.

Jahrling, P. B., Hesse, R. A., Eddy, G. A., Johnson, K. M., Callis, R. T., and Stephen, E. L. (1980). *J. Infect. Dis.* **141**, 580–589.

Jahrling, P. B., Smith, S., Hesse, R. A., and Rhoderick, J. B. (1982). *Infect. Immun.* **37**, 771–778.

Johnson, B. K., Gitan, L. G., Gichogo, A., Tukei, P. M., Else, J. G., Suleman, M. A., Kimani, R., and Sayer, P. D. (1982). *Trans. R. Soc. Trop. Med. Hyg.* **76**, 307–310.

Johnson, K. M., Webb, P. A., Lange, J. V., Murphy, F. A. (1977). *Lancet* **1**, 569.

Johnson, K. M., Elliott, L. H., and Heymann, D. (1981a). *J. Clin. Microbiol.* **14**, 527–529.

Johnson, K. M., Taylor, P., Elliott, L. H., and Tomori, O. (1981b). *Am. J. Trop. Med. Hyg.* **30**, 1291–1293.

Kark, J. D., Aynor, Y., Peters, C. J. (1982). *Am. J. Epidemiol.* **116**, 808–820.

Kiley, M. P., Lange, J. V., and Johnson, K. M. (1979). *Lancet* **2**, 738.

Kiley, M. P., Bowen, G. A., *et al.* (1982). *Intervirology* **18**, 24–32.

Kitamura, T., Komatsu, T., Sugiyama, K., Kohara, T., Ogata, M., Akao, Y., Shiga, S., Imaizumi, K., Morita, C., Oya, A., Takeda, H., Hashimoto, N., Arikawa, J., and Urasawa, S. (1982). *Proceedings of Congress Meeting of the Association of Japanese Virologists,* Kyoto.

Knobloch, J., Albiez, E. J., and Schmitz, H. (1982). *Ann. Virol.* **133E**, 125–128.

Kurata, T., Aqyama, Y., Tsai, T., and McCormick, J. B. (1982). *Abstracts of 4th International Conference on Comparative Virology,* Banff, Canada, p. 100.

"La Fièvre de la Vallée du Rift" (1981). Office International des Epizooties **1**, 3–68.

Lähdevirta, J., Enger, E., Hunderi, O. H., Traavik, T., Lee, H. W. (1982). *Lancet* **2**, 806.

LeDuc, J. W., Smith, G. A., and Bagley, L. R. (1982). *N. Engl. J. Med.* **307**, 623–624.

Lee, H. W. (1982). *Prog. Med. Virol.* **28**, 96–113.

Lee, H. W., and Johnson, K. M. (1982). *J. Infect. Dis.* **146**, 645–651.

Lloyd, G., Bowen, E. T. W., and Slade, J. H. R. (1982). *Lancet* **8**, 1046–1048.

Lupton, H. W., Lambert, R. D., Bumgardner, D. L., Moe, J. B., and Eddy, G. A. (1980). *Lancet* **1**, 1294–1295.

McCormick, J. B., Sasso, D. R., Palmore, E. P., and Kiley, M. P. (1982). *Lancet* **1**, 765–768.

McCormick, J. B., *et al.* (1983). *J. Inf. Dis.* **147**, 264–267.

Maiztegui, J. I. (1982). *Abstracts of 4th International Conference on Comparative Virology,* Banff, Canada, p. 102.

Martini, G. A. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 1–9. Springer-Verlag, Berlin and New York.

Moe, J. B., Lambert, R. D., and Lupton, H. W. (1981). *J. Clin. Microbiol.* **13**, 791–793.

Monath, T. P., and Casals, J. (1975). *Bull. W.H.O.* **52**, 707–715.

Muroid Virus Nephropaties (1982). *Lancet* **18**, 1375–1377.

Murphy, F. A. (1977). *In* "Comparative Diagnosis of Viral Diseases (E. Kurstak and C. Kurstak, eds.), Vol. 1, pp. 759–788. Academic Press, New York.

Richman, D. D., Cleveland, P. H., Owman, M., and Johnson, K. M. (1982). *J. Immunol.* **128**, 2300–2305.

Ruggiero, H. A., Milani, C., Magnoni, H. A., and Frigerio, M. (1981). *Arch. Virol.* **70**, 165–168.

Saluzzo, J. F., Gonzalez, J. P., Georges, A. J., and Johnson, K. M. (1981). *C. R. Hebd. Seances Acad. Sci.* **292**, 29–31.

Shope, R. E., Peters, C. J., Davies, F. G. (1982). *Bull W. H. O.* **60**, 299–446.

Siegert, R., and Schlenczka, W. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.) Springer-Verlag, Berlin and New York.

Simpson, D. I. H. (1978). *Bull W. H. O.* **56**, 819–832.

Simpson, D. I. H., and Metselaar, D. (1982). *In* "Practical Virology for Medical Students and Practitioners in Tropical Countries." Oxford Univ. Press, London and New York.

Smith, D. H., Isaacson, M., Johnson, K. M., Bagshawa, A., Johnson, B. K., Swaenepoel, R., Killey, M., and Siongok, T. (1982). *Lancet,* 816–820.

Spence, I. M., and Gear, J. H. S. (1982). *S. Afr. Med. J.* **62**, 796.

Stansfield, S. V., Scribner, C. L., Kaminski, R. M., *et al.* (1982). *J. Infect. Dis.* **146**, 483–486.

Swaenepoel, R. (1981). *In* "La Fièvre de la Vallée du Rift, Office International des Epizooties." Vol. 1, 15–23.

Swaenepoel, R. (1982). *Abstracts of 4th International Conference on Comparative Virology*, Banff, Canada, p. 96.

Swaenepoel, R., Struthers, J. K., McGillivray, G. M., Nel, M., Shepherd, A., and Jupp, P. G. (1982). *Abstracts of 4th International Conference on Comparative Virology*, Banff, Canada, p. 96.

Tkachenko, E. A. (1982). Abstracts of 4th International Conference on Comparative Virology, Banff, Canada, p. 116.

Tkachenko, E. A., Invanov, A. P., Dzagurova, T. K., *et al.* (1981). *Lancet* **1**, 257–258.

Umenai, T., Watanabe, M., Sekino, H., *et al.* (1981). *J. Infect. Dis.* **144**, 460–463.

van der Groen, G. (1982). *Abstracts of 4th International Conference on Comparative Virology*, Banff, Canada, p. 99.

van der Groen, G., and Pattyn S. R. (1979). *Ann. Soc. Belge Med. Trop.* **59**, 87–92.

van der Groen, G., and Trexler, P. C. (1982). *Prog. Med. Virol.* **28**, 192–207.

van der Groen, G., Johnson, K. M., Webb, P. A., Wulff, H., and Lange, J. (1978). *In* "Ebola Virus Haemorrhagic Fever" (S. R. Pattyn, ed.), pp. 203–205. Elsevier/North-Holland Biomed. Press, Amsterdam.

van der Groen, G., Kurata, T., and Mets, G. (1983). *Lancet* **1**, 654–655.

Walker, D. H., Johnson, K. M., Lange, J. V., Gardner, J. J., Kiley, M. P., and McCormick, J. B. (1982). *J. Inf. Dis.* **146**, 360–368.

Webb, P. A., Krebs, J. B., Trippel, S. J., McCormick, J. B., *et al.* (1982). *Abstracts of 4th International Conference on Comparative Virology*, Banff, Canada.

WHO (1980). WHO Technical Advisory Committee on Dengue Haemorrhagic Fever for the South-East Asian and Western Pacific Regions. "Guide for Diagnosis, Treatment and Control of Dengue Heamorrhagic Fever," 2nd ed.

WHO (1982a). Dengue vaccine development. *Bull. W. H. O.* **60**, 36.

WHO (1982b). Regional Office for the Western Pacific. Tokyo WPR/RPD/WG/82.16.

Winn, W. C., Monath, T. P., Murphy, F. A., and Whitfield, S. G. (1975). *Arch. Pathol.* **99**, 599.

Wulff, H., Lange, J. V., and Webb, P. A. (1978). *Intervirology* **9**, 344–350.

20

Structure and Variation among Arenaviruses

COLIN R. HOWARD AND P. R. YOUNG

Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London, England

I. INTRODUCTION

It is almost 50 years since the first description by Armstrong and Lillie (1934) of an agent isolated in monkeys from a patient who had died from what appeared to be St. Louis encephalitis. However, passage of material into animals immune to the St. Louis encephalitis agent produced a lymphocytic choriomeningitis. This virus has been intensively studied as an important model for the study of immunological tolerance, persistent infection, and virus-induced immunopathology (for review, see Lehmann-Grube, 1971), primarily as a result of the discovery that the domestic mouse *Mus musculus* could become chronically infected shortly after birth.

The discovery of a relationship between Machupo virus isolated from Bolivia and lymphocytic choriomeningitis (LCM) virus by thin-section electron microscopy (Murphy *et al.*, 1969) proved to be a major factor in classifying the agent of Lassa fever shortly after it came to the attention of the virologists at the Yale Arbovirus Research Unit in 1969. Inoculation of positive Lassa material into adult mice was found to induce a fine tremor, felt by holding the animals by the tail, and tonic convulsions characteristic of "classical" LCM infection (Buckley and Casals, 1970). The prolonged excretion in the urine of infectious Lassa virus also closely resembled the viremia seen in Machupo-infected mice, a relationship more adequately

ISBN 0-12-429601-7

confirmed by subsequent electron microscopy (Speir *et al.*, 1970). An anecdotal account of the initial studies surrounding the isolation and preliminary characterization of Lassa virus has been published (Fuller, 1974).

II. NATURAL HISTORY AND DISTRIBUTION

All arenaviruses are enveloped, single-stranded RNA viruses and can cause acute or persistent infections in rodents. The taxon Arenaviridae now contains 13 members, all possessing a unique morphology and sharing certain antigenic components. A summary of rodent species known to be associated with arenaviruses is given in Table I, and their distribution in the Old and New Worlds is illustrated in Figs. 1 and 2, respectively. In addition to Lassa, both Junin and Machupo viruses cause severe hemorrhagic fever in man with an associated mortality rate of 10–15%. These principal pathogenic arenaviruses pose, in areas of either South America or Africa, major public health problems resulting from human contact with soil and foodstuffs contaminated by infected domestic and peridomestic rodents. The remaining arenaviruses described so far do not cause human illness in a natural setting, although many show biological and biochemical properties similar to those of members of the group pathogenic for man. In particular, Pichinde, Amapari, and Tacaribe viruses isolated from rodents originating in tropical rain forests of South America are closely related to both Junin and Machupo viruses (Casals, 1975) and provide suitable models of arenaviruses and their infections. In addition, the pathology of Pichinde virus in small mammals closely resembles that of experimental Lassa infection (Murphy *et al.*, 1976). In recent years, several arenavirus isolates from Mozambique have shown the existence of arenaviruses related to Lassa virus and share the same natural rodent host, *Mastomys natalensis* (Wulff *et al.*, 1977). This Mozambique virus, now renamed Mopeia, apparently does not cause human illness, but protects susceptible primates from experimental Lassa infection (Kiley *et al.*, 1979).

Although there are some 1700 species of rodents worldwide grouped into 34 families, arenaviruses are associated with only two families (the Muridae and the Cricetidae), although it has been pointed out that these constitute the bulk of rodent life (Arata and Gratz, 1975). The cricetid rodents of importance in the natural history of arenaviruses are found in the South American subcontinent and represent rather recent additions to the rodent fauna of that continent, having crossed the Panamanian isthmus sometime in the Miocene or Pliocene era (Hershkovitz, 1966). The original rodent fauna of South America was largely composed of members of the New World hystricomorphs, whose present-day members include the guinea pigs.

TABLE I
Species of Mammals Known To Be Associated with Arenaviruses[a]

Virus	Species	Location	Habitat
Old World			
LCM[b]	*Mus musculus*	Worldwide	Peridomestic
Lassa[b]	*Mastomys natalensis*	West Africa	Savannah
Mopeia	*Mastomys natalensis*	Southern Africa	Savannah
Mobala	*Praomys* spp.	Central African Republic	Savannah
New World			
Junin[b]	*Calomys musculinus, C. laucha, Akodon azerae*	Argentina	Grasslands
Machupo[b]	*Calomys callosus*	Bolivia	Grasslands, peridomestic
Tacaribe	*Artibeus literatus, A. jamaicensis*	Trinidad	Forest, periurban
Amapari	*Oryzomys goeldii, Neacomys guinanae*	Paraguay, Brazil	Tropical forest
Flexal		Paraguay, Brazil	Tropical forest
Pichinde	*Oryzomys albigularis, Thomasomys fuscatus*	Colombia	Tropical forests, river valleys
Latino	*Calomys callosus*	Bolivia, Brazil	Grasslands, peridomestic
Parana	*Oryzomys buccinatus*	Paraguay	Tropical forest, savannah
Tamiami	*Sigmodon hispidus*	United States (Florida), Paraguay, Brazil	Tropical forest

[a]Data were collected from Arata and Gratz (1975), Johnson (1981), and Gonzales *et al.* (1983).

[b]Human pathogens.

Of particular note here is that although laboratory-bred guinea pigs are susceptible to Junin and Machupo infections, there is no evidence that this species plays any significant role in the natural history of either virus.

Both Machupo and Junin may be transmitted to man from rodents with very different ecological patterns. Machupo is restricted to *Calomys callosus,* a mouse which favors the upper savannah regions in eastern Bolivia and neighboring South American countries (Hershkovitz, 1966). In the absence of other peridomestic rodents it inhabits gardens and houses, presenting frequent opportunity for human contact. Unlike *C. callosus* in

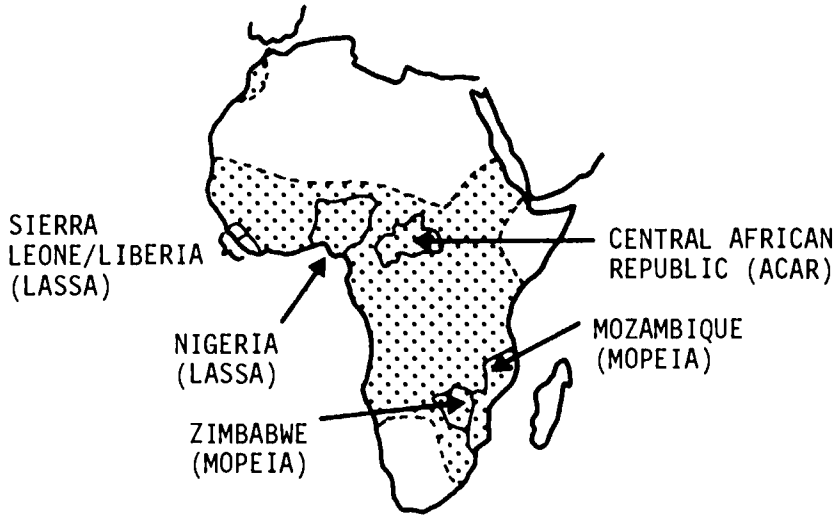


Fig. 1. Geographical locations of Lassa and related viruses from Africa. The stippled area represents the distribution of the single major rodent host (*Mastomys natalensis*) in this continent.

Bolivia, the three species of Argentinian rodent which carry Junin virus do not generally invade human dwellings, thereby making rodent control programs more difficult to effect. Human contact is therefore predominantly among agricultural workers who tend and harvest the annual maize crop in this region. Rodent control is more appropriately effected by restricting weed growth among maturing maize plants and attempting to restrict the rodent habitat at the periphery of cultivated areas. Changing agricultural practice, in particular the replacement of maize by soybean crops, may be responsible for a decline and/or spread of Junin infection in recent years as the habitat for field rodents becomes altered.

Lassa virus is found only associated with *Mastomys natalensis,* a murid rodent found extensively throughout the savannah region of the African continent. Although Lassa virus has been found only in *M. natalensis* populations of West Africa, a related arenavirus originally recovered in Mozambique from species of the closely related *Apodemus* family was reported (Lehmann-Grube, 1971), but such reports are rare. Much more serious has been the finding of LCM in laboratory-bred Syrian hamsters (*Mesocricetus quaratus*), which may be readily transmitted to man, with serious consequences. However, LCM virus is not known to be associated with hamsters in the wild, although the infection may be readily transmitted from mice to hamsters. One problem in assessing the degree of zoonotic human LCM infection may relate to inadequate clinical awareness and/or the general

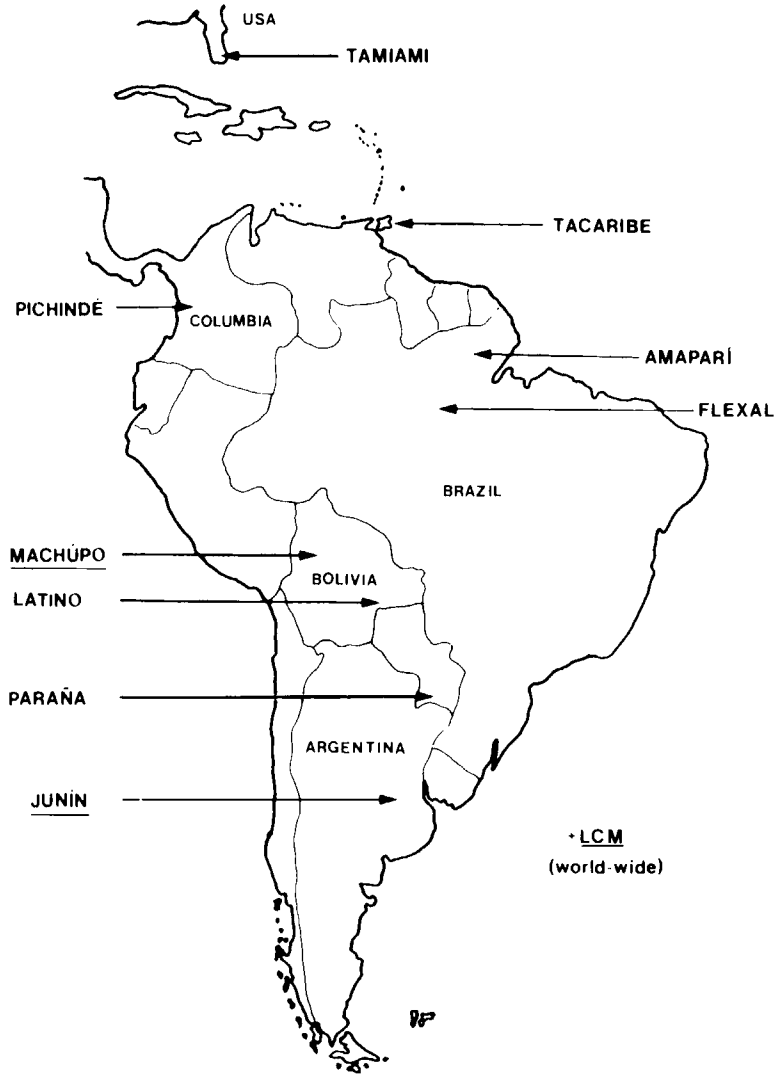


Fig. 2. Geographical locations of arenavirus isolates in the New World. All viruses with the exception of LCM virus are serologically defined as being members of the Tacaribe complex. From Howard and Simpson (1980).

"influenza-like" nature of the symptoms. Human LCM infection has been diagnosed within the endemic area of Argentine hemorrhagic fever, and Maiztegui (1975) has indicated the necessity of careful dissection of serological responses in epidemiological studies where LCM coexists in the local rodent population together with a second arenavirus pathogenic for man.

III. MORPHOLOGY AND ULTRASTRUCTURE

The morphology and morphogenesis of all arenaviruses are remarkably similar, with the result that it is not possible to distinguish any individual family member either by application of morphological criteria or by the appearance of virus-related structures seen within infected cells (Murphy and Whitfield, 1975). The first comparative study performed by Murphy *et al.* (1969) found striking morphological similarities between Machupo, Tacaribe, and LCM viruses, confirming previously reported biological and physical similarities between Machupo and LCM (Justines and Johnson, 1969; Webb, 1965). All contained 1–10 electron-dense granules approximately 20 nm in diameter with a well-defined unit membrane, giving a distinct "sandy" appearance in thin section. This granularity prompted the use of the name arenovirus by derivation from *arenosus* (Latin, sandy), a name "chosen to reflect the characteristic granules seen in the virion in ultrathin sections" (Rowe *et al.*, 1970). The name was modified soon after to arenavirus in order to avoid possible confusion with members of the Adenoviridae. The presence of intravirion particles together with an apparent excessive pleomorphism clearly distinguished arenaviruses in early studies from retroviruses, many arboviruses, and other membrane-bound viruses (Rowe *et al.*, 1970; Murphy *et al.*, 1969; Abelson *et al.*, 1969).

Cell cultures infected with arenaviruses manifest distinct intracytoplasmic inclusion bodies. These inclusions have been described as consisting of a moderately electron-dense, smooth matrix within which are embedded numerous dense granules similar to the ribosomelike structures seen within maturing and extracellular virus particles and are clearly distinguishable from glycogen granules (Murphy *et al.*, 1970). During the course of infection the inclusions become progressively denser and assume smoother margins paralleling closely the development of inclusion bodies detected by light microscopy (Buckley, 1965; Abelson *et al.*, 1969; Buckley and Casals, 1970). These changes in the fine structure of infected cells precede gross cytopathological alterations.

There is a significant difference in morphology and ultrastructure between virus examined in *in vitro* cell culture and tissue taken from infected humans or animals at necropsy (Maiztegui *et al.*, 1975; Speir *et al.*, 1970). The demonstration by electron microscopy of arenavirus particles in human tissue, particularly in the liver, has been of value in confirming the viral specificity of those lesions observed by electron microscopy. In Lassa fever, most particles are seen where hepatocyte damage is less severe (Winn and Walker, 1975). Individual cells show dilation of the endoplasmic reticulum together with the accumulation of electron-dense material within the mitochondria, although the extensive matrices containing ribosomal aggregates appear to be absent.

Carballal *et al.* (1977) found in guinea pigs experimentally infected with Junin virus that extracellular accumulations of virus particles were low and virus was rapidly transported away from infected bone marrow tissue via extracellular channels. Again there was no evidence of inclusion bodies consisting of ribosomal aggregates. This was associated with lesions of the reticular monocytes and surrounding cells exhibiting focal lysis of the plasma membranes, cytoplasmic condensation, and necrosis (Carballal *et al.*, 1981). These ultrastructural changes were also found in lymphatic tissue and are in accord with similar findings in tissue obtained at necropsy from patients infected with Junin virus, and they give further support to the role of direct virus infection of the reticuloendothelial system in the pathogenesis of Argentine hemorrhagic fever. Likewise, Tamiami virus, an arenavirus nonpathogenic for man, has a tropism for megakaryocytes and mononuclear cells in infected cotton rats (Murphy *et al.*, 1976). Typical arenavirus particles were seen in platelet demarcation channels, although there was no evidence of cytoplasmic changes or the formation of virus-induced inclusion bodies.

Immunofluorescent staining of infected cells often correlates with the detection of virus structures by electron microscopy, although this is not always the case. Occasionally the use of specific antibody may produce staining indicative of viral antigen present in large amounts, but complete virus particles are not always discerned (Murphy *et al.*, 1973). Several possibilities may account for this: either small samples might not contain involved sites of virus production or these observations reflect an underlying mechanism controlling viral gene expression within different cell types. Murphy and colleagues suggested that this disparity might reflect variation in the capacity of cells either to accumulate viral antigen, as detected serologically, or to produce the potentially infectious virus seen in the electron microscope. Of importance is that such a difference may reflect ultimately in the extent of direct cytopathology seen in different arenavirus infections *in vivo*.

Organelle destruction and membrane breakdown occur together with a general condensation of the cytoplasm. Although arenavirus replication is known to require an intact cell nucleus, there is no apparent morphological change in nuclear structure, and nuclear inclusion bodies are absent.

The arenavirus envelope is formed from the plasma membrane of infected cells, rarely from intracytoplasmic membranes into vacuoles. During virus maturation there is a clear increase in staining density of both leaflets of the membrane bilayer at the site of viral budding. Insertion of viral glycoproteins appears to precede morphological changes as demonstrated by the binding of specific antibody to unaltered plasma membrane early in infection (Mannweiler and Lehmann-Grube, 1973). As the particle forms by extrusion, surface projections become visible and ribosome granules can

become regularly arranged immediately beneath the thickened membrane (Fig. 3c).

Electron microscopy of negatively stained preparations has shown that arenavirus particles are pleomorphic enveloped structures with an average diameter in the range of 80–150 nm (Dalton *et al.*, 1968; Mannweiler and Lehmann-Grube, 1973; Murphy and Whitfield, 1975), although particles up to 500 nm are not uncommon (Pedersen, 1979). A careful study of Machupo virus size by Murphy *et al.* (1969) showed a skewed size distribution from 60 to 320 nm with a median value of 110–120. In this study, there appeared to be no significant difference in size distribution between virus grown *in vitro* and Machupo virus identified in the lymphoid organs of infected rodents. The viral envelope, formed from the plasma membrane of the infected cell, contains surface projections 5–10 nm long which appear club-shaped with a hollow central axis when viewed end-on (Fig. 3a). At high magnification, these projections can be seen as consisting of discrete globular subunits, although no information exists as to the number or type of glycopeptides present in individual structures. Considerable variation is observed in the spacing of these projections, some virions containing a tightly packed lattice of surface spikes whereas others show a more widely spaced random arrangement (Murphy *et al.*, 1970). These differences almost certainly reflect varying degrees of swelling by osmosis between different virus preparations. Although generally present as random arrays, particles with projections ordered in a symmetrical fashion may be seen in fresh, unfrozen virus preparations (Fig. 3a). Attempts to examine by negative staining techniques the viral envelope of highly pathogenic arenaviruses such as Lassa are hampered if the virus is first exposed to glutaraldehyde or osmium tetroxide (Murphy *et al.*, 1970).

Nucleocapsids of both Tacaribe and Pichinde viruses have been isolated free of contaminating host ribosomes (Palmer *et al.*, 1977; Vezza *et al.*, 1977; Young and Howard, 1983). Strands with a "beads-on-a-string" appearance that are 5–10 nm in diameter have been described representing a linear array of nucleosomal subunits. The size of each structural unit is consistent with the presence of a single molecule of the major nucleocapsid (N) polypeptide. Different organizational levels of this linear nucleosome-like ribonucleoprotein structure have been reported (Young and Howard, 1983). Disruption of purified Pichinde virus by osmotic shock releases fiberlike structures 12 nm in diameter with occasional globular condensations which may reflect enhanced association between neighboring turns of an underlying helical structure, as has been described for cellular chromatin (Thoma *et al.*, 1979). Rotary shadowing techniques of isolated ribonucleoprotein showed the spread of 15-nm strands from discrete core structures. When sufficiently dispersed the nucleoprotein appeared as closed circles

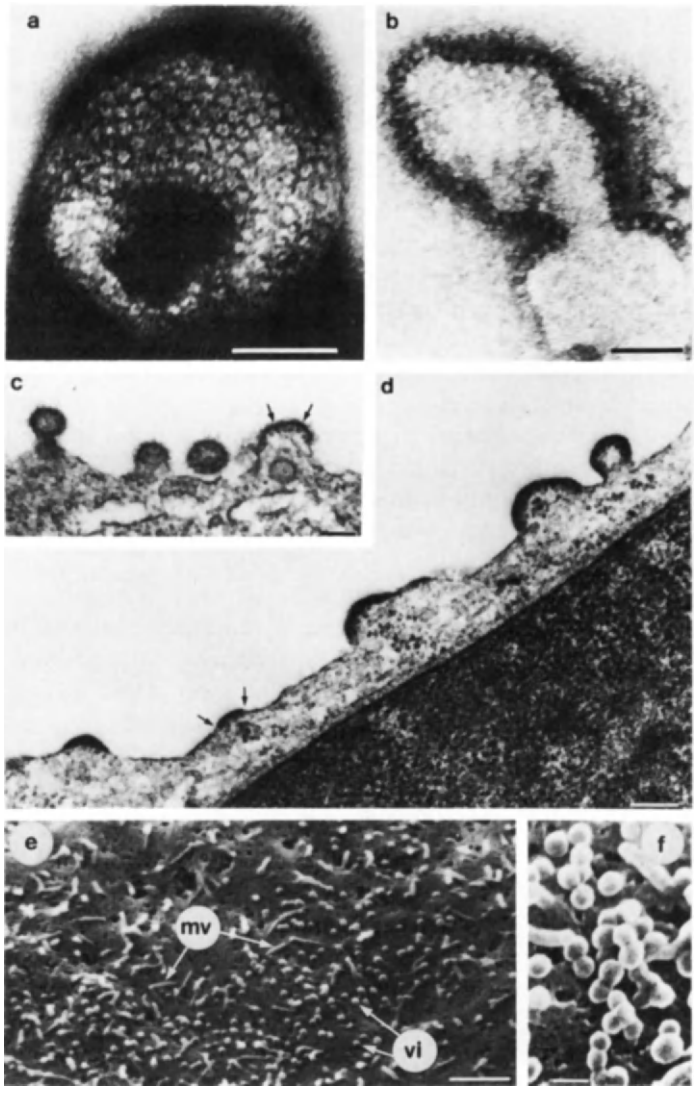


Fig. 3. Electron micrographs of (a) Pichinde virus and (b) purified virus negatively stained with 2% PTA, pH 6.8, showing surface spike morphology. Arrangement of the spikes in five- and sixfold symmetry can be seen (arrowed). (c) and (d) Ultrathin sections of infected Vero cells revealing the alignment of ribosomes beneath the membrane of budding viruses (arrowed in c) and discrete areas of membrane thickening associated with these viral buds (arrowed in d). (e) and (f) Scanning electron microscopy of infected Vero cells. A uniform population of budding viruses (vi) particles 110–130 nm in diameter can be seen among host-cell microvilli (mv). Bar represents 50 nm in (a) and (b), 200 nm in (c) and (d) and (f), and 1 μm in (e).

varying in length from 450 to 1300 nm. However, this variability appears to show no direct relationship between the two unique viral RNA segments. The frequent appearance in electron micrographs of panhandled structures and the observation that denatured viral RNA is predominantly linear (Vezza *et al.*, 1978) would suggest that circular nucleocapsid forms may arise as a result of base pairing at the termini of RNA molecules, not dissimilar to the observation that complementation exists between the 3′ and 5′ terminal ends of RNA segments isolated from the bunyavirus Uukuniemi.

The packaging of nucleocapsids during the process of virus assembly and maturation may evolve in degrees of supercoiling of circular ribonucleoprotein structures. This is supported by the finding of regularly sized 20-nm strands in virus cores and is compatible with the presence of supercoiled nucleocapsid fibers seen released from spontaneously disrupted virions. The core component thus appears to be organized as a result of the extensive convolution of these supercoiled fibers. Young and Howard (1983) have pointed out that variability in the amount of nucleocapsid material packaged into any one virus particle indicates that specific control of this process may be limited. Variation in the specific arrangement of nucleosomes within each fiber of the core structures may reflect a variable number of nucleosomes per unit turn of the helix, with an increase in the number of nucleosomes per turn accompanied by a loss in resolution of individual subunits as they become more closely associated. The most stable helical configuration appears to be a 12-nm fiber structure regularly seen in spontaneously disrupted virus preparations and may contain between six and eight nucleosomes per turn of the helix. Any further structural condensation results in little increase in the diameter of this structure. The extent of condensation appears to be directly related to ionic strength; as salt concentration is reduced, a progressive unfolding of higher order structures occurs, ultimately giving rise to the linear nucleosome array.

IV. SEROLOGICAL RELATIONSHIPS

Each member of the arenavirus group possesses some antigenic relationship to other members, although the degree of cross-reactivity largely depends on the assay system employed. The complement fixation test exhibits the broadest relationships (Casals, 1975). All the New World members, often referred to as the Tacaribe complex, are more or less related by complement fixation, with Junin, Machupo, Amapari, Parana, Latino, and Tacaribe viruses being most closely related. Pichinde and Tamiami are not so closely related to each other or to other members of the Tacaribe complex

by this test. All the available evidence indicates that the complement-fixing antigen is associated with the internal nucleoprotein. On the basis of this test, LCM and Lassa are only very distantly related to the Tacaribe complex, but they do show some distant relationship with each other. Similar patterns of serological relatedness can also be seen by the more sensitive immunofluorescence methods. The test generally stains cytoplasmic antigens. It is important that, by the indirect fluorescent antibody method, LCM and Lassa viruses are clearly related to each other, but only distantly to members of the Tacaribe complex. A relationship has also been shown by this method between Lassa and the Mopeia isolates from Mozambique (Wulff *et al.*, 1977). However, Buchmeier and Oldstone (1978) have shown that, using immunofluorescent staining of the surface of infected cells, no cross-reactivity was found among members of the Tacaribe complex and between those viruses and LCM.

The neutralization test is considerably more specific, although the ease of quantifying neutralizing antibodies differs sharply between Lassa virus and the South American human arenaviruses. These difficulties are summarized in Table II. In plaque reduction tests using human convalescent sera no cross-neutralization has been observed between Junin and Machupo viruses, although a close relationship exists by complement fixation. Neutralizing antibodies to both viruses persist for many years at high titer. In the study of Webb and colleagues (1967), neutralizing antibodies appeared simultaneously with complement-fixing antibodies in more than 30 cases of Bolivian hemorrhagic fever; however, the complement fixation titers dropped significantly over the first 12 months of convalescence and only 5.5% were positive. In contrast, all remained positive for neutralizing antibody over this same period. Follow-up studies of patients involved in several epidemics of Argentinian hemorrhagic fever have similarly shown that complement-fixing antibodies to Junin virus are comparatively short-lived and therefore indicative of recent or ongoing infections.

A similar marked specificity by neutralization tests has been demonstrated with LCM and Lassa, and both viruses are readily distinguishable

TABLE II
Ease of Detection *in Vitro* of Neutralizing Antibodies to Arenaviruses

Old World	Detection	New World	Detection
LCM	+ + +	Tacaribe	+ + + +
Lassa	+	Machupo	+ + + +
Mopeia	+	Junin	+ + + +
		Pichinde	+
		Tamiami	+
		Amapari	+

from one another by this technique. The sensitivity of the neutralization test for LCM virus can be increased by incorporating either complement or anti-γ-globulin into the test system (Oldstone, 1975; Lehmann-Grube and Anbrassat, 1977). Neutralizing antibodies to Lassa virus in convalescent human sera can be detected only with great difficulty. However, specific antibodies may be detected in acute infection by immunofluorescence from 7 days after the onset of illness (Wulff and Lange, 1975), and this procedure is currently the method of choice for the detection of specific Lassa virus antibodies.

It is well recognized that potent antisera against Pichinde and Lassa viruses possess minimal neutralizing capacity when examined by the mouse neutralization test (Lehmann-Grube *et al.*, 1979) or by tissue culture tests (Buckley and Casals, 1970; Sengupta and Rawls, 1979). The use of an alternative and simple method for detecting antibodies to Pichinde virus has been described whereby the incorporation of antiserum into the overlay of an infected cell culture results in plaque size reduction (Chanas *et al.*, 1980). In the absence of neutralizing activity, such antisera may be deficient in antibodies directed against an essential virus surface component, with the result that the covering of critical areas required for virus neutralization does not occur. Whether this may be the case in convalescent sera to Lassa virus is unclear, but the studies of Chanas *et al.* (1980) showed clearly that aggregation and extensive sensitization of Pichinde virus particles does occur after reaction with homologous antiserum. Despite the presence of antibodies directed against all the major polypeptides, adsorption and penetration of virus–antibody complexes was not prevented.

Several studies have shown that all arenaviruses contain a major 54,000–68,000 molecular weight nucleocapsid protein (N) together with one (G) or two (G1, G2) major glycoprotein species in the outer envelope (Rawls and Leung, 1979; Howard and Simpson, 1980). It is likely that one or both glycoproteins induce a protective antibody response *in vivo;* sera containing neutralizing antibodies to LCM virus have been shown to immunoprecipitate the two surface glycoproteins of the homologous virus (Buchmeier *et al.*, 1980). In the case of Pichinde virus, a monospecific antiserum prepared against the G2 component of the virus particle failed to neutralize infectivity (Young *et al.*, 1981). However, incorporation of the G2 antiserum in the agar overlay of infected cell cultures produced a significant reduction in plaque size, and electron microscopy showed virions budding from infected cells and the formation of immune aggregates in much the same manner as was previously observed with the antiserum prepared against whole virus.

In contrast, detection of neutralizing antibodies in immune ascitic fluid prepared against either Tacaribe or Junin viruses is readily accomplished by conventional plaque number reduction methods (e.g., Henderson and Downs, 1965). Junin and Machupo viruses are quite distinct by these meth-

ods (Johnson *et al.*, 1973), although there is some evidence to suggest that Junin virus is weakly neutralized by Tacaribe immune serum (Henderson and Downs, 1965; Weissenbacher *et al.*, 1975/1976).

The development of monoclonal antibodies against both LCM and Pichinde viruses has been pursued in order to obtain defined reagents suitable for the dissection of the serological interrelationships between arenaviruses. Buchmeier *et al.* (1981) analyzed six monoclonal antibodies to Pichinde virus for cross-reactions against seven New World and three Old World arenaviruses. Four of these antibodies were cross-reactive with one or more heterologous viruses within the Tacaribe complex, one of these four cross-reacting also with LCM and Lassa viruses from the Old World. All the cross-reactive antibodies were directed against the internal N polypeptide of the virus and illustrate that there exists at least one common antigenic epitope between arenaviruses considered as being either Old World or New World in origin. Of considerable interest in the same study is the finding that among 46 monoclonal antibodies prepared against LCM virus six cross-reacted with Lassa and/or Mopeia viruses. As with the cross-reacting monoclonal antibodies prepared against Pichinde virus, five of these were directed against common or related epitopes on the major nucleocapsid protein. Uniquely, the sixth reagent, identified by immunoprecipitation as recognizing the G2 protein of LCM virus, reacted only with the Mopeia virus. These studies confirm previous reports of a close relationship between LCM and Lassa viruses, and also indicate that limited homology of antigenic structure may exist between the envelope glycoproteins of these viruses in addition to common epitopes on the inner nucleocapsid. It should be recognized, therefore, that the preparation of monoclonal antibodies will be of increasing use in determining the course of genetic evolution undergone by these viruses and further emphasize that reagents reactive for highly dangerous arenavirus pathogens may be prepared safely by selecting closely related viruses which are nonpathogenic for man.

ACKNOWLEDGMENTS

The arenavirus research program at the London School of Hygiene and Tropical Medicine is generously supported by the Medical Research Council. We thank Mrs. S. Giles for assistance in the preparation and typing of the manuscript.

REFERENCES

Abelson, H. T., Smith, G. H., Hoffman, H. A., and Rowe, W. P. (1969). *J. Natl. Cancer Inst.* **42**, 497–502.

Armstrong and Lillie (1934). *Public Health Rep.* **49**, 1019–1027.

Arata, A. A. and Gratz, N. G. (1975). *Bull. W. H. O.* **52**, 621–627.
Buchmeier, M. J., and Oldstone, M. B. A. (1978). *In* "Negative Strand Viruses and the Host Cell" (B. W. J. Mahy and R. D. Barry, eds.), pp. 91–97. Academic Press, New York.
Buchmeier, M. J., Welsh, R. M., Dutko, F. J., and Oldstone, M. B. A. (1980). *Adv. Immunol.* **30**, 275–331.
Buchmeier, M. J., Lewicki, H. A., Tomori, O., and Oldstone, M. B. A. (1981). *Virology* **113**, 73–85.
Buckley, S. M. (1965). *Am. J. Trop. Med. Hyg.* **14**, 792–794.
Buckley, S. M., and Casals, J. (1970). *Am. J. Trop. Med. Hyg.* **19**, 680–691.
Carballal, G., Rodriguez, M., Frigerio, M. J., and Vasquez, C. (1977). *J. Infect. Dis.* **135**, 367–373.
Carballal, G., Cossio, P. M., Laguens, R. P., Ponzinibbio, C., Oubiña, J. R., Meckert, P. C., Rabinovich, A., and Arana, R. M. (1981). *J. Infect. Dis.* **143**, 7–14.
Casals, J. (1975). *Yale J. Biol. Med.* **48**, 115–140.
Chanas, A. C., Mann, G., Young, P., Stanford, S., Ellis, D. S., and Howard, C. R. (1980). *Arch. Virol.* **65**, 157–167.
Dalton, A. J., Rowe, W. P., Smith, G. H., Wilsnack, R. E., and Pugh, W. E. (1968). *Virology* **2**, 1465–1478.
Fuller, R. (1974). "Fever! The Hunt for a Killer Virus." Hart, Davis, MacGibbon. London.
Gonzalez, J. P., McCormick, J. B., Saluzzo, J. F., Herve, J. P., Georges, A. J., and Johnson, K. M. (1983). *Intervirology* **19**, 105–112.
Henderson, J. R., and Downs, W. G. (1965). *Am. J. Trop. Med. Hyg.* **14**, 796–797.
Hershkovitz, P. (1966). *In* "Ectoparasites of Panama" (R. L. Wenzel and V. J. Tipton, eds.). Field Museum of Natural History, Chicago, Illinois.
Howard, C. R., and Simpson, D. I. H. (1980). *J. Gen. Virol.* **51**, 1–14.
Johnson, K. M., Webb, P. A., and Justines, G. (1973). *In* Lymphocytic Choriomeningitis Virus and Other Arenaviruses" (F. Lehmann-Grube, ed.), pp. 241–258. Springer-Verlag, Berlin and New York.
Johnson, K. M., Taylor, P., Elliot, L. H., and Tomori, O. (1981). *Am. J. Trop. Med. Hyg.* **30**, 1291–1293.
Justines, G., and Johnson, K. M. (1969). *Nature (London)* **222**, 1090–1091.
Kiley, M. P., Lange, J. V., and Johnson, K. M. (1979). *Lancet* **2**, 738.
Lehmann-Grube, F. (1971). "Lymphocytic Choriomeningitis Virus," Virology Monographs, Vol. 10. Springer-Verlag, Vienna.
Lehmann-Grube, F., and Ambrassat, J. (1977). *J. Gen. Virol.* **37**, 85–92.
Lehmann-Grube, F., Killay, M., Ibscher, B., and Schwartz, R. (1979). *J. Med. Virol.* **4**, 125–136.
Maiztegui, J. I. (1975). *Bull. W. H. O.* **55**, 567–575.
Maiztegui, J. I., Laguens, R. P., Cossio, P. M., Casanova, M. B., de la Vega, M. T., Ritacco, V., Segal, A., Fernandez, N. J., and Arana, R. M. (1975). *J. Infect. Dis.* **132**, 35–43.
Mannweiler, K., and Lehmann-Grube, F. (1973). *In* "Lymphocytic Choriomeningitis Virus and Other Arenaviruses" (F. Lehmann-Grube, ed.), pp. 37–48. Springer-Verlag, Berlin and New York.
Murphy, F. A., and Whitfield, S. G. (1975). *Bull. W. H. O.* **52**, 409–419.
Murphy, F. A., Webb, P. A., Johnson, K. M., and Whitfield, S. G. (1969). *J. Virol.* **4**, 535–541.
Murphy, F. A., Webb, P. A., Johnson, K. M., Whitfield, S. G., and Chappell, W. A. (1970). *J. Virol.* **6**, 507–518.
Murphy, F. A., Whitfield, S. G., Webb, P. A., and Johnson, K. M. (1973). *In* "Lymphocytic Choriomeningitis Virus and Other Arenaviruses (F. Lehmann-Grube, ed.), pp. 273–285. Springer-Verlag, Berlin and New York.

Murphy, F. A., Winn, W, Walker, D. H., Flemister, M. R., and Whitfield, S. G. (1976). *Lab. Invest.* **34,** 125–140.

Oldstone, M.B.A. (1975). *Prog. Med. Virol.* **19,** (1977). 84–119.

Palmer, E. L., Obijeski, J. F., Webb, P. A., and Johnson, K. M. (1977). *J. Gen. Virol.* **36,** 541–545.

Pedersen, I. R. (1979). *Adv. Virus Res.* **24,** 277–330.

Rawls, W. E., and Leung, W. C. (1979). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 157–192. Plenum, New York.

Rowe, W. P., Pugh, W. E., Webb, P. A., and Peters, C. J. (1970). *J. Virol.* **5,** 289–292.

Sengupta, S., and Rawls, W. E. (1979). *J. Gen. Virol.* **42,** 141–148.

Skinner, H. H., and Knight, (1969). *Lab. Anim.* **3,** 175–184.

Speir, R. W., Wood, D., Liebhaber, H., and Buckley, S. M. (1970). *Am. J. Trop. Med. Hyg.* **19,** 692–694.

Vezza, A. C., Gard, G. P., Compans, R. W., and Bishop, D. H. L. (1977) *J. Virol.* **23,** 776–786.

Vezza, A. C., Clenley, J. P., Gard, G. P., Abraham, N. Z., Compans, R. W., and Bishop, D. A. L. (1978). *J. Virol.* **26,** 485–497.

Webb, P. A. (1965). *Am. J. Trop. Med. Hyg.* **14,** 799–802.

Webb, P. A., Johnson, K. M., Mackenzie, R. B., and Kuns, M. L. (1967). *Am. J. Trop. Med. Hyg.* **16,** 531–538.

Weissenbacher, M. C., Coto, C. E., and Calello, M. A. (1975/1976). *Intervirology* **6,** 42–49.

Winn, W. C., and Walker, D. H. (1975). *Bull. W. H. O.* **52,** 535–545.

Wulff, H., and Lange, J. V. (1975). *Bull. W. H. O.* **52,** 429–436.

Wulff, H., McIntosh, B. M., Hamner, D. B., and Johnson, K. M. (1977). *Bull. W. H. O.* **55,** 441–444.

Young, P. R., and Howard, C. R. (1983). *J. Gen. Virol.* **64,** 833–842.

Young, P. R., Chanas, A. C., and Howard, C. R. (1981). *Dev. Cell Biol.* **7,** 15–22.

21

Diarrhea Viruses: Diagnosis as Prerequisite for Epidemiological Study

T. H. FLEWETT

Regional Virus Laboratory, East Birmingham Hospital, Birmingham, England

I. WORLDWIDE PROBLEM

Acute infectious diarrhea every year takes an enormous toll of children up to the age of 5 throughout the developing world (Ashworth and Waterlow, 1982). Walsh and Warren (1979) have published figures showing that diarrhea, as a cause of death, almost equals all the other causes combined, even if respiratory tract infections are included. The World Health Organization (WHO) estimates about 750 million episodes of acute infectious diarrhea every year, although nobody knows the exact number of cases. Recent WHO figures for worldwide mortality for young children with diarrheal diseases indicate 4.3 million annual deaths, excluding China. Even these figures are only crude estimates.

II. WHO DIARRHEAL DISEASES CONTROL PROGRAM

Recognizing, however, the importance of these illnesses, the WHO has set up a Diarrheal Diseases Control Program concerned with three areas: one dealing with virus diseases; another dealing with bacterial infections; and the third dealing with the development and application of drugs and

ISBN 0-12-429601-7

other treatments to relieve the symptoms and reduce the mortality of the diarrhea. I have been closely associated with the virological section. All the evidence up to date indicates that, at least in countries with a temperate climate and in most of the parts of the tropical world where comprehensive investigations have been possible, rotaviruses have been the greatest single cause of acute infectious diarrhea, by far exceeding in importance the toxigenic and invasive *Escherichia coli*, *Campylobacter*, *Shigella*, and protozoal agents (Flewett, 1982). This has certainly been true in Asia. However, some recent investigations have shown that among black African children (in Gabon and elsewhere) rotaviruses may be very much less important as a cause of acute gastroenteritis (Sitbon, 1983). In South Africa Schoub *et al.* (1982) found rotaviruses to be responsible for a much higher proportion of cases of acute infectious diarrhea among white infants than among black infants. They attributed this difference to the fact that in conditions where fecal/oral contamination was very high, as in the comparatively poor hygiene in the black communities, most children were subclinically infected with rotaviruses at a stage when they were still protected by transplacental maternal antibody and by such antibody as might be present in breast milk, and so became immunized within the first 6 months of life; the white children, however, did not get their infections until somewhat later, when this protection had worn off. This explanation may very well be true. There is also the intriguing possibility that black children may be less susceptible to rotaviruses than white children or Asian children, that there may be an ethnic difference in susceptibility to infection. Such ethnic differences have already been discovered in susceptibility to hepatitis B virus; for example, if a child is born to a Chinese hepatitis B carrier mother, he is very much more likely to become a carrier than an African child born to an African carrier mother, who is, however, more likely to become a carrier than a child born to an Indo-European carrier mother (Derso *et al.*, 1978). However, studies of this type need to be continued over several years. In Birmingham we have had "rotavirus years," when these have been almost the only winter enteric pathogen; and winters such as 1980–1981, when adenoviruses caused more diarrhea in some months than did rotaviruses.

III. DIAGNOSTIC METHODS

Recognizing that detailed information about the epidemiology and etiology of acute infectious diarrhea in the developing world was extremely deficient, WHO has instituted numerous projects, both at global and regional levels, to determine the epidemiology and etiology of acute infectious diarrhea. To fulfill the aims of these projects, a suitable diagnostic system was essential.

The electron microscope is not very sensitive in detecting viruses—it requires about 10^6 virus particles per gram of feces before viruses are reasonably detectable. This method does have the great advantage of being the only method so far capable of detecting all the known agents found in feces from children with acute infectious diarrhea. Such instruments, however, are very expensive and are rarely encountered in developing countries, and even when they are, they very often do not work properly because good servicing agents are not available locally. It has, therefore, been necessary to develop other diagnostic systems.

It became clear to the WHO that a simpler method of detecting rotaviruses was required. The Collaborating Centre at the East Birmingham Hospital, therefore, set out to develop a simple and sensitive ELISA system for detecting rotaviruses. ELISA systems had indeed previously been published, but all appeared to have serious disadvantages, as did, e.g., the system published by Brandt *et al.* (1981), which gave about 73% false positives. Our work at East Birmingham resulted in the development of the WHO ELISA kit.

IV. ELISA TEST

For the development of the WHO ELISA test, it was necessary to test a large number of different "solid phase" microtiter plates to determine which one was the most economical and gave the most consistent results. Quality checks are made on each batch before sending out the plates. The reagents are made in part in the United States and in part in our own laboratory. The capture antibody to be attached to the microtiter plates is a rabbit antiserum made in our laboratory; it is the most critical part of the reaction. When a sample of feces is added, this antibody captures the rotavirus present in the feces. After capture, the rotaviruses can be detected by adding a guinea pig anti-rotavirus antibody which sticks to the rotaviruses. Then, an anti-guinea pig globulin antibody which is coupled to an enzyme is added. When the substrate to the enzyme is subsequently added, color develops. This system has indeed worked very well and has proved to be sensitive and accurate. We are now working on the production of reagents to detect the enteric adenoviruses, which sometimes cause diarrhea. The details of these various techniques will be published elsewhere.

We have been able to produce in our laboratory a monoclonal antibody with remarkably wide range of reactivity with different rotaviruses. These include human as well as many different animal rotaviruses (even mouse rotavirus), and so the antibody can be used in a diagnostic system for all of these. As before, the rabbit antibody as a capture antibody attached to the solid phase of the microtiter plate is used. After the virus has been

captured, we put on the monoclonal antibody (derived from mouse cells) and then an anti-mouse antibody conjugated with enzyme. When the substrate for the enzyme is added, positive cases give a blue color with the enzyme substrate of choice, tetramethylbenzidine. Other substrates for peroxidase-labeled antibodies are also available, but we find that the tetramethylbenzidine is the easiest to read visually and also appears to give the most sensitive reaction. A study of the stability of these reagents (a very important aspect when kits have to be air-freighted around the world) indicates that there is no significant difference in stability between peroxidase and alkaline phosphatase conjugates, and that both are much more stable in the freeze-dried state than in liquid form. So we are now preparing them in the freeze-dried state. The test with the monoclonal antibody and peroxidase-labeled anti-mouse antibody is still under assessment and is not yet approved as an official WHO test.

V. OTHER METHODS

Other methods for detecting virus antigen in feces are reverse passive hemagglutination, latex agglutination, and radioimmunoassay. The last is inappropriate for field work in developing countries (or indeed in developed countries) because most hospital laboratories do not have multichannel gamma counters readily available. An excellent reverse passive hemagglutination test for rotavirus antigen in feces has been developed by the Japanese (Sanekata *et al.*, 1979), but it has some disadvantages. It is expensive and the reagents lack stability on storage, so that it too would have to be air-freighted. In studying some latex agglutination systems we have found that they are not quite as sensitive or as reliable as the ELISA test. They are also liable to give false-positive reactions, and, as in the ELISA test, positives have to be confirmed by a blocking test. They do, however, have the great advantage of speed, being the most rapid method for detecting antigen in feces. Finally, Kapikian's group at the National Institutes of Health in the United States have devised a radiolabeled nucleic acid probe which can be used for spot diagnosis by autoradiography (Flores *et al.*, 1983). This is a very sensitive and specific technique, but is too esoteric for general application in most laboratories.

VI. SUBGROUPING AND SEROTYPING OF ROTAVIRUSES

Subgrouping and serotyping of rotaviruses have been carried out in a few laboratories (Beards *et al.*, 1980; Sato *et al.*, 1982). Subgrouping by the complement fixation test and by ELISA has long been possible, and we

hope before very long to be able to supply subgrouping reagents to the principal diagnostic laboratories in the WHO program around the world. Serotyping hitherto has been done by neutralization of infectivity in tissue culture. This is most easily done when the virus has been adapted to tissue culture, but it is a slow and lengthy process. We have, in collaboration with Dr. Margaret Thouless in Seattle, developed an ELISA serotyping test which enables the rotavirus in feces to be serotyped readily and has the great advantage that the serotyping can be done even when all the virus in the specimen have died (Thouless *et al.,* 1982). It has been difficult to prepare the specific ELISA serotyping antisera, but a new method demonstrated by Dr. Thouless at the Fourth International Conference on Comparative Virology in Banff offers great promise that suitable reagents can be much more easily made. We invite researchers to send us samples of feces, both those found to be positive, as well as negative ones. We need the positives for serotyping and also because we would like to get more strains of rotavirus from different parts of the world established in tissue culture for comparison. That, after all, is one of the functions of a WHO Collaborative Center. A by-product is that we should then have these tissue culture-adapted strains for distribution to other laboratories wishing to work with them.

VII. ELECTROPHORESIS OF ROTAVIRUS RNA GENOMIC SEGMENTS

Finally, I would like to mention another diagnostic procedure, reasonably sensitive, though not as sensitive as the ELISA test, that has the great advantage of giving us a sort of "fingerprint" of the rotavirus, i.e., a method in which the nucleic acids of the rotavirus genome are separated by electrophoresis through polyacrylamide gel, giving 11 bands. This method has been used very successfully in Mexico, Brazil, and Australia (Espejo *et al.,* 1980; Rodger *et al.,* 1981) and in many other parts of the world as well, both for detecting rotaviruses in feces and for comparing different viruses in the same epidemic and between one epidemic and another. The method has also revealed the existence of rotaviruses which have no serological reaction whatever with antisera against ordinary rotaviruses. Hitherto, these have been found only in three children, two in Brazil and one in Melbourne (personal communications from Ian Holmes and Helio Pereira). These rotaviruses occasionally have been found also in pigs (Saif *et al.,* 1980). Because they are serologically so different, they will not be detected at all by the standard ELISA test. So far those from pigs and children have not been established in tissue culture. They can be recognized by polyacrylamide gel electrophoresis. Because the pattern of molecular weight distribution of the genomic segment is quite different from that seen

with ordinary rotaviruses, they have been termed pararotaviruses (Saif *et al.*, 1980). I agree with Pedley *et al.* (1983) that these new varieties be called rotaviruses A, B, C, etc., by analogy with influenza, where influenza B is serologically totally different from influenza A, but is biologically very similar.

REFERENCES

Ashworth, A., and Waterlow, J. C. (1982). *Arch. Dis. Child.* **11,** 882.

Beards, G. M., Pilfold, J. N., Thouless, M. E., and Flewett, T. H. (1980). *J. Med. Virol.* **5,** 231.

Brandt, C. D., Ki, H. W., Rodriguez, W. J., Thomas, L., Yolken, R. H., Arrobio, J. O., Kapikian, A. Z., Parrott, R. H., and Chanock, R. M. (1981). *J. Clin. Microbiol.* **13,** 976.

Derso, A., Tarlow, M. J., Boxall, E. H., and Flewett, T. H. (1978). *Br. Med. J.* **1,** 949.

Espejo, R. T., Munoz, O., Serafin, F., and Romero, P. (1980). *Infect. Immun.* **27,** 351.

Flewett, T. H. (1982). *In* "Virus Infections of the Gastrointestinal Tract" (D. A. J. Tyrrell and A. Z. Kapikian, eds.), pp. 125–145. Dekker, New York.

Flores, J., Boeggeman, E., Purcell, R. H., Sereno, M., Perez, I., White, L., Wyatt, R. G., Chanock, R. M., and Kapikian, A. Z. (1983). *Lancet* **1,** 555.

Pedley, S., Bridger, J. C., Brown, J. F. and McCrae, M. A. (1983). *J. Gen. Virol.* **64,** 2093–2101.

Rodger, S. M., Bishop, R. F., Birch, C., McLean, B., and Holmes, I. H. (1981). *J. Clin. Microbiol.* **13,** 272.

Saif, L. J., Bohl, E. H., Theil, K. W., Cross, R. F., and House, J. A. (1980). *J. Clin. Microbiol.* **12,** 105.

Sanekata, T., Yoshida, Y., and Oda K. (1979). *J. Clin. Path.* **32,** 963.

Sato, K., Inaba, Y., Miura, Y., Tokuhisa, S., and Matumoto, M. (1982). *Arch. Virol.* **73,** 45.

Schoub, B. D., Cohen, F., Thompson, D., Koornhof, H. J., Miliotis, M. D., Still, C. S., Berkowitz, F. E., Miller, S., and Kushlick, E. (1982). *J. Med. Virol.* **10,** 171.

Sitbon, M. (1983). Thèse d'Etat de Biologie Humaine (Virologie), University of Paris, 7.

Thouless, M. E., Beards, G. M., and Flewett, T. H. (1982). *Arch. Virol.* **73,** 45.

Walsh, J. A., and Warren, K. S. (1979). *N. Engl. J. Med.* **301,** 967.

22

Virus-Induced Gastroenteritis in Animals

LORNE A. BABIUK

Department of Veterinary Microbiology, Western College of Veterinary Medicine, and Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

I. INTRODUCTION

Diarrhea is one of the leading causes of morbidity and mortality in infants and young animals both in developing and in developed countries. It has been estimated that over 500×10^6 cases of diarrhea occur annually in humans, resulting either directly or indirectly in approximately 10×10^6 deaths (Editorial, 1978). Accurate estimates of numbers of diarrhea cases in animal species are not available, but it would appear to be at least as high as in humans, thus indicating the economic importance of this disease syndrome.

Until recently the specific agent responsible for most cases of nonbacterial gastroenteritis was not identified. However, since the discovery in 1969 by Mebus (Mebus *et al.*, 1969) that a virus was present in feces of calves suffering from diarrhea, it has been proved that rotaviruses can infect and

ISBN 0-12-429601-7

cause diarrhea in calves. This discovery also prompted the search for a similar or related virus as a cause of diarrhea in other animals as well as in humans. As a result of these investigations, rotavirus has been found to be a major cause of nonbacterial gastroenteritis in most mammals and in fowl (Flewett and Woode, 1978; McNulty, 1978; Woode, 1982), and it is now accepted that up to 60% of nonbacterial gastroenteritis cases may be caused by rotavirus, the remaining cases being caused by a variety of viral agents which may include coronavirus, calicivirus, parvovirus, enterovirus, adenovirus, astrovirus, mini-reovirus, or rotalike viruses (Table I). However, the exact role and prevalence of some of these etiological agents in causing diarrhea have not been firmly established in all mammals.

Although as microbiologists we try to identify a specific etiological agent in causing diarrhea, it must be emphasized that diarrhea is often multifactorial and that interactions between various factors with infectious agents can often exacerbate the disease. These factors can be broadly grouped into immunological, environmental, and nutritional. In each category there are large numbers of individual components that can interact and alter both the degree of diarrhea and the final outcome of the disease.

The viruses which cause gastroenteric infections can generally be divided into two groups. In the first group, replication is restricted to the gastrointestinal tract and induce disease as a result of their direct effects only on the cells of the intestine. In this case most of these agents generally enter the host directly, via the oral cavity, into the gastrointestinal tract. The second group in viruses can enter the host via the oral cavity and replicate in the gastrointestinal tract, but do not remain localized. These viruses may

TABLE I
Some Enteric Viruses of Animals

Virus	Site of replication[a]	
	Horizontal	Longitudinal
Rota	Enterocytes, villous tip	Small intestine
Rota-like	Enterocytes, villous tip	Small intestine
Corona	Enterocytes, top half	Small and large intestine, colon
Corona-like	Enterocytes, top half	Small intestine
Breda	Mid villus, crypts	Small intestine, colon
Astro	?	?
Calici	?	?
Parvo	Crypts, lymphoid	Small and large intestine
Adeno	Enterocytes	Small
Reo	?	?
Entero	?	?

[a]Insufficient data available for definitive statements to be made.

spread to other target organs such as lymphoid tissue (Kahn, 1978) or even the central nervous system (Brown, 1973; Nathanson and Martin, 1979).

At least 11 different viruses can cause some degree of intestinal damage and diarrhea under appropriate conditions (Table I). In most cases all these agents produce the most severe clinical signs during the first few weeks of life (Colloquium on selected diarrheal diseases of the young, 1978; Little and Shadduck, 1982; Woode and Bridger, 1975). However, there are reports of virus shedding associated with diarrhea in older animals as well (Jones *et al.*, 1979; McNulty *et al.*, 1978; Von Bonsdorff *et al.*, 1976). In most cases the infection of older animals results in a subclinical infection, and it has been suggested that these animals can serve as carriers of the virus and be a source of infection for younger susceptible animals. The reasons for increased severity of diarrhea in younger animals and higher mortality is that the viruses generally causes greater villous atrophy in these younger animals.

II. AGENTS INVOLVED

A. Rotaviruses

Rotavirus-induced disease was first described by Cheever and Mueller (1948) and later established as an important infectious agent in mice by Kraft (1957, 1958). However, it was not recognized as an important pathogen in domestic animals until Mebus *et al.* (1969) identified it as a cause of neonatal diarrhea in calves. Since then it has been demonstrated to play a major role in nonbacterial gastroenteritis in most mammalian species. In most outbreaks the disease occurs suddenly and spreads rapidly to other susceptible individuals. The reasons for this rapid spread is that the concentration of virus in feces can reach 10^{11} particles per gram of feces, which is equivalent to 10^7 infectious doses of virus (Flewett and Woode, 1978; Woode *et al.*, 1976a,b). Experimental inoculation of bacteria-free filtrates containing rotavirus causes diarrhea in 12–24 hr in susceptible young animals. Associated with diarrhea is also anorexia and vomiting. The reasons for such rapid clinical signs is that, in the absence of passive antibody or local acquired immunity, the virus infects the enterocytes of the villi, rapidly killing them. The replication cycle of rotavirus is approximately 12 hr (Carpio *et al.*, 1981).

In rotavirus infections, infection is generally limited to the small intestine in calves, pigs, and humans (McAdaragh *et al.*, 1980; Mebus and Newman, 1977; Middleton *et al.*, 1974), but antigen can be found in the colon of lambs (Snodgrass *et al.*, 1977), pigs (Theil *et al.*, 1978), and mice (Little and Shadduck, 1982). Viral infection occurs in the enterocytes of the upper

half of the villi of the small intestine, resulting in rapid cell death and sloughing of the cells as they die. With death, the villi become shortened and lose their adsorptive capacity (Woode and Crouch, 1978). Furthermore, the cells at the tips of the villi are responsible for production of lactase, which aids in digestion of lactose. Thus the combination of reduced adsorptive capacity and reduced enzyme activity accounts for the diarrhea. Since the crypt cells are not damaged, regeneration of the enterocytes and recovery of the villi is generally rapid after the infection is overcome. Those animals that recover from the disease return to normal body weight within 10–28 days after infection.

B. Coronaviruses

Coronaviruses can cause both respiratory and gastrointestinal infections in humans and animals (Robb and Bond, 1979). Transmissible gastroenteritis of swine was one of the first coronaviruses identified as a cause of diarrhea in animals (Doyle and Hutchings, 1946). In young pigs, less than 2 weeks of age, diarrhea, rapid weight loss, and dehydration is dramatic and often results in death (Smith, 1956). As animals get older the degree of villus shortening, virus shedding, and death is not as high, but morbidity remains high. As animals reach 6 months of age approximately 10,000 times as much virus is required to infect animals as it does to infect a 2-day-old piglet. Virus replication occurs in the epithelial cells covering primarily the villi of the jejunum and ileum, with subsequent death, denudation, and shortening of the villi. The new cells that migrate from the crypts to replace the denuded cells are not infected by the virus. The reason for this resistance is not fully known, but it can be that these immature undifferentiated cells are not able to replicate the virus (Moon *et al.*, 1976; Thake *et al.*, 1973).

Other porcine corona and corona-like viruses have been identified which produce clinical signs similar to those of transmissible gastroenteritis but are serologically distinct (Garwes, 1982).

Coronaviruses have also been identified as one of the major causes of calf diarrhea (Mebus, 1978; Stair *et al.*, 1972; Storz *et al.*, 1978a). Bovine coronavirus, like rotavirus diarrhea, occurs in 15–24 hr after infection. Early in infection the villous epithelial cells appear morphologically normal but contain large amounts of antigen. Since diarrhea occurs before denudation and loss of enterocytes, it is postulated that the initial diarrhea occurs as a result of infection of the cell and redirection of cellular functions from adsorption to virus replication. If adsorption does not occur, there is accumulation of digestive fluids. As the infection proceeds cells are lost from the villi and are replaced by immature squamous to cuboidal epithelial cells which lack the enzymes required for digestion of milk and also have reduced

adsorptive capacity, as is the case in all other virus infection of the gastrointestinal tract.

C. Other Enteric Viruses

Although rota- and coronavirus infections are generally considered to be of major importance in inducing nonbacterial gastroenteritis in animals, other viruses such as calici- and astroviruses (Woode and Bridger, 1978) and parvovirus (Storz and Bates, 1973) may be responsible for causing gastroenteritis in animals and may account for at least a significant portion of the cases of diarrhea which are not caused by rota or coronaviruses. However, there are still 20–30% of diarrhea cases for which no etiological agent has been observed. As the search continues for etiological agents, it will not be surprising if other viruses are discovered. One such new agent the "Bredavirus" (Woode *et al.*, 1982) has been identified. In the case of Bredavirus infection, the lesions may at first appear similar to those of coronavirus infections with respect to location. However, on closer examination it becomes obvious that the disease is different, since the lesions and virus-infected cells are visible in the lower 50% of the villi and in the crypts of the small intestine. In the colon, infected cells are present throughout the villi and in the crypts.

Parvoviruses can infect a wide variety of animals ranging from pets to large domestic animals (Kahn, 1978; Storz *et al.*, 1978b). In contrast to the other viruses discussed so far, this virus can produce systemic disease as well as enteritis. Since the virus generally replicates in rapidly dividing cells, the lesions are seen in the crypts of both the small and large intestines as well as in lymphoid tissue. Because of the replication in lymphoid tissue this disease can be more severe, especially in small animals, than other viral infections because of interference with immune responses and damage to the crypts.

One common feature of all the agents described to date is that infection generally occurs by ingestion of the virus. Since the virus does not have to spread systemically the incubation period is extremely short, with villus shortening and reduced fluid adsorption, and dehydration and death if the diarrhea is severe enough. Therefore, for a differential diagnosis of the actual cause of diarrhea, attempts must be made to demonstrate the presence of the specific agent rather than merely to look at the clinical signs.

Although these viruses have been associated with diarrhea, also in many instances viruses are present but no disease occurs. Furthermore, it is not always possible to reproduce the disease in conventional animals. No good explanation exists as to why this occurs, but it is possible that too few cells are infected to disrupt absorption. This may be due to the presence of avir-

ulent strains or of strains that are extremely localized (Woode, 1982). Another explanation could be related to immunity. If low levels of active or passive immunity are present, this would keep the virus infection rate low so as to allow only few viruses to initiate new infections, as is often the case with many persistent local infections. However, during stress the immune system may be reduced and virus shedding and diarrhea may occur. When the immune system returns to normal, diarrhea stops but virus may continue to be shed. This type of carrier state would ensure the continued presence of the virus in the environment and be present to reinfect susceptible neonatal animals. Such carrier states have been demonstrated to occur both *in vivo* (Benfield *et al.*, 1982; Leece and King, 1980) and *in vitro* (Misra and Babiuk, 1980).

III. DIAGNOSIS

Since many of the viral agents involved in causing diarrhea are not easily cultivable *in vitro* by conventional methods, a large variety of tests have been developed to diagnose these agents (Yolken, 1982). Most of these tests are based on the observation that there are high levels of virus particles present in the feces of diarrheic animals and humans (Flewett and Woode, 1978; McNulty, 1978; Woode *et al.*, 1976a,b). With this large amount of virus present it is very easy to observe virus in feces by electron microscopy techniques. However, direct observation is less efficient than if combined with serological tests, as is the case with immune electron microscopy (IEM), where the virus is aggregated by specific sera and can be visualized much more easily. The availability of specific antisera and monoclonal antibodies to numerous viruses makes this a very attractive means of diagnosis. Although many enteric viruses do not grow in culture, some may infect cells without causing a cytopathic effect or producing infectious virus. In these cases the viruses may be identified by culturing *in vitro* and testing for the presence of viral antigens within virus-infected cells.

There are various advantages and disadvantages to each test, and a number of points which must be considered when choosing a test include efficiency, speed, relative costs, and the specific purpose for making a diagnosis (Table II). If trained personnel and equipment are available, then immune electron microscopy (IFM), radioimmunoassays (RIA), or ELISA assays appear to be very good with respect to specificity and speed. ELISA assays are especially useful for automation and diagnosing large numbers of samples. Furthermore, ELISA tests can be read by eye, if ELISA readers are not available, making this test very attractive, If serotyping is also desired, then many tests listed in Table II can be adapted if specific antisera and

TABLE II

Efficiency and Practicality of Some Diagnostic Techniques for Detection of Viruses Causing Gastroenteritis in Animals

Method	Efficiency	Speed	Relative cost	Practicality	
				Single sample	Multiple sample
IEM	Very good	Hour	High	Yes	Limited
RIA	Very good	Day	Moderate	Yes	Yes
ELISA	Very good	Day	Moderate to low	Yes	Yes
Fluorescence	Good	Hour	Moderate	Yes	Yes
Culture	Good	Days	Low to moderate	Yes	Yes
CIEP[a]	Good	Hour	Low	Yes	Yes
Complement fixation	Poor to good	Day	Low	Yes	Yes
Immune adsorption Hemagglutination	Average	Hours to days	Low	Yes	Yes
Hemagglutination	Poor	Hour	Low	Yes	Yes
Gel diffusion	Poor	Day	Low	Yes	Yes

[a]Counterimmunoelectrophoresis.

preferably monoclonal antibody produced against each serotype are prepared.

IV. PATHOLOGY AND PATHOPHYSIOLOGY OF ENTERIC VIRUS INFECTIONS

In most virus infections of the gastrointestinal tract, regardless of whether the virus has a predilection for the epithelial cells at the tips of the villi or in the crypts, there is severe shortening and occasional fusion of adjacent villi (Fig. 1) resulting in reduced amounts of adsorptive surface of the intestine (Keenan *et al.*, 1976; Leece *et al.*, 1976; Pearson and McNulty, 1977). Infection generally begins in the proximal part of the small intestine and spreads progressively to the jejunum and ileum and sometimes to the colon (Mebus *et al.*, 1973; Snodgrass *et al.*, 1977). This will depend, however, on the initial infective dose, the virulence of the specific virus (Woode and Crouch, 1978), and the host's immunological status. Thus in the presence of passive antibody, infection can occur but the degree of replication is limited to such an extent that either no diarrhea or only mild diarrhea oc-

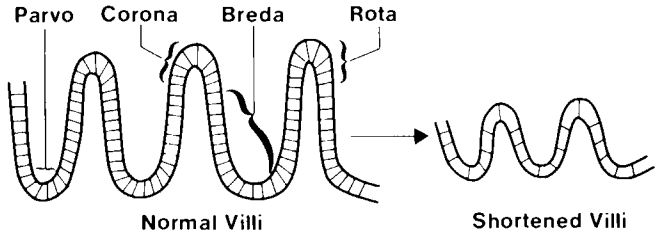


Fig. 1. Effect of enteropathic viruses on villous atrophy.

curs. In rota- and coronaviruses, which infect the cells at the tips of the villi, as infection progresses the adsorptive cells are replaced with immature squamous to cuboidal epithelial cells. Until these cells mature their adsorptive capacity and enzymatic activity is greatly reduced. Since these immature cells also appear to be relatively resistant to virus infection, the disease is often self-limiting if dehydration is not so significant as to cause death (Woode, 1982; Garwes, 1982; Moon *et al.*, 1976). Since the crypt cells are not damaged, the rate of recovery is generally rapid. In contrast, in the case of viruses that infect the crypt cells and also lead to shortening of villi, there are a limited number of new cells available to migrate up the villi, and recovery tends to take longer. It should be stressed that although the degree of villous damage may be influenced by the virulence of the virus and the immunological status of the animal, the rate of regeneration of enterocytes and of enterocyte maturation may also vary with the age, the species, and the site of virus infection. Since glucose and sodium adsorption are highest in the proximal and middle part of the jejunum (Bachmann and Hess, 1982; Shephard *et al.*, 1979), damage here would cause most severe diarrhea.

In viral infections, the mechanism of fluid loss is considered to be different from that in bacterial infections; however, the net losses may be the same. In viral infections fluid loss is predominantly from the extracellular fluid due to impaired adsorption, and osmotic loss is due primarily to the presence of undigested lactose in the lumen rather than active secretion (Graham *et al.*, 1982; Lewis and Philips, 1972; Philips and Lewis, 1973, Tennant *et al.*, 1978). However, replacement of mature adsorptive cells with immature cells, which retain some of their secretory functions, also increases the rate of secretion (Butler *et al.*, 1974; Kerzner *et al.*, 1977; Pansaert *et al.*, 1970). As the virus kills the adsorptive cells there is also a loss of enzymes responsible for digestion of disaccharides. Furthermore, loss of differentiated villi cells diminishes glucose, sodium carrier, and Na^+, K^+-ATPase activities which aid in loss of sodium, potassium, chloride, bicarbonates, and water. The loss of bicarbonate leads to the development of acidosis. However, acidosis also develops as a result of increased microbial activity in response to fermentation of undigested milk (Lewis and Philips, 1978), as well as the increased lactic acid production and decreased utili-

zation in dehydrated animals (Lewis *et al.*, 1975; Tennant *et al.*, 1972). Acidosis can create a K^+-H^+ ion exchange across the cellular membrane and inhibit cellular functions required for maintaining normal potassium concentration with a net loss of potassium from cells. The next step that occurs is hypoglycemia due to decreased intestinal adsorption, minimal glycogen reserves in young animals, inhibited glyconogenesis, and increased glycolysis (Lewis and Phillips, 1978). This series of complex pathophysiological changes if not promptly corrected results in death of the animal.

Effective management of diarrhea in animals requires prompt action to prevent continued loss of fluids and electrolytes. This is most economically achieved by removal of milk from the diet. This reduces the amount of undigested lactose in the lumen and, therefore, reduces fluid loss and acidosis. Therapy should include administration of balanced electrolyte solutions either orally or by the intravenous route. The use of intravenous fluid replacement and careful monitoring of animals could save a large percentage of severely affected animals; however, the costs are generally too high to recommend this as a standard procedure.

V. MIXED INFECTIONS

Severity of diarrhea is related not only to the virulence and age of the animal, but also to the presence of multiple infections. It has been shown in a number of studies that only a minority of cases of diarrhea in animals are caused by a single virus pathogen (House, 1978). Furthermore, it has been suggested that even if a single pathogen is involved in an infection there may be heterogeneity within the pathogen (Sabara *et al.*, 1982; Spencer *et al.*, 1983). Therefore, if two viruses can coinfect an animal and have different sites of replication, their combined effect may be much more severe than if they infected the animal individually. This may help explain why it is difficult to reproduce enteric infections in conventional calves with single plaque-purified virus isolates. Another important factor is the presence of viral-bacterial synergistic interactions. There is accumulating evidence that many bacterial infections can be more severe if combined with a virus infection (Gouet *et al.*, 1978; Leece *et al.*, 1982; Runnels *et al.*, 1980). Thus *E. coli* generally produces scours only during the first few days of life. However, if an animal is infected with a virus, *E. coli* can colonize and produce a more severe disease at a later age. This combination of *E. coli* with rota- or coronaviruses ensures that the severity of the disease is increased (Fig. 2). The exact mechanisms by which this occurs is unknown; however, it is possible that viruses may alter fluid transport by virtue of infecting some cells. This alteration allows the buildup of toxin by bacteria that are normally nonpathogenic. Thus the combination of toxin buildup and decreased mobility of the intestine results in diarrhea. In addition, re-

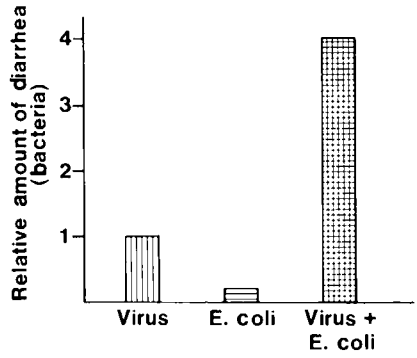


Fig. 2. Effect of the combined infection of virus plus bacteria (rotavirus–*E. coli*) on bacterial shedding and diarrhea.

duced adsorption of nutrients occurs as a result of virus infection. This provides a more suitable nutritional environment for bacteria to grow, adhere, and secrete more toxin.

Additionally, virus infection may alter the maturity of cells by reducing the rate of maturation. Since it is known that the physiological state of cells may alter adherence, it appears feasible to postulate that bacteria can actually colonize virus-infected intestines but not normal ones. In conjunction with the altered physiological state of being able to allow bacterial colonization, the virus itself may alter the cell surface in such a way as to allow direct attachment of the bacteria to the virus glycoproteins expressed on the surface or altered host-cell glycoproteins on these virus-infected cells, as has been convincingly shown for viral–bacterial interactions in the respiratory tract (Davison and Sanford, 1981; Sanford *et al.*, 1978).

Finally, it is possible that some viruses induce Fc receptors on the surface of host cells. If this occurs, then antibody-coated bacteria could easily bind via the Fc receptor and anchor the cell, allowing secretion of toxin, activation of cyclic AMP, and increased fluid loss and diarrhea. Although herpesviruses are the only viruses reported to induce Fc receptors (Costa *et al.*, 1977; Lehner *et al.*, 1975; Westmoreland and Watkins, 1974), preliminary evidence suggests that bovine coronaviruses may also induce Fc receptor on cells (L. A. Babiuk, unpublished results).

VI. IMMUNITY AND IMMUNOLOGICAL CONTROL OF GASTROENTERIC VIRUS INFECTIONS

One of the major problems with controlling gastroenteritis infections in animals is the age at which the animals get the disease, and second, the site of infection dictates that local immunity be present. Thus even if the adult

animals are immune and if they transfer antibody to the young, the antibody must be present continuously in the lumen of the intestine to prevent infection, since serum antibodies are not protective (Snodgrass and Wells, 1976; Woode and Bridger, 1975). Since many mammalian species do not continue to secrete high levels of antibody in their milk after parturition, the antibody in the intestinal lumen drops rapidly and the animal becomes fully susceptible even if it has acquired high levels of serum antibody. Thus in cattle, antibody levels in colostrum are generally high to most enteric viruses since the infection rate in adults is high. The young calf suckling a cow with high levels of antibody is protected only during the period when there are high levels of antibody in milk (Fig. 3). However, within 5–7 days after parturition antibody levels drop below the threshold required to neutralize virus in the lumen. This is the reason why most enteric virus infections causing neonatal diarrhea in mammals do so after 1 week of age.

The observed requirement for local immunity has stimulated interest in immunizing the newborn. There is presently an oral vaccine marketed for use in newborn calves to provide protection against rota- and coronaviruses. Unfortunately, if the colostral antibodies can protect against virulent viruses they will also prevent the attachment of vaccine virus to intestinal enterocytes. Thus the supposed early nonspecific protection, possibly by interferon, and the later specific immunological protection do not occur unless the animals are immunized prior to ingestion of colostrum. This is often not possible, and therefore this vaccine has not proved to be as successful as hoped. To overcome this problem a few attempts have been made at *in utero* immunization, but this is totally impractical under field situations (Newman *et al.*, 1978).

The most recent trend to overcome the requirement of local immunity in gastrointestinal virus infection is hyperimmunization of the dam. This re-

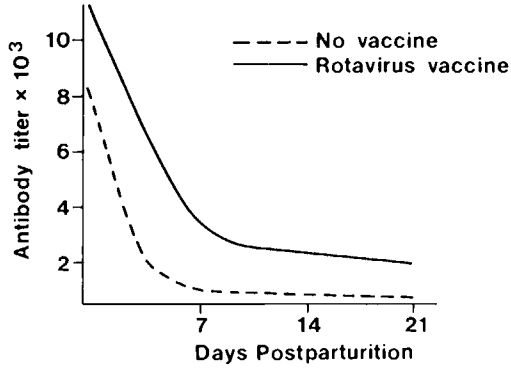


Fig. 3. The effect of hyperimmunization on levels of antibody to rotavirus in milk of cattle.

sults in a much higher initial level of antibody in the colostrum, which provides excellent early protection (Fig. 3). More important, even though antibody levels do drop they remain above a threshold level which is protective against normal virus challenge doses. The final method of providing high levels of antibody in the lumen is by feeding monoclonal antibody to the animal. This has proved to be very effective in preventing *E. coli*-induced diarrhea in calves (Sherman *et al.*, 1982). The combination of various antiviral monoclonal antibodies with anti-*E. coli* antibody should be very effective under certain situations, but is probably of limited value in field situations where animals cannot be handled routinely. Furthermore, the presence of a variety of serotypes, especially in rotaviruses (Woode *et al.*, 1983), dictates that each serotype be represented either in the vaccine or in the monoclonal antibody mixture.

The recent advances in recombinant DNA technology have great potential for helping control gastroenteric infections in animals. These approaches are especially relevant to producing vaccines against viruses which do not replicate well in culture, as is the case in many gastrointestinal viruses. This lack of replication to high levels makes it difficult to prepare sufficient quantities of virus for use as conventional vaccines. Identification of the antigens involved in protection and the genes coding for them should make it feasible to produce sufficient antigen for immunization. Furthermore, it should be possible to identify the sequences involved in protection and synthesize them (Lerner, 1982) for immunization of dams during pregnancy so as to elevate colostral and milk antibodies.

A final method of reducing enteric infections is by proper management. Since it is assumed that infections occur either as a result of virus shedding from small numbers of adults or from virus in the environment, animals should not be crowded into contaminated areas. Movement of young into clean environments, away from other animals, will greatly reduce the rate of infection and economic loss.

VII. ECONOMIC IMPACT OF NEONATAL DISEASE IN ANIMALS

Although it is difficult to estimate the exact economic losses due to virus-induced diarrhea in animals, especially if one considers that there are numerous agents involved, there have been estimates that approximately 5% of the calves born in North America die from diarrhea before they reach 1 month of age. Based on these findings it has been estimated that losses in the United States average $100 million annually (House, 1978). No reports

are available for losses occurring in developing countries, but it is believed that similar losses would occur worldwide on an animal-to-animal basis.

VIII. CONCLUSIONS

Although there are a wide variety of viruses that can cause infections of the gastrointestinal tract of animals, most of them are all localized in either the crypts or the enterocytes of the intestine. Most virus infections are initiated in the proximal part of the small intestine and progress sometimes to the colon. Infections result in loss of adsorptive cells, villous atrophy, fluid loss, and ion imbalance. These pathophysiological events lead to anorexia, dehydration, and death. Since young animals do not have large reserves of fluids and glycogen, mortality can reach 50–80% in severe outbreaks. However, removal of milk and administration of oral electrolytes can significantly reduce losses.

Effective immunization requires that local immunity be present at an early age. Oral immunization with live attenuated vaccines is difficult due to the high levels of maternal antibody in the milk during the first few days of life. To overcome this problem the trend is to immunize the dam so as to increase the level of antibody in milk above the threshold level required to prevent infection with field strains of virus. As more serotypes and agents involved in gastroenteritis are identified, it is becoming obvious that vaccines will have to combine various pathogens and serotypes for achievement of adequate protection. Although at least some of these vaccines may be produced by conventional methods, recombinant DNA technology may aid in providing sufficient quantities of antigens to vaccinate against viruses which do not replicate well in culture.

REFERENCES

Bachmann, P. A., and Hess, R. G. (1982). "Viral Infections of the Gastrointestinal Tract" (D. A. J. Tyrell and A. Z. Kapikian, eds.), pp. 361–397. Dekker, New York.

Benfield, D. A., Stotz, I., Moore, R., and McAdaragh, J. P. (1982). *J. Clin. Microbiol.* **16,** 186–190.

Brown, E. H. (1973). *Br. Med. J.* **1,** 169–171.

Butler, D. G., Gall, D. G., Kelly, M. H., and Hamilton, J. R. (1974). *J. Clin. Invest.* **53,** 1335–1340.

Carpio, H. M., Babiuk, L. A., Misra, V., and Blumenthal, R. M. (1981). *Virology* **113,** 86–47.

Cheever, F. S., and Mueller, J. H. (1948). *J. Exp. Med.* **88,** 309–316.

Colloquium on selected diarrheal diseases of the young. (1978). *J. Am. Vet. Med. Assoc.* **173,** 511–676.

Costa, J., Rabson, A. S., Lee, C., and Tralka, T. S. (1977). *Nature (London)* **269**, 251–252.
Davison, V. E., and Sanford, B. A. (1981). *Infect. Immun.* **32**, 1118–1126.
Doyle, L. P., and Hutchings, L. M. (1946). *J. Am. Vet. Med. Assoc.* **108**, 257–259.
Editorial. (1978). *WHO Chron.* **32**, 369–372.
Flewett, T. H., and Woode, G. N. (1978). *Arch. Virol.* **51**, 1–23.
Garwes, D. J. (1982). *In* "Virus Infections of the Gastrointestinal Tract" (D. A. J. Tyrrell and A. Z. Kapikian, eds.), pp. 315–359. Dekker, New York.
Gouet, P., Contrepois, M., Dubourgier, H. C., Riou, Y., Scherrer, R., Laporte, J., Vautherot, J. F., Cohen, J., and L'Haridan, R. (1978). *Ann. Rech. Vet.* **9**, 433–440.
Graham, D. Y., Estes, M. K., and Sackman, J. W. (1982). *Gastroenterology* **82**, 1072–1079.
House, J. A. (1978). *J. Am. Vet. Med. Assoc.* **173**, 573–576.
Jones, R. C., Hughes, C. S., and Henry, R. R. (1979). *Vet. Rec.* **104**, 22.
Kahn, D. E. (1978). *J. Am. Vet. Med. Assoc.* **173**, 628–630.
Keenan, K. P., Jervis, H. R., Marchwicki, R. H., and Binn, L. N. (1976). *Am. J. Vet. Res.* **37**, 247–256.
Kerzner, B., Kelly, M. H., Gall, D. G., Butler, D. G., and Hamilton, J. R. (1977). *Gastroenterology* **72**, 457–462.
Kraft, L. M. (1957). *J. Exp. Med.* **101**, 743–755.
Kraft, L. M. (1958). *Yale J. Biol. Med.* **31**, 123–137.
Leece, J. G., and King, M. W. (1980). *Proc. Int. Symp. Neonatal Diarrhea, 3rd* (S. D. Acres, A. J. Forman, and H. Fast, eds.), pp. 21–36.
Leece, J. G., King, M. W., and Mock, R. (1976). *Infect. Immun.* **14**, 816–825.
Leece, J. G., Balsbaugh, R. K., Clare, D., and King, M. W. (1982). *J. Clin. Microbiol.* **16**, 715–723.
Lehner, T., Wilton, J. M. A., and Shillitoe, E. J. (1975). *Lancet* **2**, 60–62.
Lerner, R. A. (1982). *Sci. Am.* **248**, 66–84.
Lewis, L. D., and Philips, R. W. (1972). *Cornell Vet.* **62**, 596–607.
Lewis, L. D., and Philips, R. W. (1978). *J. Am. Vet. Med. Assoc.* **173**, 636–642.
Lewis, L. D., Philips, R. W., and Elliott, E. D. (1975). *Am. J. Vet. Res.* **36**, 413–416.
Little, L. M., and Shadduck, J. A. (1982). *Infect. Immun.* **38**, 755–763.
McAdaragh, I. P., Bergeland, M. E., Meyer, R. C., Johnshoy, M. W., Stotz, I. L., Benfeld, D. A., and Hammer, R. (1980). *Am. J. Vet. Res.* **41**, 1572–1581.
McNulty, M. S. (1978). *J. Gen. Virol.* **40**, 1–18.
McNulty, M. S., Allan, G. M., and Stuart, J. C. (1978). *Vet. Rec.* **103**, 319–320.
Mebus, C. A. (1978). *J. Am. Vet. Med. Assoc.* **173**, 631–632.
Mebus, C. A., and Newman, L. E. (1977). *Am. J. Vet. Res.* **38**, 553–558.
Mebus, C. A., Underdahl, N. R., Rhodes, M. B., and Twiehaus, M. J. (1969). *Agric. Exp. Sta. Res. Bull.* No. 233. College of Agriculture, Univ. Nebraska, Lincoln.
Mebus, C. A., Stair, E. L., Rhodes, M. B., and Tweihaus, M. J. (1973). *Vet. Pathol.* **10**, 45–64.
Middleton, P. J., Szymanski, M. T., Abbott, G. D., Bortolussi, R., and Hamilton, J. R. (1974). *Lancet* **1**, 1241–1244.
Misra, V., and Babiuk, L. A. (1980). *In* "Animal Virus Genetics" (B. N. Fields, R. Jaenisch, and C. F. Fox, eds.), pp. 727–738. Academic Press, New York.
Moon, H. W., Kemeny, L. J., and Lambert, G. (1976). *Proc. Int. Congr. Int. Pig Vet. Soc. 4th, Ames,* K12.
Nathanson, N., and Martin, J. R. (1979). *Am. J. Epidemiol.* **110**, 672–692.
Newman, L. E., Whitehair, C. K., and Mullaney, T. P. (1978). *Proc. Int. Symp. Neonatal Diarrhea 2nd* (Acres, S. D., ed.), pp. 457–464.
Pearson, G. R., and McNulty, M. S. (1977). *J. Comp. Pathol.* **87**, 363–373.
Pensaert, M., Haelterman, E. O., and Hinsman, E. J. (1970). *Arch. Ges. Virusforsch.* **31**, 335–351.

Philips, R. W., and Lewis, L. D. (1973). *Ann. Rech. Vet.* **4**, 87–98.
Robb, J. A., and Bond, C. W. (1979). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 193–248.
Runnels, L. P., Moon, H. W., Whipp, S. C., Mathews, P. J., and Woode, G. N. (1980). *Proc. Int. Symp. Neonatal Diarrhea, 3rd* (S. D. Acres, A. J. Forman, and H. Fast, eds.), pp. 343–358.
Sabara, M., Deregt, D., Babiuk, L. A., and Misra, V. (1982). *J. Virol.* **44**, 813–822.
Sanford, B. A., Shelokov, A., and Ramsay, M. A. (1978). *J. Infect. Dis.* **37**, 176–181.
Shephard, R. W., Butler, D. G., Cutz, E., Gall, D. G., and Hamilton, J. R. (1979). *Gastroenterology* **76**, 770–781.
Sherman, D. M., Acres, S. D., Sadowski, P. L., and Muscoplat, C. C. (1982). C.R.W.A.D. Abstr. No. 240.
Smith, H. C. (1956). *Vet. Rec.* **51**, 425–426.
Snodgrass, D. R., and Wells, P. W. (1976). *Arch. Virol.* **52**, 201–205.
Snodgrass, D. R., and Wells, P. W. (1978a). *Vet. Rec.* **102**, 146–148.
Snodgrass, D. R., and Wells, P. W. (1978b). *J. Am. Vet. Med. Assoc.* **173**, 565–569.
Snodgrass, D. R., Angus, K. W., and Gray, E. W. (1977). *Arch. Virol.* **55**, 263–274.
Spencer, G., Avendano, L. F., and Garcia, B. (1983). *Infect. Immun.* **39**, 569–574.
Stair, E. L., Rhodes, M. B., White, R. G., and Mebus, C. A. (1972). *Am. J. Vet. Res.* **33**, 1147–1156.
Storz, J., and Bates, R. C. (1973). *J. Am. Vet. Med. Assoc.* **163**, 884–886.
Storz, J., Leary, J. J., Carlson, J. H., and Bates, R. C. (1978a). *J. Am. Vet. Med. Assoc.* **173**, 624–627.
Storz, J., Doughri, A. M., and Hajer, I. (1978b). *J. Am. Vet. Med. Assoc.* **173**, 633–635.
Stotz, I. J., Benfield, D. A., and Hammer, R. (1980). *Am. J. Vet. Res.* **41**, 1572–1581.
Tennant, B., Harrold, D., and Reina-Geurra, M. (1972). *J. Am. Vet. Med. Assoc.* **161**, 993–1007.
Tennant, B., Ward, D. E., Braun, R. K., Hunt, E. L., and Baldwin, B. H. (1978). *J. Am. Vet. Med. Assoc.* **173**, 654–661.
Thake, D. C., Moon, H. W., and Lambert, G. (1973). *Vet. Pathol.* **10**, 330–341.
Theil, K. W., Bohl, E. H., Cross, R. F., Kohler, E. M., and Agnes, A. G. (1978). *Am. J. Vet. Res.* **39**, 213–220.
Van Bonsdorff, C. H., Hovi, T., Makela, P., Hovi, L., and Tevalvoto-Aarnio, M. (1976). *Lancet* **2**, 423.
Westmoreland, D., and Watkins, J. J. (1974). *J. Gen. Virol.* **24**, 167–178.
Woode, G. N. (1982). *In* "Virus Infections of the Gastrointestinal Tract" (D. A. J. Tyrrell and A. Z. Kapikian, eds.), pp. 295–311. Dekker, New York.
Woode, G. N. and Bridger, J. C. (1975). *Vet. Rec.* **96**, 85–88.
Woode, G. N. and Bridger, J. C. (1978). *J. Med. Microbiol.* **11**, 441–452.
Woode, G. N., and Crouch, C. F. (1978). *J. Am. Vet. Med. Assoc.* **173**, 522–526.
Woode, G. N., Bridger, J., Hall, G. A., Jones, J. M., and Jackson, G. (1976a). *J. Med. Microbiol.* **9**, 203–209.
Woode, G. N., Bridger, J. C., Jones, J. M., Flewett, T. H., Bryden, A. S., Davies, H. A., and White, G. B. B. (1976b). *Infect. Immun.* **14**, 804–810.
Woode, G. N., Reed, D. E., Runnels, P. L., Herrig, M. A., and Hill, H. T. (1982). *Vet. Microbiol.* **7**, 221–240.
Woode, G. N., Kelso, N. E., Simpson, T. F., Gaul, S. K., Evans, L. E., and Babiuk, L. A. (1983). *J. Clin. Microbiol.* **18**, 358–364.
Yolken, R. H. (1982). "Virus Infections of the Gastrointestinal Tract" (D. A. J. Tyrrell and A. Z. Kapikian, eds.), pp. 51–87. Dekker, New York.

23

Viral Hepatitis A: Virus, Disease, and Control

F. DEINHARDT, J. ABB, V. GAUSS-MÜLLER,
K. VON DER HELM, M. ROGGENDORF, AND R. SCHEID

Max von Pettenkofer Institute for Hygiene and Medical Microbiology, University of Munich, Munich, Federal Republic of Germany

I. INTRODUCTION

Viral hepatitis is a major public health problem in all parts of the world. Many millions of people are infected with hepatitis viruses each year with enormous impact on health and national economies. At least three etiologically distinct forms of the disease are recognized, and these are referred to as hepatitis A (HA), hepatitis B (HB), and hepatitis non-A, non-B (HNANB). The nomenclature of the various virus components and antibodies as suggested by the World Health Organization (WHO) is explained in Table I (Deinhardt and Gust, 1982). The etiological agents of HA and HB have been identified and characterized, and specific diagnostic tests have

ISBN 0-12-429601-7

TABLE I
Nomenclature of Components and Corresponding Antibodies of the Hepatitis Viruses[a]

Virus component or antibody	Definition
HA	Hepatitis A
HAV	Hepatitis A virus
HAAg	Hepatitis A virus antigen
Anti-HAV	Antibody to hepatitis A virus without differentiation into immunoglobulin classes
Anti-HAV IgG	Antibody to hepatitis A virus of the IgG class
Anti-HAV IgM	Antibody to hepatitis A virus of the IgM class
Anti-HAV IgA	Antibody to hepatitis A virus of the IgA class
HB	Hepatitis B
HBV	Hepatitis B virus, Dane particle
HBsAg	Hepatitis B surface antigen
HBsAg: *adw, ayw, adr, ayr*	Subtypes of hepatitis B surface antigen
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
Anti-HBs	Antibody to hepatitis B surface antigen without differentiation into immunoglobulin classes
Anti-HBc	Antibody to hepatitis B core antigen without differentiation into immunglobulin classes
Anti-HBc IgG	Antibody to hepatitis B core antigen of the IgG class
Anti-HBc IgM	Antibody to hepatitis B core antigen of the IgM class
Anti-HBe	Antibody to hepatitis B e antigen without differentiation into immunoglobulin classes
HBDNAP	Hepatitis B DNA-dependent DNA polymerase
HNANB	Hepatitis non-A, non-B: hepatitis that is caused neither by HAV or HBV nor by hepatitis occurring in association with other common infectious agents, such as Epstein–Barr virus or cytomegalovirus

[a]From Deinhardt and Gust (1982).

been developed. In view of the difficulty of differentiating between the various forms of viral hepatitis on the basis of clinical findings and biochemical parameters, the advent of specific serological tests for the laboratory diagnosis of infections with both hepatitis A and B viruses has assumed therefore great importance, and widespread application of these tests has increased considerably the knowledge of the epidemiology, pathogenesis, and immunology of HA and HB (for review, see Szmuness *et al.*, 1982; Deinhardt and Deinhardt, 1983; Overby *et al.*, 1983). However, at present there are no precise virological criteria nor specific laboratory tests of HNANB (Gerety, 1981; Kryger, 1983).

II. CHARACTERISTICS OF HEPATITIS A VIRUS

Hepatitis A virus (HAV) is a single-stranded RNA virus belonging to the family Picornaviridae. It has now been classified as enterovirus type 72 (Siegl *et al.*, 1981; Coulepis *et al.*, 1982; Melnick, 1982; Gust *et al.*, 1983a) (Table II). The virus is ether resistant, stable at pH 3.0, and relatively more heat stable than other enteroviruses. It is partially inactivated by exposure to heat at 60°C for 10 hr and completely inactivated by heat at 85°C for 5 min. Partially purified virus preparations are inactivated by treatment with formalin (1:4000) at 37°C for 72 hr. Treatment of HAV-containing materials with chlorine at a concentration of 1 mg/liter for 30 min has also been reported to inactivate HAV. HAV isolates from various parts of the world (Australia, China, Europe, North and South America, and Russia) appear all to be antigenically identical or so closely related that antigenic differences cannot be detected with standard immunological methods. Hepatitis A has been transmitted to various nonhuman primate species, and HAV can be propagated in cell cultures (Frösner *et al.*, 1979; Provost and Hilleman, 1979; Flehmig, 1980, 1981; Daemer *et al.*, 1981; Deinhardt *et al.*, 1981; Gauss-Müller *et al.*, 1981; Kojima *et al.*, 1981; Locarnini *et al.*, 1981; Provost *et al.*, 1981; Gust *et al.*, 1983b). However, replication of HAV in cell cultures is much slower and less efficient than that of other picornaviruses. Studies of *in vitro* translation of the HAV genome therefore were undertaken to prove beyond question the positive strandedness of HAV RNA and to evaluate the efficiency of protein processing in the hope of finding some reason for the slow replication of HAV in cell cultures (V. Gauss-Müller, K. von der Helm, and F. Deinhardt, unpublished data). These studies have proved that the HAV RNA is present in the virion as a single positive-strand RNA, that the primary translation products are similar in size to those of other enteroviruses, and that the processing of precursor molecules yields proteins corresponding to the HAV structural proteins, although the latter occurs apparently much more slowly than, for example, in similar parallel experiments with poliomyelitis virus. However, this needs further study before it can be concluded that slow processing of precursor proteins is the reason for the slow replication of HAV.

In addition, attempts are being made currently to clone the HAV genome in plasmids, with subsequent amplification in bacteria, and to determine the base sequence of the genome. In these studies, HAV RNA from virus present in stool or grown in cell cultures is transcribed with avian myeloblastosis virus reverse transcriptase into double-stranded complementary DNA. This is difficult because the HAV RNA is about 8000 base pairs long (2.5 times longer than HBV DNA and much longer than the length of mes-

TABLE II
Characteristics of Hepatitis A Virus in Comparison to Other Picornaviruses

Characteristics	HAV[a]	Picornaviruses
Biophysical		
Size (nm)	27–29	26–34
Density in CsCl (g/ml)		
Incomplete virions	1.30–1.32	
Major component	1.33–1.34	1.33–1.45
Minor dense component	1.39–1.44	
Sedimentation coefficient in sucrose	156–160	140–165
Capsid symmetry	Cubic, 32 capsomeres	Cubic
Virion	Naked	Naked
Capsid assembly	Cytoplasm	Cytoplasm
Biochemical		
Genome		
Type	RNA	RNA
Structure	Single-stranded	Single-stranded
Polarity	Positive	Positive
Sedimentation coefficient in sucrose	32.5	32–35
Density (g/ml)	1.64	1.64
Poly(A) content	Present	Present
Molecular weight		
Under nondenaturating conditions	$2.25–2.3 \times 10^6$	$2.3–2.8 \times 10^6$, 7600 nucleotides
Fully denaturated conditions	2.8×10^6, 8100 nucleotides	
Polypeptide composition		
VP0	40,000	41,000
VP1	30,000–33,000	33,000–35,000
VP2	24,000–27,000	28,000–31,000
VP3	21,000–23,000	24,000–26,000
VP4	7,000–14,000	5,000–14,000
Stability		
Ether (20%, 4°C, 18 hr)	Stable	Stable
Acid (pH 3.0)	Stable	Stable
Formalin (1:4000, 37°C, 72 hr)	Inactivated	Inactivated
Heat		
60°C for 60 min	Stable	Generally inactivated
60°C for 10 hr	Partially inactivated	—
85°C for 3 min	Inactivated	—
100°C for 5 min	Inactivated	Inactivated
Chlorine (1 mg/liter, 30 min)	Inactivated	—

[a]Natural host: man; experimental host: Old and New World monkeys.

senger RNA measuring 2000–3000 nucleotides) and because the secondary structure of the HAV RNA apparently hinders the transcription of the full length of the genome. Nevertheless, DNA complementary to HAV RNA has been prepared using oligo(dT) and partially digested calf thymus DNA as random primers for the reverse transcription (von der Helm *et al.*, 1981; von der Helm and Deinhardt, 1984; R. Ostermayr and K. von der Helm, unpublished data).

These cDNAs were cloned into the plasmid pBR322; although all individual clones were smaller than the full length genome, they covered the entire HAV genome. The cDNA clones were mapped by hybridization and restriction enzyme analysis. Nucleotides were sequenced by the method of Maxam and Gilbert beginning with the clones proximal to the 3′ end of the RNA genome. So far, about 2000 nucleotides at the 3′ end of the genome have been sequenced (A. Lezius, K. von der Helm, E. Winnacker, E. Wimmer, and F. Deinhardt, unpublished data).

Expression of HAV antigens by some HAV cDNA clones in bacteria has been obtained, but further analysis of the nature of these antigens and their relevance in virus neutralization is needed. In addition, more productive systems for HAV genome expression must be found before proteins produced by these techniques can be applied to the production of antigens to be used in diagnostic tests or as vaccines.

III. DISEASE

Infection occurs by ingestion of virus-containing food or water, but a primary site of virus replication in the gastrointestinal tract has not been found. Virus is first detectable in the liver and appears to be excreted via the bile into the gut. Over 60% of all infections are entirely subclinical or are associated with only minor gastrointestinal symptoms, so that a specific diagnosis is not made. Hepatitis A is usually a relatively mild disease although fulminant hepatitis with death occasionally occurs. Hepatitis A never develops into chronic liver disease even in those cases with a prolonged course in which abnormal biochemical values and some histopathological changes (occasional liver cell necrosis and minor mononuclear cell infiltrations) persist for several months to a year. Chronic carriers of HAV likewise do not exist, and no prolonged virus excretion beyond maximal 4 weeks after onset is observed regardless of whether abnormal biochemical values persist or not. Immunity develops regularly after clinical or subclinical infections and persists for many years and in most cases is lifelong. HAV

infection in any part of the world will protect against an infection in the same or any other location because all HAV isolates are antigenically identical or very closely related (see above).

IV. DIAGNOSIS

The typical course of virus excretion and the development of specific antibody (anti-HAV) are shown in Fig. 1. A specific laboratory diagnosis of HA can be obtained by demonstration of HAV particles or specific viral antigens (HAAg) in the feces or by detection of anti-HAV of the IgM class (anti-HAV IgM). HAV is excreted in the feces for 1–2 weeks before the onset of disease and for up to 4 weeks thereafter. As a chronic carrier state is unknown, the detection of HAV particles or HAAg in the feces is evidence of current infection. HAV can be detected in approximately 50% of patients with HA tested within 1 week after onset of clinical disease. The chance of detecting the virus falls to 10–25% in the second week and to less than 10% in the third. Thus while the detection of HAV or HAAg is evidence of current infection, their absence does not preclude the diagnosis.

Anti-HAV IgM appears early in the illness and persists for a limited time (usually for 3–4 months after the onset of illness). This assay is the test of choice for the rapid and accurate diagnosis of acute HA. Anti-HAV IgG appears more slowly, persists for many years, and indicates immunity to the disease.

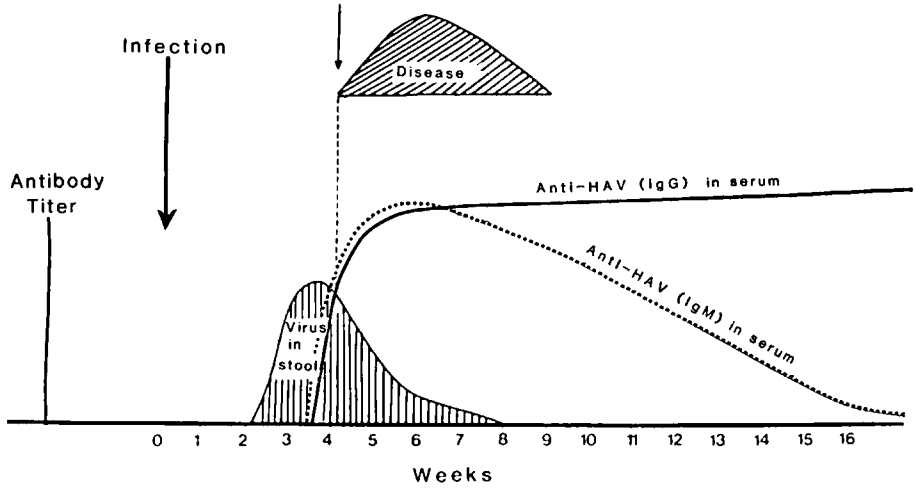


Fig. 1. Typical course of hepatitis A infection.

HAV-specific antibodies of the IgM or IgG classes can be detected by both radioimmunoassay (RIA) or enzyme immunoassay (ELISA) (Roggendorf *et al.*, 1980). Two principles of the ELISA technique for the determination of subclass-specific viral antibodies are depicted in Fig. 2.

1. Viral antigen (e.g., HAAg) is fixed to a solid-phase matrix, such as polystyrene balls or the wells of microtiter plates. Patient's serum is added and allowed to react with the viral antigen. Virus-specific antibody in the patient's serum will bind to the solid phase. Excess serum is removed by washing, and in a second step this immune complex formation is made visible by the addition of enzyme-coupled antibody against human IgM or IgG.
2. Anti-human IgM (anti-μ) is fixed to a solid-phase matrix. Patient's serum is added, and IgM is bound to the anti-μ fixed to the solid phase. Excess serum is washed out; specific IgM is marked by the addition of virus antigen. The solid phase is again washed, and the immune complex formation is made visible by the addition of enzyme-coupled antibody against the viral antigen.

In both test systems the amounts of bound enzyme activity are measured in a final step by a substrate reaction. The ELISA technique provides a sensitive, inexpensive, and reliable method for the detection of class-specific antibodies against viral antigens.

V. TRANSMISSION AND EPIDEMIOLOGY OF HEPATITIS A

Hepatitis A virus is spread by the fecal–oral route, usually by person-to-person contact, and infections are particularly common in conditions associated with poor standards of hygiene and sanitation. Common source outbreaks result most frequently from fecal contamination of drinking water and food. The consumption of raw or inadequately cooked shellfish cultivated in sewage-contaminated tidal or coastal water and the consumption of raw vegetables grown in soil fertilized with untreated human feces are associated with a high risk of infection with HAV. HAV infection is frequently contracted by travelers from areas of low prevalence to areas of high endemicity. The incubation period of HAV is between 3 and 5 weeks with a mean of 28 days.

Infections with HAV may occur at all ages among susceptible individuals. In young children the infections tend to be mild or asymptomatic; clinical severity increases with increasing age. The patterns of disease vary considerably from population to population and depend on the standards of hygiene and sanitation. In developed countries, the pattern of reported cases

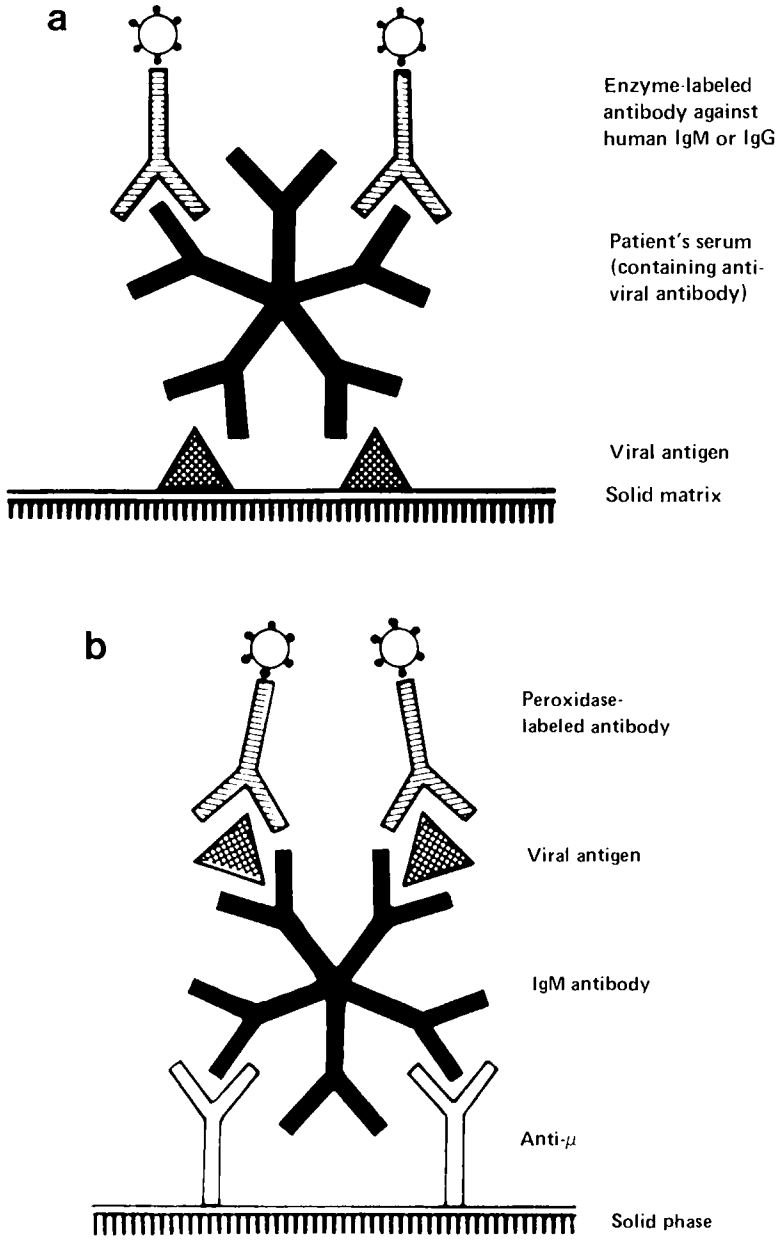


Fig. 2. Enzyme immunoassay for determination of viral antibodies. (a) Enzyme-labeled antibody against human IgG or IgM; (b) peroxidase-labeled antibody. From Meurman (1979) with permission.

is shifting from early childhood to older ages, paralleling improvements in socioeconomic conditions.

In temperate zones, epidemic waves have been observed with peaks in late autumn and early winter. In many tropical countries the peak of reported infections tends to occur during the rainy season with low incidence in dry periods.

Evidence based on serological surveys indicates that HAV infection has a worldwide distribution and that a higher prevalence of antibody is found among lower socioeconomic groups. The prevalence of HA in industrialized countries, particularly in Northern Europe, North America, and Australia, is decreasing, whereas the infection is virtually universal in most other regions, particularly in countries with a warm climate.

VI. PREVENTION

A. Hygienic Measures against Hepatitis A

The treatment of patients with HA generally does not require isolation. A private room is indicated for infants or patients with fecal incontinence. Gloves should be worn when handling excretions, as well as surfaces, materials, and objects exposed to them. Gowns should be worn when clothing may be soiled with excretions. Hands should be washed thoroughly after removing gowns and gloves. Disposable syringes and needles are preferred. Contaminated items should be placed in puncture-resistant containers. Disposable items should be decontaminated, preferably by autoclaving. Reusable items should be carefully disinfected. Patient's specimens should be labeled prominently with a special warning, such as "Hepatitis A."

B. Passive Immunization

The long-standing use of pooled normal human immunoglobulin (NIG, γ-globulin) for prophylaxis or prevention of HAV infection has demonstrated the efficacy of this approach to control HAV transmission (for review, see Deinhardt and Gust, 1982; Deinhardt and Deinhardt, 1983).

The intramuscular administration of pooled normal human immunoglobulin (16% solution in a dose of 0.02–0.12 ml/kg of body weight) before exposure to the virus or early during the incubation period may prevent or attenuate a clinical illness while not necessarily preventing infection and excretion of the virus. Protection by passive immunization lasts for a period for 3–4 months. Individuals entering highly endemic areas or institutions for a long stay should receive repeat doses of immunoglobulin every 3–4

months. If possible, their immune status should be monitored for signs of a subclinical infection, (i.e., appearance of anti-HAV IgM), because no further immunoglobulin inoculations are needed if an infection has occurred and the individual has acquired active immunity.

C. Active Immunization

As HAV is a major public health problem in many parts of the world, there is a clear need for efforts to develop effective vaccines. The successful adaptation of HAV to growth in cell cultures has opened the way for the development of both killed and live attenuated vaccines (Hilleman *et al.*, 1982; Provost *et al.*, 1983).

Recent advances in recombinant DNA technology suggest that it will soon be feasible to produce HA vaccines by genetic engineering (see above; von der Helm and Deinhardt, 1983). The ideal HA vaccine would be a live attentuated vaccine, which could be administered orally, perhaps in combination with oral polio vaccine on a large scale. For maximum effectiveness, it would need to be given early in life after the decline of maternally derived antibodies. Immunization with live attenuated vaccines may, however, face the same problems which exist with vaccination in early childhood with oral live attenuated polio vaccines in subtropical and tropical regions where interference with frequently occurring other enteroviruses prevent or diminish the immunizing effect of the vaccine if given orally. Also the extent of excretion of the attenuated HA vaccine virus and its genetic stability after several passages in man are not known as yet, and no markers for attenuation have been established. Therefore, the parallel development of inactivated noninfectious HAV vaccines appears justified. Production of such vaccines in cell cultures would be prohibitively expensive unless more productive cell culture systems for the growth of HAV can be developed. Production of HAV antigens by genetic engineering (see above) and ultimately by manufacturing the critical antigenic epitopes by entirely synthetic methods may solve these problems.

VII. TREATMENT

A specific therapy for HA is not available. Since exposure to HAV usually results in an acute self-limiting infection—with the exception of rare cases of fulminant HA—and never results in chronic liver disease, a specific treatment of patients with HA appears unnecessary.

VII. HEPATITIS A-LIKE EPIDEMIC HEPATITIS NON-A, NON-B

This disease resembles HA clinically and has been reported during the last years from parts of Asia and the Middle East. Epidemic hepatitis non-A, non-B (HNANB) is an acute, self-limiting disease occurring predominantly among young adults. It is more severe in pregnant women, in whom it is associated with a high mortality. Epidemic HNANB is thought to be spread by ingestion of contaminated water or food, and fecal–oral spread is assumed but not proved. The epidemic nature of this hepatitis and its presumed fecal–oral transmission separate it from the previously recognized endemic HNANB, which has epidemiological characteristics similar to those of HBV. Epidemic HNANB may be caused by an agent distinct from HAV or alternatively by a distinct but previously unrecognized serotype of HAV. Viruslike particles similar to picornaviruses have been demonstrated in acute-phase stools and have been specificially agglutinated by convalescent sera but not by anti-HAV. The disease has been transmitted to nonhuman primates, but serial passage in primates appears to be difficult so far. The virus has yet not been grown in cell cultures (Balayan *et al.*, 1983), and the final classification of this disease must await a definite identification of its causative agent.

IX. CONCLUSION

Hepatitis A is an orally transmitted self-limiting acute disease which does not become chronic and does not lead to chronic virus-carrier states. Fulminant courses with acute liver failure and death are rare. The causative agent is the enterovirus type 72 (picornavirus), and all virus isolates from HA cases appear to be antigenically identical; immunity following infection is practically lifelong. Vaccines against HA are under development and should become generally available within a few years. The relationship of a newly recognized form of orally transmitted epidemic HNANB to HA is not entirely known, but no antigenic relationship seems to exist between the causative agents of HA and this form of HNANB.

REFERENCES

Balayan, M. S., Andjaparidze, A. G., Savinskaya, S. S., Ketiladze, E. S., Braginsky, D. M., Savinov, A. P., and Polesschuk, V. F. (1983). *Intervirology* **20**, 23–31.

Coulepis, A. G., Locarnini, S. A., Westaway, E. G., Tannock, G. A., and Gust, I. D. (1982). *Intervirology* **18**, 107–127.

Daemer, R. J., Feinstone, S. M., Gust, I. D., and Purcell, R. H. (1981). *Infect. Immun.* **32**, 388–393.

Deinhardt, F., and Deinhardt, J., eds. (1983). "Viral Hepatitis: Laboratory and Clinical Science" Dekker, New York.

Deinhardt, F., and Gust, I. D. (1982). *Bull. W.H.O.* **60**, 661–691.

Deinhardt, F., Scheid, R., Gauss-Müller, V., Frösner, G. G., and Siegl, G. (1981). *Prog. Med. Virol.* **27**, 109–113.

Flehmig, B. (1980). *Med. Microbiol. Immunol.* **168**, 239–248.

Flehmig, B. (1981). *Med. Microbiol. Immunol.* **170**, 73–81.

Frösner, G. G., Deinhardt, F., Scheid, R., Gauss-Müller, V., Holmes, N., Messelberger, V., Siegl, G., and Alexander, J. J. (1979). *Infection* **7**, 303–305.

Gauss-Müller, V., Frösner, G. G., and Deinhardt, F. (1981). *J. Med. Virol.* **7**, 233–239.

Gerety, R. J., ed. (1981). "Non-A, Non-B Hepatitis." Academic Press, New York.

Gust, I. D., Coulepis, A. G., Feinstone, S. M., Locarnini, S. A., Moritsugu, Y., Najera, R., and Siegl, G. (1983a). *Intervirology* **20**, 1–7.

Gust, I. D., Locarnini, S. A., Coulepis, A. G., and von der Helm, K. (1983b). *In* "Viral Hepatitis: Laboratory and Clinical Science" (F. Deinhardt and J. Deinhardt, eds.) pp. 35–56. Dekker, New York.

Hilleman, M. R., Buynak, E. B., McAleer, W. J., McLean, A. A., Provost, P. J., and Tytell, A. A. (1982). *In* "Viral Hepatitis" (W. Szmuness, H. J. Alter, and J. E. Maynard, eds.), pp. 385–397. Franklin Inst. Press, Philadelphia, Pennsylvania.

Kojima, H., Shibayama, T., Sato, A., Suzuki, S., Ichida, F., and Hamada, C. (1981). *J. Med. Virol.* **7**, 273–286.

Kryger, P. (1983). *Liver* **3**, 176–198.

Locarnini, S. A., Coulepis, A. G., Westaway, E. G., and Gust, I. D. (1981). *J. Virol.* **37**, 216–225.

Melnick, J. L. (1982). *Intervirology* **18**, 105–106.

Meurman (1979). *Laboratoriumsblätter* **4**, 133–148.

Overby, L. R., Deinhardt, F., and Deinhardt, J., eds. (1983). "Viral Hepatitis." Dekker, New York.

Provost, P. J., and Hilleman, M. R. (1979). *Proc. Soc. Exp. Biol. Med.* **160**, 213–221.

Provost, P. J., Giesa, P. A., McAleer, W. J., and Hilleman, M. R. (1981). *Proc. Soc. Exp. Biol. Med.* **167**, 201–206.

Provost, P. J., Conti, P. A., Giesa, P. A., Banker, F. S., Buynak, E. B., McAleer, W. G., and Hilleman, M. R. (1983). *Proc. Soc. Exp. Biol. Med.* **172**, 357–363.

Roggendorf, M., Frösner, G., Deinhardt, F., and Scheid, R. (1980). *J. Med. Virol.* **5**, 47–62.

Siegl, G., Frösner, G. G., Gauss-Müller, V., Tratschin, J. D., and Deinhardt, F. (1981). *J. Gen. Virol.* **57**, 331–341.

Szmuness, W., Alter, H. J., and Maynard, J. E. eds. (1982). "Viral Hepatitis" (Int. Symp. 1981). Franklin Inst. Press, Philadelphia, Pennsylvania.

von der Helm, K., and Deinhardt, F. (1984). *In* "Advances in Hepatitis Research" (F. Chisari, ed.) pp. 263–267. Masson Publ., New York.

von der Helm, K., Winnacker, E. L., Deinhardt, F., Frösner, G. G., Gauss-Müller, V., Bayerl, B., Scheid, R., and Siegl, G. (1981). *J. Virol. Methods.* **3**, 37–43.

24

Impact of Arthropod-Borne Virus Diseases on Africa and the Middle East

THOMAS P. MONATH

Division of Vector-Borne Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, U.S. Public Health Service, Department of Health and Human Services, Fort Collins, Colorado

I. INTRODUCTION

At least 42 arthropod-borne viruses which occur in Africa and the Middle East have been associated with disease in humans and/or domesticated animals (Table I). For the majority of these viruses, the true impact on human or veterinary health in the region has not been defined, and only superficial insights into the transmission cycles and epidemiology have been obtained. Associations with disease are often established on the basis of a single patient or a few patients, from whose blood the agent has been isolated, and descriptions of the clinical features are therefore sketchy. Infections with some of these viruses are probably relatively rare events, representing chance intrusions on a basic zoonotic transmission cycle; these disease entities will

APPLIED VIROLOGY ISBN 0-12-429601-7

TABLE I
Arthropod-Borne Viruses Associated with Disease in Humans (H) and/or Domesticated Animals (A) in Africa and the Middle East

Disease	Occurrence
African horse sickness	A
African swine fever	A
Akabane	A
Bangui	H
Banzi	H
Bhanja[a]	H
Bunyamwera	H
Bwamba	H
Chikungunya	H
Chandipura[a]	H
Crimean HF-Congo	H
Dengue 1	H
Dengue 2	H
Dhori[b]	H
Dugbe	H
Germiston[b]	H
Ilesha	H
Kemerovo[a]	H
Lebombo	H
Lunyo[a]	H
Middelburg	A
Ndumu	A
Nyando	H
O'nyong nyong	H
Orungo	H
Quaranfil	H
Rift Valley fever	H, A
Sandfly fever—Naples	H
Sandfly fever—Sicilian	H
Semliki forest	H,[b] A
Shuni	H
Sindbis	H
Soldado[a],[b]	H
Spondweni	H
Tataguine	H
Thogoto	H
Usutu	H
Wesselsbron	H, A
West Nile	H, A
Yellow fever	H
Zika	H
Zirqa	H

[a]Virus was isolated in the region, but disease was recorded elsewhere.
[b]Laboratory infection.

remain medical curiosities. However, in the case of other viruses only rarely associated with disease, there is serological evidence for widespread infection; examples are the sandfly fever viruses in countries surrounding the Mediterranean and several agents of Sub-Saharan Africa transmitted by *Anopheles* mosquitoes, such as Tataguine, Orungo, Bwamba, and Nyando viruses. The disease produced may be very mild, and it may occur principally in children; however, future ecological changes or intrusion of nonimmune population groups may change the epidemiology of such viruses from an endemic to an epidemic pattern.

A few arthropod-borne viruses are presently regarded as having major importance, generally because they cause epidemics or epizootics with high morbidity rates. In this chapter, I shall focus on viruses having a known or potentially significant impact on human health; these viruses are shown in Table II.

II. YELLOW FEVER

Yellow fever occurs throughout much of tropical Sub-Shaharan Africa, but, within this region, intense virus activity may be intermittent and quite localized. Between 1965 and 1981, a total of 2082 cases were officially notified to the World Health Organization (Fig. 1), the annual incidence vary-

TABLE II
Arthropod-Borne Viruses with Significant Adverse Impact on Human Health in Africa and the Middle East

Virus	Known geographic distribution within region	Basis for adverse impact
Yellow fever	West, Central, East Africa	Recurring epidemics
Rift Valley fever	Sub-Saharan Africa, Egypt	Recurring epidemics, recent previously unaffected area
Dengue	West, East Africa	Recent outbreaks in previously unaffected area
Chikungunya	Sub-Saharan Africa	Recurring epidemics
O'nyong nyong	Sub-Saharan Africa	Past outbreaks; epidemic potential
Sindbis	Africa, Middle East	Past outbreaks; epidemic potential
West Nile	Africa, Middle East	Past outbreaks; epidemic potential
Crimean HF-Congo	Africa, Middle East	Nosocomial epidemics

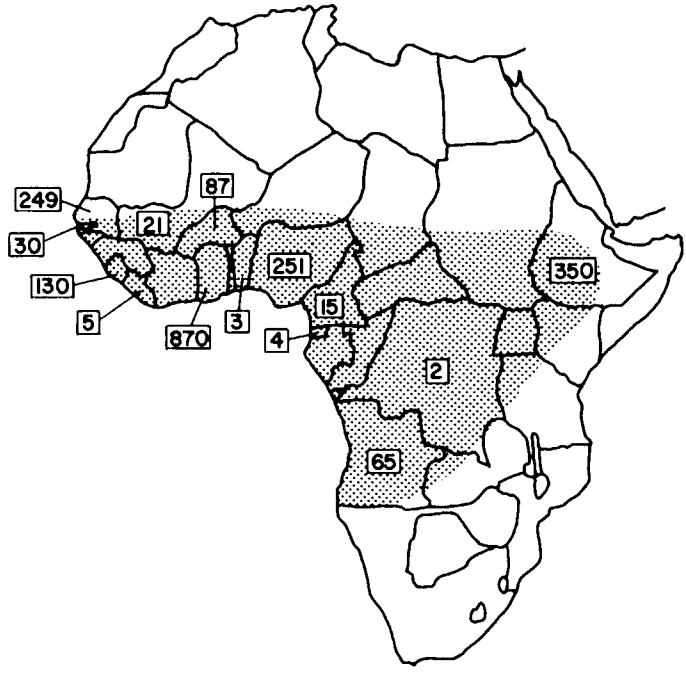


Fig. 1. Number of cases of yellow fever officially reported to the World Health Organization, 1965–1981. The shaded area represents the region of known or potential enzootic-endemic virus activity.

ing between 5 and 500 cases. This distribution of reported cases gives only a partial picture of the natural circulation of yellow fever virus, because inadequate surveillance and reporting systems, high levels of vaccine-induced and natural immunity, low human population densities, and other factors result in an apparent absence of disease from areas with a high level of enzootic viral transmission. This lesson was vividly taught in 1979, when two unvaccinated French tourists acquired fatal yellow fever infections in southwestern Senegal; only 1 case occurred among the indigenous population of Senegal in 1979, where large-scale vaccination campaigns had been completed the previous year.

The official reports (Fig. 1) represent a gross underestimate of the true morbidity associated with yellow fever. This is best shown by investigations of various epidemics (Table III). The ratio of officially notified cases to the number determined by epidemiological investigation has ranged from 1:10 to 1:500. Some localized outbreaks would have gone completely undiscovered, had it not been for chance and the active interest of local medical virologists (ORSTOM, 1982; Monath *et al.*, 1972, 1973). Moreover, a relatively high rate of endemic transmission occurs annually in the wet savan-

TABLE III
Discrepancies between the Number of Officially Notified Yellow Fever Cases and Deaths and Estimates of Morbidity and Mortality from Direct Investigations of Epidemics[a]

Country	Year(s)	Number of cases (deaths)	
		Officially notified	Determined by epidemiologic investigation
Ethiopia	1960–1962	—[b] (3000)	100,000 (30,000)
Senegal	1965	243 (216)	2,000–20,000 (200–2,000)
Upper Volta	1969	87 (44)	3000 (100)
Nigeria	1969	208 (60)	100,000 (—[b])
Nigeria	1970	4 (1)	786 (15-40)
The Gambia	1978–1979	30 (3)	5,000-8,000 (1,000-1,700)

[a]From T. P. Monath, *in* "Tropical and Geographical Medicine" (K. S. Warren and A. A. F. Mahmoud, eds.), Chapter 68. McGraw-Hill, New York, 1983.
[b]No estimate available.

nahs of West Africa; yellow fever is nevertheless rarely recognized due to a lack of laboratory diagnostic facilities and clinical confusion with viral hepatitis, malaria, and other infections.

Human populations in many areas within the affected region are relatively unprotected by vaccination. Mass vaccination (with the French neurotropic vaccine) in French West Africa between 1940 and 1960 resulted in the virtual disappearance of the disease, which remained epidemic in anglophone countries. Systematic vaccination in francophone West Africa was suspended in 1960, resulting in major outbreaks in 1965 and 1969, affecting principally the susceptible childhood population. Prophylactic immunization is now practiced in some West African countries on a limited scale. The approach in most areas, however, is to undertake mass vaccination in response to the occurrence of disease outbreaks, such as was done in Nigeria (1969–1970), in the Gambia (1978–1979), and in Ghana (1970 and 1977–1979).

A challenge for the future is the inclusion of yellow fever 17D vaccine in expanded programs of immunization (EPI) in Africa. This challenge cannot be made glibly, for the world's present supply of vaccine may be inadequate to the task, and the cost both of vaccine and of delivery under cold-chain conditions are high. Although yellow fever has been an epidemic problem only in West Africa in the last 20 years, we must not lose sight of the past history of severe outbreaks in the Sudan and Ethiopia and of the potential for spread to *Aedes aegypti* areas outside the endemic zone, such as Asia. Improvements in yellow fever 17D vaccine production, including the use of a cell culture substrate (instead of hen's eggs) for growth of the virus and the development of effective stabilizers, are priorities of international health agencies.

The epidemics of the last few years have led to a resurgence in research and major breakthroughs in our understanding of yellow fever epidemiology and ecology. Only a few highlights of this research can be presented here.

Longitudinal field studies by scientists of the Office de la Recherche Scientific et Technique Outre-Mer (ORSTOM) and the Pasteur Institutes in Senegal, Ivory Coast, Upper Volta, and the Central African Republic have clarified many aspects of the maintenance and epidemic cycles of yellow fever (Fig. 2). In the humid, equatorial forests, year-round transmission of virus occurs in a cycle involving monkeys and *Aedes africanus* mosquitoes. The level of viral activity in the enzootic high forest zone is generally low, reflected by low immunity rates in human and monkey populations and absence of large epidemics. This is explained by the extreme dilution of vectors and hosts in a continuous, uniformly favorable forest environment

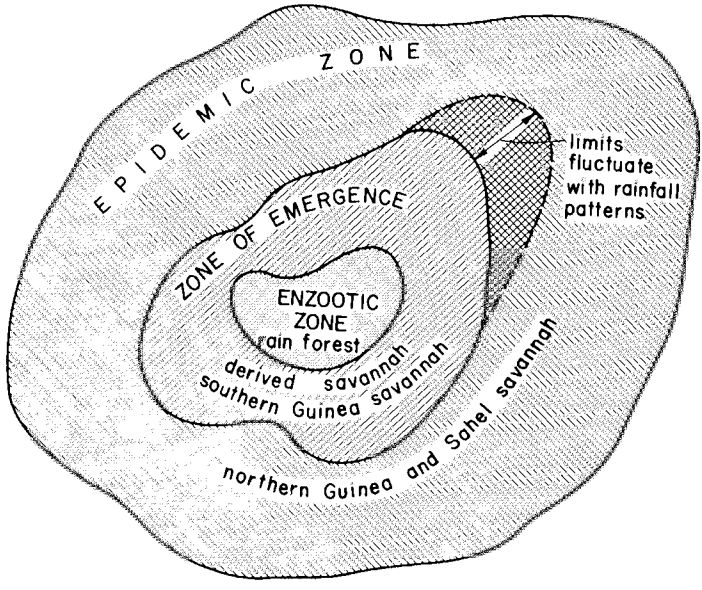


Fig. 2. Current concepts of yellow fever ecology in West Africa. The virus is maintained year round at low levels in an enzootic cycle involving *Aedes africanus* and monkeys in equatorial forests. In surrounding savannah-mosaic and moist Guinea savannah (zone of emergence), high rates of transmission occur during the rainy season, involving monkeys, man, and sylvatic *Aedes* species, in which dry-season maintenance is assured by transovarial transmission. The limits of this zone may fluctuate, depending on weather cycles. Extending farther into dry savannah, yellow fever is not endemic, but periodic introductions of the virus can result in sharp epidemics, with *Aedes aegypti* (breeding in domestic water containers) as the principal vector.

(Germain *et al.*, 1981). In areas where extensive modification of the forest zone has resulted from human agricultural development, sporadic cases and miniepidemics may occur (Cordellier *et al.*, 1982).

Surrounding the enzootic forest zone in West Africa are the relatively wet savannah–forest mosaic and progressively drier Guinean and southern Sudan savannah vegetational zones, including riverine forests. This extensive ecosystem supports large and *concentrated* populations of monkeys, humans, and sylvatic vector species, including *Ae. africanus, Ae. luteocephalus, Ae. furcifer-taylori,* and others. Virus transmission intensifies during the rainy season and wanes during the dry season, when adult vector populations virtually disappear. Human immunity rates are high, and epidemic transmission may occur, especially at the fringes of the zone, where viral activity may subside for intervals of some years due to meterologic phenomena (Germain *et al.*, 1980). This ecosystem has been appropriately named the Zone of Emergence by Germain and his colleagues (Germain *et al.*, 1981).

Surrounding the Zone of Emergence are areas subject to extreme drying (northern Sudan savannah and Sahel vegetational zones). In these areas, yellow fever epidemics occur intermittently as a result of virus introductions. Water storage is widely practiced and domestic *Ae. aegypti* populations are high. Certain drought-resistant sylvatic vector species (e.g., *Ae. vittatus, Ae. metallicus, Ae. luteocephalus*) also may play a role in interhuman virus transmission.

These concepts allow, in theory at least, more rational approaches to epidemic prediction by (1) surveillance of viral activity (in vectors and/or monkeys in man) in the Zone of Emergence, and (2) monitoring of *Ae. aegypti* density and of immunological susceptibility of human population in the epidemic zone.

The recrudescence of yellow fever after quiescent periods or after the annual long dry season has long defied explanation. Recent observations by French workers have provided clarification. Yellow fever virus has been shown to be transovarially transmitted in *Ae. furcifer-taylori* mosquitoes (Cornet *et al.*, 1979), this assuring survival over periods adverse to horizontal spread. In addition, recent isolations of yellow fever virus from *Amblyomma variegatum* ticks collected from cattle in the Central African Republic (Germain *et al.*, 1979) raises the interesting possibility that ticks may play a role in dry season maintenance or dispersal of the virus.

Several other technical advances should be noted. The rapidity with which the virus can be isolated has been greatly improved by the use of mosquito cell cultures. Specific identification of the virus in infected cultures may be made as early as 48 hr by means of immunofluorescence or ELISA. Yellow fever monoclonal antibodies have been developed (Schlesinger *et al.*, 1983),

some of which are yellow fever-specific in the immunofluroescence test (T. P. Monath, unpublished data). The ELISA method is also useful for detection of IgM yellow fever antibodies, which show little cross-reaction with heterologous flaviviruses. Improvements in the surveillance and control of yellow fever will depend on practical application of these and other techniques, which now should be transferred to regional and local laboratories. It must be emphasized that surveillance of yellow fever is rudimentary or nonexistent in many areas, with the result that the true impact of the disease in Africa cannot be accurately assessed. Improved laboratory diagnostic services, active surveillance, and reporting are central to future preventive and control strategies.

Finally, a very brief consideration of the clinical disease is appropriate. The pathophysiology of yellow fever is incompletely understood. Over 50% of patients with severe disease die, and overall, the case-fatality ratio is 10–20%. In Africa, patients with the disease are rarely hospitalized, and those that are do not benefit from modern intensive care. The total reversibility of the hepatic lesion in sublethal infections and the suggestion from animal experiments that renal damage is secondary to hypotension (and therefore preventable) (Monath *et al.*, 1981) indicate that aggressive clinical care may reduce mortality (as in dengue hemorrhagic fever). There is a need for more sophisticated clinical research in future outbreaks. A corollary is the need for further experimental study of potential antiviral chemotherapeutic agents and interferon.

III. RIFT VALLEY FEVER

Rift Valley fever was first clinically described during an epizootic in Kenya in 1912, and the virus was subsequently isolated from a sick sheep in 1930 (Daubney *et al.*, 1931). In 1979, the virus was shown to be serologically related to other members of the phlebotomus fever group (genus *Phlebovirus*) of the family Bunyaviridae (Shope *et al.*, 1980).

During the period of 45 years between isolation of the virus and 1976, a picture emerged of recurring epizootics in eastern and southern Africa. Outbreaks occurred at intervals of 5–15 years and tended to be localized to foci characterized by moist grasslands surrounding forest or woodland. Studies in eastern and southern Africa showed that the virus was enzootic in these areas and that the the periodic epizootic intensification followed periods of exceptionally heavy rainfall. Notable epizootics during the period 1931–1976 occurred in Kenya, the United Republic of Tanzania, Zimbabwe, South Africa, and Namibia (Fig. 3). In addition, the virus was isolated in Nigeria, Cameroon, and Chad, and serological evidence was obtained for infections

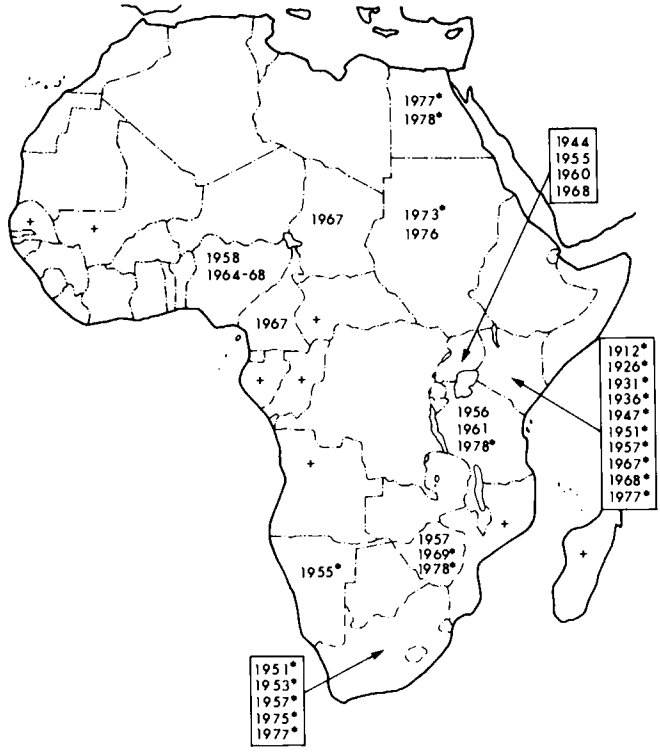


Fig. 3. Distribution of Rift Valley fever virus in Africa. Modified fram Fig. 1 in Bres (1981), with permission. Dates with asterisk: reported epizootics; dates without asterisk: first evidence of enzootic situations; +, enzootic situations evidenced without precise date.

in many other areas of Africa. In 1982, a fortuitous observation by Dr. James M. Meegan at the Yale Arbovirus Research Unit showed Rift Valley fever virus to be identical to Zinga virus, an agent first isolated in equatorial moist forest in the Central African Republic (Digoutte *et al.*, 1974), where it has been associated with febrile illness in humans. Further isolations of Zinga virus have been made from infected humans and mosquitoes in Senegal and from mosquitoes in Guinea and Madagascar. These observations widen the known distribution of Rift Valley fever in Africa.

During epizootics, the disease in domesticated animals has resulted in serious losses. Young lambs are most susceptible, and mortality approaches 100% (Erasmus and Coetzer, 1981). Older lambs and adult sheep have a mortality rate of up to 30%, and abortions in affected ewes are usual. Only 10% of affected bovines die. Calves are more susceptible than adults, abortion is common, and milk production in dairy cows is severely reduced. Goats develop high viremias, but clinical disease is rare. Camels and water

buffaloes become infected; mortality is probably low, although abortion may occur. Horses are refractory to infection.

Prior to 1976, human disease was considered of relatively minor consequence. Small numbers of cases were associated with epizootics, and these were generally self-limited, influenza-like infections, with only rare complications of retinitis, encephalitis, and hemorrhagic diathesis and few fatalities. Persons having close contact with infected animals or carcasses (butchers, veterinarians, ranchers) were at highest risk, and they acquired the disease by contact spread.

These concepts were shattered in 1977, with the unprecedented extension of Rift Valley fever to Egypt (Meegan, 1981). In 1977 an epidemic of Rift Valley fever appeared unexpectedly in the Nile Delta and Valley of Egypt; this outbreak had been preceded by reports of the disease in the Sudan the previous year, but the relationship between these events and the exact mode of introduction of the virus to Egypt are not known. Egypt experienced a second epizootic in 1978, and persistent virus activity was detected in 1979 and 1980. Losses of domesticated animals were severe; however, the major medical revelation was the extent and severity of human disease. Official reports for 1977 include 1000 clinical cases and 598 deaths (Meegan, 1981). However, other estimates indicated that at least 200,000 cases occurred. Case-fatality rates ranged from 0.2% in military recruits to as high as 14% among civilian hospitalized patients. Deaths were due to the severe forms of the disease complicated by hemorrhagic fever or encephalitis. Ocular disease was seen in over 800 patients in 1977; permanent visual loss resulted in some cases.

Retrospective surveys of banked sera showed that Rift Valley fever had been absent from Egypt prior to 1977. Surveys conducted after the epidemics in areas affected showed that, overall, 8.5% of the human population and 22.3% of surviving domesticated animals had been infected (WHO, 1981). The surveys also indicated that many infections occurred in areas that had not reported disease, an indication that the size of the epidemic was underestimated.

The extent of human involvement in these epidemics was unprecedented, since previous epizootics had been associated with a maximum of only about 100 human cases. Severe human disease, with encephalitis, ocular involvement, hemorrhage, and fatal outcome had been previously described in South Africa (Van Velden *et al.*, 1977), and it is probable that the large number of severe and fatal infections in Egypt reflects the high overall incidence of human infection rather than emergence of a virus strain with increased virulence. However, in comparative pathogenesis studies, Peters and Anderson (1981) noted that Egyptian strains of the virus caused lethal infections in Wistar–Furth rats, whereas virus strains from eastern and

southern Africa did not. It should be emphasized that the choice of experimental host was critical to the outcome of these experiments, since *all* virus strains were fully virulent for adult mice and hamsters. The difficulty in making correlations between virulence for laboratory and clinical hosts was further illustrated by the Lunyo (Uganda) strain, which is pathogenic for humans but not for adult mice. RNA oligonucleotide fingerprinting of Rift Valley fever virus strains has shown considerable genetic variation between strains from different geographic areas and between strains from the same area isolated several years apart (Cash *et al.*, 1981). Given the potential for genetic drift and genetic reassortment of this group of viruses, the emergence of strains with high virulence characteristics deserves further study. At present, there is no conclusive evidence that this occurred in Egypt in 1977.

The Egyptian epidemic raised the threat of further spread of the disease from Africa to receptive areas of Mediterranean Basin or Southwest Asia, particularly to irrigated river valley areas having abundant mosquito vectors and large, concentrated populations of domesticated animals (Bres, 1981; Shope *et al.*, 1982). Although the disease did not spread from Africa in the late 1970s, this possibility remains a concern for the future.

The epidemiology and mode of transmission of Rift Valley fever must be considered from two perspectives: (1) epizootic–epidemic spread and (2) enzootic maintenance mechanisms. The former is reasonably well understood, whereas elucidation of enzootic transmission remains a major challenge in arbovirology.

Isolations of the virus have been made from mosquitoes captured during outbreaks in domestic ruminants. Many of the mosquito species implicated have also been shown in the laboratory experiments to be reasonably efficient biological vectors (Table IV). Since domesticated animals and humans sustain very high viremias, they undoubtedly serve as effective hosts for mosquitoes. Mechanical transmission by biting flies and even by nonbiting muscoid flies which feed on carcasses, abortuses, etc. must be considered a potential mode of spread during epizootics. Direct contact and aerosol spread (Yedloutschnig *et al.*, 1981; Brown *et al.*, 1981) are important modes of infection, especially under conditions of crowding and close contact between animals and animals and man.

The basic means by which Rift Valley fever virus survives in nature between epizootics has defied years of study. Isolations from arthropods (mosquitoes, culicoid midges) have occasionally been made in enzootic areas (Table IV). However, no wild vertebrate host species have been clearly implicated by seroepidemiological or experimental studies, although antibodies (to Zinga virus) have been found in wart hogs and water buffalo in the Central African Republic (Berge, 1975).

TABLE IV
Arthropods Implicated as Biological Vectors of Rift Valley Fever Virus[a]

Species	Isolation from field-collected arthropods in situations considered		Experimental infection (I) and transmission (T)
	Epizootic	Enzootic	
Mosquitoes			
Aedes dentatus	+ (Zimbabwe)		
Ae. tarsalis		+ (Uganda)	
Ae. circumluteolus		+ (Uganda)	I
Ae. lineatopennis	+ (Zimbabwe, Africa)	+ (Kenya)	I, T
Ae. caballus	+ (S. Africa)		I
Ae. juppi	+ (S. Africa)		
Ae. cinereus	+ (S. Africa)		
Ae. africanus		+ (Uganda)	
Ae. dendrophilus		+ (Uganda)	
Anopheles coustani	+ (Zimbabwe)		I
Coquillettidia fuscopennata		+ (Uganda)	I
Culex theileri	+ (Zimbabwe, S. Africa)		I, T
Culex pipiens	+ (Egypt)		
Eretmopodites spp.		+ (Uganda)	
E. quinquevittatus	+ (S. Africa)		I, T
Mansonia africana		+ (Uganda, Central African Republic)	
M. uniformis		+ (Uganda)	I
Midges and blackflies			
Culicoides sp.		+ (Nigeria)	
Simulium sp.	+ (S. Africa)		

[a]Many other mosquito species and one species of tick were shown to become infected and to transmit the virus under laboratory conditions; however, natural infection is not documented (Meegan *et al.*, 1980; McIntosh and Jupp, 1981).

Very recent and dramatic new observations on the enzootic cycle in Kenya can be reported. While on an aerial survey in Kenya, Dr. Charles Bailey of the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) was impressed by a natural geological characteristic of enzootic areas, the presence of large depressions (locally called "dambos"), which flood during periods of heavy rainfall. Similar formations were known to exist in southern Africa. The possibility that these formations could periodically yield large numbers of *Aedes* mosquitoes which might be transovarially infected with Rift Valley fever virus stimulated further research. In recent

months, Dr. Kenneth Linthicum, also of USAMRIID, and Dr. F. G. Davies of the Kenya Ministry of Agriculture, Veterinary Research Laboratories, Kabete have demonstrated that, after flooding, these dambos produce a succession of large populations of *Aedes,* followed by *Culex* and *Anopheles.* Rift Valley fever virus was isolated from adult *Ae. lineatopennis,* a univoltine species, shortly after emergence. Moreover, the virus was isolated from male and female *Ae. lineatopennis* reared from larvae collected from a dambo. These very exciting observations indicate that inherited transmission in *Aedes* mosquitoes is fundamental to virus maintenance and periodic recrudescence.

Finally, efforts to prevent and control Rift Valley fever have focused on the development and use of veterinary vaccines. A live, attenuated vaccine developed in South Africa is successfully and widely used there and in Kenya; however, reversion to virulence appears to occur occasionally, and the live vaccine is unsuitable for use in areas free of the disease. Killed vaccines are used to protect livestock in Egypt, South Africa, and other areas (Eddy *et al.,* 1981) and have also been used in limited trials in man (Barnard, 1979). Research is currently under way in the United States on the development of a genetically engineered Rift Valley fever vaccine. Although vaccination offers an important means of preventing spread of the virus to receptive areas and even of restricting enzootic transmission, other possibilities for control must also be considered. In particular, the recent demonstration of transovarial transmission in floodwater *Aedes* suggests the targeted use of biological or chemical larvicides in enzootic areas.

IV. WEST NILE FEVER

Relatively little attention has been paid to this disease in recent years. The virus was first isolated in the West Nile District of Uganda in 1937 from the blood of woman with a mild febrile illness. Subsequent serological surveys and virus isolations revealed a wide geographic distribution of West Nile virus throughout Africa, the Middle East, parts of Europe and the USSR, India, and Indonesia. In the 1950s classical studies on the epidemiology and ecology of the virus in Egypt and the Sudan revealed that human infections were extremely common in the Nile Delta (Taylor *et al.,* 1956). Nearly three-quarters of the population in these areas were shown to acquire the infection by early adulthood. This hyperendemic virus circulation precludes sharp epidemics, but places a high burden of infection on the childhood population, which experiences largely unrecognized and clinically undifferentiated febrile disease. The Egyptian studies also proved that wild birds are the principal viremic hosts and *Culex univittatus* the main vector of West Nile virus.

Summertime epidemics caused by West Nile virus were recognized as early as 1950 in Israel and recurred there at frequent intervals during the decade (Marberg *et al.*, 1956). These outbreaks involved hundreds of recognized cases, but their true extent was probably much greater; an attack rate of over 60% was reported in one instance. The disease was generally mild, resembling dengue fever. However, an outbreak of disease in a group of elderly persons was characterized by a high incidence of severe central nervous system (CNS) infections and a case-fatality rate of nearly 10% (Clarke and Casals, 1965). The CNS infections were also subsequently confirmed in Egypt (Abdel Wahab, 1970), where 4 of 133 patients with aseptic meningitis or encephalitis admitted to Abbassia Fever Hospital between 1966 and 1968 were shown to have West Nile virus.

The epidemics in Israel in the 1950s were the result of amplified virus transmission and spillover to a human population with a low background of immunity. Development of irrigated agricultural areas associated with human settlement, breeding of *Culex* vectors, and an expanded avian population favored epidemic transmission. Little recent information is available regarding West Nile virus activity in these areas. The absence of epidemics in the last 20 years may relate to changing patterns of human immunity, to vector control activities, or to changing agricultural practices.

In 1974, South Africa experienced an epidemic of West Nile fever (McIntosh *et al.*, 1976). Sporadic cases had been recognized earlier, and relatively low antibody prevalences of 13–20% had been determined in the human population. The outbreak involved an area of some 2500 km^2 of central and northern Cape Province; 55% of the population was infected with West Nile virus, and hundreds to thousands of clinical cases occurred.

The 1974 epidemic was of mixed etiology, involving both West Nile and Sindbis viruses. As in Egypt, both viruses are enzootically maintained in local reservoirs, but in South Africa, the enzootic cycle is much more restricted, and endemic human infections relatively infrequent. Conditions favoring virus amplification and high population of *Cx. univattatus,* including unusually high rainfall and abnormally high temperatures, were responsible for the epidemic.

Outbreaks in areas of The Middle East and temperate areas of southern Africa may be anticipated in the future. The critical elements favoring outbreaks are (1) a limited immune barrier in the human population; and (2) conditions supporting virus amplification, especially expansion of *Cx. univittatus,* which serves as both the enzootic maintenance vector and the epidemic vector. The development of irrigation projects in arid areas may favor such conditions.

Although West Nile virus occurs in tropical Africa, few clinical human cases have been recognized (Tomori *et al.*, 1978), and antibody prevalences are relatively low. The vectors involved in transmission are not known, but

it is probable that enzootic transmission cycles are not linked so closely with man as in temperate zones. Geographic variation in disease prevalence may also be explained by the occurrence of virus strains with differing host ranges and virulence. Antigenic differences between West Nile virus strains have been repeatedly demonstrated. Strains from Africa, the Middle East, Pakistan, and the USSR are distinct from Indian viruses (Hammam and Price, 1966), possibly indicating restricted gene flow in areas with separate bird migration patterns; however, considerable antigenic variation also occurs between strains isolated in individual countries (Odelola and Fabiyi, 1978; Umrigar and Pavri, 1977). Moreover, West Nile virus strains vary in their pathogenicity for man and laboratory animals (Parks *et al.*, 1958). A similar plasticity of flavivirus genomes has also been demonstrated for St. Louis encephalitis and Japanese encephalitis viruses. In tropical America, St. Louis encephalitis is rarely associated with overt human disease, and many strains have reduced pathogenicity for animal models (Monath *et al.*, 1980; Trent *et al.*, 1980). Whether similar strain variation explains the relative absence of human cases of West Nile fever in tropical Africa, or whether vector–host relationships, a low level of medical surveillance, or other factors are responsible, remains to be determined.

V. SINDBIS VIRUS DISEASE

The epidemiology and ecology of this alphavirus in Africa are closely linked to that of West Nile virus. Both viruses share a common vector (*Cx. univittatus*) and viremic avian hosts in a manner analogous to the dual circulation of western equine encephalitis and St. Louis encephalitis in *Cx. tarsalis* and birds in the western United States.

Sindbis virus was first recovered in 1952 from *Cx. univittatus* in the Nile Delta. The virus has been isolated subsequently in West, Central, East, and South Africa, in Eastern Europe and in many areas of Asia and Australia. Human infections in Egypt are less prevalent than those due to West Nile virus and usually escape medical attention. Central nervous system infections occur occasionally (Abdel-Wahab, 1970), but the usual disease is mild, dengue-like, and cannot be clinically distinguished from West Nile fever. In 1974, a mixed West Nile–Sindbis epidemic occurred in South Africa (McIntosh *et al.*, 1976). Sindbis virus infections were less frequent than those due to West Nile virus, and the infection rate in *Cx. univittatus* was one-sixth that of West Nile. *Culex univittatus* has been shown experimentally to be a signficantly more efficient vector of West Nile than of Sindbis virus (Jupp, 1976). Other, less extensive epidemics of Sindbis occurred in South Africa in 1967 and 1979.

Although the epidemic spread of Sindbis virus in South Africa is a unique

event for the continent, information from other areas emphasizes the need for vigilance and further study. In the late 1960s, a febrile summertime epidemic disease with rash and arthralgia appeared in east central Sweden, and was given the name of the village first affected, "Ockelbo disease" (Skogh and Espmark, 1982). In 1974 a similar disease occurred in Finland and was given the name "Pogosta disease." In 1981, the same condition was recognized in Karelia (USSR), and was called "Karelian fever" (Lvov *et al.*, 1982). Serological studies in Sweden, Finland, and the USSR clearly implicate Sindbis virus (or a close antigenic relative). Summertime outbreaks in northern Europe appear to be quite common; they present interesting new challenges in arbovirus ecology, since the transmission and overwintering mechanisms require definition. The virus has not been isolated as yet. It will be interesting to see whether strains from the far North are similar to other strains from the Palearctic–Ethiopian zoogeographic region which have been compared and shown to be similar by RNA hybridization (Rentier-Delrue and Young, 1980). From the perspective of this volume, the important point is that the disease in northern Europe probably escaped attention for many years due to its relatively mild and undifferentiated clinical presentation.

What is the distribution and prevalence of Sindbis virus infections in the Middle East? Will changes in environment and human demography lead to future emergence of Sindbis fever as a public health problem? Recent observations (Wills *et al.*, 1984) illustrate the need for future surveys. Collections of mosquitoes in the Eastern Province of Saudi Arabia were made in heavily irrigated agricultural areas and areas with date palm gardens, ponded sewage, and other features which provided large populations of birds and mosquitoes. The area is on the path of bird migrations between East Africa and the USSR. Sixteen strains of Sindbis virus were isolated from three species of *Culex*, the majority (13 strains) from *Cx. univattatus*. The infection rate was high (nearly 5/1000). Nothing is presently known about the proclivity of *Cx. univattatus* to feed on man in Saudi Arabia or about the prevalence of human infection.

VI. CHIKUNGUNYA

Chikungunya, an alphavirus, was first isolated in the United Republic of Tanzania in 1952 during a prolonged epidemic of disease characterized by fever and severe joint pains (Ross, 1956). Subsequently, the virus has been repeatedly associated with epidemics in Uganda, Zaire, Zimbabwe, South Africa, Angola, Senegal, Nigeria, Southeast Asia, and the Philippines (Fig. 4). The disease is moderately severe; patients may be prostrate during the

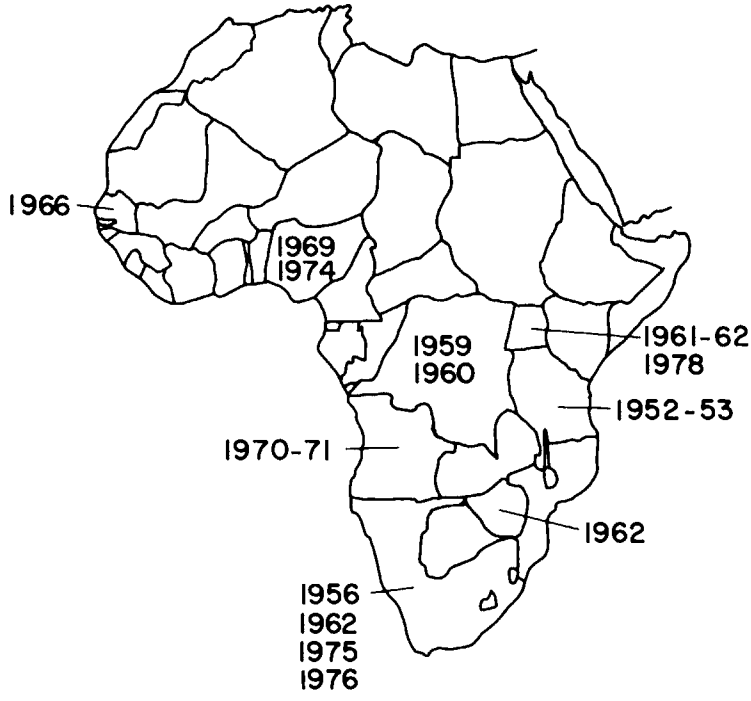


Fig. 4. Epidemics of chikungunya reported in Africa.

acute phase, and there is often a prolonged convalescence with persisting joint pains. In Asia, hemorrhagic manifestations and a few deaths have been described, but in Africa chikungunya has not been associated with these complications.

The disease incidence in most outbreaks has not been accurately determined. During the 1952–1953 epidemic in Tanzania more than 40% of inhabitants were affected. In Ibadan, Nigeria, an outbreak in 1969 resulted in serological evidence for infection in 78% of the susceptible population under 5 years of age (Moore *et al.*, 1974). Inapparent infections are rare with chikungunya.

In considering the epidemiology of chikungunya, the following observations are relevant.

1. In African towns and villages, transmission is by the agency of *Aedes aegypti* mosquitoes, and humans probably serve as the viremic hosts.
2. Mixed urban (*Ae. aegypti*-borne) outbreaks of chikungunya and dengue (Carey *et al.*, 1969) or chikungunya and yellow fever (Filipe *et al.*, 1973) are described.
3. In between urban epidemics, chikungunya virus activity ceases (or re-

mains at very low, i.e., undetectable, levels), due principally to exhaustion of immunologically susceptible persons. Antibody prevalence rates reach nearly 100% in West African adults. Outbreaks occur after sufficient time for a susceptible childhood population to appear e.g., every 5 years (Tomori *et al.*, 1975).

4. Antibody prevalence rates in East African adult populations are lower (30–35%) (Henderson *et al.*, 1970), indicating that these areas are receptive to future outbreaks involving all age groups.

5. Enzootic maintenance involves nonhuman primates and forest *Aedes* species (McCrae *et al.*, 1971; McIntosh *et al.*, 1964), principally *Ae. furcifer-taylori, Ae. luteocephalus, Ae. africanus*, and possibly *Mansonia africana*, in a cycle analogous to sylvatic yellow fever. The level of enzootic virus activity waxes and wanes, reflecting changes in the relative proportions of infective vectors and susceptible hosts in a stable focus or periodic reintroductions from a "wandering epizootic." The concepts previously developed with regard to yellow fever maintenance cycles (Fig. 2) probably apply to chikungunya as well, but transovarial transmission has not been demonstrated (Jupp *et al.*, 1981).

On seroepidemiological evidence alone, chikungunya must be the most widespread and frequent arboviral pathogen of man in Africa. Its importance is likely to increase in concert with human population growth and migration to urban centers. The medical impact on young children, the main target for reasons already described, is largely unknown, and the viral insult remains to be assessed in the context of parasitic infections and nutritional deficiencies affecting this age group.

VII. O'NYONG NYONG

This fascinating disease is considered here because it caused one of the largest arbovirus epidemics on record. Between 1959 and 1962 an estimated 2 million persons were affected in East Africa, with attack rates of up to 90% (Haddow *et al.*, 1960). During this epidemic, the virus (an alphavirus closely related to chikungunya) was isolated. The disease was unknown to East Africa prior to 1959, and all age groups were affected; clinically it resembles chikungunya, but has marked lymphadenopathy as a characteristic feature (Shore, 1961). No subsequent outbreaks have been described. Where did the virus come from? Where did it go?

Serological surveys are complicated by cross-reactions with chikungunya, and differential titers often must be used to distinguish these infections, a fact that has been overlooked, even in recent studies (Marshall *et al.*, 1982). The serological evidence indicates that o'nyong nyong virus, or closely re-

lated antigenic variants, exists in Nigeria, Ghana, Liberia, Senegal, Upper Volta, Ivory Coast, Cameroon, and the Central African Republic (Berge, 1975; Chippaux and Chippaux-Hyppolite, 1968). The virus has been isolated in Senegal from sentinel mice (Bres *et al.*, 1963), and a nearly identical agent (Igbo-Ora virus) is known from Nigeria. This wide distribution (Fig. 5) suggests that the 1959–1962 epidemic in East Africa was the result of emergence or introduction from a cryptobiotic transmission cycle, rather than rapid evolution of a new viral genome (e.g., from chikungunya).

A highly significant feature of o'nyong nyong is epidemic transmission by *Anopheles* mosquito vectors (Williams *et al.*, 1965), a unique association among the alphaviruses. Interhuman transmission by the agency of *Anopheles funestus* and *Anopheles gambiae* accounted for spread of the epidemic 20 years ago. No other vectors have been incriminated, nor have wild vertebrate reservoir-hosts been demonstrated.

Studies in western Kenya after the epidemic suggested that o'nyong nyong virus transmission continued until at least 1969 (Marshall *et al.*, 1982), but apparently declined thereafter. No seropositives were found in children un-

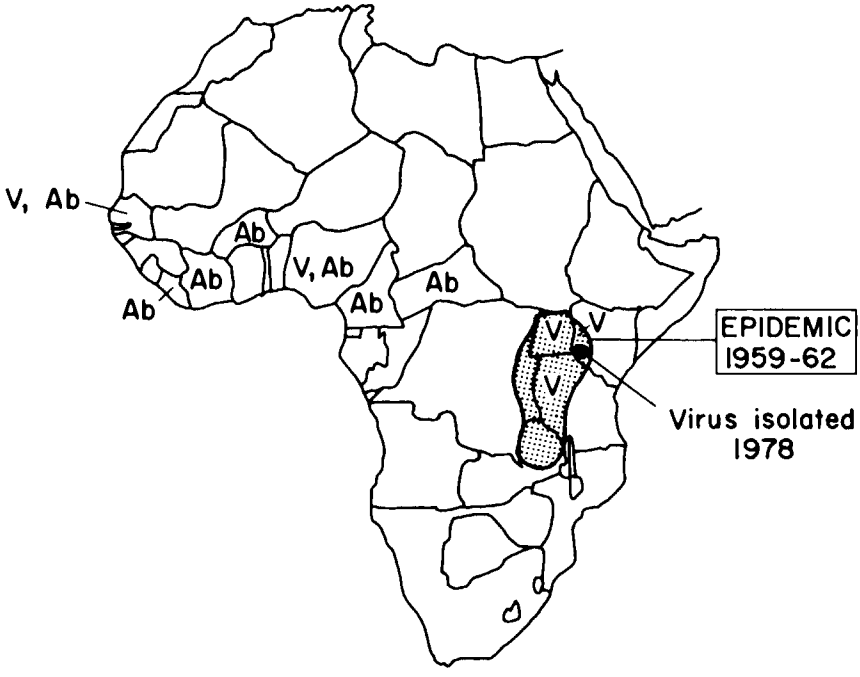


Fig. 5. Geographic distribution of o'nyong nyong virus and antibodies in Africa. The shaded area of East Africa was affected in large epidemic involving over 2 million cases between 1959 and 1962.

der 6 years bled in 1975, and no virus was isolated from a large sample of mosquitoes between 1971 and 1975. In 1978 Johnson and colleagues isolated a single strain of o'nyong nyong virus from *An. funestus* in western Kenya (Johnson *et al.*, 1981). Apparently, *Anopheles* populations in 1978 were unusually large. Disappearance of the virus for intervals sufficient to allow the accumulation of nonimmunes and its reemergence or reintroduction in 1978 indicate the potential for future epidemics of o'nyong nyong disease.

There is a need for laboratory research. Considerable difficulty has been encountered in primary isolation of the virus, which is nonpathogenic for suckling mice on early passage. This difficulty might be overcome by use of newer techniques, especially mosquito cell cultures, but requires further study. Experimental infection studies with vectors and vertebrates are conspicuously lacking. The 1959–1962 and 1978 isolates from East Africa and the strains from West Africa have not been compared by RNA fingerprinting, hybridization, or other techniques which might provide clues as to their origins.

VIII. DENGUE

Outbreaks of disease clinically resembling dengue were reported in Africa as early as 1870 (Christie, 1881) and were described frequently through World War II. No virological basis for implicating dengue virus in the etiology of these epidemics exists, however; historical evidence suggests that the disease described in these outbreaks more closely resembles chikungunya than dengue (Carey, 1971).

The modern history of dengue in the region begins in 1971 when Carey *et al.* (1971) reported the first isolations of dengue types 1 and 2 viruses from febrile patients in Nigeria; the earliest isolate (identified retrospectively) was made in 1964. Dengue viruses have been subsequently repeatedly isolated from febrile patients in Nigeria both in the absence of and during recognized epidemics (Fagbami and Fabiyi, 1975; Fagbami *et al.*, 1977). Serological studies showed high prevalences of dengue neutralizing antibodies, with the highest rates in the derived savannah zone. Monkeys in gallery forests of Nigeria have dengue-specific antibodies indicating the possibility of a sylvatic transmission cycle (Fagbami *et al.*, 1977; Monath *et al.*, 1974). In 1978, dengue type 2 virus was isolated from a sick human in Senegal.

Two highly interesting and important events have occurred since 1980, which restructure our view of dengue in Africa. These events also illustrate the practical application of new techniques in molecular virology to the investigation of dengue virus epidemiology.

A. Sylvatic Dengue

Since 1980 French scientists in Senegal, Ivory Coast, and Upper Volta have succeeded in isolating several hundred strains of dengue 2 virus from forest *Aedes* mosquitoes (principally *Ae. furcifer-taylori, Ae. luteocephalus, Ae. opok,* and *Ae. africanus*) (Institut Pasteur Dakar, 1981). Monkeys are apparently involved in transmission, but the roles of human hosts and of transovarial transmission in mosquitoes remain to be elucidated. Clinical human disease has not been associated with the high rates of infection in mosquitoes. The high rate of success in isolation of dengue virus strains is due in large measure to the use of sensitive assay systems, in particular mosquito cell cultures and intrathoracic inoculation of mosquitoes, and the identification of dengue strains to serotype has been greatly facilitated by use of monoclonal antibodies (Henchal *et al.*, 1983).

The importance of these recent observations lies in the potential for endemic maintenance and epidemic expansion in a fashion analogous to yellow fever and independent of the classical vector, *Ae. aegypti.* Dengue is probably hyperendemic in savannah-woodland zones of West Africa; the disease is rarely recognized because it occurs most often in children and is confused readily with malaria and other fevers, and because the associated rash is difficult to discern. With future improvements in rural health care, more attention will undoubtedly be paid to this and other presently ignored infections. It is also possible that, given the right sequence of infections, dengue hemorrhagic fever could occur in West Africa. These events will underscore the difficulty posed by zoonotic dengue, since vaccination (not elimination of *Ae. aegypti*) will be the only effective means of prevention and control.

B. Epidemic Dengue in East Africa

In early 1982, our laboratory received serum samples from several Americans who had acquired a dengue-like illness while in Somalia. Diagnosis was complicated by cross-reactive antibodies indicating flavivirus superinfection, but the data strongly implicated dengue type 2 virus. Anecdotal information indicated that a large number of cases of clinical dengue had occurred in Mogadishu in 1981.

Between March and October, 1982, Johnson and colleagues succeeded in isolating dengue type 2 virus from 7 patients who acquired the disease in Mogadishu or in several localities in coastal Kenya (Johnson *et al.*, 1982). Antibody prevalence rates in coastal Kenya rose from 7.6% in June to 52% in October 1982. Medical personnel working in the area had not noticed a marked increase in unusual febrile illness during the outbreak, but did describe a rise in cases diagnosed as "clinical malaria." This again illustrates

the recurring theme of underrecognization of epidemic virus diseases in Africa.

The probable vector in the affected area was *Ae. aegypti,* which occurs in two genetically and behaviorally distinct forms, *Ae. aegypti aegypti,* which is highly anthropophilic and breeds in domestic containers, and the feral *Ae. aegypti formosus,* which breeds in natural containers (e.g., tree holes) and is weakly arthropophilic.

What was the origin of this first confirmed epidemic of dengue in East Africa? Comparative analysis of the RNA oligonucleotide fingerprints of a 1982 Kenyan isolate and 36 other dengue 2 virus strains from various geographic areas (Trent *et al.,* 1983; Trent, personal communication, 1982) showed that the Kenyan strain was distinct from all other strains including viruses from Nigeria, Upper Volta, and the Seychelle islands.

It is possible that the appearance of dengue 2 in Somalia and Kenya in 1981–1982 may be the result of introduction from India or from the Seychelles, which experienced outbreaks in 1976–1977 and 1978–1979 (Metselaar *et al.,* 1980; Calisher *et al.,* 1981). If so, extensive changes must have occurred in the viral genome, since RNA fingerprints of the Kenyan and Seychelles isolates were distinct. However, another possibility deserves consideration: extension of dengue from an enzootic transmission cycle. Forested areas with large populations of nonhuman primates and feral *Aedes (Stegomyia)* species exist along coastal Kenya. In the light of recent findings in West Africa discussed previously, this possibility deserves study.

REFERENCES

Abdel-Wahab, K. S. E. (1970). *Acta Virol.* **14,** 501–506.

Barnard, B. J. H. (1979). *J. S. Afr. Vet. Med. Assoc.* **50,** 155–157.

Berge, T. O. (ed.) (1975). International Catalogue of Arboviruses. DHEW *Publ.* (CDC) (U.S.) **75,** 8301.

Bres, P. (1981). *Contrib.* Epidemiol. *Biostat.* (Karger, Basel) **3,** 178–190.

Bres, P., Chambon, L., Pape, Y., and Michel, R. (1963). *Bull. Soc. Med. Afr. Noire Lang. Fr.* **8,** 710–712.

Brown, J. I ., Dominik, J. W., and Morissey, R. L. (1981). *Infect. Immun.* **33,** 848–853.

Calisher, C. H., Nuti, M., Lazuick, J. S., Ferrari, J. D., and Kappus, K. D. (1981). *Bull. W.H.O.* **59,** 619–622.

Carey, D. E. (1971). *J. Hist. Med.* **26,** 243–262.

Carey, D. E., Myers, R. M., DeRanitz, C. M., Jadhav, M., and Reuben, R. (1969). *Trans. R. Soc. Trop. Med. Hyg.* **63,** 434–445.

Carey, D. E., Causey, O. R., Reddy, S., and Cooke, A. R. (1971). *Lancet* **1,** 105–106.

Cash, P., Robeson, G., Erlich, B. J., and Bishop, D. H. L. (1981). Contrib. *Epidemiol. Biostat.* (Karger, Basel) **3,** 1–20.

Chippaux, A., and Chippaux-Hyppolite, C. (1968). *Med. Trop. (Marseille)* **28,** 345–362.

Christie, J. (1881). *Glasgow Med. J.* **16,** 161–176.

Clarke, D. H., and Casals, J. (1965). *In* "Viral and Rickettsial Infections of Man" (F. L. Horsfall and I. Tamm, eds.), pp. 606–658. Lippincott, Philadelphia, Pennsylvania.

Cordellier, R., Bouchite, B., Roche, J.-C., and Akoliba, P. (1982). *Med. Trop. (Marseille)* **42**, 269–273.

Cornet, M., Robin, Y., Herve, G., Adam, C., Renaudet, J., Valade, M., and Eyraud, M. (1979). *Med. Maladies Infect.* **9**, 63–66.

Daubney, R., Hudson, J. R., and Garnham, P. C. (1931). *J. Pathol. Bacteriol.* **34**, 545–579.

Digoutte, J.-P., Cordellier, R., Robin, Y., Pajot, F. X., and Geoffroy, B. (1974). *Ann. Microbiol. (Paris)* **125B**, 107–118.

Eddy, G. A., Peters, C. J., Meadors, G., and Cole, F. E., Jr. (1981). *Contrib. Epidemiol. Biostat.* (Karger, Basel) **3**, 124–141.

Erasmus, B. J., and Coetzer, J. A. W. (1981). *Contrib. Epidemiol. Biostat.* (Karger, Basel) **3**, 77–82.

Fagbami, A. H., and Fabiyi, A. (1975). *Trop. Geogr. Med.* **27**, 59–62.

Fagbami, A. H., Monath, T. P., and Fabiyi, A. (1977). *Trans. R. Soc. Trop. Med. Hyg.* **71**, 60–65.

Filipe, A. R., Pinto, M. R., Serra, M. L., and Champalimaud, J. L. (1973). *Trans. R. Soc. Trop. Med. Hyg.* **67**, 400–404.

Germain, M., Saluzzo, J. F., Cornet, J. P., Herve, J.-P., Sureau, P., Camicas, J. L., Robin, Y., Salaun, J.-J., and Heme, G. (1979). *C. R. Hebd. Seances Acad. Sci. Ser. D* **239**, 635–637.

Germain, M., Francy, D. B., Monath, T. P., Ferrara, L., Bryan, J., Salaun, J.-J., Herve, G., Renaudet, J., Adam, C., and Digoutte, J.-P. (1980). *Am. J. Trop. Med. Hyg.* **29**, 929–940.

Germain, M., Cornet, M., Mouchet, J., Herve, J.-P., Robert, V., Camicas, J. L., Cordellier, R., Digoutte, J.-P., Monath, T. P., Salaun, J.-J., Deubel, V., Robin, Y., Coz, J., Tauffbels, R., Saluzzo, J. F., and Gonzales, J. P. (1981). *Med. Trop. (Marseille)* **41**, 31–43.

Haddow, A. J., Davies, C. W., and Walker, A. J. (1960). *Trans. R. Soc. Trop. Med. Hyg.* **54**, 517–522.

Hammam, H. M., and Price, W. H. (1966). *Am. J. Epidemiol.* **83**, 113–122.

Henchal, E. A., McCown, J. M., Seguin, M. C., Gentry, M. K., and Brandt, W. E. (1983). *Am. J. Trop. Med. Hyg.* **32**, 164–169.

Henderson, B. E., Kirya, B. G., and Hewitt, L. E. (1970). *Bull. W.H.O.* **42**, 797–805.

Inst. Pasteur Dakar (1981). Annu. Rep.

Johnson, B. K., Chichogo, A., Gitau, G., Patel, N., Ademba, G., Kirui, R., Nighton, R. B., and Smith, D. H. (1981). *Trans. R. Soc. Trop. Med. Hyg.* **75**, 239–241.

Johnson, B. K., Musoke, S., Ocheng, D., Gichogo, A., and Rees, P. H. (1982). *Lancet* **1**, 208–209.

Jupp, P. G. (1976). *Mos. News* **36**, 166–173.

Jupp, P. G., McIntosh, B. M., Dos Santos, I., and De Moor, P. (1981). *Trans. R. Soc. Trop. Med. Hyg.* **75**, 15–19.

Lvov, D. K., Skvortsorv, T. M., Kondrashina, N. G., Vershinsky, B. V., Lesnikov, A. L., Derovyansky, V. S., Berezina, L. K., Gromashevsky, V. L., Andrianova, D. P., and Yakovlev, V. I. (1982). *Vopr. Virusol.* **6**, 50–52.

McCrae, A. W. R., Henderson, B. E., Kirya, B. G., and Sempala, S. D. K. (1971). *Trans. R. Soc. Trop. Med. Hyg.* **65**, 152–168.

McIntosh, B. M., and Jupp, P. G. (1981). *Contrib. Epidemiol. Biostat.* (Karger, Basel) **3**, 92–99.

McIntosh, B. M., Paterson, H. E., McGillivray, G., and DeSousa, J. (1964). *Ann. Trop. Med. Parasitol.* **58**, 45–51.

McIntosh, B. M., and Jupp, P. G., Dos Santos, I., and Menehan, G. M. (1976). *S. Afr. J. Sci.* **72**, 295–300.

Meegan, J. M. (1981). Contrib. *Epidemiol. Biostat.* (Karger, Basel) **3**, 110–113.

Meegan, J. M., Khalil, G. M., Hoogstraal, H., and Adham, F. K. (1980). Am. J. Trop. Med. Hyg. **29**, 1405–1410.

Metselaar, D., Grainger, C. R., Oei, K. G., Reynolds, D. G., Pudner, M., Leake, C. J., Tukei, P. M., D'Offay, R. M., and Simpson, D. I. H. (1980). *Bull. W.H.O.* **58**, 937–943.

Monath, T. P., Smith, E. A., Onejeme, S. E., Okeke, G. N., Francis, T. I., and Fabiyi, A. (1972). *Niger. Med. J.* **2**, 178–186.

Monath, T. P., Wilson, D. C., Lee, V. H., Stroh, G., Kuteyi, K., and Smith, E. A. (1973). *Bull. W.H.O.* **49**, 113–121.

Monath, T. P., Lee, V. H., Tomori, O., and Wilson, D. (1974). *Trans. R. Soc. Trop. Med. Hyg.* **68**, 30–38.

Monath, T. P., Cropp, C. B., Bowen, G. S., Kemp, G. E., Mitchell, C. J., and Gardner, J. J. (1980). *Am. J. Trop. Med. Hyg.* **29**, 948–962.

Monath, T. P., Brinker, K. R., Chandler, F. W., Kemp, G. E., and Cropp, C. B. (1981). *Am. J. Trop. Med. Hyg.* **30**, 431–443.

Moore, D. L., Reddy, S., Akinkugbe, F. M., Lee, V. H., David-West, T. S., Causey, O. R., and Carey, D. E. (1974). *Ann. Trop. Med. Parasitol.* **68**, 59–68.

Odelola, H. A., and Fabiyi, A. (1978). *Arch. Virol.* **58**, 291–295.

ORSTOM (1982). *Inst. Pasteur, Abidjan, Ivory Coast Annu. Rep.*

Parks, J. J., Ganaway, J. R., and Price, W. H. (1958). *Am. J. Hyg.* **68**, 106–119.

Peters, C. J., and Anderson, G. W., Jr. (1981). *Contrib. Epidemiol. Biostat.* (Karger, Basel) **3**, 21–41.

Rentier-Delrue, F., and Young, N. A. (1980). *Virology* **106**, 59–70.

Ross, R. W. (1956). *J. Hyg.* **54**, 177–191.

Schlesinger, J. J., Brandriss, M. W., and Monath, R. P. (1983). *Virology* **125**, 8–17.

Shope, R. E., Peters, C. J., and Walker, J. S. (1980). *Lancet* **1**, 886–887.

Shope, R., Peters, C. J., and Davies, F. G. (1982). *Bull. W.H.O.* **60**, 299–304.

Shore, H. (1961). *Trans. R. Soc. Trop. Med. Hyg.* **55**, 361–373.

Skogh, M., and Espmark, A. (1982). *Lancet* **1**, 795–796.

Taylor, R. M., Work, T. H., Hurlbut, H. S., and Rizk, F. (1956). *Am. J. Trop. Med. Hyg.* **5**, 579–620.

Tomori, O., Fagbami, A., and Fabiyi, A. (1975). *Trop. Geogr. Med.* **27**, 413–417.

Tomori, O., Fagbami, A., and Fabiyi, A. (1978). *Trans. R. Soc. Trop. Med. Hyg.* **72**, 103–104.

Trent, D. W., Monath, T. P., Bowen, G. S., Vorndam, A. V., Cropp, C. B., and Kemp, G. E. (1980). *Ann. N.Y. Acad. Sci.* **354**, 219–237.

Trent, D. W., Grant, J. A., Rosen, L., and Monath, T. P. (1983). *Virology* (in press).

Umrigar, M. D., and Pavri, K. M. (1977). *Indian J. Med. Res.* **65**, 603–612.

Van Velden, D. J. J., Meyer, J. D., Olivier, J., Gear, J. H. S., and McIntosh, B. (1977). *S. Afr. Med. J.* **51**, 867–871.

Williams, M. C., Woodall, J. P., Corbet, P. S., and Gillet, J. D. (1965). *Trans. Roy. Soc. Trop. Med. Hyg.* **59**, 300–306.

Wills, W. M., Jakob, W. L., Francy, D. B., Oertley, R. E., Anani, E., Calisher, C. H., and Monath, T. P. (1984). *Trans. R. Soc. Trop. Med. Hyg.* (in press).

WHO (1981). *Weekly Epidemiological Record* No. **27**, 212.

Yedloutschnig, R. J., Dardiri, A. H., and Walker, J. S. (1981). *Contrib. Epidemiol. Biostat.* (Karger, Basel) **3**, 53–59.

25

Congenital Viral infections and Malformations: Problems, Prophylaxis, and Diagnosis

P. D. GRIFFITHS* AND W. AL-NAKIB†

**Virus Laboratory, Royal Free Hospital and School of Medicine, Hampstead, London, England*

†Department of Microbiology, Faculty of Medicine, Kuwait University, Safat, Kuwait

ISBN 0-12-429601-7

I. INTRODUCTION

Most women become infected with several viruses during pregnancy, but most of these have no discernible effect on fetal well-being. However, in some cases, a maternal virus infection can have devastating effects on the normal development of the fetus and can produce damage incompatible with continuing intrauterine life. In other cases the infection may impair fetal organogenesis without inducing fatal lesions, with the result that gross congenital malformations are apparent at birth. In yet more cases, the virus may gain access to and infect the fetus but may take longer to produce clinically apparent lesions so that the neonate appears to be normal but is destined to develop adverse sequelae during infancy or childhood. Finally, a fetus may be infected *in utero,* may be excreting virus at birth, and may continue to excrete virus for many months or years, yet can remain entirely asymptomatic. Fortunately, this last presentation appears to be rather more common than the preceding ones.

It would be useful to be able to explain these various clinical presentations in terms of the pathogenetic factors involved, but, unfortunately, this knowledge is not available at present. However, to illustrate the complexity of the problem, some putative pathological factors can be listed: the type of infection (primary or recurrent), the stage of gestation, the quantity and quality of the maternal and fetal immune responses, the ability of the virus to cross the placenta, the dose of virus transmitted *in utero,* and the tissue tropisms of the virus. From this list, it will be apparent that the lessons learned about the effects of one virus on the developing fetus cannot necessarily be assumed to apply to another virus. Furthermore, when one considers that some of the viruses concerned tend to segregate within certain socioeconomic subsections of a population, it will be obvious that attempts to control each virus must be based upon different strategies in different countries.

In this chapter we attempt to illustrate some of these points by reference to four different viruses: rubella, cytomegalovirus, varicella-zoster, and influenza. These four have been chosen because they illustrate the wide spectrum of viruses proved or thought to cause congenital infection. Rubella is the simplest example; its pathogenesis is sufficiently well understood to allow prophylactic measures to have been introduced. Cytomegalovirus has a more complex natural history, which is only just becoming unraveled to present the possibility that immunization may become a practical proposition within a few years. Varicella-zoster is an example of a definite fetal pathogen which damages so rarely that widespread immunization could not be justified for this reason alone. Finally, influenza is a virus whose fetal pathogenicity, if any, is a subject of current controversy.

II. RUBELLA

The brilliant clinical observations of the ophthalmologist Sir Norman Gregg, which led to the identification of the teratogenic properties of rubella virus (Table I), are well known. Suffice it to say that his description (Gregg, 1941) of the association between maternal German measles in early pregnancy and the development of congenital cataracts, with or without heart defects, is a classical publication, which also contains the following remarkably prophetic statement: "It is difficult to forecast the future for these unfortunate babies. We cannot at this stage be sure that there are not other defects present which are not evident now but which may show up as development proceeds."

Thus, although the teratogenic effects of rubella were the most obvious at birth, Gregg suspected that further lesions, best termed congenital defects (Hanshaw and Dudgeon, 1978), might appear with prolonged clinical follow-up. This was subsequently shown to be the case when, following the extensive rubella pandemic of 1964–1965, more clinical sequelae (Table I) of congenital rubella syndrome (CRS) were noted. These lesions are not teratogenic in nature but probably result from continuing lytic virus replication in the target organs concerned together with a degree of immune mediated cytolysis.

Rubella Vaccination

The 1964–1965 pandemic had a further effect in that it stimulated research into rubella infection with the result that, at the remarkably early time of 1969, the first live attenuated rubella vaccine was licensed in the United States. The rationale behind the use of this vaccine was that only primary maternal rubella infection could lead to intrauterine transmission, and it was assumed that the presence of maternal virus-specific antibody

TABLE I
Major Clinical Manifestations of Congenital Rubella Syndrome

Teratogenic	
	Eye: Cataract
	Heart: Patent ductus arteriosus, pulmonary artery lesions, aortic stenosis, ventricular septal defect
Lytic/immunopathological	
	Ear: Sensorineural deafness
	CNS: Mental retardation, late-onset panencephalitis
	General: Intrauterine growth retardation, thrombocytopenic purpura, pneumonitis, osteopathy, chronic rash

was the factor responsible for fetal protection. This assumption has turned out to be correct, and the vaccines currently available have been shown to be highly successful at preventing CRS in the offspring of those women who receive them. The problem remains, however, who should be given the vaccine? This remains a highly controversial subject which requires detailed consideration. Essentially, there have, to date, been two main approaches, which may be termed the United States and the United Kingdom strategies.

1. The United States Strategy

The United States strategy recognizes that many pregnant women become infected through contact with children and aims to interrupt this transmission by rendering most children immune before they reach school age. Following an initial vaccination program aimed at all prepubertal children, the United States strategy has settled into a maintenance phase where it is aimed to vaccinate all children aged around 15 months.

It must be accepted that this program has been remarkably successful in reducing the incidence of reported rubella in the general population of the United States (Hinman *et al.*, 1983). However, as these authors accept, the reduction in the number of cases of CRS has as yet been less impressive than the reduction in the overall incidence of postnatal rubella. While outbreaks of congenital rubella continue to occur in socially disadvantaged sections of urban communities (Lamprecht *et al.*, 1982), the United States approach cannot be said to have been entirely successful.

Furthermore, it is possible that the initial success of the United States strategy could be followed by a long-term failure when the noncompliant children, and those who fail to respond to the vaccine, reach childbearing age, since a pool of young girls who enter pregnancy unprotected against rubella might be produced (Knox, 1980). In fact, there has been some increase in the incidence of reported rubella in teenagers in the United States; although the figures appear to be declining again (Hinman *et al.*, 1983), this is obviously a factor which must be kept under constant review.

2. The United Kingdom Strategy

The United Kingdom strategy does not aim to interrupt rubella transmission within the community but hopes to prevent CRS by immunizing a target population of females about to reach childbearing age. This involves the vaccination of all girls aged 10–14 years, irrespective of their serological status. The fact that 70–80% of these girls will already have experienced natural rubella is considered an advantage, since natural immunity to rubella is known to be lifelong whereas vaccine-induced immunity has been monitored for only a little over 10 years.

One disadvantage of the United Kingdom strategy is the time lag before

a reduction in CRS would be expected. Immunization was begun in 1970 in girls aged around 13 years. The mean age of women attending for antenatal care is around 26 years, so by 1983 we should, in theory, have induced immunity in the younger half of our antenatal population. In fact, vaccine uptake has been poor, and epidemics of CRS have continued to occur despite large increases in the number of therapeutic abortions performed on women with laboratory-confirmed rubella infection (Banatvala, 1982).

3. *Modified Strategies*

In practice, the stated aims of the United States and United Kingdom strategies have altered over the years. In the United Kingdom, special emphasis was placed in 1974 on the vaccination of adult women, and since that time vaccine has been widely available through general practitioners, occupational health clinics, and family planning clinics. Before vaccinating these adult women it was considered prudent to determine their serological status. By this means, vaccine would actually be given to only the small seronegative minority (10–15%) of women requesting it, so that vaccination of women who were, unknowingly, in the early stages of pregnancy could be kept to a minimum. It is now becoming clear that, although rubella vaccine virus can infect fetal tissues, the theoretical risk of producing CRS is low (Centers for Disease Control, 1982). If more extensive data from women who elect to go to term following vaccination in early pregnancy become available for the least attenuated RA27/3 vaccine strain, then it should be possible to dispense with our requirement for a preliminary screening test for adult women.

In the United States the vaccination effort has intensified over the last few years as peripubertal schoolchildren have been given combined rubella–measles vaccine. This deviation from the original aim of the United States strategy has probably contributed to the dramatic decline seen in the reported incidence of rubella (Hinman *et al.*, 1983). Thus, the contrasts between the United States and United Kingdom strategies have lessened progressively, and it has been suggested recently that we should consider a somewhat more rational approach which combines features of both strategies (Hinman *et al.*, 1983). Hinman *et al.* are now convinced that in the United States a one-time widespread vaccination of all women of childbearing age followed by the continuation of the maintenance vaccination of all 15-month-old children would provide a "complete program" for the prevention of CRS. In the United Kingdom strategy would be initially to vaccinate all women of childbearing age together with all children and then settle into a maintenance schedule of immunization of all children as they reached 15 months of age.

We must admit to being rather sceptical about the efficacy of such a

program. The problem in the United Kingdom is poor uptake of the vaccine, and we doubt whether altering the theoretical strategy would persuade many complacent people to attend for vaccination. The United Kingdom population currently exhibits a perverse refusal to volunteer themselves, or their children, for prophylactic measures. Unless this aspect of human nature shows a distinct improvement, it seems unlikely that any vaccination program will be completely effective.

4. A Neglected Alternative

One additional way of increasing immunity in women of childbearing age is to screen serologically those women attending antenatal clinics and to vaccinate, after delivery, those shown to be susceptible. The efficacy of this approach has only recently been evaluated and, much to our surprise, was shown to be remarkably effective (Griffiths and Baboonian, 1982). One thousand women were studied when they returned for further antenatal care after having had rubella screening during a previous pregnancy. Of the 108 women who were initially seronegative, 90 (83%) had been rendered immune and so were protected during their subsequent pregnancies. The 18 program failures were attributed to false-positive hemagglutination inhibition reactions (8 cases), failure to administer vaccine (7 cases), and true vaccine failures (3 cases).

As the single radial hemolysis test has now improved the screening system, even better results should be attainable in the future. Although this approach cannot protect primigravidas it could reduce the number of cases of CRS since, in the United Kingdom, 60% are born to women who have had a previous pregnancy (Peckham, 1978). We believe, therefore, that this part of the vaccination program should be encouraged and expanded, especially since it has the pragmatic advantage of providing a captive population, who, in our experience, are very unlikely to refuse vaccination once its importance has been explained.

5. Strategies for Developing Countries

While the debate continues over the optimum rubella vaccination program to be used in developed countries, our colleagues in developing countries are beginning to formulate their own programs. It is essential that such decisions be based upon the local prevalence of rubella infection, since this may be quite different from that found in other parts of the world.

In general, around 80% of women residing in urban areas in Europe, the United States, Canada, Australia, South America, and the Middle or Far East are immune to rubella by the time they enter the childbearing age (Rawls *et al.*, 1967; Dowdle *et al.*, 1970; Saidi, 1972; Khalifa *et al.*, 1973). In island communities such as Trinidad, Jamaica, Hawaii, Japan, and Ma-

laysia, much lower prevalence rates averaging only 50% have been reported (Rawls *et al.*, 1967; Halstead *et al.*, 1969; Dowdle *et al.*, 1970; Lam, 1972). For all these countries, a rubella vaccination program would be expected to be well worthwhile. In contrast, in Kuwait 95% of adult women are already immune to rubella so that a program of vaccinating the whole community would not be necessary for the prevention of CRS (Hathout *et al.*, 1978). Since these authors also report that premarital sexual intercourse is almost nonexistent, so that illegitimate births are rare, rubella screening as a routine premarital test would appear to be the basis of an efficient and cost-effective vaccination program. This is an excellent example of adaptation of a program to the local needs of a country. However, as the authors acknowledge, it will be necessary to keep the social as well as the virological characteristics of the population under surveillance, since a liberalization of sexual mores could profoundly affect the efficacy of such a program.

III. CYTOMEGALOVIRUS

Cytomegalovirus (CMV) was first isolated in 1956 from a neonate with the severe form of congenital infection termed "cytomegalic inclusion disease" (Smith, 1956; Rowe *et al.*, 1956; Weller *et al.*, 1957). From that moment it was assumed by many workers that the pathogenesis and natural history of CMV infection would be similar to that of rubella and that control of this infection would be accomplished readily by vaccination. Unfortunately, these assumptions have turned out to be false, and CMV is now known to differ from rubella in many respects, as described below.

A. CMV Is Not a Teratogen

Unlike rubella, CMV does not impair normal organogenesis, so that structural malformations are not produced. The damage attributable to intrauterine CMV infection is largely the result of lytic infection in target organs such as the brain, ear, and bone marrow together with responses in organs with immunological function. Thus, signs such as microcephaly, cerebral calcification, sensorineural deafness, thrombocytopenic purpura, and hepatosplenomegaly are classical manifestations of the infection.

Although the clinical presentation is striking and has therefore received much attention, cytomegalic inclusion disease probably represents only 5% of all cases of congenital CMV infection (Hanshaw and Dudgeon, 1978). A further 5% of infected neonates exhibit some nonspecific signs at birth, but the vast majority are completely asymptomatic (Alford *et al.*, 1980). Despite this clinically silent presentation a certain proportion, perhaps 15–

20%, of these neonates will develop symptoms during infancy or early childhood, progressive damage to the brain and ear being paramount (Melish and Hanshaw, 1973; Reynolds *et al.*, 1974; Stagno *et al.*, 1975; Hanshaw *et al.*, 1976; Alford *et al.*, 1980; Saigal *et al.*, 1982). Thus, long-term follow-up of infected infants is required to identify those who will ultimately suffer as a result of CMV transmission *in utero*.

Although the lesions produced by CMV infection may be described generally as lytic, it is becoming clear that several different but interrelated, mechanisms can produce this cell destruction. One might imagine that the greater the initial dose of virus transmitted *in utero,* the greater would be the chance of CMV being disseminated to, and initiating infection in, multiple fetal tissues. To prove this, virus titers in biopsies taken from target organs should be determined, but this is clearly impractical due to their intrauterine location. The best that can be done is to quantitate the viruria found in samples collected soon after birth. This approach has the theoretical disadvantages of not testing the target organs and of obtaining the specimens many weeks after the initial virus insult. Nevertheless, it has been shown that symptomatic neonates have, in general, a higher titer of virus in the urine than do those who are asymptomatic (Stagno *et al.*, 1975). This difference undoubtedly reflects intrauterine replication of virus, as well as the level of the initial viral inoculum, but clearly implicates lytic virus infection as being responsible for some of the clinically apparent defects.

In addition, it has been well documented that cord sera from symptomatic babies show evidence of heightened fetal immune response to CMV, measured by cord blood titers of specific IgM antibodies, levels of total IgM, and the presence of rheumatoid factor (Griffiths *et al.*, 1982a). Since, in the same study, it was shown that the level of fetal humoral immune response was not simply a reflection of the level of antigenic stimulation, it is suggested that immune-mediated lysis of cells may also be important in the pathogenesis of some tissue damage. It is also known that immune complexes are found in sera from congenitally infected babies. Although similar complexes are found also in children with perinatal infection, who are asymptomatic, it is possible that they may contribute to the pathogenesis of this infection (Stagno *et al.*, 1977a).

B. Infection Is Asymptomatic in Pregnant Women

Unlike the case with rubella, most women delivering a baby congenitally infected with CMV do not report an illness during pregnancy (Starr, 1970). Primary CMV infection during pregnancy may produce heterophile-negative infectious mononucleosis, but this occurs only rarely (Stern and Tucker, 1973; Griffiths *et al.*, 1980a; Grant *et al.*, 1981; Griffiths, 1981;

Ahlfors *et al.*, 1982). Thus it is instructive to note that if Gregg had been presented with several cases of congenital CMV, rather than congenital rubella infection, he would have been unable to discern the underlying viral etiology of the condition due to the lack of maternal history.

Since the infection is entirely asymptomatic, maternal infections have to be detected by laboratory surveillance programs. The usual approach is to detect primary CMV infections by monitoring the initially seronegative women for seroconversions as pregnancy progresses. This is technically simple to perform and such studies have been carried out, using reliable complement-fixation tests, by several groups of workers (Stern and Tucker, 1973; Griffiths *et al.*, 1980a; Grant *et al.*, 1981; Ahlfors *et al.*, 1982). The major disadvantage of this approach is that maternal primary infections cannot be detected early in pregnancy since most women have already completed the first trimester before they present for blood to be drawn at the antenatal clinic. Fortunately this defect can now be overcome by testing these acute sera by radioimmunoassay for CMV-specific IgM, since the presence of this class of antibody in a single serum is clearly diagnostic of recent primary infection (Griffiths, 1981; Griffiths *et al.*, 1982b).

C. Trimester of Fetal Vulnerability

The general assumption that CMV would behave like rubella is reflected in an editorial which states that maternal infection early in pregnancy represents the greatest risk to the fetus (Editorial, 1977). At the time the editorial was written, no cases of primary CMV infection had been identified in the first trimester. Now that a small number of cases has been detected, the speculation of the editorial writer has been confirmed, but only in a general sense as described below.

The results presented in Table II show that, overall, CMV is transmitted *in utero* in about one-third of cases and that transmission is not more likely early in pregnancy. Despite this, in the United Kingdom population, fetal death has resulted frequently (5/23; 22%) from maternal infection in the early stages of pregnancy but has not occurred following maternal seroconversion late in pregnancy (0/28 cases). When these figures are compared to a fetal loss rate of 3% in the control group, it will be clear that the fetus is indeed most susceptible to early primary maternal infection. However, it appears that the vulnerability is manifest in terms of fetal loss rather than in the production of severe congenital defects as originally predicted. Thus, it would appear that the birth of the potentially most severely affected individuals is being naturally prevented, while no adverse sequelae have yet developed in those babies who were born alive following first trimester maternal infection.

TABLE II
Relationship between the Timing during Pregnancy of Primary Maternal CMV Infection and Intrauterine Transmission of Virus

Reference	No. of CMV infections detected		No. of babies[a] congenitally infected/No. born to women infected in each trimester of pregnancy			
	Primary maternal	Congenital	First	Second	Third	Either second or third
Stern and Tucker (1973)	11	5 (45%)	0	4/6[b]	1/5	0
Griffiths *et al.* (1980a)[c]	27	7 (26%)	0	0/11	6/15	1/1
Grant *et al.* (1981)	13	5 (38%)	0	3/4	2/7	0/2
Griffiths, (1981)[d]	23	7[a] (30%)	3/12	4/11	0	0
Ahlfors *et al.* (1982)	14	6 (43%)	0	1/1	3/8	2/5
Total	88	30 (34%)	3/12 (25%)	12/33 (36%)	12/35 (34%)	3/8 (38%)

[a]Includes five cases of fetal loss.
[b]Two possible first trimester infections.
[c]Results are updated to July 1982. Five further cases were excluded because the neonates were not tested for congenital infection.
[d]Three further cases where the neonates were not tested for congenital CMV infection are not included.

These results do not support the further contention in the same editorial (Editorial, 1977) that women with early primary infections should be offered termination of pregnancy. Cytomegalic inclusion disease may be the result of primary maternal infection early in pregnancy (Stagno *et al.*, 1982), but the risk of any individual fetus developing this severe syndrome appears to be so low that intervention could not be justified. Furthermore, in our series, severe sequelae have resulted, in two cases, from maternal infection in the late second and early third trimesters so that, unlike rubella, termination of pregnancy is definitely not capable of controlling the childhood damage attributable to congenital CMV infection.

Clearly, further cases need to be identified to provide more reliable data on which to base an emotive decision like the recommendation of termination of pregnancy. Any recommendation made today could rapidly become outdated if we came to understand how two-thirds of women prevent CMV from being transmitted *in utero* (Table II). If we could identify such

fortunate women, we might have the basis for labeling the others as being at high risk of delivering a baby affected by CMV. For example, women delivering congenitally infected babies appear to have impaired cell-mediated immunity, as measured by responses in lymphocyte transformation assays (Gehrz *et al.*, 1977; Reynolds *et al.*, 1979; Starr *et al.*, 1979). It is tempting to speculate that this impairment may have predisposed them to allow intrauterine transmission, with the corollary that women with normal lymphocyte transformation responses may be able to protect their fetuses from congential infection. This hypothesis seems plausible but must be confirmed by carefully controlled studies. The example of the *Lancet* editorial discussed above should illustrate that the natural history of congenital CMV infection is so complex that our therapeutic strategies must be based upon hard data, not upon theoretical considerations, however plausible such theories may appear to be at first glance.

D. Type of Maternal Infection

Another assumption made about CMV infection was that, like rubella, only primary maternal infection could produce intrauterine transmission (Starr and Gold, 1969; Stern and Tucker, 1973; Grant *et al.*, 1981). This is clearly not so, since individual case reports have documented congenital CMV infection in consecutive siblings (Embil *et al.*, 1970; Krech *et al.*, 1971; Stagno *et al.*, 1973; Yeager *et al.*, 1977; Griffiths and Baboonian, 1984). Clearly, the second fetus in each case cannot have been infected *in utero* by primary maternal infection, since its mother was known to have been infected at the time of her earlier pregnancy. In some cases, strains of CMV from the siblings have been typed by restriction endonuclease mapping (Huang *et al.*, 1980) and have been shown to be identical, thus implicating either reactivation of latent virus or maternal reinfection with homologous virus during pregnancy.

Far from being a rare event, these recurrent (nonprimary) maternal infections seem to be the predominant means by which intrauterine transmission of virus occurs in the one population which has been studied so far (Stagno *et al.*, 1977b). The important point is whether childhood damage can result from this type of transmission *in utero,* and there is now some evidence to suggest that intrauterine infection resulting from primary maternal infection may have a greater pathological potential for the fetus than does recurrent maternal infection (Stagno *et al.*, 1982). This difference is clearly not absolute, since a single case report has shown that sequelae can result from recurrent maternal infection with transmission *in utero* (Ahlfors *et al.*, 1981). It now remains to be shown how frequently damage, either overt or more subtle, results from this type of infection.

It is important that studies comparing the relative contributions of primary and recurrent maternal infections to the overall incidence of congenital infection are carried out in different parts of the world. Developing countries, or areas of low socioeconomic status within developed countries, have a high incidence of congenital infection despite having a high prevalence of CMV-IgG antibodies among women of childbearing age (Li and Hanshaw, 1967; Monif *et al.*, 1970; Stern and Tucker, 1973; Stagno *et al.*, 1977b; Schopfer *et al.*, 1978; Cabau *et al.*, 1979). In such populations, congenital CMV infection will result predominantly from recurrent infection, and, since this may have a low risk of causing fetal damage (Stagno *et al.*, 1982), these babies may be relatively spared from adverse sequelae. In contrast, developed countries, or local areas of high socioeconomic status, have a lower overall incidence of congenital infection, but more of these will have resulted from the relatively more pathogenic primary maternal infection. We thus have a paradox, where damage attributable to congenital CMV infection may be most apparent in those populations which have the lowest incidence. This situation is somewhat reminiscent of poliomyelitis, where increasing standards of hygiene resulted in a decrease in prevalence of infection but a paradoxical increase in adult paralysis. The two examples are similar in that adverse effects result from infection occurring in adults, not from infection in childhood.

It must be clear, from the foregoing oversimplified version of the natural history of congenital CMV infection, that much more work will be needed before a vaccination strategy can be planned. It might be thought, by analogy with poliomyelitis, that natural childhood CMV infection should be encouraged as a means of ensuring that most females enter the childbearing years with previous immunological experience of CMV infection. However, as mentioned above, communities with this pattern of acquisition of CMV infection have a high incidence of recurrent maternal infection with intrauterine transmission, which is not entirely without risk to the fetus (Ahlfors *et al.*, 1981). Perhaps CMV is like hepatitis B virus in that infection during infancy, when the immune system is immature, may lead to poor control of virus replication, failure to eradicate the virus, and subsequent reactivation of latent CMV when pregnancy supervenes many years later.

IV. VARICELLA-ZOSTER VIRUS

Chickenpox occurs frequently during childhood so that most females have already been infected by the time they reach childbearing age. Nevertheless, chickenpox does still occur with some regularity during pregnancy. The clinical diagnosis of chickenpox is reliable enough to provide a good esti-

mate of the incidence of this infection, and one study estimated this to be 0.7 per thousand pregnancies (Sever and White, 1968). The clinical effects resulting from intrauterine transmission of varicella-zoster virus can be conveniently classified according to the stage of pregnancy at which maternal chickenpox occurs. As far as is known, recurrent varicella-zoster infection (shingles) during pregnancy has no adverse effects on the fetus.

A. Early Infection

Cases of congenital varicella syndrome have resulted from maternal chickenpox occurring between 8 and 19 weeks of pregnancy (Frey *et al.*, 1977; Hanshaw and Dudgeon, 1978; Brunell and Kotchmar, 1981). All 10 cases reported so far had cutaneous scars often appearing in a dermatomal distribution. In some cases extensive healing by fibrosis has been associated with limb hypoplasia or paralysis. The babies have been of rather low birth weight, and some have exhibited mental retardation or chorioretinitis. Clearly, the virus has caused extensive tissue damage in these fetuses, and it is not surprising to find a mortality during infancy of around 50%.

Fortunately, this severe syndrome occurs only rarely and should be put into perspective by emphasizing that over 400 cases of maternal chickenpox, at varying stages of pregnancy, have been identified in different studies and that not one case of congenital varicella syndrome has occurred (Bradford Hill *et al.*, 1958; Manson *et al.*, 1960; Siegel, 1973). Thus, it appears that the syndrome is a serious, but rare, manifestation of maternal infection, and so it seems reasonable to reassure pregnant women who contract chickenpox during early pregnancy.

B. Late Infection

Maternal chickenpox at 6–7 months of pregnancy has been associated with zoster developing in infancy or early childhood. It is interesting to note that these children had not had an episode of chickenpox prior to their zoster, which provides strong evidence for intrauterine acquisition of the virus (Brunell and Kotchmar, 1981). The fact that zoster developed during infancy in these cases, rather than the more usual late adult age, suggests that productive virus infection is not as well suppressed following intrauterine transmission as it is following postnatal chickenpox (Brunell and Kotchmar, 1981).

Zoster during infancy does not result only from intrauterine infection late in pregnancy. It may occur as a late event in children who have been infected at an early stage of gestation and who have congenital varicella syndrome (Dodian-Fransen *et al.*, 1973; Brunell and Kotchmar, 1981).

C. Infection Close to Term

Maternal chickenpox occurring in the few days before delivery can have serious consequences for the neonate with the development of a severe disseminated infection which may be fatal. In most cases, however, this severe disease does not develop, and it is now clear that the outcome is related to the timing between the onset of chickenpox and the delivery of the baby (Gershon, 1975).

If delivery occurs less than 5 days after the onset of chickenpox then severe infection may ensue with a case fatality rate of 30%. In contrast, if the time between chickenpox and delivery is greater than 5 days, then severe perinatal infection is avoided and no deaths should be expected (Gershon, 1975). The striking difference between these two outcomes is presumably related to the time taken for maternal humoral immunity to be produced and to passively immunize the fetus. If the time between transmission (viremia and onset of clinical chickenpox) and delivery is short, then virus will have been transmitted *in utero* but specific IgG antibody will not. The neonate is thus born incubating chickenpox with little or no passive immunity from its mother. In contrast, if a longer time is allowed between transmission and delivery then sufficient antibody can be transferred to the fetus to ameliorate the effects of the virus transmitted previously.

V. INFLUENZA

The example of influenza infection has been chosen to illustrate the many difficulties which may be involved when one attempts to demonstrate whether a particular virus is a fetal pathogen.

Several epidemiological studies of influenza infection during pregnancy have been carried out with varying results. Hardy *et al.* (1961) reported an excess of fetal loss, Griffith *et al.* (1972) postulated that influenza infection could cause low birth weight, and several workers have associated the infection with congenital abnormalities (Coffey and Jessop, 1959; Hakosalo and Saxen, 1971). An excess of childhood cancer has also been found in separate studies. Fedrick and Alberman (1972) using data from the 1958 perinatal mortality study found nearly 2000 women who reported clinical influenza during pregnancy. They found an increased incidence of leukemia and neoplasms of lymphatic and hematopoietic tissue but not of other cancers among the offspring and estimated the relative risk to the fetus to be not less than fourfold. Data from an Oxford survey of childhood cancers have similarly been related to maternal influenza. The initial results of Stewart *et al.* (1958) have been updated by Bithell *et al.* (1973) to include over 9000 case/control pairs. This study estimated the risk to the fetus to be 1.5-fold, but the tumors were not restricted to hematopoietic tissue.

These epidemiological studies have provoked much interest but doubt must be expressed about the specificity of a clinical diagnosis of "influenza." Inevitably, many of the volunteers in the study will have reported "influenza-like" illnesses not caused by influenza viruses, whereas others will have had mild clinical attacks of true influenza during pregnancy which they had forgotten. This last point is illustrated by a study in which only 16% of women with serologically proven influenza infection during pregnancy had had an "influenza-like" illness mentioned in their antenatal medical records (Griffiths *et al.*, 1980b). Furthermore, 4% of women without serological evidence of influenza infection reported an "influenza-like" illness during pregnancy. It seems reasonable to conclude that many of the cases reported in epidemiological studies were not suffering from influenza and that most of the women infected by influenza viruses were not identified by the studies.

Nevertheless, the interesting associations found by the epidemiological studies should stimulate us to use more specific virological techniques to test the hypotheses put forward. Intrauterine transmission of influenza viruses has only been documented rarely (Yawn *et al.*, 1971; Ruben *et al.*, 1975) but might occur more frequently than we realize, especially if it is manifest as fetal loss. It is also possible that the infection could have a constitutional effect upon the mother's well-being which could impair fetal nutrition and explain the association with low birth weight. Furthermore, the marked fever which often accompanies cases of influenza might act as a teratogen although this suggestion is controversial (Editorial, 1978; Hunter *et al.*, 1979). A more likely explanation for some of the adverse fetal effects attributed to influenza is the vast quantities and varieties of drugs which people take in an attempt to gain symptomatic relief from this infection (Hakosalo and Saxen, 1971). Rather more difficult to explain is the association between maternal influenza and childhood cancer. Perhaps, by analogy with animal models of leukemogenesis, a human retrovirus infection of a pregnant woman can endow the fetus with the potential to develop leukemia during childhood. If the retrovirus infection produced an "influenza-like" illness, then the association between influenza and childhood cancer could be explained. The possibility that intrauterine events could initiate oncogenicity should not be dismissed since it seems clear that maternal stilbestrol ingestion during pregnancy can produce adenocarcinoma of the vagina many years later when the offspring reach adolescence (Herbst *et al.*, 1971).

Ideally, all these hypotheses should be tested in cohort studies of women with or without laboratory-proven influenza infections. Such studies should attempt to diagnose those infections occurring early in the first trimester in order to detect teratogenic effects. One group of workers has attempted this serologically by using an immunofluorescence test for specific IgM an-

tibodies, but the ability of this technique to provide a retrospective diagnosis has not been proved (Warrell *et al.*, 1981). The only reliable way at present is to wait for the next influenza pandemic, when the presence in the first available antenatal serum of antibodies against the novel virus would provide clear evidence of infection early in pregnancy (Wilson *et al.*, 1959).

Pending the arrival of the next pandemic we have attempted to detect influenza infections serologically in the second and third trimesters in a group of 1600 women (Griffiths *et al.*, 1980b). Overall 77 women were infected, but their babies did not have lower birth weights than those born to the control, uninfected, women. Interestingly, the cases delivered more babies with congenital malformations, even though the infections occurred outside the first trimester. This association has been found before (Hardy *et al.*, 1961), and we wonder if there might be some truth in the suggestion (Leck, 1963) that a woman bearing an abnormal fetus might have an increased susceptibility to influenza.

VI. LABORATORY DIAGNOSIS OF INFECTION

Congenital viral infections with consequent effect on the fetus or the newborn may vary according to the type of virus, the population in question, and whether the virus is in an epidemic or interepidemic state, as is the case, for example, with rubella. Thus, the frequency of congenital cytomegalovirus (CMV) infections may vary between 0.5 and 2.9% depending on location whereas the rate of rubella virus (RV) infection during pregnancy for an epidemic state is 0.4–3.0%, and for an interepidemic state (0.005%) (Stagno *et al.*, 1981). Herpes simplex virus (HSV) infection, on the other hand, has a generally low incidence of infection in the mother (0.15–0.7%), and congenital HSV infections are generally accepted to be rare (Stagno *et al.*, 1981). The other virus of importance with regard to perinatal infection is hepatitis B virus (HBV). Over 170 million people are carriers of hepatitis B surface antigen (HBsAg). Carriership rate and hence the so-called "vertical" transmission vary according to the ethnic group of the population, the region of the world, and whether the mother is hepatitis B e-antigen positive or not (Stevens and Szmuness, 1980). Thus, transmission rates may vary from as low as 6% for a European to as high as 70% for a Chinese in Taiwan (Boxall, 1980). The laboratory diagnosis of congenital viral infection can be summarized in seven different approaches.

1. Direct isolation of virus from tissues and other clinical material from patients
2. Demonstration of characteristic cytology

3. Direct visualization of virus by demonstrating characteristic morphology
4. Direct detection of viral antigens employing specific immunological methods
5. Demonstration of elevated total cord IgM (>20 mg/100 ml) as a nonspecific indicator of intrauterine infection
6. Demonstration of persistence of virus-specific IgG in the infant beyond the life of maternal IgG (i.e., 4–6 months)
7. Demonstration of virus-specific IgM as an indicator of intrauterine viral infection

A. Direct Isolation of Virus

Virus isolation from the patient provides the most specific procedure for the demonstration of intrauterine viral infection. This is particularly the case with cytomegalovirus (Stagno *et al.,* 1980). Thus virus isolation of CMV from the urine of an infant during the first week of life provides the most specific indication of an intrauterine infection, whether primary or recurrent (or reactivation) infection during pregnancy. However, not all laboratories have tissue culture facilities. Furthermore, in the case of CMV, cytopathic effect (CPE) may not be demonstrated before 4–6 weeks after virus inoculation in cell cultures such as those of human foreskin fibroblasts (Stagno *et al.,* 1980). Therefore, cultures cannot be declared negative for at least 6 weeks. This makes such a diagnosis prolonged. Stagno *et al.* (1980) have coupled CMV isolation in human foreskin fibroblast with anticomplement immunofluorescence (ACIF) to detect CMV antigen in cultures as early as 24–72 hr.

With HSV, virus isolation is relatively rapid with clear and characteristic CPE being demonstrated as early as 24 hr after inoculation and in a variety of cell cultures (HEL, HeLa, HEP-2) (Hanshaw and Dudgeon, 1978).

Virus isolation of rubella can be useful in providing evidence of congenital rubella in tissues and material obtained from fetuses following spontaneous abortions, therapeutic abortion, or postmortem (Hanshaw and Dudgeon, 1978). It may also play an important role in providing evidence of congenital rubella in infants infected *in utero* and who may excrete virus for prolonged periods of time (Hanshaw and Dudgeon, 1978). In most other situations, demonstration of rubella virus-specific IgM in the mother's serum during pregnancy or cord or early neonatal serum provides the most specific and rapid method for the demonstration of congenital rubella infection (Pattison, 1978). This may especially be the case with a pregnant contact presenting within the first 12 weeks of pregnancy and thus awaiting a decision as to whether therapeutic abortion should be conducted or not (Pattison, 1978).

B. Demonstration of Characteristic Cytology

Looking for specific changes in cells infected by viruses such as inclusions or multinucleated giant cells has a limited application today, especially since considerable progress has been achieved in the development of more specific procedures for the diagnosis of viral infections. However, interesting and important results have been obtained in certain centers where such procedures have been in routine use. Thus, nuclear hypertrophy and multinucleation with nuclear molding are considered diagnostic for HSV infection (Naib, 1981). The procedure is claimed to be inexpensive, rapid, and simple to perform.

Coleman (1980) and co-workers have also used cytological examination of specimens of urine sediments extensively as a method for primary screening of women who may have had a BK virus (a polyomavirus) infection during pregnancy. These studies, however, remain very limited in their scope and application and hence are conducted only by certain laboratories and certainly require specific virological confirmation before a definitive diagnosis could be reached.

C. Direct Visualization of Virus

Although visualization of virus by demonstrating characteristic morphology is relatively simple provided electron microscopic (EM) facilities are available, it is applicable, at this stage, only to HSV and to a lesser extent CMV with regard to the diagnosis of perinatal infection. Its main advantage is the rapidity with which a diagnosis can be reached. For HSV, scraping from the bottom of vesicular lesions or aspiration of vesicular fluids from vesicles can provide the most suitable material for EM diagnosis provided the samples are taken early. HSV has a characteristic morphology and size. This, coupled with the clinical picture of the patient, can offer a very rapid and simple procedure for the diagnosis of HSV infections.

Diagnosis of CMV by direct visualization of virus in the urine of infected infants, however, has not been very successful, since virus is present in relatively low concentrations and since EM is usually negative if virus concentration is $< 10^6$ particles/ml. Thus direct negative staining of urine and visualization of CMV by EM often would not reveal any virus. However, recent reports on the application of the pseudoreplica EM are more encouraging. Thus, some workers have reported EM positivity rate of about 90% when compared to cell culture (Macris *et al.*, 1981; Stagno *et al.*, 1980). Although the morphology of CMV is similar to that of other herpesviruses and hence cannot be distinguished morphologically from HSV, VZV, or EBV, these latter viruses are rarely excreted in the urine. Nevertheless, this procedure is still not generally applied in many laboratories, especially since sensitivity has been shown to vary from one laboratory to another and the

rate of detection appears to be highly dependent on the expertise of a particular laboratory.

D. Direct Detection of Viral Antigens

The use of specific immunological methods to directly detect viral antigens can be a most interesting, specific, and very rapid method for identifying viruses from infected material obtained from either the mother or the newborn. So far, its application has been mostly limited to the direct detection and identification of HSV in vesicular material and HBsAg in the serum. A variety of procedures have been applied in the direct detection of HSV: immunofluorescence, radioimmunoassay, reverse passive hemagglutination, and ELISA. Most promising are the studies of Vestergaard and Jensen (1981) with their three-layer ELISA procedure, which can not only detect HSV but also type HSV isolates whether HSV-1 or HSV-2. This procedure has been shown to detect HSV at the very small concentration of 0.2 ng and hence provides a procedure far more sensitive than tissue culture.

Typing of HSV isolates can also be done by a number of procedures such as neutralization (NT), radioimmunoassays (RIA), immunofluorescence (IF), or reverse passive hemagglutination (RPHA). Most have been shown to be satisfactory depending on the experience of the particular group and availability of facilities in a particular laboratory.

Direct CMV detection by rapid specific immunological methods in the urine of infants during the first week of life can also provide the most desirable approach in the screening and identifying infants at risk. Such a procedure if available could overcome many of the shortcomings of virus isolation in cell culture, which still presents the most specific method for providing evidence of congenital CMV infection, as was discussed earlier. Early identification of infants at risk may be extremely important not only from the viewpoint of early clinical supervision and hence possible developmental correction, but also from the viewpoint that with the recent advances in the development of more specific and less toxic antivirals, early therapeutic help may prove to be not only possible, but also essential for halting further damage to the newborn by inhibiting viral multiplication. At present there are only two preliminary reports of ELISA procedures that are capable of direct detection of CMV in the urine (Yolken and Stopa, 1980; Sundqvist and Wahren, 1981). Both these procedures, however, would require considerable development and assessment against standard and specific procedures. They would require clinical trials and evaluation. For example, whether CMV could be detected equally well among symptomatic and asymptomatic infected infants, whether the mother had primary or recurrent infection. Furthermore, such procedures should be evaluated in relation to other markers of infection such as virus-specific IgM detection in

the cord, elevated cord IgM or RF, and whether a combination of such markers are more useful in predicting the prognostic outcome of such infants. A number of laboratories are in the process of developing and evaluating such methods with regard to these parameters.

The other virus of significance with regard to perinatal infection and where early identification is very important is HBV (Boxall, 1980). Although it is still not clear whether infection of the fetus or newborn is transplacental or occurs through ingestion of fluids containing the virus during fetal development or during delivery, the newborn becomes infected from the blood of the mother who is HBsAg positive (Boxall, 1980). Early identification of such an HBsAg mother by demonstration of HBsAg and whether she is e-antigen positive or not by RIA or ELISA is essential in order that infants may be given specific hepatitis B immunoglobulin (HBIG) within 48 hr of birth with a repeated dose at monthly intervals (Boxall, 1980). Recent studies also show that infants born to an HBsAg carrier mother could be prevented from developing hepatitis B virus infection and hence chronic carriership by early prophylactic vaccination with the new hepatitis B vaccine. Again this should be done within 48 hr of birth (Boxall, 1980).

E. Demonstration of Elevated Total Cord IgM

Maternal IgM does not normally cross the intact placenta. Presence of IgM in the cord blood at concentration of <20 mg/100 ml is generally considered to be normal. Furthermore, early studies by Alford *et al.* (1967) have shown that elevated cord IgM (>20 mg/100 ml) could be a very useful indicator of intrauterine infection, since elevation may reflect antigenic stimuli in the fetus due to fetal infection. Once such infants are shown to have been born with elevated cord serum IgM, they may then be investigated in depth for a particular viral or other infection. Such an approach could provide an interesting and simple method for the selection of infants who may have had an infection *in utero*. Later studies, however, failed to show a good correlation between elevation of cord IgM and the selection of cases infected with viruses such as CMV (Reynolds *et al.*, 1974). Recently, we employed a latex agglutination procedure calibrated to be positive at 16.4 mg/100 ml. The procedure is rapid (5 min) and hence allowed the testing of large number of specimens routinely with great ease. Once cord serum was positive, neonatal blood was requested in order to confirm elevation of the cord IgM and eliminate the possibility of maternofetal blood contamination, for example, due to placental leakage, which could result in false-positive results. Using such a procedure, we were able to select 10 out of 65 (15.4%) infants who had clinical symptoms and 16 out of 42 (38.1%) of infants with serological evidence of intrauterine viral infection,

of whom 14 (33.3%) were due to CMV and 2 (4.8%) to RV. Only in 4 cases could we correlate the serological evidence with the clinical symptoms during our year of follow-up (Al-Nakib *et al.*, 1984). However, many of these infants could have been asymptomatic during the period of follow-up. What these studies have demonstrated is that elevation of cord IgM could still be a very important and useful nonspecific marker for intrauterine viral infection and should further be properly investigated, especially with other parameters of infection. Indeed, very recently, studies by Griffiths *et al.* (1982a) showed that the presence of RF, elevated cord IgM, and CMV-specific IgM could provide the best indicator for the prognostic potential of infants who had a CMV infection *in utero*.

With the availability of computerized instruments such as laser nephelometry which provides quantitative estimation of total IgM, a large number of cases could easily be screened for elevated total IgM in the cord. This marker could also be investigated with other parameters in the cord serum, including RF, and can be assessed in relation to more specific indicators of intrauterine infection, such as virus-specific IgM and/or virus isolation from clinical material of the newborn, which is urine in the case of CMV. Such studies could be valuable and applicable especially in the case of CMV and are the subject of intensive investigation in our laboratory.

F. Demonstration of Persistence of Virus-Specific IgG in the Infant

In normal infants maternal antibodies generally decline to undetectable levels by about 4–6 months of life. However, in infants who did have an intrauterine viral infection, viral antibodies would not only persist beyond the life of maternal antibodies but may show a continuous rise by 8–9 months of life (Hanshaw and Dudgeon, 1978; Al-Nakib *et al.*, 1984). Therefore, contrasting the decay of maternal antibodies with the persistence of viral antibody in the infant serum has generally been accepted to represent evidence of intrauterine viral infection (Hanshaw and Dudgeon, 1978). Our own studies have shown that this could be a useful approach to provide further evidence of congenital infection. However, among certain populations where some viral infections such as CMV are common very early in life, exclusion of early postnatal infection is important. Demonstration of virus-specific IgM in cord or early neonatal serum provides good evidence that these infections were congenital rather than postnatal. A serious limitation with this approach is the fact that serial specimens would have to be taken from the infants. Furthermore, it would take up to 6 months to reach a final diagnosis. Therefore, this approach, although important and perhaps interesting as part of a long-term follow-up, is of limited diagnostic value at present, especially with the recent development of a more rapid

and sensitive procedure capable of detecting virus-specific IgM, as will be discussed in the next section.

G. Demonstration of Virus-Specific IgM

Intrauterine viral infection during pregnancy can be established either by demonstrating virus-specific IgM in the serum of the patient, as is the case with RV infection during pregnancy and to a lesser extent with CMV, or by demonstrating virus-specific IgM in the cord or early neonatal sera (Hanshaw and Dudgeon, 1978). This immunoglobulin (IgM) does not normally cross the intact placenta, and therefore detection of virus-specific IgM in the cord or early neonatal serum is a good indicator of a congenital viral infection. Presence of virus-specific IgM in fetal serum is considered to be a normal response to an antigenic stimulus due to a viral infection. Figure 1 clearly demonstrates the events regarding the fetal immune response due to a viral infection during pregnancy. This approach has been exploited extensively in the case of rubella, toxoplasma, and recently CMV, whereas the situation with HSV is not clear.

With a rubella infection taking place early in pregnancy, the rate of congenital malformation developing in a fetus of a susceptible mother who acquires primary infection during the first 12 weeks of pregnancy may vary between 15.3 and 24.6% (Hanshaw and Dudgeon, 1978). Often these pregnant mothers present very late in the first trimester, and hence accurate and rapid laboratory diagnosis is essential if a decision is to be taken as to whether or not therapeutic abortion is to be conducted. In contrast, dem-

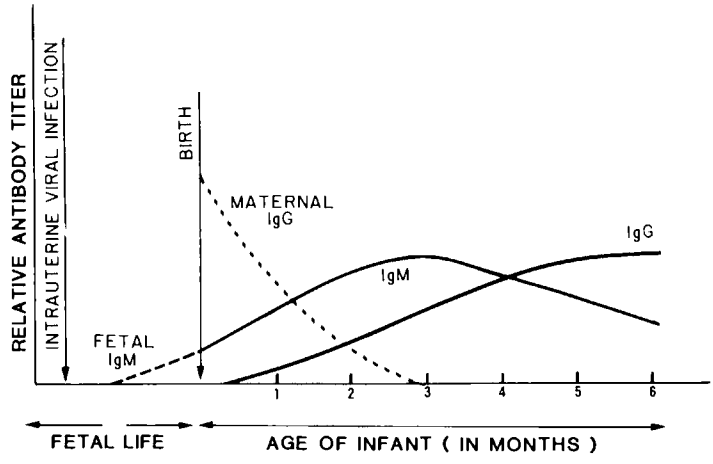


Fig. 1. The immune responses following viral infection in pregnancy.

onstration of virus-specific IgM in cord serum or neonatal sera are mainly to provide evidence of a congenital rubella infection (Pattison, 1978). Of the procedures employed in the detection of rubella-specific IgM, clearly the most specific procedure is that of serum fractionation on a sucrose density gradient (SDG) followed by detection of viral antibody in the IgM fractions. Detection of specific IgM indicates recent infection by rubella, whereas the presence of specific IgG in the absence of specific IgM indicates past exposure and immunity (Vesikari and Vaheri, 1968). Detection of rubella antibody in the IgM fractions can be done using standard HAI procedure (Vesikari and Vaheri, 1968). The sensitivity of this HAI procedure can be further enhanced by incubating antigen–antibody mixtures overnight at 4°C instead of at 37°C for 1 hr (Al-Nakib *et al.*, 1974). The use of trypsinized human O erythrocytes can also further increase the sensitivity of such a procedure (Al-Nakib and Lilley, 1978). Alternatively, RV antibody activity in the IgM fraction can be detected by other procedures such as IF (Cradock-Watson *et al.*, 1972, 1976) or RIA (Meurman *et al.*, 1977; Kangro *et al.*, 1978). The main advantage of serum fractionation prior to testing is to eliminate "false" negative results due to competition of the often more prevalent viral IgG antibodies with the viral IgM antibodies for the same antigen binding sites or for the presence of rheumatoid factor, which is an IgM anti-IgG and hence "false" positive test (Meurman and Ziola, 1978; Schmitz *et al.*, 1980).

Both these problems are particularly inherent in the more practicable tests of the indirect solid-phase immune assays such as IF, RIA, or ELISA (Meurman and Ziola, 1978; Vejtrop, 1980; Schmitz *et al.*, 1980). Although more specific serum fractionation on SDG prior to testing is not very practicable for large-scale testing and requires complex and expensive equipment such as an ultracentrifuge. Hence, considerable research has gone into the development of a more practicable procedure, such as the indirect solid-phase immune assays or the recently developed direct solid-phase immune assays based on the method of Krech and Wilhelm (1979).

In the indirect assays, the solid phase is first coated with viral antigens, then the patient's serum is allowed to react with the antigen and excess is washed away. The type of viral immunoglobulin in reaction with the specific viral antigen is then identified by the addition of antispecies immunoglobulin, i.e., antihuman IgM or IgG (Fig. 2). In contrast, in the direct method the solid phase is coated with antihuman IgM (anti-μ), which selects the IgM fractions in the patient's serum. Excess, including all other immunoglobulins, is washed away. Viral antigens are then added; they react with those IgM antibodies that are virus specific. Excess is washed away, and then the virus is identified either with erythrocytes which produce a hemagglutination pattern (Krech and Wilhelm, 1979; van der Logt *et al.*,

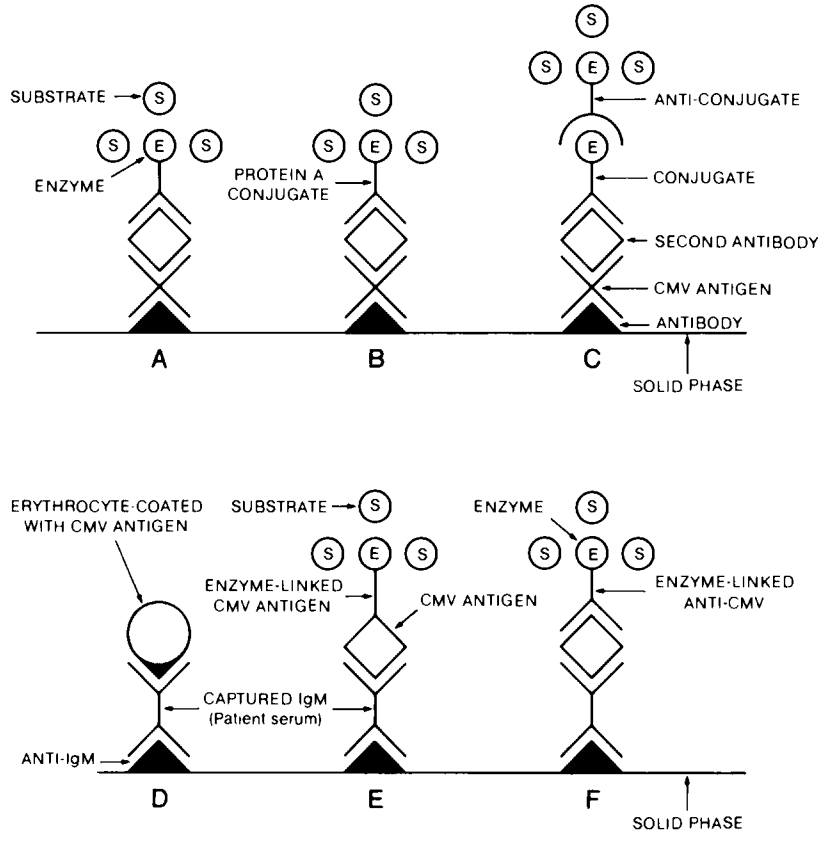


Fig. 2. Solid-phase enzyme immunoassays for the demonstration of CMV-specific IgM and direct detection of cytomegalovirus (CMV) or its antigens in urine. A, B, and C represent several possible systems which are being developed for the direct detection of CMV antigens in clinical specimen. D, E, and F represent also several other possible systems that are being evaluated in the detection of CMV-specific IgM using the concept of a capturing antibody (anti-IgM) bound to the solid phase.

1981) or a hemadsorption pattern (Goldwater and Banatvala, 1981) or with antiviral antibody conjugated to ^{125}I (Mortimer *et al.*, 1981) or enzyme (Vejtorp, 1980; Chantler *et al.*, 1982). These direct assays for rubella have clearly been shown to be superior to the indirect methods in the detection of rubella-specific IgM and to overcome the problems of both false positive due to RF and false negative due to competition from viral IgG with viral IgM for the same antigen binding sites (Vejtrop, 1980). They have a clear advantage over the SDG fraction procedure, since the fractionation step is no longer necessary and now a larger number of sera could be investigated

routinely for rubella, including rubella contacts during pregnancy, which often represent the bulk of referral of cases.

With CMV infection the question of a case during pregnancy being established as having had a recent infection by CMV or having had contact with a case of symptomatic CMV infection does not normally arise since most CMV infections are asymptomatic (Starr, 1970) unless there is a frank heterophil antibody-negative infectious mononucleosis, which is relatively uncommon (Ahlfors *et al.*, 1982). Furthermore, even if such an infection is established, the question of therapeutic abortion does not normally arise since the whole question regarding the type of CMV infection, whether primary or recurrent, and the time of infection during pregnancy and their relative effect on the developing fetus is not yet clear by any means (Stern and Tucker, 1973; Krech *et al.*, 1971; Stagno *et al.*, 1973; Ahlfors *et al.*, 1982). Depending on the procedure followed, CMV-specific IgM may also appear frequently following recurrent infection, and hence the question of therapeutic abortion using specific IgM as the diagnostic criterion may thus confuse the picture further, since recurrent infection has not often been associated with the development of congenital defects (Stagno *et al.*, 1982). Nevertheless, recent studies by Griffiths *et al.* (1982a) using a sensitive indirect RIA have shown that such a procedure has considerable accuracy in the diagnosis of a congenital infection and provides good correlation with CMV excretion in the urine of infected infants. In contrast, application of the indirect IF or ACIF in the detection of CMV-specific IgM in the cord did not show a similar correlation with virus excretion in the urine of infected infants and did result in a considerable proportion of false positive and/or negative results (Stagno *et al.*, 1980). ELISA procedures have been developed with the solid phase being coated with antihuman IgM (direct method), and these have shown very promising results (Van Loon *et al.*, 1981). Nevertheless, these procedures require considerable clinical evaluation, as was done by Griffiths *et al.* (1982a), before a firm statement can be made regarding their potential as highly specific tools in the diagnosis of congenital CMV infection. Therefore, with the present limitation of virus isolations in cell culture and the direct detection of virus or viral antigens, it is essential that further and considerable effort be made to develop and evaluate procedures for the detection of CMV-specific IgM in the cord as possible methods for the large-scale screening and identification of infants at risk.

Detection of HSV-specific IgM in the cord, although advocated by Palmer *et al.* (1977), has not received wide investigation and evaluation. This may have been due to the fact that the incidence of congenital HSV infections is very small since disseminated neonatal herpes infections are frequently symptomatic (Hanshaw and Dudgeon, 1978; Visintine *et al.*,

1978; Whitley and Alford, 1979) and perhaps since other methods for the diagnosis of HSV—such as virus isolation and direct detection of virus by EM or of viral antigen by immunological methods—are satisfactory.

This review demonstrates that the question of laboratory diagnosis of congenital or perinatal infection not only is complex but requires complete understanding of the methodology applied in such diagnosis, especially the limitation of many of the procedures that are used today. Nevertheless, in recent years we have seen considerable improvement in the area of rapid virus diagnoses and solid-phase immunoassays, which thus holds considerable promise for the future. Rapid diagnosis of congenitally infected infants should now become especially important since in parallel there has also been considerable improvement in the development of more specific and less toxic antivirals. It is thus envisaged that the application of such drugs in the future will require early detection and identification of such infants.

REFERENCES

Ahlfors, K., Harris, S., Ivarsson, S., and Svanberg, L. (1981). *N. Engl. J. Med.* **305,** 284.

Ahlfors, K., Ivarsson, S.-A., Johnsson, T., and Svanberg, L. (1982). *Acta Paediatr. Scand.* **71,** 109–113.

Alford, C. A., Schaefer, J., Blakenship, W. J., Stanmfjord, J. V., and Cassidy, G. L. (1967). *N. Engl. J. Med.* **277,** 437–449.

Alford, C. A., Stagno, S., and Pass, R. F. (1980). *In* "Perinatal Infections" pp. 125–147. Ciba Foundation–Excerpta Medica, Amsterdam.

Al-Nakib, W., and Lilley, H. (1978). *J. Clin. Pathol.* **31,** 730–734.

Al-Nakib, W., Best, J. M., and Banatvala, J. E. (1974). *Br. Med. J.,* **3,** 579.

Al-Nakib, W., Deverajan, L. W., Thorburn, H., Ibrahim, M. E. A., Moussa, M. A. A., Hathout, H., Yousos, A. M., and Soufi, S. (1984). *Bull. W.H.O.*, in press.

Banatvala, J. E. (1982). *Br. Med. J.* **284,** 1285–1286.

Bithell, J. F., Draper, G. J., and Gorbach, P. D. (1973). *Br. Med. J.* **1,** 706–708.

Boxall, E. H. (1980). *In* "Recent Advances in Clinical Virology" (A. P. Waterson, ed.), pp. 17–29. Churchill-Livingstone, Edinburgh.

Bradford Hill, A., Doll, R., Galloway, T. M., and Hughes, J. P. (1958). *Br. J. Prev. Soc. Med.* **12,** 1–7.

Brunell, P. A., and Kotchmar, G. S. (1981). *J. Pediatr.* **98,** 71–73.

Cabau, N., Labadie, M. D., Vesin, C., Feingold, J., and Bone, A. (1979). *Arch. Dis. Child.* **54,** 286–290.

Centers for Disease Control (1982). *Morbidity and Mortality Weekly Report* **31,** 477–481.

Chantler, S., Evans, C. J., Mortimer, P. P., Cradock-Watson, J. E., and Ridehalgh, K. S. (1982). *J. Virol. Methods* **4,** 305–313.

Coffey, V. P., and Jessop, W. J. E. (1959). *Lancet* **2,** 935–938.

Coleman, D. V. (1980). *In* "Recent Advances in Clinical Virology" (A. P. Waterson, ed.), pp. 89–111. Churchill-Livingstone, Edinburgh.

Cradock-Watson, J. E., Bourne, M. S., and Vandervelde, E. M. (1972). *J. Hyg.* **70,** 473–485.

Cradock-Watson, J. E., Ridehalgh, M. K. S., and Chantler, S. (1976). *J. Hyg.* **76,** 109–123.

Dodion-Fransen, J., Dekegel, D., and Thire, L. (1973). *Scand. J. Infect. Dis.* **5**, 149–153.
Dowdle, W. R., Ferreira, W., De Salles, Gromes, L. F., King, D., Kourany, M., Madalengoitia, J., Pearson, E., Swanston, W. H., Tosi, H. C., and Vilches, A. M. (1970). *Bull W.H.O.* **42**, 419–422.
Editorial (1977). *Lancet* **2**, 541.
Editorial (1978). *Br. Med. J.* **2**, 1586.
Embil, J. A., Ozere, R. L., and Haldane, E. V. (1970). *J. Pediatr.* **77**, 417–421.
Fedrick, F., and Alberman, E. D. (1972). *Br. Med. J.* **2**, 485–488.
Frey, H. M., Bialkin, G., and Gershon, A. A. (1977). *Pediatrics* **59**, 110–112.
Gehrz, R. C., Marker, S. C., Knorr, S. O., Kalis, J. M., and Balfour, H. H. (1977). *Lancet* **2**, 844–847.
Gershon, A. A. (1975). *Prog. Clin. Biol. Res.* **3**, 79–95.
Goldwater, P. N., and Banatvala, J. E. (1981). *J. Virol. Methods* **2**, 241–247.
Grant, S., Edmond, E., and Syme, J. (1981). *J. Infect.* **3**, 24–31.
Gregg, N. M. (1941). *Trans. Ophthalmol. Soc. Aust.* **3**, 35–46.
Griffith, G. W., Adelstein, A. M., Lambert, P. M., and Weatherall, J. A. C. (1972). *Br. Med. J.* **3**, 553–556.
Griffiths, P. D. (1981). *Br. J. Obstet. Gynaecol.* **88**, 582–587.
Griffiths, P. D., and Baboonian, C. (1982). *J. Clin. Pathol.* **35**, 1340–1344.
Griffiths, P. D., Campbell-Benzie, A., and Heath, R. B., (1980a). *Br. J. Obstet. Gynaecol.* **87**, 308–314.
Griffiths, P. D., Ronalds, C. J., and Heath, R. B. (1980b). *J. Epidemiol. Community Health* **34**, 124–128.
Griffiths, P. D., and Baboonian, C. (1984). *J. Hyg.* **92**, 89–95.
Griffiths, P. D., Stagno, S., Pass, R. F., Smith, R. J., and Alford, C. A. (1982a). *Pediatrics* **69**, 544–549.
Griffiths, P. D., Stagno, S., Pass, R. F., Smith, R. J., and Alford, C. A. (1982b). *J. Infect. Dis.* **145**, 647–653.
Hakosalo, J., and Saxen, L. (1971). *Lancet* **2**, 1346–1347.
Halstead, S. B., Diwan, A. R., and Oda, J. (1969). *J. Am. Med. Assoc.* **210**, 1881–1883.
Hanshaw, J. B., and Dudgeon, J. A. (1978). "Viral Diseases of the Fetus and Newborn." Saunders, Philadelphia, Pennsylvania.
Hanshaw, J. B., Scheiner, A. P., Moxley, A. W., Gaev, L., Abel, V., and Scheiner, B. (1976). *N. Engl. J. Med.* **295**, 468–470.
Hardy, J. M. B., Azarowicz, E. N., Mannini, A., Medearis, D. N., and Cooke, R. E. (1961). *Am. J. Public Health* **51**, 1182–1188.
Hathout, H., Al-Nakib, W., Lilley, H., Abo-Ahmed, H. S., and Nosseir, A. F. (1978). *Int. J. Epidemiol.* **7**, 49–53.
Herbst, A. L., Ulfelder, H., and Poskanzer, D. C. (1971). *N. Engl. J. Med.* **284**, 878–881.
Hinman, A. R., Bart, K. J., Orenstein, W. A., and Preblud, S. R. (1983). *Lancet* **1**, 39–41.
Huang, E.-S., Alford, C. A., Reynolds, D. W., Stagno, S., and Pass, R. F. (1980). *N. Engl. J. Med.* **303**, 958–962.
Hunter, W. S., Ellert, M. S., Nequin, L. G., and Suarez, W. A. (1979). *Br. Med. J.* **1**, 887–888.
Kangro, H. O., Pattison, J. R., and Heath, R. B. (1978). *Br. J. Exp. Pathol.* **59**, 577–583.
Khalifa, A. S., Abdel-Wahab, K. S. E., and Imam I. Z. E. (1983). *J. Infect. Dis.* **127**, 567–570.
Knox, E. G. (1980). *Int. J. Epidemiol.* **9**, 13–23.
Krech, U. H., Konjajev, Z., and Jung, M. (1971). *Helv. Paediatr. Acta* **26**, 355–362.
Krech, U. H., and Wilhelm, J. A. (1979). *J. Gen. Virol.* **44**, 281–286.
Lam, S. K. (1972). *Bull. W.H.O.* **47**, 127–129.

Lamprecht, C., Schauf, V., Warren, D., Nelson, K., Northrop, R., and Christiansen, M. (1982). *J. Am. Med. Assoc.* **247**, 1129–1133.

Leck, I. (1963). *Br. J. Prev. Soc. Med.* **17**, 70–80.

Li, F. P., and Hanshaw, J. B., (1967). *Am. J. Epidemiol.* **86**, 137–147.

MacCracken, G. H., Hardy, J. B., Chen, T. C., Hoffman, L. S., Gilkeson, M. R., and Sever, J. L. (1969). *J. Pediatr.* **74**, 383–392.

Macris, M. P., Nahmais, A. J., Bailey, P. D., Lee, F. K., Visintine, A. M., and Brann, A. W. (1981). *J. Virol. Methods* **2**, 315–320.

Manson, M. M., Logan, W. P. D., and Loy, R. M. (1960). *Rep. Public Health Med. Subj.* No. 101 (HMSO, London).

Melish, M. E., and Hanshaw, J. B. (1973). *Am. J. Dis. Child.* **126**, 190–194.

Meurman, O. H., and Ziola, B. R. (1978). *J. Clin. Pathol.* **31**, 483–487.

Meurman, O. H., Viljanen, M. K., and Granforns, K. (1977). *J. Clin. Microbiol.* **5**, 257.

Monif, G. R. F., Hildebrandt, R. J., and Weber, J. M. (1970). *Am. J. Obstet Gynecol.* **108**, 372–374.

Mortimer, P. P., Edwards, J. M. B., Porter, A. D., Tedder, R. S., Mace, J. E., and Hutchinson, A. (1981). *J. Hyg.* **87**, 131–138.

Naib, Z. M. (1981). *In* "The Human Herpesviruses (A. J. Nahmias, W. R. Dowdle, R. F. Schinazi, eds.). Elsevier, New York, 381–386.

Palmer, D. F., Cavalloro, J. J., Herrmann, K., Stewart, J. A., and Walls, K. W. (1977). "Serodiagnosis of Toxoplasmosis, Rubella, Cytomegalic Inclusion Disease, Herpes Simplex." Immunology Series No. 5. United States Department of Health Education and Welfare, Public Health Service, Center for Disease Control, Atlanta.

Pattison, J. R. (1978). *In* "Modern Topics in Infection" (J. D. Williams, eds.), pp. 184–255. Heinemann, London.

Peckham, C. S. (1978). *J. R. Coll. Physicians* **12**, 250–255.

Rawls, W. E., Melnick, J. L., Bradstreet, C. M. P., Bailey, M., Ferris, A. A., Lehmann, N. I., Nagler, F. P., Furesz, J., Kono, R., Ontawara, M., Halonen, P., Stewart, J., Ryan, J. M., Strauss, J., Zdrazilek, J., Leerhoy, J., Von Magnus, H., Sohier, R., and Ferreira, W. (1967). *Bull. W.H.O.* **37**, 79–88.

Reynolds, D. W., Stagno, S., Stubbs, K. G., Dahle, A. J., Livingstone, M. M., Saxon, S. S., and Alford, C. A. (1974). *N. Engl. J. Med.* **290**, 291–296.

Reynolds, D. W., Dean, P. H., Pass, R. F., and Alford, C. A. (1979). *J. Infect. Dis.* **140**, 493–499.

Rowe, W. P., Hartley, J. W., Waterman, S., Turner, H. C., and Huebner, R. J. (1956). *Proc. Soc. Exp. Biol. Med.* **92**, 418–424.

Ruben, F. L., Winkelstein, A., and Sabbagha, R. E. (1975). *Proc. Soc. Exp. Biol. Med.* **149**, 881–883.

Saidi, S. (1972). *Bull W. H. O.* **46**, 563–565.

Saigal, S., Lunyk, O., Larke, R. P. B., and Chernesky, M. A. (1982). *Am. J. Dis. Child.* **136**, 896–901.

Schmitz, H., Von Deimbing, U., and Flehming, B. (1980). *J. Gen. Virol.* **50**, 59–68.

Schopfer, K., Lauber, E., and Krech, U. (1978). *Arch. Dis. Child.* **53**, 536–539.

Sever, J., and White, L. R. (1968). *Annu. Rev. Med.* **19**, 471–486.

Siegel, M. (1973). *J. Am. Med. Assoc.* **226**, 1521–1524.

Smith, M. G. (1956). *Proc. Soc. Exp. Biol. Med.* **92**, 424–430.

Stagno, S., Reynolds, D. W., Lakeman, A., Charamella, J. L., and Alford, C. A. (1973). *Pediatrics* **52**, 788–794.

Stagno, S., Reynolds, D. W., Tsiantos, A., Fuccillo, D. A., Long, W., and Alford, C. A. (1975). *J. Infect. Dis.* **132**, 568–577.

Stagno, S., Volanakis, J. E., Reynolds, D. W., Stroud, R., and Alford, C. A. (1977a). *J. Clin. Invest.* **60,** 838–845.

Stagno, S., Reynolds, D. W., Huang, E.-S., Thames, S. D., Smith, R. J., and Alford, C. A. (1977b). *N. Engl. J. Med.* **296,** 1254–1258.

Stagno, S., Pass, R. F., Reynolds, D. W., Moor, M., Nahmias, A. J., and Alford, C. A. (1980). *Pediatrics* **65,** 251–257.

Stagno, S., Pass, R. F., and Alford, A. (1981). *Birth Defects,* **17**(1), 31–50.

Stagno, S., Pass, R. F., Dworsky, M. E., Henderson, R. E., Moore, E. G., Walton, P. D., and Alford, C. A. (1982). *N. Engl. J. Med.* **306,** 945–949.

Starr, J. G. (1970). *N. Engl. J. Med.* **282,** 50.

Starr, J. G., and Gold, E. (1969). *J. Pediatr.* **74,** 815–816.

Starr, S. E., Tolpin, M. D., Friedman, H. M., Paucher, K., and Plotkin, S. A. (1979). *J. Infect. Dis.* **140,** 500–5.

Stern, H., and Tucker, S. M. (1973). *Br. Med. J.* **2,** 268–270.

Stevens, C. E., and Szmuness, W. (1980). *In* "Virus and the Liver" (Bianchi *et al.*, eds.), pp. 285–293. MTP Press, Lancaster, England.

Stewart, A., Webb, J., and Hewitt, D. (1958). *Br. Med. J.* **1,** 1495–1508.

Sundqvist, V. A., and Wahren, B. (1981). *J. Virol. Methods* **2,** 301–312.

Van Loon, A. M., Heesen, F. W. A., Van der Logt, J. T. M., and Van der Veen, J. (1981). *J. Clin. Microbiol.* **13,** 416–422.

Van der Logt, J. T. M., Van Loon, A. M., and Van der Veen, J. (1981). *J. Clin. Microbiol.* **13,** 410–415.

Vejtrop, M. (1980). *J. Virol. Methods* **1,** 1–9.

Vesikari, T., and Vaheri, A. (1968). *Br. Med. J.* **1,** 221–223.

Vestergaard, B. F., and Jensen, A. E. (1981). *In* "The Human Herpes Viruses" (A. J. Nahmias, W. R. Dowdle, and R. F. Schinazi, eds.), pp. 391–398. Elsevier, Amsterdam.

Visintine, A. M., Nahmias, A. J., and Jose, W. E. (1978). *Perinatal Care* **2,** 32–41.

Warrell, M. J., Tobin, J., O'H., and Wald, N. J. (1981). *J. Med. Microbiol.* **14,** 159–162.

Weller, T. H., Macaulay, J. A., Craig, J. M., and Wirth, P. (1957). *Proc. Soc. Exp. Biol. Med.* **94,** 4–12.

Whitley, R. J., and Alford, C. A. (1979). *In* "Antiviral Agents and Viral Diseases of Man" (G. Y. Galasso *et al.*, eds.), pp. 541–604. Raven, New York.

Wilson, M. G., Heins, H. L., Imagawa, D. T., and Adams, J. M. (1959). *J. Am. Med. Assoc.* **171,** 638–41.

Yawn, D. H., Pyeatte, J. C., Joseph, J. M., Eichler, S. L., and Giarcia-Bunnel, R. (1971). *J. Am. Med. Assoc.* **216,** 1022–1023.

Yeager, A. S., Martin, H. P., and Stewart, J. A. (1977). *Clin. Pediatr.* **16,** 455–458.

Yolken, R. H., and Stopa, P. J. (1980). *J. Clin. Microbiol.* **11,** 546–551.

26

Viral–Bacterial Synergistic Interactions in Respiratory Infections

LORNE A. BABIUK

Department of Veterinary Microbiology, Western College of Veterinary Medicine, and Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

I. INTRODUCTION

Bacterial colonization of the lower respiratory tract of healthy animals and man is a relatively rare event. However, in individuals suffering from a variety of diseases, including virus infection, colonization occurs rapidly. Thus it is estimated that 90% of bacterial pneumonias develop after a viral infection. Furthermore, individuals suffering from a viral pneumonia have a 40% chance of developing bacterial pneumonia (Finland *et al.*, 1942; Jakab, 1982a,b). The reasons for the increase in colonization has been shown to be related to the surface properties of epithelial cells lining the respiratory tract as well as the physiological environment of the respiratory tract. These two conditions allow greater numbers of bacteria to adhere to the surface of cells as well as possibly to replicate at a faster rate. This increased rate of adherence and replication results in the production of microcolonies which can then enter the lower respiratory tract (Amberson, 1937; Lam *et al.*, 1980). These microcolonies are difficult to phagocytize and are resistant

ISBN 0-12-429601-7

to attack by antibodies or antibiotics (Costerton *et al.*, 1981). In many cases infection with viruses may play a major role in alteration of epithelial cell surfaces, thus allowing bacteria to adhere (Aly *et al.*, 1977; Davison and Sanford, 1981; Fainstein *et al.*, 1980; Nugent and Pesanti, 1982; Ramphal *et al.*, 1980; Sanford *et al.*, 1978) or by killing cells which control bacterial growth by releasing bacteriocidal factors (Pijoan *et al.*, 1980), or, of more importance, lysis and death of cells results in the release iron, which enhances bacterial growth and encourages colonization (Miles and Khimji, 1975).

In addition to colonization, the rate of clearance of bacteria from the lung will determine whether disease occurs or not.

Alveolar macrophages are considered to be the primary defense mechanism of the lung infections caused by gram-positive bacteria (Green and Kass, 1964). Viral infection has been shown to affect ingestion and phagosome–lysosome fusion with subsequent increased susceptibility to bacterial pneumonia (Jakab and Warr, 1981; Shanley and Pesanti, 1980; Warr and Jakab, 1979). In contrast neutrophils are primarily involved in clearing gram-negative bacteria. As in the case of virus-induced alteration of macrophage function it is becoming evident that viruses can also affect neutrophil function (Abramson *et al.*, 1981; Bale *et al.*, 1982; Jackson *et al.*, 1967; Yourtee *et al.*, 1982). It appears that the rate of clearance of either group of bacteria, whether by macrophages or neutrophils, will determine whether they get established in the lung or not. Thus, if the virus alters either the number or functional activity of alveolar macrophages or neutrophils sufficiently to provide a replicative advantage for the bacteria, then the chances of secondary bacterial infection are greatly increased (Gardner and Kung, 1980). In most cases all the interactions may not occur simultaneously, or indeed some may not occur at all; however, they have been included to help explain the reasons for viral–bacterial synergistic interactions in respiratory infections as well as to demonstrate the complexities of the virus–bacteria–host interactions and the need for a multidisciplinary approach to help understand and control respiratory infections.

II. BACTERIAL ADHERENCE

It is becoming increasingly evident that bacterial adherence to mucosal surfaces is often required for colonization by the bacteria, and that colonization appears to be a prerequisite for production of disease (Beachey, 1981; Costerton *et al.*, 1981). This is especially true in the respiratory and gastrointestinal tract, where bacteria are rapidly removed from the mucosal surfaces unless they adhere. Whether bacteria adhere or not can be influ-

enced by the organism as well as by its environment. Even though the bacterium may have the genetic potential to produce the adhesins or pili whereby attachment is mediated, the environment may influence the amount of adhesins produced or activity in adhesion. Thus in bacteria that produce a capsule, the extent of adhesion may be influenced by the quantity and quality of the capsule. A hyaluronic acid capsule will reduce adhesion because of its high charge density and net repulsion effect between the bacteria and the animal cell (Beisel, 1976). However, in the presence of divalent cations such as zinc these charges may be reduced so that adhesion is enhanced (Sugarman, 1980, Sugarman *et al.,* 1982), and this may explain how zinc acts as a bacterial virulence factor. It is known that zinc levels are altered during infections, but whether specific viral infections increase available zinc at the mucosal surfaces remains to be determined (Beisel, 1976; Falchuk, 1977).

Viral infections appear to enhance adherence and colonization of both gram-positive (Davison and Sanford, 1981; Fainstein *et al.,* 1980; Sanford *et al.,* 1978) and gram-negative (Nugent and Pesanti, 1982) bacteria. The exact mechanism whereby virus infection leads to increased adherence is probably multifactorial ranging directly from surface alteration of the host cell, induced receptors on exposed surfaces for bacterial attachment, to alteration of the extracellular environment, which indirectly allows bacterial attachment.

In some individuals increased colonization has been correlated with altered levels of fibronectin on the surface of epithelial cells (Woods *et al.,* 1981). The quantities of cell surface fibronectin do vary with the physiological state of the cell as well as the environment (Zetter *et al.,* 1979). Viral infection would tend to cause the death of mature cells which have high levels of fibronectin on their surface. Replacement with immature dividing cells would favor lower levels of fibronectin and also colonization (Hynes and Bye, 1974; Woods *et al.,* 1981). Furthermore, viral infection enhances cell surface proteases, and cell death would release cell proteases which would degrade fibronectin on adjacent cells and further favor adherence. Once sufficient quantities of bacteria are present they themselves could produce proteases and further decrease fibronectin levels and favor colonization (Woods *et al.,* 1981; Yamada and Weston, 1974).

Iron is known to play a significant role in bacterial growth and pathogenesis (Hatch *et al.,* 1981; Miles and Khimji, 1975), and organisms produce iron chelators to help trap the iron required for their growth. Although the production of iron chelators is not correlated directly with virulence factors, the presence of these chelators or abundance of iron offers a definite replicative advantage for the organism. If a virus infects the respiratory tract and induces extensive hemorrhages and tissue damage, this would

presumably increase the levels of iron compounds available for utilization by the bacteria and provide the bacterium with a favorable milieu for rapid growth and possibly even increase adherence, especially if in some cases the iron does increase the levels of adhesins or pili formation on the bacteria.

Virus infection has been suggested to alter secretions of mucosal epithelial cells which are bacteriocidal (Pijoan *et al.*, 1980). The absence of these bacteriocidal or bacteriostatic substances in the respiratory tract encourages rapid growth of the adherent bacteria. Once large quantities of bacteria are present in the upper respiratory tract, they can form microcolonies which can enter the lung by aspiration (Johanson *et al.*, 1979) or as a result of reduced mucociliary clearance (Jakab *et al.*, 1980). Once these microcolonies reach the lung, they are difficult to phagocytize and are resistant to attack by antibodies and antibiotics (Costerton *et al.*, 1981). In fact, when a phagocyte encounters such microcolonies in its attempt to phagocytize this large microcolony, it gets "frustrated" and discharges its enzymes, which are toxic to the surrounding tissue (Slauson, 1982). Furthermore, the bacteria may release cytotoxins which are toxic to phagocytic cells even if they are phagocytized, thus further preventing clearance and inducing lung injury; this in turn creates a better environment for the bacteria to replicate.

Most of the hypotheses that have been proposed regarding adherence have been supported by *in vitro* studies. However, it must be pointed out that *in vivo* a mucous layer covers the epithelial cells, and in order for a bacterium to adhere to the epithelial cells it must first adhere to and penetrate this mucous layer. As well as providing a physical barrier, this mucous layer may contain substances which may block adherence (Williams and Gibbons, 1975). Adherence can increase in various situations where there is injury to the epithelial and mucous layer and appears to be a prerequisite for bacterial colonization (Johanson *et al.*, 1980; Ramphal *et al.*, 1980). Viral infections may be just the trigger to increase or decrease the quantity as well as the quality of this mucous layer and cause the epithelial damage required for the primary colonization. Substances such as lactoferrin, lysozyme, or local antibodies may be greatly altered due to virus infection and also favor colonization and bacterial growth.

III. EXPERIMENTAL MODELS

In many instances it has been shown that the initial viral dose as well as the route of infection may influence the extent of viral–bacterial interactions. Thus in bovine herpesvirus-1–*Pasteurella haemolytica* infections an aerosol dose of at least 10^6 PFU per animal is required to get sufficient damage by 4 days postinfection to allow the bacteria to produce pneumonia

(Yates, 1982a). Similarly, in influenza and Sendai virus the severity of the disease is directly correlated to the input virus (Jakab, 1982a). In most of these models the maximum susceptibility is at least partially correlated to anatomical or pathological changes occurring throughout the airways. Thus maximum damage at high viral doses as well as maximum susceptibility to bacteria occurs between 4 and 7 days after infection. In the case of the BHV-1–*Pasteurella haemolytica* model, this time also correlates well with the maximum temperature response of calves. Thus the peak temperature response occurs between 3 and 5 days and starts decreasing rapidly thereafter if animals are infected only with virus (Fig. 1). If, however, animals are challenged with bacteria at this time, the temperature response does not decrease but increases approximately 1°C and remains elevated until the animals die. In animals that die early there is still evidence of viral lesions and tracheobronchiolitis, but those that die later (>12 days after virus infection) have very little evidence of viral infection and virus may not be isolated from infected lungs. Thus in many cases the role of the virus, or which specific virus was involved, may go unrecognized in natural field situations.

IV. VIRUS-INDUCED IMMUNOSUPPRESSION

Viral damage has been shown to lower the activity of a variety of bacteriocidal mechanisms of the lung (Goldstein *et al.*, 1972; Jakab, 1982b, Jakab and Dick, 1973) especially those involving the macrophage (Table I).

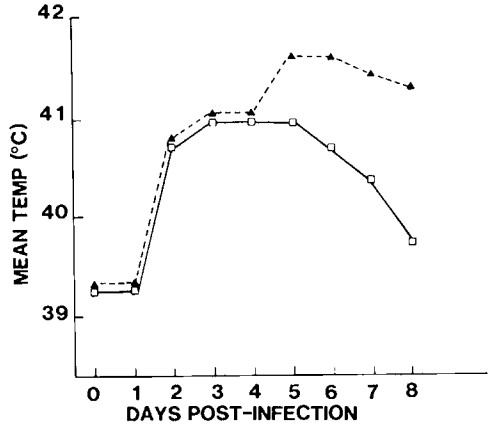


Fig. 1. Temperature response of calves infected via an aerosol with BHV-1 alone (□) and with BHV-1 plus *Pasteurella haemolytica* 4 days later (▲).

TABLE I
Effect of Viral Infection on Macrophage and Neutrophil Activity

Macrophage dysfunction
Chemotaxis
Fc receptors
Phagocytosis
Phagosome–lysome fusion
Intracellular killing
Decreased lysosome levels
Decreased monokine production
Neutrophil dysfunction
Chemotaxis
Altered maturity
H_2O_2 release
Killing
Chemiluminescence

There are basically two phagocytic cells involved in clearing bacteria from the lung. The macrophage is probably more important in clearing gram-positive bacteria and the polymorphonuclear neutrophil in clearing gram-negative bacteria. However, both cells often cooperate *in vivo*. The Fc receptor has been and continues to be one of the parameters used to study macrophage activity, and it is correlated with the macrophage's ability to ingest bacteria. In the case of viral infections the alveolar macrophage present in lavage fluids from virus-infected lungs often has decreased Fc receptor activity. However, probably more important is that even if the bacteria are ingested they are not killed as efficiently by these virus-infected macrophages (Jakab, 1982a; Jakab *et al.*, 1980; Jakab and Green, 1976; Silverberg *et al.*, 1979; Taylor *et al.*, 1974; Warr *et al.*, 1979; Warr and Jakab, 1979). This decreased killing is initially compensated for by an increased influx into the lung of phagocytic cells, but eventually even the 10- to 100-fold increase in phagocytic cells is not adequate to control the bacteria (Jakab, 1982a; McGuire and Babiuk, 1983). The decreased killing is due at least in part to inhibition of phagosome–lysosome fusion. Reduced killing may also be explained by the presence of local edema, which creates anaerobic conditions detrimental to the phagocytic and bacteriocidal capacity of the alveolar macrophage (Green and Kass, 1965). In addition to bacterial activity, the alveolar macrophage is very important in aiding recruitment of neutrophils by production of chemotactic factors (Gadek *et al.*, 1980; Hunninghake *et al.*, 1978; Merril *et al.*, 1980). It has been shown that virus infection can reduce chemotactic factor production by alveolar macrophages and, therefore, could dramatically influence neutrophil recruitment into the lung (Bale *et al.*, 1982; McGuire and Babiuk, 1983; Yourtee *et al.*, 1982).

Although the mechanism(s) whereby viruses reduce neutrophil migration is presently unknown, it is possible that, in addition to reduced chemotactic factor production by alveolar macrophages, which would alter neutrophil infiltration, other factors are also involved such as interferon or prostaglandin synthesis by a variety of cells in response to a virus infection. Both of these mediators have varied effects on neutrophil function. Furthermore, it is possible that circulating neutrophils which can migrate rapidly have already done so in response to the virus infection, and the cells present in the circulation can no longer respond either *in vivo* to the bacteria or in *in vitro* assays to chemotactic factors. This possibility may at first seem feasible since neutropenia can occur following virus infections (Yates, 1982b) and there are a considerable number of neutrophils in the lungs after virus infection, but before bacterial challenge (Fig. 2). This suggests an alteration in neutrophil populations. Such alterations in neutrophil populations have been suggested to occur in mice infected with murine cytomegalovirus (Bale *et al.*, 1982). A second possibility is that virus infection directly alters neutrophil mobility. This has been previously shown to occur with numerous viruses (Nelson *et al.*, 1978; Ruutu *et al.*, 1977). In some cases the neutrophil could be infected by the virus (Rinaldo *et al.*, 1979) or mere interaction of the virus with the neutrophil would be sufficient to induce neutrophil dysfunction (Abramson *et al.*, 1982a; Faden *et*

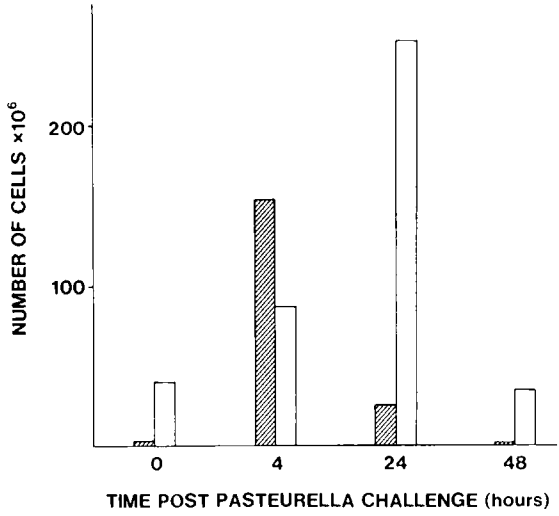


Fig. 2. Neutrophils present in lung lavages of cattle. Normal cattle have very low levels of neutrophils; however exposure to *Pasteurella haemolytica* (hatched bars) causes a rapid increase into the lung. Viral infection indicates a increased number of neutrophils (□). Challenge with bacteria results in a slower rate of infiltration than in control animals.

al., 1979). If sufficient numbers of neutrophils are infected, this could easily explain neutropenia and altered mobility *in vivo*. Since many viruses can spread systemically, causing viremia, it is possible that the presence of virus or viral glycoproteins may trigger oxidative metabolism of the neutrophil and then cause subsequent neutrophil dysfunction, as has been suggested to occur in influenza virus infections (Abramson *et al.*, 1982a,b; Yourtee *et al.*, 1982).

The importance of neutrophils in clearing gram-negative bacteria has been shown in other model systems not involving viruses wherein animals or humans that are rendered neutropenic show a decreased migration rate of PMNs into the lung and develop severe pneumonia (Kurrle *et al.*, 1981; Rehm *et al.*, 1980). Thus any decrease in chemotaxis (Martin *et al.*, 1981), phagosome–lysosome fusion (Abramson *et al.*, 1982a), or alterations in oxidative metabolism and bacteriocidal capacity would greatly increase the chance of bacterial colonizaton.

The final immunological parameter that may be affected by virus infection is the lymphocyte. Although this cell population has not received much attention to date with respect to respiratory infections, it is well known that cytotoxic T cells can be very important both in recovery and in inducing immunopathology (Rouse and Babiuk, 1979). If the alveolar macrophages express viral antigens on their surface either due to replication or phagocytosis of the antigen, the cytotoxic T cells could be directed against these cells and further impair their functions (Forman and Babiuk, 1982; Nugent and Pesanti, 1979; Shanley and Pesanti, 1980; Yilma *et al.*, 1979). Finally, it is possible that certain sub-populations of T cells, i.e., suppressor cells, may influence susceptibility and reactivity against a virus infection. Whether it is coincidental or not, T suppressor cell activity appears to peak at approximately the time that maximum susceptibility to bacterial superinfection occurs. Furthermore, treatment of animals with low levels of cyclophosphamide does circumvent viral–bacterial synergistic interactions (Jakab and Warr, 1981). These doses of cyclophosphamide used mainly affect the induction of thymus-derived T suppressor cells (Chiorazzi *et al.*, 1976). Moreover these suppressor cells have been implicated in the depression of cell-mediated immune responses following infection with influenza and respiratory syncytial virus (Roberts, 1982), and susceptibility more closely resembles the induced suppression of the cell-mediated immunity (CMI) than of virus shedding and lesion development.

In vitro blastogenic responses to mitogens and antigens are also dramatically reduced at the peak of susceptibility to the bacteria (Filion *et al.*, 1983). Whether this indicates the presence of suppressor cells or a generalized depression of lymphocyte activity remains to be determined, as does the significance of this observation on increased susceptibility to bacterial infection.

V. INJURY TO LUNGS VIA INFLAMMATORY PROCESSES

Viruses may act as initiators allowing the bacteria to colonize and in combination induce a considerable amount of damage through killing various cells. Viruses generally kill the cells they infect, and some bacteria release cytotoxins that are directly toxic to selected cell populations (Koehler *et al.*, 1980; Markham and Wilkie, 1980a,b). However, a vast amount of tissue damage occurring in pneumonia is due to the host's response to the agents. Leukocytes are one of the major mediators of tissue injury due to their accumulation and release of biological mediators at the sites of injury (Slauson, 1982). Some of these same mediators that are required for bacteriocidal activity in the cell, when released extracellularly, are detrimental to the host. The release of lysosomal enzymes can occur most frequently when the leukocyte is overwhelmed with a bacterial load such as occurs in pneumonia (Hawkins, 1980) or by extracellular bacterial cytotoxins released into the environment (Berggren *et al.*, 1981; Maheswaran *et al.*, 1980; Markham and Wilkie, 1980a,b). However, release is induced also by membrane perturbation and membrane potential changes (Henson *et al.*, 1979; Korchak and Weissman, 1978; Weissman *et al.*, 1979). These changes result in a "respiratory burst" and release of toxic oxygen metabolities and arachidonic acid (DeChatelet, 1978; Samuelsson *et al.*, 1979).

It is not the intent to review in detail all the various mediators of inflammation, enzymes, mediators of increased permeability, vasoactive substances, etc., that are released into the lung during an inflammatory process since this has been done recently (Slauson, 1982), except to say that these can help to clear the organism but if allowed to continue in an uncontrolled fashion may lead to further lung pathology and possibly death of the individual.

VI. IMPACT OF VIRAL–BACTERIAL INFECTIONS

In all cases of viral–bacterial pneumonia the speed with which antibiotic therapy is initiated will influence the eventual outcome. In developing countries where immediate veterinary or medical care is not available death may occur very quickly even during antibiotic therapy, since the damage is already done and the inflammatory process continues. Furthermore, the antibiotics generally cannot get into the microcolonies produced, and therefore the bacteria continue to replicate. Even if the animal does not die, it generally does not perform very well and any further stressful insults such as diet deprivation or adverse environmental stresses may result in death. This is especially important in developing countries, where seasonal dietary stresses are often present. Another important consideration is to be sure

that the correct antibiotics are used early. The emergence of drug-resistant organisms, over the past number of years, dictates that drug sensitivities be determined before initiation of antibiotic therapy. This is not always possible in remote areas.

VII. CONCLUSIONS

Viruses can alter the host's susceptibility to bacterial infections by a number of independent or interdependent methods (Fig. 3). Thus the virus can infect the animal and alter the host defense mechanism as well as cause tissue damage. These two events can lead to increased bacterial colonization, growth, and microcolony formation. In addition, the virus itself, or through tissue damage in combination with the bacteria, can initiate the inflammatory process, which causes further damage, and pathology which may actually be an ultimate cause of death. This multifactorial enhancement of bacterial growth by viral infections emphasizes the need for proper control of not only the bacteria but the virus as well. Control of the viral disease appears to be more important than the bacterial components of this complex since the bacteria involved are often those present normally in the upper respiratory tract of individuals, and the bacteria usually cannot cause disease in the absence of a viral infection. Thus susceptibility correlates with virus-induced immunosuppression and phagocyte dysfunction (Fig. 4). Unfortunately there are numerous viruses that can predispose animals and individuals to bacterial superinfection, indicating that control will not be achieved by one single vaccine.

Although our ultimate goal may be either to eliminate or to eradicate the predisposing viral agents, in many cases this is not feasible or economically possible at present; therefore, our emphasis must be directed at reduction of the extent of virus replication and transmission. This should be possible

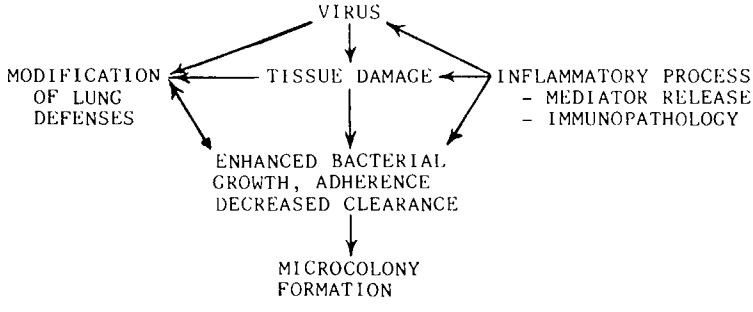


Fig. 3. Interactions between various events during the development of respiratory disease.

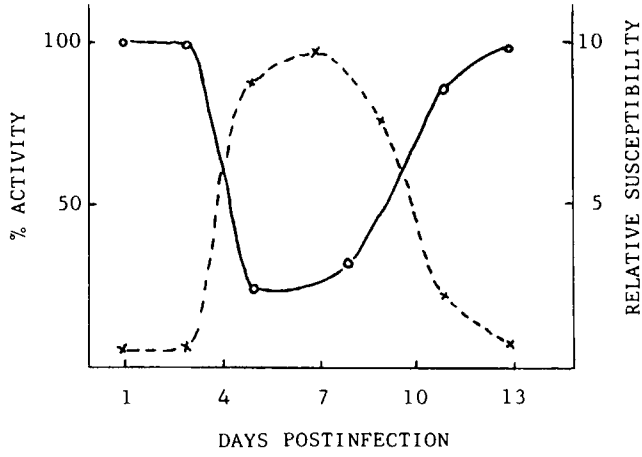


Fig. 4. Interrelationship between depression of defense mechanisms (○) of animals infected with viruses and susceptibility (×) to bacterial superinfection.

to achieve in most cases by identification of the agents involved, determining the patterns of pathogenesis and epidemiology of the virus and subsequent immunization regimes implemented. If this is not done, losses will continue to be enormous both with respect to human and animal suffering as well as economically. However, before this can be achieved there is a need for cooperation between livestock owners, who feel a need to "do something," and governments and industry, who can assist in providing both financial and human resources for such an endeavor as well as research institutions with the scientific personnel required to help unravel the complexities of the host–parasite interactions. Once control in an area is achieved, eradication is possible as long as all parties involved do not become indifferent.

REFERENCES

Abramson, J. S., Giebink, G. S., Mills, E. L., and Quie, P. G. (1981). *J. Infect. Dis.* **143**, 836–845.

Abramson, J. S., Lewis, J. C., and Lyles, D. S. (1982a). *J. Clin. Invest.* **69**, 1393–1397.

Abramson, J. S., Mills, E. L., Gienbink, G. S., and Quie, P. G. (1982b). *Infect. Immun.* **35**, 350–355.

Abramson, J. S., Lyles, D. S., Heller, K. A., and Bass, D. A. (1982c). *Infect. Immun.* **37**, 794–799.

Amberson, J. B. (1937). *Int. Anesthesiol. Clin.* **3**, 126–138.

Aly, R., Shinefield, H. I., Strauss, W. G., and Maibach, H. I. (1977). *Infect. Immun.* **17**, 546–549.

Bale, J. F., Kern, E. R., Overall, J. C., and Glasgow, L. A. (1982). *J. Infect. Dis.* **145**, 525–531.
Beachey, E. H. (1981). *J. Infect. Dis.* **143**, 325–345.
Beisel, W. R. (1976). *Med. Clin. North Am.* **60**, 831–849.
Berggren, K. A., Baluyat, C. S., Simonson, R. R., Bemrick, W. J., and Maheswaran, S. K. (1981). *Am. J. Vet. Res.* **42**, 1383–1388.
Chiorazzi, N., Fox, D., and Katz, D. H. (1976). *J. Immunol.* **117**, 1629–1635.
Costerton, J. W., Irvin, R. T., and Cheng, K. J. (1981). *CRC Crit. Rev. Microbiol.* **8**, 303–338.
Davison, V. E., and Sanford, B. A. (1981). *Infect. Immun.* **32**, 1118–1126.
DeChatelet, L. R. (1978). *J. Reticuloendothel. Soc.* **24**, 73–91.
Faden, H., Sutyla, P., and Ogra, D. L. (1979). *Infect. Immun.* **24**, 673–678.
Fainstein, V., Musher, D. M., and Cates, T. R. (1980). *J. Infect. Dis.* **141**, 172–176.
Falchuk, K. H. (1977). *N. Engl. J. Med.* **296**, 1129–1134.
Filion, L., McGuire, R., and Babiuk, L. A. (1983). *Infect. Immun.* (in press).
Finland, M., Peterson, O. L., and Strauss, E. (1942). *Arch. Intern. Med.* **70**, 183–205.
Forman, A. J., and Babiuk, L. A. (1982). *Infect. Immun.* **35**, 1041–1047.
Gadek, J. E., Hunninghake, G. W., Zimmerman, R. L., and Crystal, R. G. (1980). *Am. Rev. Respir. Dis.* **121**, 723–733.
Gardner, I. D., and Kung, T. M. (1980). *Br. J. Exp. Pathol.* **61**, 415–420.
Goldstein, E., Buhles, W. C., Akers, T. C., and Vedros, N. (1972). *Infect. Immun.* **6**, 398–402.
Green, G. M., and Kass, E. H. (1964). *J. Exp. Med.* **119**, 167–175.
Green, G. M., and Kass, E. H. (1965). *Br. J. Exp. Pathol.* **46**, 360–366.
Hatch, G. E., Slade, E., and Boykin, E. (1981). *Am. Rev. Respir. Dis.* **124**, 167–173.
Hawkins, H. K. (1980). *In* "Pathobiology of Cell Membranes" (B. F. Trump and A. U. Arstila, eds.), Vol. 2, pp. 251–285. Academic Press, New York.
Henson, P. M., Hollister, J. R., Musson, R. A., Webester, R. O., Dreisin, R. B., King, T. C., and Shaw, J. O. (1979). *Am. J. Pathol.* **97**, 93–110.
Hunninghake, G. W., Gallin, J. I., and Fauci, A. S. (1978). *Am. Rev. Respir. Dis.* **117**, 15–23.
Hynes, R. O., and Bye, J. M. (1974). *Cell* **3**, 113–120.
Jackson, A. E., Southern, P. M., Pierce, A. K., Fallis, B. O., and Sanford, J. P. (1967). *J. Lab. Clin. Med.* **69**, 833–841.
Jakab, G. J. (1982a). *Am. Rev. Respir. Dis.* **126**, 778–782.
Jakab, G. J. (1982b). *Adv. Vet. Sci. Comp. Med.* **26**, 155–171.
Jakab, G. J., and Dick, E. C. (1973). *Infect. Immun.* **8**, 762–768.
Jakab, G. J., and Green, G. M. (1976). *J. Clin. Invest.* **57**, 263–270.
Jakab, G. J., and Warr, G. A. (1981). *Am. Rev. Respir. Dis.* **123**, 524–528.
Jakab, G. J., Warr, G. A., and Sannes, P. L. (1980). *Infect. Immun.* **27**, 960–968.
Johanson, W. G., Woods, D. E., and Chaudhuri, T. (1979). *J. Infect. Dis.* **139**, 667–673.
Koehler, K. L., Markham, R. J. F., Muscoplat, C. C., and Johnson, D. W. (1980). *Am. J. Vet. Res.* **41**, 1690–1693.
Korchak, H. M., and Weissman, G. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3818–3822.
Kurrle, E., Bhaduri, S., Krieger, D., Gaus, W., Heimple, H., Pflieger, H., Arnold, R., and Vanek, E. (1981). *J. Infect. Dis.* **144**, 128–136.
Lam, J., Chan, R., Lam, K., and Costerton, J. W. (1980). *Infect. Immun.* **28**, 546–556.
McGuire, R., and Babiuk, L. A. (1983). *Vet. Immunol. Immunopathol.* (in press).
Maheswaran, S. K., Berggren, J. C. C., Simonson, R. R., Ward, G. E., and Muscoplat, C. C. (1980). *Infect. Immun.* **30**, 254–262.

Markham, R. J. F., and Wilkie, B. N. (1980a). *Am. J. Vet. Res.* **41**, 18–22.
Markham, R. J. F., and Wilkie, B. N. (1980b). *Am. J. Vet. Res.* **41**, 443–446.
Martin, R. B., Couch, R. B., Greenberg, S. B., Cate, T. R., and Warr, G. A. (1981). *J. Infect. Dis.* **144**, 279.
Merril, W. W., Naegeel, G. P., Matthay, R. A., and Reynolds, H. Y. (1980). *J. Clin. Invest.* **65**, 268–276.
Miles, A. A., and Khimji, I. (1975). *J. Med. Microbiol.* **8**, 477–490.
Nelson, R. D., McCormack, R. T., Fiegel, V. D., and Simmons (1978). *Infect. Immun.* **22**, 441–444.
Nugent, K. M., and Pesanti, E. L. (1979). *Infect. Immun.* **26**, 651–657.
Nugent, K. M., and Pesanti, E. L. (1982). *Am. Rev. Respir. Dis.* **125(S)**, 173–185.
Pijoan, C., Campos, M., and Ochoa, G. (1980). *Rev. Latinoam. Microbiol.* **22**, 69–72.
Ramphal, R., Small, P. M., and Sands, J. W. (1980). *Infect. Immun.* **27**, 614–619.
Rehm, S. R., Gross, G. N., and Pierce, A. K. (1980). *J. Clin. Invest.* **66**, 194–199.
Rinaldo, C. R., Stussel, T. P., Black, P. H., and Hirsch, M. S. (1979). *Clin. Immunol. Immunopathol.* **12**, 331–334.
Roberts, N. J. (1982). *Infect. Immun.* **35**, 1142–1146.
Rouse, B. T., and Babiuk, L. A. (1979). *Adv. Vet. Sci. Comp. Med.* **23**, 103–136.
Ruutu, P., Vaheri, A., and Kosunen, T. U. (1977). *Scand. J. Immunol.* **6**, 897–907.
Samuelsson, B., Hammarström, S., and Brugeat, P. (1979). *Adv. Inflammation Res.* **1**, 405–412.
Sanford, B. A., Shelokov, A., and Ramsay, M. A. (1978). *J. Infect. Dis.* **137**, 176–181.
Shanley, J. D., and Pesanti, E. L. (1980). *Infect. Immun.* **29**, 1152–1159.
Silverberg, B. A., Jakab, G. J., Thomson, R. G., Warr, G. A., and Boo, K. S. (1979). *J. Reticuloendothel. Soc.* **25**, 405–416.
Slauson, D. O. (1982). *Adv. Vet. Sci. Comp. Med.* **26**, 99–154.
Sugarman, B. (1980). *J. Med. Microbiol.* **13**, 351–354.
Sugarman, B., Epps, L. R., and Stenback, W. A. (1982). *Infect. Immun.* **37**, 1191–1199.
Tannock, G. W. (1974). *Infect. Immun.* **9**, 591–598.
Taylor, R. N., Dietz, T. M., Maxwell, K. W., and Marcus, S. (1974). *Immunol. Commun.* **3**, 439–455.
Warr, G. A., and Jakab, G. J. (1979). *Infect. Immun.* **26**, 492–497.
Warr, G. A., Jakab, G. J., and Hearst, J. E. (1979). *J. Reticuloendothel. Soc.* **26**, 357–366.
Weissman, G., Korchak, H. M., Perez, H. D., Smolem, J. E., Goldstein, I. M., and Hoffstein, S. T. (1979). *Adv. Inflammation Res.* **1**, 95–112.
Williams, R. C., and Gibbons, R. J. (1975). *Infect. Immun.* **11**, 711–718.
Woods, D. E., Strauss, D. S., Johanson, W. G., and Bass, J. A. (1981). *J. Infect. Dis.* **143**, 784–790.
Yates, W. D. G. (1982a). Ph.D. Thesis, University of Saskatchewan, Saskatoon.
Yates, W. D. G. (1982b). *Can. J. Comp. Med.* **46**, 225–263.
Yamada, K. M., and Weston, J. A. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3492–3496.
Yilma, T., Zee, Y. C., and Osebold, J. W. (1979). *J. Infect. Dis.* **139**, 458–464.
Yourtee, E. L., Bia, F. L., Griffith, B. P., and Root, R. K. (1982). *Infect. Immun.* **36**, 11–16.
Zetter, B. R., Daniels, T. E., Quadra-White, C., and Greenspan, J. S. (1979). *J. Dent. Res.* **58**, 484–488.

27

Importance and Control of Fish and Mollusk Viruses

BRUCE L. NICHOLSON

Department of Microbiology, University of Maine at Orono, Orono, Maine

I. INTRODUCTION

While the artificial culture of fish as a source of food and recreation has been practiced in many parts of the world for many years, the scale, intensity, and worldwide interest in aquaculture and fish farming has increased dramatically during the last 20 years. Furthermore, such practices are no longer localized to one country or part of the world. Modern transportation has enabled the final product as well as brood fish and eggs for breeding purposes to be shipped anywhere in the world. In addition, in some parts of the world, the culture of non-native fishes is increasing. As the scale, intensity, and variety of fish culture increases, the risk of disease problems is also increased. Furthermore, the transportation of fish and fish products from one locality to another, or even from one part of the world to another,

ISBN 0-12-429601-7

greatly enhances the spread of diseases. Losses from communicable fish diseases, either endemic or imported, present a real threat to any aquaculture or fish farming venture. Indeed, effective control of diseases is frequently the major determinant in the success of such operations. Because of the difficulty in treating and controlling viral diseases, these can present major problems.

The continued development of new fish cell cultures during the last 20–25 years has provided the means for the isolation and characterization of increasing numbers and varieties of fish viruses. Approximately 50 viruses or viruslike agents of fish have been recognized, and about half this number have been isolated (Wolf and Mann, 1980). Most of these viruses have been demonstrated, or at least implicated, as the etiological agents of specific diseases. Although viruses and virus diseases are known to occur in natural fish populations, most information has been obtained for the most economically important species, particularly those that are artificially propagated for food or sport purposes. In general, fish viruses are similar in most characteristics to viruses of homoiotherms, differing only in host range and optimum temperatures of replication. Much less information is available about viruses of mollusks and other shellfish. Although viruses or viruslike particles have been reported in at least 14 species of shellfish, most of these agents have not been isolated and classification is based almost exclusively on electron microscopy (Hill, 1982).

Several recent, comprehensive reviews of fish and shellfish viruses have been published (Anderson *et al.*, 1983; Hill, 1982; McAllister, 1979; Pilcher and Fryer, 1980; Wolf, 1983). Therefore, this chapter concentrates on those viruses which are likely to cause problems in aquaculture, fish farming, or sea ranching. Little information is available on viral diseases of fish and shellfish in the Middle East and Africa; therefore, this discussion is concerned largely with diseases that have been well documented in other parts of the world. However, many of the fish species discussed are propagated in this region too. Therefore, implications for food fish production in the countries of the Middle East and Africa are emphasized whenever possible.

II. FISH VIRUSES

Although the known fish viruses can be classified into many taxonomic groups, the majority represent the following families: Rhabdoviridae, Reoviridae, Birnaviridae, Herpesviridae, and Iridoviridae.

Most reviewers of the viral diseases of fish have chosen to organize their discussion into a separate consideration of each major virus group. However, the purpose of this report might be better served by considering the diseases as they relate to specific species or groups of fish.

A. Salmonid Species

Salmonid fishes such as trout and salmon have long been artificially propagated on a large scale in Europe and North America, and more recently in Japan and other parts of the world. Therefore, probably more is known about diseases of these species than of others. Although the specific diseases of salmonids may not occur in warm water fishes, they may serve as useful models of viral diseases of fish.

One of the most significant viral diseases of salmonid fishes is infectious pancreatic necrosis (IPN). Recent reviews of IPN include those of Dorson (1982), McAllister (1979), and Pilcher and Fryer (1980). Geographically, IPN is widespread with occurrences in North America, Europe, and Japan. Although IPN viruses (IPNV) and IPN-like viruses have recently been isolated from numerous nonsalmonid fishes as well as from marine invertebrates, the major effect of this disease has been in populations of salmonid fishes subjected to intensive artificial propagation. The most susceptible species include brook trout (*Salvelinus fontinalis*), rainbow trout (*Salmo gairdneri*), brown trout (*Salmo trutta*), amago trout (*Oncorhynchus rhodurus*), sockeye salmon (*O. nerka*), and cutthroat trout (*Salmo clarki*). The virus has been implicated in epizootics of Atlantic salmon (*S. salar*) (Munro *et al.*, 1981), but most work indicates that this species usually fails to exhibit clinical signs of the disease. However, some histopathological lesions are observed, and Atlantic salmon apparently can become carriers of the virus for extended periods of time (Swanson and Gillespie, 1979). A similar situation may exist for most other salmonid species since apparently healthy carriers have been detected in populations of chinook salmon (*O. tshawytsha*), humpback salmon (*O. gorbuscha*), coho salmon (*O. kisutch*), yamake (*O. masou*), huchen (*Hucho hucho*), and grayling (*Thymallus thymallus*).

Infectious pancreatic necrosis and/or IPN-like viruses have also been isolated from numerous nonsalmonid species. Specifically, IPN-like viruses have been shown to be responsible for eel branchionephiritis (Sano *et al.*, 1981) and the spinning disease of the marine clupeid menhaden (*Brevoortia tyrannus*) (Stephens *et al.*, 1980). In addition, the virus has been isolated from other nonsalmonid species without signs of disease. These include roach (*Rutilus rutilus*), carp (*Cyprinus carpio*), minnow (*Phoxinus phoxinus*), barbel (*Barbus barbus*), white sucker (*Catostomus commersoni*), perch (*Perca fluviafilis*), pike (*Esox lucius*), and zebra danio.

IPNV causes high levels of mortality (10–90%) in susceptible salmonids less than 6 months of age. Survivors and adults frequently become lifelong carriers of the virus and serve to disseminate the disease to other fish via transmission through urine, feces, and sex products. Sac fry often die without distinctive clinical signs. In feeding fry, typical symptoms include whirling or corkscrew swimming, swelling of the stomach, darkened coloration, exophthalmia, and the production of fecal casts.

Although originally classified as a reovirus-like agent, IPNV is now classified together with the avian infectious bursal disease virus and the insect *Drosophila* X virus in a new virus family termed the Birnaviridae (Dobos *et al.*, 1979). Fundamental characteristics of these viruses include unenveloped icosahedral-like virions with a diameter of 59–60 nm and consisting of a single-layer protein capsid and a double-stranded RNA genome in two segments.

IPNV replicates in a variety of salmonid and nonsalmonid fish cell cultures with the production of a characteristic cytopathic effect (CPE) (Wolf, 1966). Replication occurs over a relatively wide temperature range of 4–26°C. The serological relationships of the many isolates of IPNV and IPNV-like viruses have not been clearly resolved; however, current evidence suggests that there are at least six distinct serogroups (Dorson, 1983).

Several rhabdoviruses are also a problem in rearing salmonid fishes. Infectious hematopoietic necrosis (IHN) is the name applied to a highly fatal viral disease occurring in a variety of salmonid species; it is endemic to the Pacific Northwest of North America. This disease has occurred in other parts of the world, but ultimately all outbreaks have been linked to fish originating in North America. Information about IHN has been summarized by Nicholson (1983) and Pilcher and Fryer (1980). Natural epizootics have been reported in various species of salmon and trout. The virus is transmitted horizontally and vertically with feces, urine, and ovarian or seminal fluids. Epizootics generally occur in young fish 3–6 months of age with mortality rates usually in excess of 90%. Mortality in fish 2–6 months old is somewhat less but still often exceeds 50%. Acute infections may occur in older fish, but mortality rates are usually very low. Survivors of IHNV infections usually become carriers. These carrier fish appear normal, and no virus can be detected in their tissues. However, high concentrations of virus do appear in ovarian and seminal fluids during spawning. The mechanism by which this carrier state is maintained in adult fish is not understood and has not been studied extensively.

Gross, clinical signs of IHN are similar to those of other virus diseases of salmonid species, including exophthalmia, distension of the abdomen, mucoid fecal casts, and hemorrhaging at the bases of pectoral and pelvic fins, at the vent, and occasionally in the skin and musculature.

The general morphology of the IHN virions is the bullet shape characteristic of rhabdoviruses in general with an average length of 160 nm and a diameter of 90 nm (Amend and Chambers, 1970; Hill *et al.*, 1975). The viral protein coat consists of five structural proteins; this is characteristic of all other fish and mammalian rhabdoviruses (Hill *et al.*, 1975; Lenoir and Dekinkeln, 1975; McAllister and Wagner, 1975). Detailed studies of the molecular characteristics of these structural proteins indicate that IHNV

resembles the mammalian rabies virus and differs in structure from the vesicular stomatitis virus (VSV) type of rhabdovirus. The virus replicates in a variety of salmonid and nonsalmonid fish cell cultures with the production of a characteristic CPE (Amend *et al.*, 1969; Nicholson and Byrne, 1973; Nims *et al.*, 1970; Wingfield, *et al.*, 1969). Replication occurs over a range of 4–30°C, but the optimal temperature range is 13–18°C (Yasutake and Amend, 1972).

A rhabdovirus similar to IHNV is responsible for viral hemorrhagic septicemia (VHS), a serious disease of salmonids, particularly rainbow trout, in Europe (Dekinkeln, 1983; Pilcher and Fryer, 1980). The disease appears to be geographically limited to continental Europe. In addition to rainbow trout, brown trout and pike fry are also natural hosts for VHS virus (Meier and Vestegard-Jorgensen, 1980; Vestegard-Jorgensen, 1980). Atlantic salmon and goldfish have been experimentally infected (Dekinkeln and Castric, 1982; Pfitzner, 1966). Coho and chinook salmon are apparently resistant to VHSV (Dekinkeln *et al.*, 1974). The external signs of VHS are similar to those of IHN and other fish virus diseases; i.e., the signs are general ones and not particularly distinctive of a specific disease. Transmission of VHS is horizontal from carrier fish. Virus shedding by carriers is discontinuous, and the factors influencing this shedding are unknown.

As with the other fish rhabdoviruses, VHS virus particles are typically bullet shaped, 180 nm in length and 60 nm in diameter (Olberding and Frost, 1975). The capsid proteins are similar to IHNV and rabies virus (Hill *et al.*, 1975).

Several representatives of the herpesvirus group have been detected in artificially propagated salmonid fishes. One virus, *Herpesvirus salmonis,* was originally isolated from adult rainbow trout in the United States (Wolf *et al.*, 1975). A similar virus (NeVTA) was isolated independently in Japan from both fry and adult landlocked sockeye salmon (Sano, 1976). Epizootics have been reported with mortality levels ranging from 50 to 100%, clinical manifestations usually being apparent at temperatures below 14°C. The principal pathological lesions occur in kidney hematopoietic tissue, liver, and pancreatic acinar cells. Epizootics of this disease, however, have not apparently been widespread, and its real threat to hatcheries and aquaculture has not been established.

Other herpesviruses have been isolated from adult landlocked masou salmon (*Onchorhynchus masou*) in Japan (Kimura *et al.*, 1981a,b). Although originally isolated from apparently healthy fish, the *O. masou* virus (OMV) has been shown to be lethal for chum salmon with mortalities of 35–60% in young fish. No mortalities have been observed in fish 240 days old. Coho salmon, kokanee salmon, and rainbow trout have also been experimentally infected. As with most other fish viruses the external signs

were general, including anorexia and exophthalmia. Marked histopathologic changes were observed in the liver.

In addition to causing death, the OMV may also be oncogenic. Among the survivors of OMV infection, more than 60% developed tumors, most frequently in the perioral and mandibular region. Also, a virus similar to NeVTA, and designated NTV, has been isolated from tumor tissue of cultured *O. masou* (Sano *et al.,* 1982). In infectivity trials NTV caused mortalities up to a level of 65% in *O. masou* and *O. keta* fry. As with OMV, tumors developed in many of the survivors.

A group of iridoviruses has been described in several species of wild marine fishes and, more recently, in populations of anadromous salmonid species, including Atlantic salmon and several species of Pacific salmon (Appy *et al.,* 1976; Evelyn and Traxler, 1978; Reno *et al.,* 1978; Walker and Sherburne, 1977; Reno and Nicholson, 1981). These viruses apparently replicate solely in erythrocytes, resulting in a viral erythrocytic necrosis (VEN). Therefore they have been designated erythrocytic necrosis viruses (ENV). There are at least three types of ENV that are similar in that they are large, icosahedral, DNA viruses that replicate in the cytoplasm of fish erythrocytes with the production of a characteristic intracytoplasmic inclusion. They differ, however, in size (ranging from 140 to 350 nm in diameter) and ultrastructural details. Although ENV has not been isolated in cell cultures, experimental infection is possible with infected blood, and transmission through the water has been demonstrated (MacMillan and Mulcahy, 1979; Reno and Nicholson, 1981). Apparently ENV does not directly result in death; however, infected fish (particularly young fish) become very anemic and are more susceptible to other infectious agents and adverse environmental stress conditions (MacMillan *et al.,* 1980; Reno *et al.,* 1982).

B. Catfish

Various species of catfish are reared in the United States, Central and South America, Europe, Asia, the Middle East, and Africa. In the United States, channel catfish (*Ictaluras punctatus*) production is the largest food fish industry. Channel catfish virus (CCV) disease is the only viral disease that has been documented as a problem in catfish farming (Fijan *et al.,* 1970; Plumb and Jezck, 1982). CCV appears to be largely limited to the United States, where the greatest concentration of channel catfish farming is located. The only known isolate from outside the United States was from feeding fry shipped from the United States to Honduras.

The channel catfish appears to be the only natural host of CCV. Blue catfish (*I. feuicatus*) have been experimentally infected (Plumb and Chappell, 1978), but brown bullheads (*I. hetulosus*) and yellow bullheads (*I. nat-*

lis) are apparently resistant (Plumb and Jezek, 1983). Differences in the susceptibility of different strains of channel catfish have been reported with mortalities ranging from 10% to over 70% (Plumb *et al.*, 1975). Transmission is horizontal, either water borne or as a result of cohabitation with infected fish (Plumb and Chappell, 1978).

Communicability of CCV is restricted by size and age of fish as well as water temperature (Plumb and Jezek, 1983). Natural infections occur in younger fry and fingerlings when the water temperature is above 23°C. Maintenance of the virus in fish populations is not understood. A carrier or latent state has not been demonstrated, and the virus has not been isolated from fish in the absence of clinical signs of the disease.

Clinical signs of CCVD are, again, similar to those of other acute viral diseases. Secondary bacterial infections frequently mask the presence of CCV in diseased fish.

The CCV is a herpesvirus with an enveloped virion 176–200 nm in diameter (Wolf and Darlington, 1971). The nucleocapsid is icosahedral and 95–105 nm in diameter.

CCV is stable when frozen but completely inactivated in 7 days at 25°C in pond water containing organic material (Wolf, 1973). The virus is sensitive to drying on concrete or fish netting and is almost immediately inactivated in pond bottom muds (Plumb and Jezek, 1983). Stability of CCV in dead fish at 22°C is poor, less than 0.1% of infectivity remaining after 48 hr. Some infectivity, however, persisted after 14 days in fish kept on ice.

CCV virus replicates in brown bullhead (BB) cell cultures and channel catfish ovary (CCO) cell cultures (Bowser and Plumb, 1980). Optimum growth occurs in the range of 25–30°C.

C. Carp

Various species of carp are farmed for food throughout the world, primarily in Europe, Asia, the Middle East, and Africa. As is the case with other fish species, most information about diseases, particularly viral diseases, comes from work in Europe, North America, and Japan. Little specific information is available about viral diseases of carp in other parts of the world.

There are two well documented, major viral diseases of concern to carp culture. Spring viremia of carp (SVC) disease has been reported in most countries of Eastern and Western Europe (Fijan, 1976). A variety of cyprinid species including *Cyprinus carpio* L. are naturally susceptible, and several noncyprinid species have been experimentally infected. Again, the clinical and histopathological signs are not unique. Fish of all ages are apparently susceptible, but the mechanism of transmission is not known. Ep-

izootics generally occur at temperatures of 12–22°C with 16–17°C the optimal temperature. Mortalities usually cease when the water temperature is 20–22°C. Losses in excess of 50% have been reported, but usual levels of mortalities range from very few to 40%.

The etiological agent of SVC is a rhabdovirus termed *Rhabdovirus carpio*. The virion has the typical bullet shape, 160 nm in length and 70 nm in diameter (Hill *et al.*, 1975). Antigenically, *Rhabdovirus carpio* is distinct from the salmonid rhabdoviruses IHNV and VHSV and another fish rhabdovirus, the pike fry rhabdovirus (PFRV). Also the virus-specific structural proteins of *Rhabdovirus carpio* are distinct from those of IHNV, VHSV, and rabies virus but similar to PFRV and vesicular stomatitis virus (VSV).

Another common disease of carp is swim bladder inflammation (SBI), which occurs in pond-reared carp of all ages. The disease can be either acute or chronic (Arshaniza and Bauer, 1973). The acute form is characterized by lack of appetite, loss of hydrostatic equilibrium and movement coordination, and a swollen body. Destruction of the swim bladder and peritonitis are common, with necrotic foci in the swim bladder walls. More commonly, SBI occurs in a chronic form. Symptoms are not distinct and are observed in only 20–30% of the population. External signs include a temporary disturbance of equilibrium and movement coordination and swelling of the body as a result of an increase in the swine bladder volume.

Interestingly, the etiological agent of SBI appears to be the same virus causing SVC, namely *Rhabdovirus carpio* (Bachman and Ahne, 1974; Hill *et al.*, 1975). Since the two diseases have been generally regarded as distinct in pathogenesis, seasonal variation, and geographical distribution, many factors must influence the initiation and course of virus infection. At this time these factors are not known.

Another agent has been isolated from moribund grass carp (*Ctenopharyngodon idella* Val.) This agent was identified as a rhabdovirus which is serologically and biochemically similar, if not identical, to pike fry rhabdovirus (PFR) (Ahne, 1975). Little information is available on species susceptibility, but common carp and silver carp (*Hypothalmichtys molitrix* Val.) are apparently resistant.

D. Eels

One of the most serious diseases of eels in Japanese fish culture is branchionephritis. This disease often results in mortalities of more than 50% in some stocks. Evidence has been presented that a virus is the causative agent (Sano *et al.*, 1981). This virus was first isolated from the European eel (*Anguilla anguilla*) and termed EVE; later it was also isolated from the Japanese eel (*A. japonica*). Subsequently, EVE has been isolated from both

healthy and diseased eels throughout Japan and Taiwan (Kou *et al.*, 1983; Sano *et al.*, 1981). Also a virus similar, and possibly identical, to EVE has been isolated from *Tilapia* (Kou *et al.*, 1983) in Taiwan. Experimental infectivity trials have demonstrated that EVE can produce high levels of mortality in eels and *Tilapia* but not in rainbow trout.

EVE is morphologically and antigenically similar to some strains of IPNV. However, EVE differs from IPNV in host range; i.e., IPNV is lethal for trout but not eels, whereas EVE kills eels but not trout.

Two rhabdoviruses have been isolated from eels shipped to Japan (Hill *et al.*, 1980; Sano, 1976; Sano *et al.*, 1977). Although mortalities occur in rainbow trout associated with a disease clinically indistinguishable from VHS resulting from experimental infection with these viruses, little is known about their ability to cause disease in eels. One of these viruses was isolated from American eels (*A. rosteata*) and termed EVA; the other was isolated from European elvers and designated EVX. The virions are bullet shaped; EVA measures 136–150 nm by 53–58 nm, and EVX measures 170–175 nm by 90–95 nm. EVA and EVX are antigenically similar to each other but not to the other fish rhabdoviruses. Similarly, the virion proteins of the two viruses are indistinguishable in acrylamide gel electrophoresis but differ from those of the other fish rhabdoviruses.

E. *Tilapia*

There is virtually no information available in the scientific literature about viral diseases of *Tilapia*. It is unclear whether this results from a lack of such diseases or from the fact that few investigations of diseases of *Tilapia* have been undertaken in the past. As a result of the popularity of *Tilapia* throughout the world as a species for artifical culture, interest in present and potential *Tilapia* diseases has increased in recent years. Several viruses have been recently isolated from cultured *Tilapia* (Kou *et al.*, 1983). As indicated previously, one of these is similar, and possibly identical, to EVE. EVE does produce high mortalities in experimentally infected *Tilapia*. The second virus has not been characterized even with respect to morphology. The possible role of these viruses in diseases of *Tilapia* and their seriousness as an adverse factor in *Tilapia* culture remain to be assessed.

F. Other Cultured Fish Species

In addition to the species discussed previously, there are several other fishes cultured in various parts of the world. These include mullet, milkfish, and grouper. There is no information in the scientific literature about viral diseases of these species, and apparently no viruses have been isolated from

these fishes. Also, few, if any, experimental infectivity investigations have been carried out using these species; therefore, it is impossible at the present time to assess the effects of viral diseases on the propagation of these species. More research in this area is required.

III. MOLLUSK VIRUSES

Viruses or "viruslike particles" have been described in at least 14 species of mollusks (Hill, 1982). However, procedures for the specific isolation of mollusk viruses are lacking in comparison to those available for fish viruses. Therefore, most mollusk viruses have not been isolated and characterized in any detail. Most available information and tentative classification is based on morphology as visualized by electron microscopy. To date, at least six virus families appear to be found in mollusks: Herpesviridae, Iridoviridae, Papovaviridae, Reoviridae, Birnaviridae, and Retroviridae.

In most cases, conclusive evidence for a viral etiology of destructive diseases of mollusks has not been obtained; however, strong circumstantial evidence suggests that viruses can produce diseases, often devastating diseases, in both natural and cultured populations of mollusks. Most work in this area has concentrated on the most economically important bivalves, including oysters, clams, and mussels. One of the first reports of a virus infecting mollusks was a herpesvirus infection in the American oyster (*Crassostrea virginica*) on the east coast of the United States (Farley *et al.*, 1972). An iridovirus was found to be associated with (and presumably the etiological agent of) epizootics in the Portuguese oyster (*C. angulata*) (Comps, 1972). This disease has virtually destroyed farming of this species in France. Another iridovirus has been linked to a disease of larvae of *C. gigas* in the United States (Comps and Bonami, 1972). While viral etiology as demonstrated by experimental transmission of disease has not been proved with these diseases, a B-type retrovirus has been isolated from the soft-shell clam (*Mya arenaria*) and demonstrated to cause a lethal hematopoietic neoplasm in this species (Hill, 1982).

Several strains of IPNV-like birnaviruses have been isolated in fish cell cultures from several species of bivalve mollusks in Europe and North America (Hill, 1976). In addition, a reovirus has been isolated in fish cell cultures from *C. virginica* in the United States (Meyers, 1980). These viruses have not been demonstrated to produce disease in mollusks; however, several of these viruses are lethal to some species of fish.

Knowledge of viruses infecting mollusks and the potential effects of these viruses in aquaculture is very limited. Increased research efforts are needed to assess the economic importance of these and other, undetected, viruses.

IV. DETECTION AND IDENTIFICATION OF FISH VIRUSES

In some instances, external clinical signs and histopathological examination can be used to make a presumptive diagnosis of viral diseases in fish, particularly if correlated with a knowledge of the past history of diseases in a given population. However, as indicated previously, the signs of many viral diseases in fish tend to be somewhat general and usually are not distinctive. Therefore, positive diagnosis requires the isolation and identification of the causative agent.

Suggested procedures to ensure some standardization of methodology have been approved by several governmental agencies and professional organizations. Typical examples of such procedures include those prepared by the Fish Health Section of the American Fisheries Society (1975) and Environment Canada (Gillespie *et al.,* 1974). In general, a statistically significant number of fish, depending on population size, are collected, and the appropriate tissues or body fluids are prepared for inoculation of fish cell cultures. During epizootics the sampling of moribund fish may require relatively few animals. When testing for virus in asymptomatic fish, the sample size should be large enough to provide a 95% level of confidence with an assumed minimum carrier incidence of 5% for killed fish and 2% if sex products (ovarian or seminal fluids) are used. Pooling of large numbers of samples is usually employed to provide a manageable number of viral assay units. The pattern of sampling should also consider the arrangement and size of rearing units and such factors as common water supplies and age of fish. Sac fry are usually assayed whole, whereas the entire viscera is collected from fingerlings up to 4 cm. With fish larger than 4–5 cm, mixtures of kidney, spleen, and pyloric ceca-pancreas are usually assayed. Samples should be inoculated into cell cultures as soon as possible after collection. If inoculations cannot be made immediately, the samples should be stored or transported at 4°C.

Tissue samples are homogenized, clarified by low speed centrifugation, and diluted in cell culture growth medium. Several dilutions of each homogenate are then inoculated onto fish cell cultures and incubated for a minimum of 14 days. The specific cell cultures used and the temperature of incubation depend on the species of fish and the virus(es) being assayed. Whenever possible it is best to use two or more different cell cultures. Occasionally, a particular virus isolate may not replicate in a cell culture normally sensitive to other isolates of the same virus. An example of this phenomenon is IPNV. The two cell cultures most frequently used in some parts of the world for the isolation of IPNV and other salmonid viruses are the rainbow trout gonad (RTG-2) and fathead minnow (FHM) lines. However, several isolates of IPNV do not replicate in FHM lines and several other isolates replicate only to a limited extent in these cells on primary

isolation (Nicholson *et al.*, 1979). The exclusive use of FHM cells for isolation of IPNV may occasionally result in failure to detect virus.

Replication of a virus in susceptible cell cultures is usually detected by the production of cell death and the destruction of the cell monolayers [cytopathic effect (CPE)]. The type of CPE produced in certain cell cultures is somewhat characteristic of many of the fish viruses, and the experienced pathologist frequently feels confident in making a presumptive identification at this point. However, it should be emphasized that positive identification requires confirmation by a specific test, usually a serological test.

The most commonly used serological test for identification of fish viruses is either the serum or virus neutralization test. The former (using several dilutions of cell culture fluids from cultures exhibiting CPE) is generally easier to perform and is most often employed in routine identification. Neutralization tests, however, suffer from several disadvantages, including cost and complexity. Also, strain differences are frequently more apparent in neutralization tests; i.e., in comparison to other serological tests this test tends to be more specific and results in less cross-reaction between strains. Therefore, the choice of positive antiserum is particularly important.

Several other serological tests widely used in human and veterinary medicine have been applied to the identification of fish viruses. These include immunofluorescence and immunohistochemical tests for IPNV (Nicholson and Henchal, 1978; Piper *et al.*, 1973; Swanson and Gillespie, 1981) and VHS (Vestergard-Jorgensen, 1972; Enzmann, 1981); complement fixation for IPNV (Finlay and Hill, 1975); and enzyme-linked immunosorbent assay (ELIZA) for IPNV (Nicholson and Caswell, 1982) and VHS (Hill, 1982). Although these tests provided significant advantages over the neutralization test, they have not gained the widespread acceptance observed in human and veterinary medicine. However, it is likely that they will be utilized to a greater extent in the not too distant future as the practices of veterinary medicine are more and more applied to aquaculture and fish farming. It should be noted that most serological tests such as the ELISA and immunofluorescence are usually not sensitive enough to detect virus directly in asymptomatic, carrier fish. In these cases, virus must still be isolated first in cell culture. However, these tests significantly simplify and economize virus identification once it has been isolated.

Although standard procedures have been developed by several sources and are used to varying degrees throughout the world, it should be noted that little standardization of reference reagents has been developed for the diagnosis of viral diseases of fish. Significant advances, however, have been made in this field so that some standardization should be possible. This, in fact, was the topic of a recent symposium (Anderson and Hennessen, 1981).

Although much work remains to clarify the antigenic relationships of strains or isolates of many fish viruses, especially IPNV, it should be possible in many cases to provide standard reference strains of viruses and

antisera. With regard to the latter, the use of monoclonal antibodies for fish viruses is very promising and is currently being developed in several laboratories (Nicholson *et al.*, unpublished data).

This discussion of detection and identification of viruses has dealt only with viruses infecting so-called "finfish" and has not considered viruses of mollusks and other "shellfish." As indicated previously, much less information exists for viral diseases of mollusks, and few mollusk viruses have been isolated. And even with these few isolates, demonstration that they result in diseases of shellfish has usually been lacking. One reason, probably the major reason, for this is the lack of mollusk cell cultures. Those few virus isolations from mollusks have utilized "finfish" cell cultures. Until mollusk cell cultures are developed, the ability to detect and identify mollusk viruses will lag behind that of other aquatic animal viruses.

V. CELL CULTURES

Because the detection and identification of most viruses infecting fish ultimately depend on the isolation of the virus by replication in a susceptible cell culture, it is important to consider the types and availability of fish cell cultures.

The first continuous fish cell culture was the RTG-2 line originated 20 years ago from rainbow trout gonads (Wolf and Quimby, 1962). Subsequently, over 60 continuous fish cell cultures representing 17 families and 36 species of fish have been developed, many in the last 10 years. It is beyond the scope of this report to discuss, or even list, all these cell cultures, especially considering that an excellent and comprehensive review of this subject has been published (Wolf and Mann, 1980). However, a brief discussion of fish cell cultures and their use in the diagnosis of viral fish diseases is warranted, particularly in view of the fact that many diagnosticians previously familiar only with mammalian cell cultures and viruses may be called upon to participate in the diagnosis of fish diseases as the scale and scope of worldwide aquaculture continues to expand.

In most respects, cell cultures from fish and other lower vertebrates are handled in a manner similar to homoiotherm cell cultures. That is, standard cell culture growth media supplemented with fetal bovine serum are routinely employed although some cultures from marine fishes exhibit a requirement for increased concentration of NaCl. Naturally, the incubation temperatures are generally lower than for mammalian cells and span a greater range. The optimal incubation temperature for a particular fish cell culture will vary depending on the species. In general the range of growth temperatures of fish cell cultures extends from about 2°C up to 35–37°C. A unique feature of most fish cell cultures is that they can be maintained

at low temperatures (4–15°C) without changing the medium for relatively long periods of time (several months to a year). This characteristic significantly facilitates the handling and maintenance of many different cell cultures, all of which may not be routinely used in diagnosis. In addition, fish cells can be stored frozen at −65°C or lower as with mammalian cells.

Most fish cell cultures have originated from species that are intensively cultivated. For example, at least 18 continuous lines exist from salmonid fishes such as salmon and trout, 9 lines from carp, and 5 from catfish.

The sensitivity of different cell cultures for a particular virus should be determined when selecting cell cultures for use in routine diagnosis. It would be expected that cell cultures derived from the same species or a species closely related to that in which the disease occurs would be the most sensitive for virus isolation. In general this is true, but several significant exceptions occur. For example, while trout or salmon cell cultures are used most often for isolation of IPNV, several investigators report that BF-2 cells derived from the bluegill are more sensitive and, therefore, more useful in isolating IPNV. An example of a more species-specific host range for fish viruses is CCV, which, as mentioned previously, replicates only in several catfish cell cultures.

Many years of experience with the diagnosis of viral diseases in some species such as the salmonids have resulted in the identification of several cell cultures that are particularly suitable for detecting viruses infecting these fish. However, where little or no information or experience with specific viral diseases exist, such as in situations where disease identification and control are being initiated for the first time, or where new species are being cultured, the development and use of cell cultures derived from the local fish species should be a high priority. Indeed, in such situations continuous cell cultures of indigenous species (both cultured and noncultured species) should be developed and used not only to isolate virus from fish during epizootics suspected to have a viral etiology, but also to screen as many healthy fish as possible. The development and use of such cell cultures will markedly improve the chances of isolating viruses from moribund fish and will also provide valuable information on the types of viruses normally found in the area and which may present problems when the appropriate situation exists.

In addition to the cell cultures listed in the review of Wolf and Mann (1980), it should be noted that several new continuous fish cell cultures have been developed as a result of intensive efforts in several parts of the world, primarily Japan and the Republic of China, to develop cell cultures from local species utilized in aquaculture and fish farming. Examples include new cell cultures from common carp (Chen *et al.*, 1983a), loach (Chen *et al.*, 1983a), *Tilapia* (Chen *et al.*, 1983b), milkfish (Tung, personal communication), perch (B. L. Nicholson and J. L. Wu, unpublished data), grouper (B. L. Nicholson and J. L. Wu, unpublished data), and eel (S. N. Chen, per-

sonal communication). These new cell lines should also find application in other parts of the world where these and/or related species of fish are reared.

The development of cell cultures from tissues of mollusks and other shellfish has proved to be one of the most difficult research problems facing shellfish biologists. Indeed, there exists only one continuous cell culture derived from an aquatic invertebrate (excluding insects), and this originated from embryos of the freshwater snail (*Biophalaria glabrata*) (Hansen, 1979). While numerous attempts have been made to develop shellfish cell cultures, the only success has been in the preparation of primary cultures from tissues of bivalve mollusks (Brewster and Nicholson, 1979; Stephens and Hetrick, 1979). These cultures have consisted primarily of hematocytes and, although such cells could be maintained in a viable state for many months, there was no evidence of cell division, and subcultivation was not possible.

The lack of continuous mollusk cell cultures has been the major obstacle in isolating viruses infecting these species. Continued efforts to develop continuous cultures should be maintained. In addition, attempts should be made to isolate viruses from mollusks using primary hematocyte cultures as well as existing fish cell cultures. Few attempts of this type have been made although some birnaviruses and a reovirus have been isolated from bivalve mollusks using fish cell cultures.

VI. CONTROL OF VIRAL DISEASES OF FISH AND SHELLFISH

No vaccines for the control of viral diseases of fish are currently available commercially. However, several vaccines are in various stages of development, and it is likely that some vaccines will eventually be licensed and commercially available for use in aquaculture (Fryer *et al.*, 1977). The use of both inactivated and live, attenuated viral vaccines is being explored.

One of the deterrents to the development of viral vaccines for fish has been the difficulty and expense incurred in obtaining licenses for such vaccines in some countries. Also, the development of an effective vaccine depends on a thorough knowledge of strain variations in antigenic properties and the prevalence of different strains in various parts of the world. This information is not available in many cases. Those viral diseases for which vaccines will most likely be developed include IPN (Dorson, 1983), IHN (Nicholson, 1983), VHS (Dekinkeln, 1983), and CCVD (Plumb and Jezek, 1983).

The most effective control measure for viral diseases in fish farms and other rearing facilities, at the present time, is the prevention of exposure. This is accomplished, whenever possible, by constructing rearing facilities in areas with water supplies free of specific diseases. Furthermore, only fish inspected and certified to be free of specific pathogens should be used for rearing. The indiscriminate transfer of diseased fish has resulted in the dis-

tribution of some diseases throughout the world. This has led to the development of laws and regulations controlling the importation and/or movement of fish, fish eggs, and even some fish products in at least 42 countries (Fryer *et al.*, 1979). Inspection and certification programs for salmonid species in particular are very extensive and rigid in some countries, including the United States and Canada. In general, however, the nature and thoroughness of inspection and certification programs varies greatly from one country to another.

While the establishment of a thorough inspection program and the enactment of laws regulating the importation of diseased fish have been demonstrated to be very effective in controlling certain viral diseases, it must be recognized that such programs require trained pathologists and facilities for testing. The cost of such programs can be very high. However, the economic impacts resulting from the loss of dead or diseased fish in the marketplace also may be substantial. The cost of the control measures employed must be weighed against the cost of the disease.

In general, however, attempts should be made to identify and understand the actual and potentially destructive viral diseases in the different species of cultured fish and shellfish in the various parts of the world. Implicit in this, therefore, is the need to develop additional cell cultures from such species and use these cultures together with other fish cell cultures to investigate epizootics of suspected viral origin and to screen cultured and wild populations for the presence of potentially destructive viruses.

REFERENCES

Ahne, W. (1975). *Arch. Virol.* **48,** 181.

Amend, D. F., and Chambers, V. C. (1970). *J. Fish. Res. Board Can.* **27,** 1285.

Amend, D. F., Yasutake, W. T., and Meade, R. W. (1969). *Trans. Am. Fish. Soc.* **98,** 796.

American Fisheries Society, Fish Health Section. (1975). Suggested Procedures for the detection and identification of certain infectious diseases of fishes.

Anderson, D. P., and Hennessen, W., eds. (1981). "Fish Biologics: Serodiagnostics and Vaccines." *Dev. Biol. Stand.* **49.**

Anderson, D. P., Dorson, M. M., and Dubourget, P., eds. (1983). "Antigens of Fish Pathogens; Proceedings of Symposium International de Talloires." Collection Foundation Marcel Merioux, Lyon.

Appy, R. C., Burt, M. D. B., and Morris, T. J. (1976). *J. Fish. Res. Board Can.* **33,** 1380.

Arshaniza, N. M., and Bauer, O. N. (1973). Symp. on the Major Communicable Fish Diseases in Europe and Their Control, *EIFAC Tech. Pap.* **17** (Suppl. 2), p. 140.

Bachman, P. A., and Ahne, W. (1974). *Arch. Gesamte Virusforsch.* **44,** 261.

Bowser, P. R., and Plumb, J. A. (1980). *J. Wildl. Dis.* **16,** 451.

Brewster, F., and Nicholson, B. L. (1979). *J. Fish. Res. Board Can.* **36,** 461.

Chen, S. N., Chen, J. H., and Kou, G. H. (1983a). *Proc. Republic of China-Japan Symposium on Fish Diseases.* In press.

Chen, S. N., Chi, S. C., Ureno, Y., and Kou, G. H. (1983b). *Proc. Republic of China-Japan Symposium on Fish Diseases.* In press.

Comps, M. (1972). *C. R. Consil. International Exploration de Mer*, p. 22.
Comps, M., and Bonami, J. P. (1977). *C. R. Hebd. Seances Acad. Sci., Ser. D* **285**, 1139.
Dekinkeln, P. (1983). *In* "Antigens of Fish Pathogens" (*Proc. Symp. Int. Talloires*) (D. P. Anderson, M. M. Dorson, and P. Durbourget, eds.), pp. 51–62. Collection Foundation Marcel Merioux, Lyon.
Dekinkeln, P., and Castric, J. (1982). *J. Fish Dis.* **5**, 57.
Dekinkeln, P., LeBerre, M., and Meurillon, A. (1974). *Bull. Fr. Pisic.* **253**, 166.
Dobos, P., Hill, B. J., Hallet, R., Kelly, D. T. C., Becht, H., and Teninges, P. (1979). *J. Virol.* **32**, 593.
Dorson, M. (1983). *In* "Antigens of Fish Pathogens" (*Proc. Symp. Int. Talloires*) (D. P. Anderson, M. M. Dorson, and P. Dubourget, eds.), pp. 7–32. Collection Foundation Marcel Merioux, Lyon.
Enzmann, P. Z. (1981). "Fish Biologics: Serodiagnostics and Vaccines," *Dev. Biol. Stand.* **49**, p. 57.
Evelyn, T. P. T. and Traxler, G. S. (1978). *J. Fish. Res. Board Can.* **35**, 903.
Farley, C. A., Banfield, W. G., Kasnic, G., and Foster, W. S. (1972). *Science* **178**, 759.
Fijan, N. (1976). *Gyobyo Kenkyu (Fish Pathol.)***10**, 129.
Fijan, N. N., Wellborn, T. L., and Naftel, J. P. (1970). *U. S. Dep. Inter. Bur. Sport Fish Wildl. Tech. Pap.* **43**, 11.
Finlay, J., and Hill, B. J. (1975). *Aquaculture* **3**, 305.
Fryer, J. L., Amend, D. F., Harrell, L. W., Novotny, A. J., Plumb, J. A., Rohovec, J. S., and Tebbit, G. L. (1977). Oregon State University Sea Grant College Program, Publ. No. ORESU-T-77-012.
Fryer, J. L., Rohovec, J. S., Pullford, E. F., Olson, R. G., Ransom, D. P., Winton, J. R., Iannon, C. N., Hedrick, R. P., and Groberg, W. J. (1979). Proc. Conf. Disease Inspection and Certification of Fish and Fish Eggs, Oregon State University Sea Grant College Program, Publ. No. ORESU-W-79001.
Gillespie, D. C., Evelyn, T. P. T., Frantsi, C., MacKelvie, R. M., and Newfield, N. (1974). Methods for Detection of Certain Pathogens of Salmonid Fishes. *Environment Can. Misc. Spec. Publ.* **23**.
Hansen, E. L. (1979). *Tissue Cult. Assoc. Man.* **5**, 1009.
Hill, B. J. (1976). *FAO Aquaculture Bull.* **8**, 13.
Hill, B. J. (1982). *Abstracts 4th International Conference on Comparative Virology*, p. 25 Banff, Canada.
Hill, B. J., Underwood, B. O., Smale, C. J., and Brown, F. (1975). *J. Gen. Virol.* **27**, 359.
Hill, B. J., Smale, C. J., Underwood, B. O., and Brown, F. (1980). *Intervirology* **14**, 208.
Kimura, T., Yoshimizu, M., Tanaka, M., and Sannohe, H. (1981). *Gyoby. Kenkyu (Fish Pathol.)* **15**, 143.
Kimura, T., Yoshimizu, M., and Tanaka, M. (1981). *Gyobyu Kenkyu (Fish Pathol.)* **15**, 149.
Kou, G. H., Chen, S. N., Yeno, Y., Hedrick, R. D., and Fryer, J. L. (1983). *Proc. Republic of China–Japan Symposium on Fish Diseases.* In press.
Lenoir, G., and Dekinkeln, P. (1975). *J. Virol.* **16**, 259.
McAllister, P. E. (1979). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.) Vol. 14, pp. 401–470. Plenum, New York.
McAllister, P. E., and Wagner, R. R. (1975). *J. Virol.* **15**, 733.
MacMillan, J. R., and Mulcahy, D. (1979). *J. Fish. Res. Board Can.* **36**, 1097.
MacMillan, J. R., Mulcahy, D., and Landolt, M. (1980). *Can. J. Fish. A.* **37**, 799.
Meier, W., and Vestegard-Jorgensen, P. E. V. (1980). *In* "Fish Diseases" (W. Ahne, ed.), p. 8–17. Springer-Verlag, Berlin and New York.
Meyers, T. R. (1980). *J. Fish Dis.* **3**, 187.
Munro, A. L. S., Smail, D. A., Waddell, I. F., and Liversidge, J. (1981). *Abstr. FAO/EIFAC, 4th COPRAQ Meeting.*

Nicholson, B. L. (1983). *In* "Antigens of Fish Pathogens" (*Proc. Symp. Int. Talloires*) (D. P. Anderson, M. M. Dorson, and P. Dubourget, eds.), pp. 63–80. Collection Foundation Marcel Merioux, Lyon.
Nicholson, B. L., and Byrne, C. (1973). *J. Fish. Res. Board Can.* **30**, 913.
Nicholson, B. L., and Caswell, P. (1982). *J. Clin. Microbiol.* **16**, 469.
Nicholson, B. L., and Henchal, E. A. (1978). *J. Wildl. Dis.* **14**, 465.
Nicholson, B. L., and Reno, P. W. (1981). *Gyobyo Kenkyu (Fish Pathol.)* **15**, 129.
Nicholson, B. L., Thorne, G. W., Janicki, C., and Hanson, A. (1979). *J. Fish. Dis.* **2**, 367.
Nims, L., Fryer, J. L., and Pilcher, K. S. (1970). *Proc. Soc. Exp. Biol. Med.* **135**, 6.
Olberding, K. P., and Frost, J. W. (1975). *J. Gen. Virol.* **27**, 305.
Pfitzner, I. (1966). *Zentralbl. Bakteriol. Parasitenk. Infectionskr. Hyg.* **201**, 306.
Pilcher, K. S., and Fryer, J. L. (1980). *CRC Crit. Rev. Microbiol.* 7(4), 287–364.
Piper, D., Nicholson, B. L., and Dunn, J. (1973). *Infect. Immun.* **8**, 249.
Plumb, J. A., and Chappell, J. (1978). *Proc. Annu. Conf. Southern Fish and Wildlife Agen.* **32**, 680.
Plumb, J. A., and Jezek, D. A. (1983). *In* "Antigens of Fish Pathogens" (*Proc. Symp. Int. Talloires*) (D. P. Anderson, M. M. Dorson, and P. Dubourget, eds.), pp. 33–50. Collection Foundation Marcel Merioux, Lyon.
Plumb, J. A., Green, O. L., Smitherman, R. O., and Pardue, G. B. (1975). *Trans. Am. Fish. Soc.* **104**(1), 140.
Reno, P. W. and Nicholson, B. L. (1981). *J. Fish Dis.* **4**, 361.
Reno, P. W., Philippon-Fried, M., Nicholson, B. L., and Sherburne, S. W. (1978). *J. Fish. Res. Board Can.* **35**, 148.
Reno, P. W., Serreze, P., Hellyer, S., and Nicholson, B. L. (1982). *Abstracts 4th International Conference on Comparative Virology,* p. 133. Banff, Canada.
Sano, T. (1976). *Gyobyu Kenkyu (Fish Pathol.)* **10**, 221.
Sano, T., Nishimura, T., Okamoto, N., and Fukuda, H. (1977). *Nippon Suisan Gakkaishi* **43**, 491.
Sano, T., Okamoto, N., and Nishimura, T. (1981). *J. Fish Dis.* **4**, 127.
Sano, T., Fukuda, H., Okamoto, N., Nakayama, Y., and Kaneko, F. (1982). *Abstracts 4th International Conference on Comparative Virology,* p. 138. Banff, Canada.
Stephens, E. B., and Hetrick, F. M. (1979). *Tissue Cult. Assoc. Man.* **5**, 991.
Stephens, E. B., Newman, M. W., Zachary, A. L., and Hetrick, F. M. (1980). *J. Fish Dis.* **3**, 387.
Swanson, R. N., and Gillespie, J. H. (1979). *J. Fish. Res. Board Can.* **36**, 587.
Swanson, R. N., and Gillespie, J. H. (1981). *J. Fish. Dis.* **4**, 309.
Vestergard-Jorgensen, P. E. (1972). *Symp. Zool. Soc. London* **30**, 333.
Vestergard-Jorgensen, P. E. (1980). *In* "Fish Diseases" (W. Ahne, ed.) p. 3–7. Springer-Verlag, Berlin and New York.
Walker, R., and Sherburne, S. W. (1977). *J. Fish. Res. Board Can.* **34**, 1188.
Wingfield, W. H., Fryer, J. L., and Pilcher, K. S. (1969). *Proc. Soc. Exp. Biol. Med.* **130**, 1055.
Wolf, K. (1966). *Adv. Virus Res.* **12**, 35–101.
Wolf, K. (1973). *In* "The Herpesviruses" (A. S. Kaplan, ed.), pp. 495–520. Academic Press, New York.
Wolf, K. (1982). *Abstracts 4th International Conference on Comparative Virology,* Banff, Canada, p. 23.
Wolf, K., and Darlington, R. W. (1971). *J. Virol.* **8**, 525
Wolf, K., and Mann, J. A. (1980). *In Vitro* **16**, 168.
Wolf, K., and Quimby, M. C. (1962). *Science* **135**, 1065.
Wolf, K., Sano, T., and Kimura, T. (1975). *U. S. Fish Wildl. Serv., Fish Dis. Leaflet* **44**.
Yasutake, W. T., and Amend, D. F. (1972). *J. Fish Biol.* **4**, 261.

28

Recent Advances in Immunodiagnosis of Viral Diseases of Crops

M. H. V. VAN REGENMORTEL

Institute of Molecular and Cellular Biology, Strasbourg, France

I. INTRODUCTION

Several surveys have shown that virus diseases of the main food crops in countries of the Middle East and Africa are responsible for considerable yield losses and low crop productivity (Guthrie, 1978; Mink, 1976; Makkouk *et al.*, 1979).

Surveys to determine which plant viral diseases are present have been carried out in only a few of the countries in this geographical area, but all available data indicate that a wide variety of virus diseases are present in food crops. In the Jordan Valley, where tomatoes and cucurbits are grown over about 40% of the cultivated area, it is common to see entire fields of these vegetables nearly 100% infected with viral diseases (Mink, 1976; Al-Musa, 1982). At least 25 different viral diseases have been recognized in Jordan, either on the basis of transmission and serology (Table I) or on the basis of field symptoms. In the Ivory Coast, 30 different viral diseases have been recognized in industrial and food crops, legumes, and vegetables (Fauquet and Thouvenel, 1980). Surveys carried out in Kenya showed that the disease caused by cassava mosaic virus reduces yields by 70% whereas the maize streak disease and groundnut virus diseases lead to losses of 25–60% and 60–70%, respectively (Bock, 1982).

ISBN 0-12-429601-7

TABLE I
Plant Viruses Isolated from Vegetable Crops in Jordan[a]

Virus	Crop	Identification by	
		Host range and properties	Serology
Bean yellow mosaic virus	Broadbean	+	
Cucumber mosaic virus	Pepper, squash	+	+
Cucumber yellow vein virus	Cucurbits	+	
Lettuce mosaic virus	Lettuce	+	
Potato virus Y	Pepper, tomato	+	
Squash mosaic virus	Squash	+	+
Tobacco mosaic virus	Broadbean, tomato	+	+
Tomato aspermy virus	Tomato	+	+
Tomato black ring virus	Cucumber, tomato	+	+
Tomato bushy stunt virus	Cucumber, tomato	+	+
Tomato yellow leaf curl virus	Tomato	+	
Watermelon mosaic virus	Squash	+	+

[a]Data from Mink (1976), Makkouk (1978), Mansour and Al-Musa (1982), and Al-Musa (1982).

A correct identification of the viruses causing diseases represents the first step of any program aimed at reducing yield losses in important food crops. Since the symptoms shown by diseased plants are often an inadequate guide to the identification of the causative agent, a reliable diagnosis usually requires a study of the serological properties of the viruses. A serological test is the most rapid and reliable way of demonstrating that the etiological agent belongs to an established group of viruses or that it corresponds to an already well-known virus. Such an identification often makes it possible to predict how the disease is transmitted, and it may also give some indication as to the type of control measures that are likely to succeed.

II. NEWLY DESCRIBED VIRUSES

Although all plant viruses cannot yet be identified serologically, an initial survey of the viruses present in a geographical area necessarily involves a considerable amount of serological testing with standard plant virus antisera. A comprehensive list of the 262 plant viruses that could be identified

by serological techniques in 1980 has been published (Van Regenmortel, 1982). The references included in that publication make it possible to locate likely sources of the virus antisera. In the period 1981–1982, a number of additional viruses were characterized serologically, and these are listed in Table II. This brings to 285 the number of different virus species that can be distinguished by means of antisera at the present time. In addition to identifying entities that are considered to be separate viruses (or virus species) by the International Committee on the Taxonomy of Viruses (Matthews, 1982), it is also possible by means of strain-specific antibodies to distinguish a large number of strains and serotypes of each virus species (Jones and Diachun, 1977; Lin *et al.*, 1981). A major advantage of serological identification methods is that the unique discriminating power of antigenic specificity allows the identification of viruses at the genus (group), species, and strain level.

Virus identification at the group level is continuously being improved as a result of the increased sensitivity of new serological methods. For instance, luteoviruses that used to be considered as distinct are now known to be serologically related (Rochow and Duffus, 1981); similarly, some whitefly-transmitted and leafhopper-transmitted members of the geminivirus group have recently been shown to be antigenically related (Thomas and Bowyer, 1980; Sequeira and Harrison, 1982). The use of indirect ELISA procedures is of particular interest in this respect since it permits the detection of many different virus strains by means of virus antiserum directed to one strain only (Van Regenmortel and Burckard, 1980; Koenig, 1981).

III. PRECIPITATION AND AGGLUTINATION METHODS

In spite of the development of very sensitive virus detection methods such as ELISA, liquid precipitin tests and immunodiffusion tests are still widely used in the serological analysis of plant viruses (see Table II). In crude extracts of infected plant tissue, the concentration of many plant viruses is often sufficient for detection by serological precipitation. For detecting viruses that diffuse poorly in gel, reliable immunodiffusion methods based on the use of dissociated viral subunits have been developed (Purcifull and Batchelor, 1977). These tests, which are carried out in the presence of sodium dodecyl sulfate, were in the past mainly applied to the detection of elongated viruses, but they have been found to be equally suitable for detecting isometric viruses (Purcifull *et al.*, 1981). Simplified procedures that eliminate tissue grinding and chemical treatment of the antigen have also been developed (Lima and Purcifull, 1980). It is possible, for instance, to cut out leaf disks from infected leaves by means of a paper punch and to

TABLE II
Serological Characterization of Plant Viruses Described in 1981–1982

Virus	Virus group	Serological method[a]	Reference	CMI/AAB Description No.[b]
American hop latent	carla	EIA	Adams and Barbara	—
Artichoke latent	poty	ID, IEM	Rana *et al.* (1982)	—
Bean rugose mosaic	como	PRE, ID, IEM	Gamez (1982)	246
Blackgram mottle		ID	Scott and Hoy (1981)	237
Carrot red leaf	luteo	ID, IEM	Waterhouse and Murant (1981)	249
Cereal chlorotic mottle	rhabdo	ID, PRE	Greber (1982)	251
Galinsoga mosaic		ID	Behncken *et al.* (1982)	252
Garlic yellow streak	poty	PRE	Mohamed and Young (1981)	—
Hibiscus latent ringspot	nepo	ID	Brunt *et al.* (1980)	233
Hop latent	carla	PRE, IEM, EIA	Adams and Barbara (1982a)	—
Lamium mild mosaic		ID, IEM	Lisa *et al.* (1982)	—
Melandrium yellow fleck	bromo	PRE, ID	Hollings and Horvath (1981)	236
Peanut green mosaic	poty	PRE, ID, PHA	Sreenivasulu *et al.* (1981)	—
Pelargonium zonate spot		ID	Gallitelli (1982)	—
Plantain virus X	potex	PRE, ID	Hammond and Hull (1981)	—
Sweet potato feathery mottle		PRE	Cali and Moyer (1981)	—
Tephrosia symptomless		ID	Bock *et al.* (1981)	256
Tobacco leaf curl	gemini	PRE, ID	Osaki and Inouye (1981)	232
Tobacco necrotic dwarf	luteo	PRE, ID	Kubo and Takanami (1979)	234
Tulip virus X	potex	PRE, ID	Mowatt (1982)	—
Ullucus mosaic	poty	PRE	Brunt *et al.* (1982a)	—
Ullucus virus C	como	ID	Brunt *et al.* (1982b)	—
Zucchini yellow mosaic	poty	PRE, ID, IEM	Lisa *et al.* (1981)	—

[a]EIA, enzyme immunoassay; ID, immunodiffusion; IEM, immunoelectron microscopy; PHA, passive hemagglutination; PRE, precipitation.

[b]Commonwealth Mycological Institute—Association of Applied Biologists Description of Plant Viruses.

use these disks as an antigen source by placing them in the wells of immunodiffusion plates (Wisler *et al.*, 1982).

In the case of viruses with double-stranded (ds) RNA genomes, it has been found possible (Francki and Jackson, 1972) to detect virus-specific dsRNA in plant extracts by means of immunodiffusion tests using antibodies to poly(I):poly(C). Attempts to use the same antibodies for detecting the dsRNA produced during infection by viruses with single-stranded RNA genomes failed, mainly because of the presence of high levels of plant dsRNA in healthy leaves (Gould and Francki, 1981).

Other useful techniques based on precipitation that have been applied in recent years are rocket immunoelectrophoresis (Juretic and Mamula, 1980; Hagen *et al.*, 1982) and counterimmunoelectrophoresis (Rohozinski *et al.*, 1981). Another useful technique is the serological detection of viral proteins in crude extracts from infected plants, following their electrophoretic migration on polyacrylamide gels. Viral protein can be transferred electrophoretically from the gels to nitrocellulose paper and their presence revealed with virus antiserum followed by labeled protein A (O'Donnell *et al.*, 1982) or antiglobulin enzyme conjugates (Rybicki and Von Wechmar, 1982).

Agglutination reactions refer to procedures where either the antigen or the antibody is attached to the surface of red blood cells or carrier particles of similar size (latex, bentonite, etc.). Because of the size of these reacting particles, visible clumping can be induced with a lower concentration of serological reactants than is needed for precipitation. In passive hemagglutination, virus particles or antibodies are coupled to erythrocytes by various chemical treatments (see Van Regenmortel, 1982). Nonspecific agglutination of erythrocytes is sometimes observed, but this can be prevented by the addition of 0.5% bovine serum albumin (Rajeshwari *et al.*, 1981; Sreenivasulu *et al.*, 1981).

The latex test, which uses commercially available polystyrene latex particles, has been improved by coupling antibody molecules to latex via an intermediate layer of protein A (Querfurth and Paul, 1979; Torrance, 1980a). This modification of the test has been called PALLAS (protein A-coated latex-linked antiserum) and is somewhat easier to perform, mainly because the amount of antibody needed for sensitization of the latex is not critical. Compared to precipitation tests, the hemagglutination and latex tests allow the detection of 100- to 1000-fold smaller quantities of virus (Van Regenmortel, 1982).

IV. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The ELISA method has become widely used for detecting and identifying plant viruses and it has replaced many of the older techniques (Clark, 1981; Bar-Joseph and Garnsey, 1981; Van Regenmortel, 1982). For several years,

plant virologists used mainly the direct "double antibody sandwich" form of ELISA described by Clark and Adams (1977) in spite of the fact that this test suffers from two disadvantages: it is extremely strain specific (Koenig, 1978; Koenig and Paul, 1982), and it requires a separate antivirus enzyme conjugate to be prepared for each virus to be tested. In recent years, indirect ELISA methods based on the use of antiglobulin conjugates or labeled protein A have become increasingly popular (Van Regenmortel and Burckard, 1980; Bar-Joseph and Malkinson, 1980; Rybicki and Von Wechmar, 1981; Devergne *et al.*, 1981; Koenig, 1981; Lommel *et al.*, 1982; Barbara and Clark, 1982). The different types of ELISA used in plant virology are described briefly in Table III.

Although indirect ELISA procedures possess several advantages over direct procedures, some researchers consider that the need for a second virus antiserum prepared in a different animal species is a major inconvenience of the indirect method. In the author's laboratory, chickens have been used routinely for producing antibodies against more than 20 different plant viruses; laying hens are very convenient animals to house, and large quantities of viral antibody can be obtained easily from the egg yolks (Polson *et al.*, 1980; Gardner and Kaye, 1982). Hens can be immunized successfully with as little as 100 μg of virus, and with most plant viruses enough antigen will generally be available for immunizing both a rabbit and a chicken.

The need for employing antibodies from two animal species can be circumvented by the use of $F(ab')_2$ fragments of rabbit immunoglobulins for coating microtiter plates (Barbara and Clark, 1982). The $F(ab')_2$ fragments derived from specific antibodies trap the virus, which is then detected with the whole immunoglobulin followed by a protein A enzyme conjugate. Protein A will bind to the Fc portion of the antibody molecule, but not to the $F(ab')_2$ fragment used for trapping the virus. This method has been used

TABLE III
Types of ELISA Used in Plant Virology[a]

1	AG, AB^R-E
2	AG, AB^R, ANTI-R^G-E
3	AG, $McAB^M$, ANTI-M^R-E
4	AB^R, AG, AB^R-E
5	AB^C, AG, AB^R, ANTI-R^G-E
6	AB^R, AG, $McAB^M$, ANTI-M^R-E
7	ANTI-M^R, $McAB^M$, AG, AB^C, ANTI-C^R-E
8	$F(AB')^R_2$, AG, AB^R, ANTI-Fc $(R)^G$-E
9	$F(AB')^R_2$, AG, AB^R-E

[a]AG, antigen; AB, antibody; R, rabbit; G, goat; E, enzyme; Mc, monoclonal antibody; M, mouse; C, chicken; $F(AB')_2$ and Fc antibody fragments.

successfully for detecting relationships among carlaviruses that were overlooked when direct ELISA was used for the serological comparisons (Adams and Barbara, 1982a,b).

Another way of avoiding the need for virus antiserum prepared in two animal species consists in coating microtiter plates with the C1q component of bovine complement. The C1q molecules will bind the virus–IgG complexes formed when virus and specific antiserum are incubated together in the wells; the complexes trapped by C1q are then detected by an enzyme-labeled antiglobulin conjugate (Torrance, 1980b). The C1q ELISA possesses the same advantage as other indirect ELISA methods, namely that it can detect several virus serotypes using an antiserum prepared to only one of them (Torrance, 1981). However, the method suffers from several disadvantages: background control readings tend to be high, many plant saps have an inhibitory effect on the assay, and C1q is not readily available commercially.

ELISA procedures are much more convenient than earlier serological techniques for large-scale testing of virus infections in crops. The major limitation in analyzing large numbers of samples by ELISA resides in the preparation of leaf extracts by tissue grinding. This problem can be overcome by replacing leaf homogenates by disks of leaf tissue cut out with a paper punch (Marco and Cohen, 1979; Romaine *et al.*, 1981). When such disks are placed in the wells of microtiter plates and buffer is added, enough virus is released from injured cells at the periphery of the disks to allow its detection by ELISA. Although the use of disks reduces the sensitivity of the assay, it is possible to compensate for this disadvantage by increasing the concentration of reactants and the incubation times. In cases where the virus concentration in infected tissue is sufficiently high, the use of leaf tissue disks should greatly facilitate large-scale indexing for virus infections.

The high sensitivity of ELISA has greatly simplified the detection of many plant viruses that are present in diseased tissue at a very low concentration. It has been possible, for instance, to detect lettuce necrotic yellows virus in plant extracts without prior concentration of the virus (Chu and Francki, 1982), whereas for detection by immunodiffusion it was necessary first to concentrate plant extracts. The conventional method of detecting lettuce necrotic yellows virus by infectivity tests was shown to be about 600 times less sensitive than detection by ELISA (Chu and Francki, 1982).

The detection of plant viruses in their insect vectors has also been considerably improved by the use of ELISA (Gera *et al.*, 1978; Clarke *et al.*, 1980; Tamada and Harrison, 1981; Carlebach *et al.*, 1982). Although insect extracts tend to produce relatively large nonspecific reactions, it was found that as little as 0.01 ng of potato leafroll virus present in a single aphid could be detected (Tamada and Harrison, 1981). Since there are large differences in virus content of individual aphids, it is necessary to use batches

of 10–20 aphids per sample to obtain consistent results. A variation of ELISA using a fluorogenic substrate instead of the usual chromogenic ones has been introduced for the detection of plant viruses (Torrance and Jones, 1982). The *p*-nitrophenyl phosphate substrate used with alkaline phosphatase-labeled antibodies in ELISA is replaced by the fluorogenic substrate 4-methylumbelliferyl phosphate in the enzyme-linked fluorescence immunoassay. Theoretically, the hydrolyzed fluorescent substrate can be detected at a 1000-fold lower concentration than that required for detecting the yellow *p*-nitrophenol by spectrophotometry (Shalev *et al.*, 1980). This assay has been used for detecting potato leafroll virus in dormant infected potato tubers and barley yellow dwarf virus in single viruliferous aphids (Torrance and Jones, 1982). The assay was found to be about eight times more sensitive for virus detection than the usual ELISA, its sensitivity being limited by the background fluorescence of the samples. The increased sensitivity of fluorescent immunoassays will be useful in diagnostic work as it will facilitate the detection of viruses in certain tissues and will also extend the period over which indexing for virus infection can be performed successfully.

It is possible to perform ELISA by coating the surface of polystyrene beads instead of the usual microtiter plates, but there seems to be little advantage to that procedure (Chen *et al.*, 1982). The particular assay conditions in direct ELISA found to be optimal by different investigators do not always agree, and some improvement in sensitivity may be obtained by varying parameters such as reactant concentrations and the length and temperature of incubation (McLaughlin *et al.*, 1981). It is also possible to gain time by incubating some of the reactants together instead of sequentially (Van Regenmortel, 1984). This is illustrated in Fig. 1. Some time may be saved this way, but the sensitivity of the assay may also be considerably reduced.

V. IMMUNOSORBENT ELECTRON MICROSCOPY (ISEM)

Virus–antibody complexes can be visualized in the electron microscope by means of three different phenomena that may occur together or separately: clumping (Milne and Luisoni, 1975), antibody coating (Langenberg, 1974), and trapping on antibody-coated grids (Derrick, 1973). The trapping technique has been called "serologically specific electron microscopy," but, since all forms of immunoelectron microscopy can be said to be serologically specific, it seems preferable to use the term immunosorbent electron microscopy (ISEM) to refer to techniques by which virus particles are attached to grids previously coated with antiserum (Roberts *et al.*, 1982).

The ISEM technique has been used extensively for identifying and de-

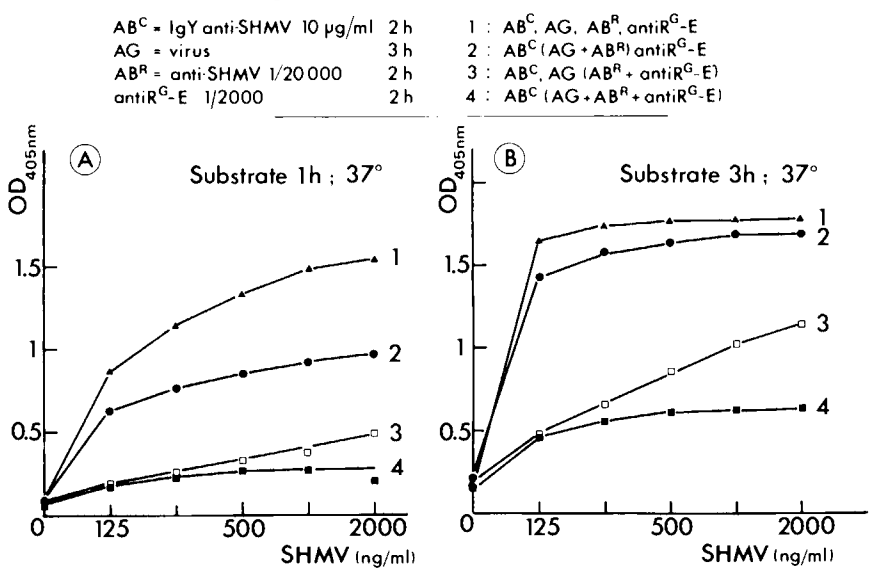


Fig. 1. Indirect ELISA with the tobamovirus, sunn hemp mosaic virus (SHMV), illustrating the influence of substrate incubation time (1 hr in A and 3 hr in B) on assay sensitivity. To obtain curves 1, all reactants were added sequentially; for curves 2, the virus and binding antibody were added together in the wells; for curves 3 the binding antibody and conjugate were added together; for curves 4 the virus, binding antibody, and conjugate were added together. From Van Regenmortel (1984).

tecting plant viruses present at a low concentration in infected cells or insect vectors (Milne and Lesemann, 1978; Roberts *et al.*, 1980; Cohen *et al.*, 1982). The main advantages of the technique are its great sensitivity and the fact that only minute volumes of both antiserum and virus-containing preparations are needed. When purified virus preparations are used in ISEM tests, nonspecific attachment of the particles to grids sometimes occurs (Lesemann *et al.*, 1980; Waterhouse and Murant, 1981); no such nonspecific reactions are found with crude sap from infected plants or when plant sap from healthy plants is added to the purified virus preparations. The ISEM technique has also been used successfully for demonstrating relationships between viruses (Nicolaïeff and Van Regenmortel, 1980), for instance, among luteoviruses (Roberts *et al.*, 1980), although the pattern of relationships revealed by ISEM is not always the same as the degree of relationship demonstrated by other serological techniques (Waterhouse and Murant, 1981).

When microscope grids are pretreated with protein A of *Staphylococcus aureus* before coating them with antiserum, it is sometimes possible to increase the sensitivity of the ISEM technique (Gough and Shukla, 1980; Le-

semann and Paul, 1980). Another useful procedure consists in trapping virus particles by means of protein A-containing *S. aureus* cells coated with a layer of viral antibodies (Nicolaïeff *et al.*, 1982).

The highest degree of specificity in immunoelectron microscopy is achieved when the phenomena of trapping, clumping, and antibody coating are combined. A double layer of antibody coating, first with a virus-specific rabbit antiserum and then with sheep anti-rabbit immunoglobulins, has been reported to improve the visualization of coated virus particles (Kerlan *et al.*, 1982).

VI. MONOCLONAL ANTIBODIES

Monoclonal antibodies produced by continuous cultures of lymphocyte hybrids (Köhler and Milstein, 1975) are rapidly replacing polyclonal antisera for many diagnostic purposes. The hybrid cell lines, known as hybridomas, are produced by fusing spleen cells from immunized mice with a continuously growing line of mouse myeloma cells. By cloning the lymphocyte hybrids, one obtains a series of clones, each producing a single antibody to one particular antigenic determinant of the virus particle. The monospecificity of the monoclonal antibody preparations makes it possible to dissect apart the complex antigenic structure of viruses and to obtain diagnostic reagents of very high specificity (Yewdell and Gerhard, 1981; Oxford, 1982). Monoclonal antibodies directed to several plant viruses have become commercially available, and it seems likely that they will be used increasingly in the future. The use of monoclonal antibodies presents the following advantages: (1) The availability of very large quantities of each antibody ensures that it can be used for reference purposes in laboratories all over the world. (2) The variability of polyclonal antisera will be eliminated and uniform results will be obtained in all laboratories, for instance, in the quantitative measurement of cross-reactivity between viruses. (3) It will be possible to identify viruses at the species or strain level in a reproducible manner. (4) Contamination problems with antibodies to other viruses or to plant antigens will be eliminated.

Hybridoma lines secreting monoclonal antibodies to plant viruses have been obtained in several laboratories (Al Moudallal *et al.*, 1982; Halk *et al.*, 1982a,b) and found to be capable of differentiating between closely related viral strains and mutants. The ability of a monoclonal antibody prepared against tobacco mosaic virus (TMV) to differentiate in ELISA between several tobamoviruses is illustrated in Fig. 2. This particular antibody is capable of differentiating between closely related strains of TMV that differ from the common strain by only a few amino acid exchanges (Briand *et al.*, 1982); for instance, the orchid strains O1 and O3 that can be distin-

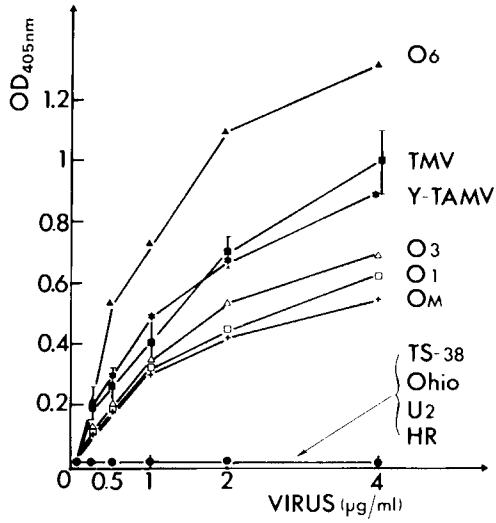


Fig. 2. Detection of tobamoviruses by indirect ELISA using a monoclonal antibody raised against TMV. Coating of the wells was done with 1 µg of goat anti-TMV globulin per milliliter. The different viruses were incubated in the coated wells for 3 hr. The monoclonal antibody (ascitic fluid) was allowed to interact for 3½ hr, and the enzyme conjugate (rabbit anti-mouse globulin) for 2 hr. Strains O1, O3, and O6 are orchid strains of TMV (Al Moudallal *et al.*, 1982) and OM is the common Japanese strain. Strain Y-TAMV is a tomato mosaic strain. From Briand *et al.* (1982).

guished from TMV (Fig. 2) were previously considered to be serologically identical with TMV when analyzed by means of polyclonal antiserum. On the other hand, some tobamoviruses that are always distinguishable from TMV when compared by means of antisera were recognized as antigenically different only by some of the TMV monoclonal antibodies that were obtained (Briand *et al.*, 1982). As shown in Fig. 2, tomato mosaic virus (Y-TAMV) was not distinguishable from TMV, presumably because the one antigenic determinant that is recognized by this monoclonal antibody is unaltered in Y-TAMV and TMV. In indirect ELISA, none of the nine TMV monoclonal antibodies described by Briand *et al.* (1982) showed any binding to tobamoviruses that differed from TMV by a serological differentiation index (Van Regenmortel, 1975) larger than 2. The small number of hybridomas studied until now is probably responsible for the fact that no antibodies recognizing a common antigenic determinant in the tobamovirus group have yet been isolated.

Although monoclonal antibodies often possess a greater discriminatory capacity for differentiating between closely related viruses than polyclonal antisera, this is not necessarily an advantage in all diagnostic applications. When identification at the strain level is not required or when a broad range of different strains should be detected, it may be necessary to prepare a

cocktail of monoclonal antibodies of different specificities. Furthermore, a large number of hybridoma may have to be produced to permit the selection of clones with the desired degree of selectivity.

Another peculiarity of monoclonal antibodies is illustrated in Fig. 2 in the case of strain O6. It is often found that certain strains and mutants are better recognized by the monoclonal antibody than the homologous virus used for immunization (Al Moudallal *et al.*, 1982). Such antibodies that recognize a related antigen better than the homologous one are called heterospecific (see Van Regenmortel, 1982) and are more easily recognized in hybridoma clones than in polyclonal antisera. It is now generally recognized that the binding sites of antibodies are polyfunctional (Talmage, 1959; Richards and Konigsberg, 1973; Cameron and Erlanger, 1977) and that the specificity of polyclonal antisera is the result of a population phenomenon that masks the potential cross-reactivities of each individual antibody (Lane and Koprowski, 1982). In the case of monoclonal antibodies, heterospecific activities are not masked by the additive effect of common antibody reactivities for the immunogen, and the superior binding to another antigen stands out more clearly.

It is clear that although monoclonal antibodies possess a number of distinct advantages compared to polyclonal antisera, they may also, at times, be too specific for the diagnostic task at hand. Detailed investigations with many different plant viruses should be carried out to determine the suitability of monoclonal antibodies as diagnostic reagents in a variety of epidemiological situations.

REFERENCES

Adams, A. N., and Barbara, D. J. (1982a). *Ann. Appl. Biol.* **101**, 483–494.

Adams, A. N., and Barbara, D. J. (1982b). *Ann. Appl. Biol.* **101**, 495–500.

Al Moudallal, Z., Briand, J. P., and Van Regenmortel, M. H. V. (1982). *EMBO J.* **1**, 1005–1010.

Al-Musa, A. (1982). Personal communication.

Barbara, D. J., and Clark, M. F. (1982). *J. Gen. Virol.* **58**, 315–322.

Bar-Joseph, M., and Garnsey, S. M. (1981). *In* "Plant Diseases and Vectors: Ecology and Epidemiology" (K. Maramorosch and K. F. Harris, eds.), pp. 35–54. Academic Press, New York.

Bar-Joseph, M., and Malkinson, M. (1980). *J. Virol. Methods* **1**, 179–183.

Behncken, G. M., Francki, R. I. B., and Gibbs, A. J. (1982). *CMI/AAB Descriptions of Plant Viruses,* No. 252.

Bock, K. R. (1982). Personal communication.

Bock, K. R., Guthrie, E. J., Figueiredo, G., and Woods, R. D. (1981). *Ann. Appl. Biol.* **97**, 277–283.

Briand, J. P., Al Moudallal, Z., and Van Regenmortel, M. H. V. (1982). *J. Virol. Methods* **5**, 293–300.

Brunt, A. A., Barton, R. J., Phillips, S., and Lana, A. O. (1980). *Ann. Appl. Biol.* **96**, 37–43.
Brunt, A. A., Phillips, S., Jones, R. A. C., and Kenten, R. H. (1982a). *Ann. Appl. Biol.* **101**, 65–71.
Brunt, A. A., Barton, R. J., Phillips, S., and Jones, R. A. C. (1982b). *Ann. Appl. Biol.* **101**, 73–78.
Cali, B. B., and Moyer, J. W. (1981). *Phytopathology* **71**, 302–305.
Cameron, D. J., and Erlanger, B. F. (1977). *Nature (London)* **268**, 763–765.
Carlebach, R., Raccah, B., and Loebenstein, G. (1982). *Ann. Appl. Biol.* **101**, 511–516.
Chen, L. -C, Durand, D. P., and Hill, J. H. (1982). *Phytopathology* **72**, 1177–1181.
Chu, P. W. G., and Francki, R. I. B. (1982). *Ann. Appl. Biol.* **100**, 149–156.
Clark, M. F. (1981). *Annu. Rev. Phytopathol.* **19**, 83–106.
Clark, M. F., and Adams, A. N. (1977). *J. Gen. Virol.* **34**, 475–483.
Clarke, R. G., Converse, R. H., and Kojima, M. (1980). *Plant Dis.* **64**, 43–45.
Cohen, J., Loebenstein, G., and Milne, R. G. (1982). *J. Virol. Methods* **4**, 323–330.
Derrick, K. S. (1973). *Virology* **56**, 652–653.
Devergne, J. C., Cardin, L., Burckard, J., and Van Regenmortel, M. H. V. (1981). *J. Virol. Methods* **3**, 193–200.
Fauquet, C., and Thouvenel, J. C. (1980). "Virus Diseases of Crop Plants in Ivory Coast" ORSTOM Publications, Paris.
Francki, R. I. B., and Jackson, A. O. (1972). *Virology* **48**, 275–277.
Gallitelli, D. (1982). *Ann. Appl. Biol.* **100**, 457–466.
Gamez, R. (1982). *CMI/AAB Descriptions of Plant Viruses*, No. 246.
Gardner, P. S., and Kaye, S. (1982). *J. Virol. Methods* **4**, 257–262.
Gera, A., Loebenstein, G., and Raccah, B. (1978). *Virology* **86**, 542–545.
Gough, K. H., and Shukla, D. D. (1980). *J. Gen. Virol.* **51**, 415–419.
Gould, A. R., and Francki, R. I. B. (1981). *J. Virol. Methods* **2**, 277–286.
Greber, R. S. (1982). *CMI/AAB Descriptions of Plant Viruses*, No. 251.
Guthrie, E. J. (1978). *Plant Dis. Rep.* **62**, 839–841.
Hagen, T. J., Taylor, D. B., and Meagher, R. B. (1982). *Phytopathology* **72**, 239–242.
Halk, E. L., Hsu, H. T., Aebig, J., and Chang, K. (1982a). *Phytopathology* **72** (Abstr.), 938.
Halk, E. L., Hsu, H. T., and Aebig, J. (1982b). *Phytopathology* **72** (Abstr.), 953.
Hammond, J. and Hull, R. (1981). *J. Gen. Virol.* **54**, 75–90.
Hollings, M., and Horvath, J. (1981). *CMI/AAB Descriptions of Plant Viruses*, No. 236.
Jones, R. T., and Diachun, S. (1977). *Phytopathology* **67**, 831–838.
Juretic, N., and Mamula, D. (1980). *Intervirology* **13**, 209–213.
Kerlan, C., Mille, B., Detienne, G., and Dunez, J. (1992). *Ann. Virol. (Inst. Pasteur)* **133E**, 3–14.
Koenig, R. (1978). *J. Gen. Virol.* **40**, 309–318.
Koenig, R. (1981). *J. Gen. Virol.* **55**, 53–62.
Koenig, R., and Paul, H. L. (1982). *J. Virol. Methods* **5**, 113–125.
Köhler, G., and Milstein, C. (1975). *Nature (London)* **256**, 495–497.
Kubo, S., and Takanami, Y. (1979). *J. Gen. Virol.* **42**, 387–398.
Lane, D., and Koprowski, H. (1982). *Nature (London)* **296**, 200–202.
Langenberg, W. G. (1974). *Phytopathology* **64**, 128–131.
Lesemann, D. E., and Paul, H. L. (1980). *Acta Hortic.* **n110**, 119–128.
Lesemann, D. E., Bozarth, R. F., and Koenig, R. (1980). *J. Gen. Virol.* **48**, 257–264.
Lima, J. A. A., and Purcifull, D. E. (1980). *Phytopathology* **70**, 142–147.
Lin, M. T., Anjos, J. R. N., and Rios, G. P. (1981). *Phytopathology* **71**, 435–438.
Lisa, V., Boccardo, G., D'Agostino, G., Dellavalle, G., and D'Aquilio, M. (1981). *Phytopathology* **71**, 667–672.

Lisa, V., Luisoni, E., Boccardo, G., Milne, R. G., and Lovisolo, O. (1982). *Ann. Appl. Biol.* **100**, 467–476.
Lommel, S. A., McCain, A. H., and Morris, T. J. (1982). *Phytopathology* **72**, 1018–1022.
McLaughlin, M. R., Barnett, O. W., Burrows, P. M., and Baum, R. H. (1981). *J. Virol. Methods* **3**, 13–25.
Makkouk, K. M. (1978). *Plant Dis. Rep.* **62**, 259–262.
Makkouk, K. M., Shehab, S., and Majdalani, S. E. (1979). *Phytopathol. Z.* **96**, 263–267.
Mansour, A., and Al-Musa, A. (1982). *Phytopathol. Z.* **103**, 35–40.
Marco, S., and Cohen, S. (1979). *Phytopathology* **69**, 1259–1262.
Matthews, R. E. F. (1982). *Intervirology* **17**, 4–199.
Milne, R. G., and Lesemann, D. E. (1978). *Virology* **90**, 299–304.
Milne, R. G., and Luisoni, E. (1975). *Virology* **68**, 270–274.
Mink, G. I. (1976). *Research Report,* Faculty of Agriculture, University of Jordan.
Mohamed, N. A., and Young, B. R. (1981). *Ann. Appl. Biol.* **97**, 65–74.
Mowatt, W. P. (1982). *Ann. Appl. Biol.* **101**, 51–63.
Nicolaïeff, A., and Van Regenmortel, M. H. V. (1980). *Ann. Virol. (Inst. Pasteur)* **131E**, 95–110.
Nicolaïeff, A., Katz, D., and Van Regenmortel, M. H. V. (1982). *J. Virol. Methods* **4**, 155–166.
O'Donnell, I. J., Shukla, D. D., and Gough, K. H. (1982). *J. Virol. Methods* **4**, 19–26.
Osaki, T., and Inouye, T. (1981). *CMI/AAB Descriptions of Plant Viruses,* No. 232.
Oxford, J. (1982). *J. Hyg.* **88**, 361–368.
Polson, A., Von Wechmar, M. B., and Van Regenmortel, M. H. V. (1980). *Immunol. Commun.* **9**, 475–493.
Purcifull, D. E., and Batchelor, D. L. (1977). *Bull.-Fla. Agric. Exp. Stn.* **788**.
Purcifull, D. E., Christie, S. R., and Lima, J. A. A. (1981). *Phytopathology* **71**, 1221–1224.
Querfurth, G., and Paul, H. L. (1979). *Phytopathol. Z.* **94**, 282–285.
Rajeshwari, R., Reddy, D. V. R., and Iizuka, N. (1981). *Phytopathology* **71**, 306–308.
Rana, G. L., Russo, M., Gallitelli, D., and Martelli, G. P. (1982). *Ann. Appl. Biol.* **101**, 279–289.
Richards, F. F., and Konigsberg, W. H. (1973). *Immunochemistry* **10**, 545–553.
Roberts, I., Tamada, T., and Harrison, B. D. (1980). *J. Gen. Virol.* **47**, 209–213.
Roberts, I., Milne, R. G., and Van Regenmortel, M. H. V. (1982). *Intervirology* **18**, 147–149.
Rochow, W. F., and Duffus, J. E. (1981). *In* "Handbook of Plant Virus Infections" (E. Kurstak, ed.), pp. 147–170. Elsevier, Amsterdam.
Rohozinski, J., Francki, R. I. B., and Chu, P. W. G. (1981). *J. Virol. Methods* **3**, 177–186.
Romaine, C. P., Newhart, S. R., and Anzola, D. (1981). *Phytopathology* **71**, 308–312.
Rybicki, E. P., and Von Wechmar, M. B. (1981). *Virology* **109**, 391–402.
Rybicki, E. P., and Von Wechmar, M. B. (1982). *J. Virol. Methods* **5**, 267–278.
Scott, H. A., and Hoy, J. W. (1981). *CMI/AAB Descriptions of Plant Viruses,* No. 237.
Sequeira, J. C., and Harrison, B. D. (1982). *Ann. Appl. Biol.* **101**, 33–42.
Shalev, A., Greenberg, A. H., and McAlpine, P. J. (1980). *J. Immunol. Methods* **38**, 125–139.
Sreenivasulu, P., Iizuka, N., Rajeshwari, R., Reddy, D. V. R., and Nayudu, M. V. (1981). *Ann. Appl. Biol.* **98**, 255–260.
Talmage, D. W. (1959). *Science* **129**, 1643–1648.
Tamada, T., and Harrison, B. D. (1981). *Ann. Appl. Biol.* **98**, 261–276.
Thomas, J. E., and Bowyer, J. W. (1980). *Phytopathology* **70**, 214–217.
Torrance, L. (1980a). *Ann. Appl. Biol.* **96**, 45–50.
Torrance, L., (1980b). *J. Gen. Virol.* **51**, 229–232.
Torrance, L. (1981). *Ann. Appl. Biol.* **99**, 291–299.

Torrance, L., and Jones, R. A. C. (1982). *Ann. Appl. Biol.* **101,** 501–509.
Van Regenmortel, M. H. V. (1975). *Virology* **64,** 415–420.
Van Regenmortel, M. H. V. (1982). "Serology and Immunochemistry of Plant Viruses." Academic Press, New York.
Van Regenmortel, M. H. V. (1984). *In* "Control of Virus Diseases" (E. Kurstak, ed.), pp. 405–422. Dekker, New York.
Van Regenmortel, M. H. V., and Burckard, J. (1980). *Virology* **106,** 327–334.
Waterhouse, P. M., and Murant, A. F. (1981). *Ann. Appl. Biol.* **97,** 191–204.
Wisler, G. C., Zettler, F. W., and Purcifull, D. E. (1982). *Phytopathology* **72,** 835–837.
Yewdell, J. W., and Gerhard, W. (1981). *Ann. Rev. Microbiol.* **35,** 185–206.

29

Enzyme Immunoassays Applied in Virology: Reagents Preparation and Interpretation

E. KURSTAK,* P. TIJSSEN,* AND C. KURSTAK†

**Comparative Virology Research Group, Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada*

†Laboratory of Virology, Hospital Hôtel-Dieu de Montreal, University of Montreal, Montreal, Quebec, Canada

ISBN 0-12-429601-7

I. INTRODUCTION

Enzyme immunoassays (EIA) are rapidly becoming one of the standard procedures in clinical and biochemical laboratories to identify and quantify antibodies, antigens, or haptens in biological or test fluids (Kurstak *et al.*, 1984). These techniques are based on two biological phenomena: (1) the extraordinary discriminatory power of antibodies to distinguish, sometimes very closely related, antigens; and (2) the amplification factor due to high enzyme activities. It is not uncommon that an enzyme has a turn number of 10^5 (Barman, 1974).

Other recent developments, such as the hybridoma technique, will further increase the potential of the EIA. It is important, however, to realize that sensitivities of EIA are limited by the affinity constant of the antigen–antibody systems (in which the affinity constant is a thermodynamic expression of the monovalent interaction). Monoclonal antibodies often lack the affinity bonus encountered with polyclonal sera. To obtain an equal avidity, a judicious selection of monoclonal antibodies is required. Disregard of this aspect might explain early unsatisfactory results obtained with EIA when monoclonal antibodies were used instead of polyclonal antisera.

Reformulation of the equilibrium equation for the affinity constant K_a [$= \exp(-\Delta F^\circ/RT)$] into the Langmuir adsorption isotherm, and numerical analysis using the maximum concentration of antibody which can be bound on a solid phase (e.g., of a microtiter plate), show that K_a^{-1} should exceed the antibody concentration in order to have a positive response. Different concentrations of immunoreactants measured at antibody concentrations close to the K_a^{-1} can be distinguished not at all or only poorly, since results will reflect mainly avidity. At higher K_a^{-1} levels (about more than 10 times the antibody concentration) the enzyme reaction obtained reflects the immunoreactant concentration and reliable dose-response curves can be obtained.

The affinity constants of the various antibodies in polyclonal antisera determine also the degree of cross or shared reactivity. This specificity is generally assessed by comparing the ability of various cross-reacting ligands to displace the labeled tracer from the antibody sites. This relative ability has been termed the relative potency (rp) in which

$$\text{rp} = \frac{\text{(cross-reacting ligand) bound}}{\text{(specific ligand) bound}} = (1 - B)\,\frac{K_a^s}{K_a^c} + B$$

in which B is the fraction of tracer-bound antibody and K_a^s and K_a^c are the affinity constants for the specific and cross-reacting immunoreactant, respectively. Thus the relative potency varies with B and the ratio of the affinity constants. This consideration is more complicated in enzyme immuno-

assays than for radioimmunoassays since the affinity constant of the immunoreactant will be affected more by attaching an enzyme than a small radiolabel. Specificity of enzyme immunoassays can be increased significantly by choosing an immobilized antibody concentration intermediate between the affinity constant of the nonspecific ligand and the specific ligand.

EIA will complement or even replace many of the other methods in current use, such as radioimmunoassays, since it is cheaper, simpler, less dangerous, and requires less expensive equipment and its reagents are stable.

The designs of EIA are numerous and depend on the goal of their application. The objectives of this chapter is to present the principles of the techniques, to demonstrate generally used designs, and to indicate how these may be modified for specific purposes. Currently about 500 papers are published annually in which EIA is applied, and many authors use different or modified designs. It is, however, relatively easy to detect a common thread.

Reviews on this subject have been published by Scharpe *et al.* (1976), Wisdom (1976), Kurstak *et al.* (1977, 1984), Voller and Bidwell (1977), Schuurs and van Weemen (1977); O'Sullivan *et al.* (1979); Oellerich (1980); and Carlier *et al.* (1981).

EIA are applied mainly in three different fields, which we shall review in this work: (1) identification and titration procedures, (2) identification and localization after electrophoresis and "Western" blotting on nitrocellulose membranes and the possibility of epitope mapping, and (3) identification and localization of antigens in cells.

Titration procedure using EIA may be performed in heterogeneous systems [i.e., with a solid phase (van Weemen and Schuurs, 1971; Engvall and Perlmann, 1971)] or in homogeneous systems (Rubenstein *et al.*, 1972). Moreover, they can be subdivided into competitive and noncompetitive systems and direct or indirect methods. It is also possible to subdivide them according to the enzymes used, the nature of the substrate (colorimetric, fluorimetric, biochemiluminimetric, radiometric), the nature of the solid phase, and the way the enzyme activity is measured (i.e., application of principles of enzyme kinetics).

Results are also affected by the enzyme kinetics. Many enzymes can be shown to obey Michaelis–Menten kinetics, which relates the actual enzyme activity to the maximum reaction velocity obtainable (V_{max}) and to the substrate concentration at which $V_{max}/2$ is obtained (the so-called Michaelis constant, K_m. The two parameters are often easily determined in homogeneous solutions, but often it is not realized that they may change considerably for immobilized enzyme. The solid phase may have a considerable zeta potential, causing a redistribution of ions near the solid phase, so that the enzyme may be in a completely different environment (ionic strength,

pH) and have an activity much lower than that potentially possible (e.g., less than 1%). Besides these partition effects, hydrophobic or ionic interactions between enzyme and solid phase may change the conformation. This may affect the activity, in particular of allosteric enzymes. Furthermore, restrictions in diffusion from and to this unstirred layer will lower enzyme activity (i.e., the product:substrate ratio will be higher near the enzyme solid phase than in the solution). These factors have to be taken into consideration for all solid-phase enzyme immunoassays.

In order to summarize this very large subject, only those topics which are essential and can be used for a large range of experiments will be discussed. The following topics are considered essential.

1. Efficient conjugation of enzyme to antibody or to antigen (hapten), and methods to obtain cheap enzyme of high activity.
2. Solid-phase immobilization principles.
3. Application of EIA in heterogeneous systems and homogeneous systems.
4. Blotting and localization of antigens both in nitrocellulose membranes and in cells.
5. Data processing and transformation of sigmoid dose–response curves.
6. Recognition of causes which may lower specificity or sensitivity.

II. CONJUGATION PROCEDURES

Different conjugation procedures have been developed. The most satisfactory procedure for the conjugation of one enzyme to an antibody is often not satisfactory for another enzyme. Three groups of conjugation procedures may be distinguished:

1. Chemical linkage of enzyme to antibody (i.e., with a covalent bond).
2. Immunological linkage, in which an antibody is prepared to an enzyme and can introduce this enzyme into the immune complex by this noncovalent bond. A requirement for this method is that enzyme activity is not abrogated by the antibody.
3. Linkage by bridge procedures. All living systems are based on recognition among molecules. Some of these recognition systems used for EIA are the avidin–biotin system and lectins.

For conjugation, four important parameters should be considered: yield, immune reactivity of complex, enzyme activity of complex, and size of conjugate. Considerable variation exists with respect to yield among the dif-

ferent conjugation methods. The most widely used techniques yield often less than 10% conjugate, but they are used because they are technically undemanding. Three different approaches can be taken for chemical linkage: direct linkage, linkage by homobifunctional reagents, and linkage by heterobifunctional cross-linkers. Two important principles, which are widely ignored in the literature, to obtain optimum yields are the law of mass action and the Poisson probability distribution (hit kinetics). Moreover, buffers should be chosen which ensure the highest activity of the reactive groups and do not result in a repulsion of the proteins (e.g., if the pH of the medium is not between the p*I*s of the proteins to be conjugated, both will have like net charges and may repel each other, preventing efficient conjugation).

On the other hand, immunological linkage demands the availability of both antienzyme antibodies and bridging antibodies. Assays using these complexes generally need more steps, but the assay is more sensitive for immunohistochemistry (less so for immunoassays).

The avidin–biotin system avoids many of these problems and has higher detectabilities than the immunologically linked probes. The particular advantages of the avidin–biotin system are that immunoreactants are very easily biotinylated, though it is not applicable for all enzymes, and the extremely high affinity constant of avidin for biotin (10^{15} M^{-1}), which equals a half-life of about 160 days (almost similar to covalent linkage). Moreover, this enables the use of the same serum in different incubations just by biotinylation of the antibodies for one of the incubations. This approach can also be achieved after haptenation and production of antibodies against this particular hapten. This method is of interest for the simultaneous detection of two or more antigens in the same cell.

The first and third methods will be discussed in more detail here. The enzyme used is peroxidase, which has high activities and is easily detected (Kurstak and Kurstak, 1974). The cost of this enzyme is lower than that for other enzymes used in EIA, such as alkaline phosphatase, β-galactosidase, or glucose oxidase, but is nevertheless high, reflecting overhead, marketing costs, etc., of the suppliers. We will show here a simple method (Tijssen and Kurstak, 1984; Kurstak *et al.*, 1984) in which a crude extract of the enzyme is purified in about an hour, saving hundreds of dollars. Moreover, the enzyme prepared by this method has a higher activity than the "pure" enzyme provided by commercial suppliers.

In a critical study, we recently reestablished the parameters of the conjugation procedure to obtain conjugates of high quality and to simplify earlier methods (Tijssen and Kurstak, 1983, 1984).

Moreover, a method is presented to obtain avidin–biotin–peroxidase complexes with high activity in EIA (Section II,D).

A. Purification of Peroxidase

1. *Materials Needed for Peroxidase Purification*

Crude peroxidase (type II from Sigma, or from other companies, the grade of enzyme with an RZ of about 1.0)

DEAE-Sepharose or equivalent, a small amount which can be used repeatedly

Sodium phosphate buffer, 2.5 m*M*, pH 8.0

A small column and a spectrophometer. It is convenient if this is equipped with a flow cell.

2. *Method of Peroxidase Purification*

i. Crude peroxidase is dissolved in the phosphate buffer.
ii. The DEAE-Sepharose is equilibrated with the same buffer (first with high-ionic strength phosphate buffer, pH 8.0, and then with 2.5 m*M* buffer) in a small column.
iii. The enzyme sample is applied (up to 5 mg of protein for milliliter of gel) to the gel.
iv. The impurities are retained while the most active isozyme of peroxidase C passes directly. The elution of pure peroxidase C can be monitored with the spectrophotometer or even visually.

3. *Analysis of Purified Peroxidase Obtained*

Peroxidase quality is usually established according to two criteria, the RZ (i.e., the optical density ratio at 401 and 275 rm, reflecting the hemin content of the protein) and by specific activity (activity per unit weight).

Peroxidase at 1 mg/ml has an optical density at 403 nm (1 cm optical path length) of 2.25 cm^2/mg. The assay mixture contains, besides 0.1 *M* sodium phosphate buffer (pH 6.5), 1 m*M* hydrogen peroxide, 30 m*M* 3-dimethylaminobenzoic acid, and 0.6 m*M* 3-methyl-2-benzothiazolinone hydrozone. The volume activity is measured at 590 nm and can be converted (by dividing by the amount of enzyme) to specific activity (U/mg). An operational molar extinction coefficient of the product is 47,600 (see Appendix, Section A).

B. Purification of Immunoglobulin

A plethora of methods exist to purify IgG from sera, such as salts or organic solvent precipitation, chromatography, electrophoresis, isoelectric focusing, isotachophoresis, ion-exchange chromatography, and affinity chromatography (Kurstak and Kurstak, 1974; Kurstak *et al.*, 1984).

Salt precipitation (i.e., 35% ammonium sulfate or 18% sodium sulfate)

remains most convenient, though some important precautions should be taken to obtain excellent instead of mediocre results. Some of the precautions are to make the proper choice of the salt concentration and the correct measurement of the pH of concentrated salt solutions.

A more recent, but very convenient, method is protein A–Sepharose affinity chromatography. This method is based on the observation that protein A isolated from *Staphylococcus aureus* (in particular the Cowan strain) has a strong affinity for the Fc fragments of IgG of many mammals, including man (except IgG3) and rabbit (Langone, 1982).

Method

i. A small column of protein A-Sepharose is prepared and equilibrated with PBS.
ii. Serum diluted once with PBS is added (1 ml of serum per 2 ml of Sepharose), and the column is washed with PBS until no protein is detected in the eluent.
iii. A gradient can be applied (with decreasing pH to about 3.0), if the subclasses of the IgG are to be recovered separately, or 0.58% acetic acid in a 0.85% sodium chloride solution if bulk desorption is desired.
iv. IgG is recovered in a test tube containing 0.2 ml of 0.2 *M* Tris-HCl, pH 8.0.
v. The approximate purity and quantity of IgG are most conveniently established by the measurement of the optical densities of the protein sample at 251 and 278 nm. The absorbance ratio at 278/251 nm should be 2.5, and the absorbance at 278 nm divided by 1.4 yields the concentration (in mg/ml).

C. Chemical Linkage of Peroxidase

1. *Principle of Chemical Peroxidase Conjugation*

The method chosen for this purpose takes advantage of the fact that the peroxidase protein is situated in a shell of carbohydrates. These carbohydrates can be oxidized gently, so that enzyme activity is hardly affected, but that nevertheless sufficient aldehyde groups are generated. These aldehyde groups are allowed to form Schiff's bases with the amino groups of the IgG (about 70 present per molecule), and subsequently the bases formed are stabilized by reduction with sodium borohydride ($NaBH_4$).

2. *Material Needed for Peroxidase Conjugation*

Sodium carbonate and sodium bicarbonate
Sodium metaperiodate

Sodium borohydride
Concanavalin A–Sepharose and α-methyl-D-mannopyranoside
Ammonium sulfate, glycerol, sodium hydroxide

3. *Method*

The method consists of four steps—peroxidase activation, conjugation, stabilization, and conjugate purification—and is performed at room temperature.

a. Activation of Peroxidase (HRPO)

i. Dissolve 5 mg of purified peroxidase in 0.5 ml of freshly prepared 0.1 *M* sodium bicarbonate (prepared in distilled water) in a small closed tube.
ii. Add 0.5 ml of 8–16 m*M* (depending on lot of HRPO) sodium metaperiodate ($NAIO_4$) and leave for 2 hr in the dark at 20°C.

b. Conjugation of Peroxidase. Formation of Schiff's Bases between Peroxidase and Antibodies

i. Prepare 15 mg of IgG in 0.1 *M* sodium carbonate buffer, pH 9.2 (1–2 ml).
ii. Add dry Sephadex G-25 to Pasteur pipette (bottom closed) fitted with glasswool filter (amount of Sephadex equals one-sixth of combined weight of HRPO and IgG samples) and containing HRPO and IgG samples.
iii. The mixture is incubated for 3 hr at room temperature to allow the Schiff's bases to form.

c. Stabilization of Conjugate

i. The conjugate is eluted from the Sephadex by chasing with the appropriate carbonate buffer.
ii. Add 1/20 volume of freshly prepared sodium borohydride (5 mg/ml $NaBH_4$ in 0.1 m*M* NaOH) and after 30 min add 3/20 volume of another freshly prepared sodium borohydride ($NaBH_4$) solution.
iii. After an incubation of 1 hr, the preparation may be purified.

d. Purification of Peroxidase Conjugate

i. Precipitate peroxidase–IgG conjugate and free IgG by the addition of an equal volume of saturated ammonium sulfate (with mixing) and equilibration for 1 hr.

ii. The precipitate is collected by low-speed centrifugation (10 min at 5000 × 8), washed with 50% ammonium sulfate. Free peroxidase remains in solution in this step and is thus removed.
iii. The collected precipitate is dialyzed against CA buffer (0.1 *M* acetate buffer, pH 6.0, supplemented with 1 *M* sodium chloride, 1 m*M* calcium chloride, 1 m*M* magnesium chloride, and 1 m*M* manganese chloride).
iv. In a second step, IgG is separated from the conjugate, an important step which may increase the detectability in EIA 10-fold (Fig. 1). Concanavalin A-Sepharose has an affinity for peroxidase, and free IgG will flow through in contrast to the conjugate.
v. After all the free IgG has eluted, CA buffer is supplemented with 0.02 *M* α-methyl-D-mannopyranoside, which competes with peroxidase for the lectin and thus desorbs the conjugate.
vi. This conjugate is analyzed by measuring the optical density at 403 and 278 nm (density at 403 nm should be about three- to four-tenths that at 278 nm).
vii. Storage of the conjugate is most convenient by adding 5 mg of bovine serum albumin per milliliter (not essential, but helpful for long-term storage) and an equal volume of glycerol; store at −20°C.

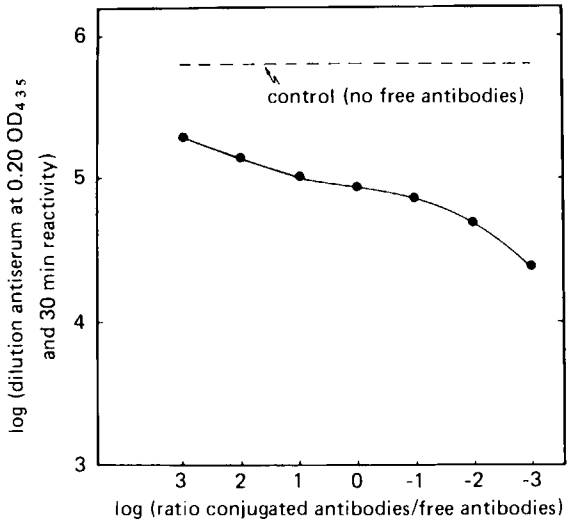


Fig. 1. Influence of competing free antibodies on the reaction with the conjugate (antibody-peroxidase).

D. Biotinylation of Enzyme and IgG

i. IgG or peroxidase concentrations in 0.2 *M* sodium bicarbonate should be about 20 mg/ml. Biotinyl-*N*-hydroxysuccinimide (BNHS) is dissolved immediately prior to use in dimethylformamide at about 0.1 *M*.
ii. The molar ratio (per amino group) in which BNHS is added to IgG or peroxidase should be 2 and 10, respectively.
iii. The mixture is incubated for a few hours with intermittent stirring.
iv. Unreacted biotin is then removed by extensive dialysis or by desalting on Sephadex G-25.
v. Biotinylated enzyme can be complexed with avidin by adding a fourfold excess of avidin and subsequently purified by affinity chromatography as described in Section II,C,3,d.

III. SOLID-PHASE IMMOBILIZATION

The importance of proper execution of this step is generally underestimated. If the concentration of macromolecules is too great, loosely bound immunoreactants will form which will desorb during subsequent incubations and compete with the bound immunoreactants for the reactants in the solution. On the other hand, too little immobilization will decrease the sensitivity of the method. Third, ionic conditions and temperature may be of importance for many systems.

The most popular solid phase is polystyrene in the form of microplates, though many other solid phases may be used (plastic, paper, nitrocellulose, Sephadex, Sepharose, polyacrylamide beads, CNBr of $NaIO_4$-activated cellulose).

Noncovalent coating is little affected by the buffer, the three most common coating buffers being PBS, Tris–saline, and carbonate. PBS and Tris are used as 0.01 *M* buffers containing 0.9% saline. It is, however, essential to prevent the presence of nonionic detergents (Tween, Triton X-100, Nonidet P-40) during coating. Overnight incubations at 4°C are common, but we use short incubation periods (from 15 min for some antigens to 1 hr for antibodies). The optimum concentration depends on the antigen (e.g., for densonucleosis virus, 10 μg/ml), but for antibody it is generally 1 μg/ml. Most antigens have optimum concentrations between these two values. If nonpurified antiserum is used, a very pronounced optimum is found at a dilution of 10,000. Nitrocellulose coating is similar to plastic coating, with the difference that very small samples (<1 μl) can be used.

IV. ENZYME IMMUNOASSAYS: TITRATION PROCEDURES

The titration methods used will be of the solid-phase type, in polystyrene plates and on nitrocellulose. The flow chart of four different methods is given in Table I.

Essentially, four different enzyme immunoassays are in current use: homogeneous noncompetitive, heterogeneous noncompetitive, homogeneous competitive, and heterogeneous competitive (Appendix, Section IX,B). Most of the homogeneous assays are employed with haptens, whereas the heterogeneous approach is usually employed for antigens. The sensitivity and specificity attainable with the competitive and noncompetitive assays are quite different. The noncompetitive assay has higher detectabilities (up to 10^6 times), but generally suffers from a lower specificity since antibodies are added in excess, and they appear to be equipotent.

For polystyrene microplates 200-μl samples are used in PBS-T buffer [8

TABLE I
Enzyme Immunoassays for Parvovirus (DNV)[a]

	Sandwich method		"Coated virus" method	
Steps	Direct	Indirect	Direct	Indirect
Sensitization (in carbonate buffer)	0.2 μg purified IgG anti-DNV/well (rabbit), 37°C, 60 min	0.2 μg purified IgG anti-DNV/well (mouse) 37°C, 60 min	DNV: 0.02 OD_{260} per well, 37°C, 30 min	DNV: 0.02 OD_{260} per well, 37°C, 30 min
Incubation with virus (in PBS-T buffer)	100 ng DNV/ml, 37°C, 100 min	100 ng DNV/ml 37°C, 100 min		
Incubation with antiserum (in PBS-T buffer)	—	1000 times diluted antiserum (rabbit); −5% PEG:	—	1000 times diluted antiserum; 37°C, 45 min
Incubation with conjugate (in PBS-T buffer)	5 μg conjugate/ml, 4% PEG 37°C, 30 min	5 μ g conjugate/ml, 4% PEG 37°C, 30 min	5 μg conjugate/ml, 37°C, 30 min	5 μg conjugate/ml, 4% PEG 37°C, 30 min

[a]Nonlimiting conditions in standardized methods. Depending on the nature of the experiment, serial dilutions were made of the step under investigation.

g of sodium chloride, 0.2 g of potassium phosphate (monobasic), 2.9 g of sodium phosphate (dibasic), 0.2 g of potassium chloride, and 0.5 g of Tween 20 per liter]. This buffer is used for both the washing and dilution steps.

Incubation periods required depend on the nature of the macromolecule to be captured by the solid-phase immunoreactant, on the temperature, and on the distance from the solid phase. Two-hour periods are generally too long but may be safe for preliminary tests (Tijssen *et al.*, 1982).

Polyethylene glycol 6000 (PEG-6000) may reduce the incubation period significantly (Fig. 2), but not for the first layer captured by the solid-phase anchored molecules. In all subsequent steps 4% polyethylene glycol will reduce incubation periods to 30 min at 37°C. Precautions are warranted for so-called cold antibodies (i.e., antibodies which derive their affinity for the antigen from ionic interactions). Incubation periods are interspersed with washings (three times for 5 min).

Nitrocellulose-bound antigen is detected in the following way.

i. After antigen is added to membrane, the filter is allowed to dry and is left for 5–10 min in the cold (4°C).
ii. The membrane is washed with 0.05 Tris-HCl, containing 0.2 *M* NaCl, pH 7.4.
iii. The remaining active sites are blocked with the same buffer con-

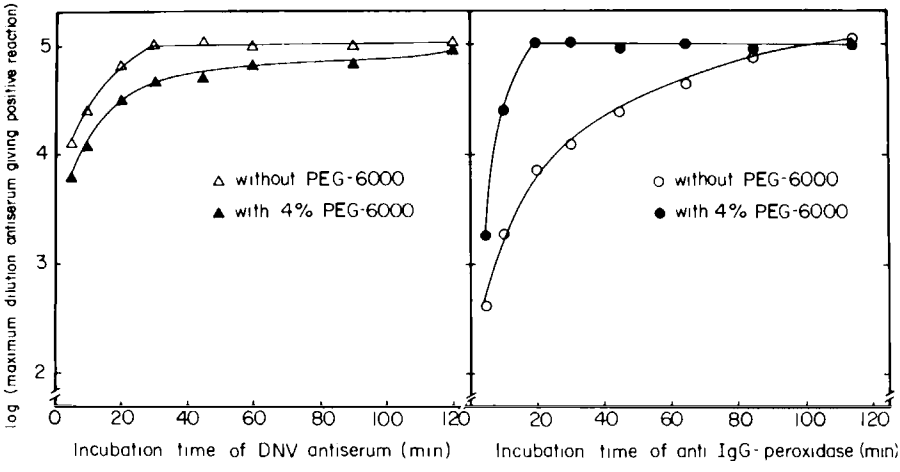


Fig. 2. Secondary plots of the results obtained with the indirect methods. The plots to the left reflect the results obtained by assaying a serial dilution of DNV antiserum against time followed by a standard incubation with anti-IgG peroxidase. The plots to the right were obtained similarly with the exception that the various incubation periods were assayed for the conjugate instead of for the DNV antiserum.

taining 3% bovine serum albumin for 15 min or by inclusion of 0.05% Tween 20 in each of the following steps.

iv. Add antibody and incubate for 2 hrs.
v. Wash the membranes for 30 min in several changes of buffer.
vi. Repeat step iii.
vii. Add conjugate (e.g., 1:1000 in blocking solution) and incubate for 2 hrs.
viii. Repeat step iv, and reveal enzyme activity.

Various hydrogen donors can be used for peroxidase (Appendix, Section IX, C). Popular is 5-aminosalicylic acid, though the commercially available product is far from pure. We purify this product in the following way.

i. About 5 g of impure 5-aminosalicylic acid and 5 g of sodium bisulfite are dissolved in 550 ml of deionized water at 80°C. The solubility of this product is poor, and the mixture is maintained for 10 min at 80°C.
ii. Activated charcoal is added, then mixed with the hot solution for about 5 min.
iii. The hot solution is filtered and then cooled to 4°C.
iv. A precipitate arises which is washed twice with 5 ml of water at 4°C and subsequently dried in the dark. This product is white, in contrast to the original purple product.
v. For use in EIA, 1 g is dissolved in 1 liter of 0.01 *M* sodium phosphate buffer, pH 6.0, containing 0.1 m*M* EDTA, and can be stored at −20°C for prolonged periods (years).

O-Phenylenediamine is dissolved in 0.1 *M* phosphate–citrate buffer, pH 5.0, at a rate of 0.4 mg/ml. Chloronaphthol is prepared by dissolving 40 mg in about 0.3 ml of absolute ethanol, then added to 100 ml of 0.05 *M* Tris-HCl, pH 7.6.

Aminosalicylic acid or phenylenediamine is used in polystyrene plates, whereas the chloronaphthol is used on nitrocellulose membranes. To all those solutions immediately prior to use hydrogen peroxide is added (1:10,000 of a 30% solution). Enzyme detection is always preceded by extensive washing.

V. IDENTIFICATION AND LOCALIZATION PROCEDURES

Identification of viral antigens on protein blots or in cells and their localization (Fig. 3) with respect to other proteins or cellular structures are of considerable importance.

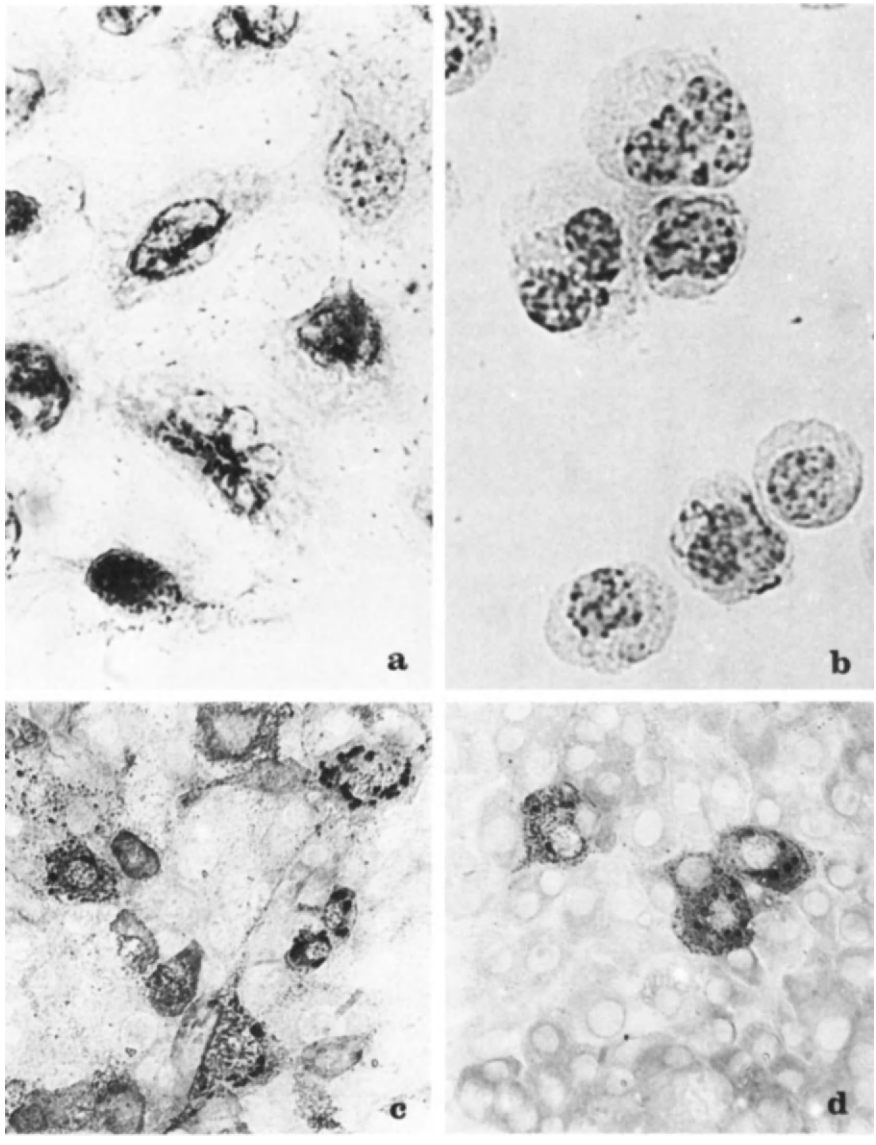


Fig. 3. Localization of virus antigens in infected cells using immunoperoxidase methods: (a) intranuclear herpes simplex virus type 2 antigens in Hep-2 cells (direct immunoperoxidase); (b) Epstein-Barr nuclear antigen (EBNA) in EBV-transformed human lymphocytes (anti-complement immunoperoxidase); (c, d) Intracytoplasmic rotavirus antigens in BSC-1 cells (direct immunoperoxidase).

The principles of these methods resemble those used in titration, with the notable exception that the product into which the soluble substrate is converted by the enzyme should be insoluble instead of soluble. This requirement of insolubility, causing the product to precipitate at the site of conversion, permits the exact localization of the antigens (Kurstak *et al.*, 1977, 1982, 1984).

This method has important advantages over the original immunofluorescence procedures: (1) a normal light microscope can be used; (2) the method can be used at the ultrastructural level (with an electron microscope); (3) cellular structure can be easily appreciated, and stained preparations are permanent; (4) background levels are diminished; and (5) the reagents have a long shelf life.

A recently developed two-dimensional electrophoresis system (Tijssen and Kurstak, 1983), in which proteins are digested by proteases in the gel between the two electrophoresis steps, yields small peptide fragments; these can be transferred by diffusion or electroelution to nitrocellulose membranes. Antibodies with a given specificity will react with their corresponding epitopes and subsequently can be eluted from these epitopes and allowed to react with other peptides or proteins subjected to limited proteolysis. In this way, epitope mapping of the complete protein can be achieved.

After fixation of the cells with methanol or acetone or transfer of the proteins to nitrocellulose membranes, it is essential to wash the cells with PBS in order to have favorable conditions for the subsequent antigen–antibody interactions. Dilution of antiserum should be about 200 times less then in quantitative EIA. Washings are with isotonic or slightly hypertonic buffer at neutral pH; incubation steps generally take 1–2 hr at 37°C. A general method is as follows:

i. Wash smears with PBS (5 min).
ii. Incubate for 2 hr with antiserum (e.g., 1:100).
iii. Wash three times for 5 min with PBS.
iv. Incubate for 2 hr with conjugate (e.g., 1:40).
vi. Wash once with 0.05 *M* Tris-HCl, pH 7.6.
vii. Incubate with diaminobenzidine solution (1 min) and supplement with 0.1% hydrogen peroxide (1 min).
viii. Wash with buffer (2 × 5 min).
ix. Examine in a light microscope.

Once the antigen–antibody–enzyme complexes have been formed, suitable ionic conditions have to be established for enzyme revelation. The most common chromogen used for peroxidase is 3,3′-diaminobenzidine tetrahydrochloride; it is used at a rate of 50 mg/100 ml in 0.05 *M* Tris-HCl (pH 7.6) in combination with 0.01% hydrogen peroxide. The preparations to be

tested should be equilibrated with this buffer without substrate, prior to revelation. Two-minute periods generally suffice for staining. Subsequently the preparations are washed twice with buffer only (5 min each time). It is also possible to localize different antigens in the same cell by contrasting the colors of two EIA experiments. The first antigen is detected in the usual way as described above. The whole procedure is then repeated for another antibody (against a second antigen), but staining is done with 40 chloro-1-naphthol. Distinct colors are obtained if the antigens are not in too close proximity.

VI. PROCESSING OF RESULTS

Two basically different situations can be distinguished: (1) EIA in which antigens are quantified and for which a precise dose-response curve can be established; and (2) EIA in which antibody activities are established. In the latter, dose-response curves for different sera often are not parallel, and activities cannot be established in a reliable fashion from single dilutions. This, in turn, requires long dilution series to find the exact titer or an otherwise established absorbance value.

Various mathematical procedures have been designed to improve the precision of curve fitting and to save time and expenditure; the most advanced of these use microcomputers which can be interfaced with microplate readers and printers. The values of the results obtained may then be transformed in such a way that linearized dose-response curves are obtained.

A basic problem with all sigmoid dose-response curves, which crops up in each EIA experiment, is the establishment of a cutoff value to discriminate positive from negative results and to decrease as much as possible type I and type II errors. Although the standard deviation (SD) and mean of negative reference sera are generally used for this purpose, it is strictly speaking not permitted since there is a positive skew of the results obtained with those sera. However, nonparametric methods, which would be more suitable, have the disadvantage that larger sample sizes (e.g., 100 experiments) are required. The discrimination level is commonly put at the mean plus 2 or 3 SD. The coefficient of variation in inter- and intra-assays should be established for continuous control.

A second decision to be made is the way in which serological EIA are to be expressed. The most common methods are (1) titers, (2) effective dose, (3) absorbance, (4) absorbance ratio of positive to negative sera or ratio of surfaces of dose-response curves from different sera, (5) percentile, (6) multiple of normal activity, and (7) units of standard curve.

All these methods have particular advantages or disadvantages, and the choice of method depends on the requirements of the communication or

use in subsequent studies. For example, titrations generally are work intensive and costly, and titers obtained generally are not accurate. The concept is simple, and the test generally is quantitative of antibody activity. The effective-dose method (Leinikki and Passila, 1977) has the same disadvantages as the titration method but the results are mostly considerably more accurate. The uncommon way of expressing the results is awkward and not readily comprehensible to clinicians (in Briggs logarithms). The same can be said of the absorbance method, which has the advantage that it is considerably less work consuming but disregards the fact that dose-response curves may not be parallel. The multiple of normal activity (MONA) (Felgner, 1978) is also a single serum dilution test.

Accuracy of antigen concentration measurements can be achieved by a plethora of curve-fitting procedures. A popular approach is the use of the logit function (introduced by Berkson, 1944) for the transformation of sigmoidal response curves in bioassays. This popularity is probably due to the fact that those in the biological or medical sciences are familiar with the logit function. Other methods to linearize dose-response curves are spline-fit or third-order polynomials (Marschner *et al.*, 1974), arc sine transformation (Soula *et al.*, 1982), Freundlich adsorption isotherm (Lehtonen and Viljanen, 1982), and the Hill equation (Dietzler *et al.*, 1980).

Internal quality control and external quality assessment are essential to maintain or improve reproducibility and repeatability of EIA. The coefficient of variation serves as a parameter for quality-control charts and may indicate trends such as deterioration of reagents. The standard deviation of between-assay results should be less than 2–3 higher than those for within-assay results, and the inter-assay variability should be less than 10%. Other parameters to be included in quality control charts are the detectability index and the specificity index.

External quality assessment in collaborative studies among laboratories serve to detect bias-type errors. Monitoring of precision is, however, of utmost importance for each laboratory in order to take full advantage of this assessment. A most common error encountered in these programs is the use of overwide working ranges.

VII. IMMUNODIAGNOSIS OF VIRUS INFECTIONS USING ENZYME TRACERS: PROBLEMS AND INTERPRETATION

Progress in enzyme immunoassays has been made at several levels: (1) faster and more efficient conjugation procedures; (2) use of more sensitive enzymes and substrates; (3) improvement of the quality of immunoreactants, especially for the preparation of monoclonal antibodies; and (4) in-

creasingly clever and sophisticated enzyme immunoassays designs with higher speed, sensitivity, and efficiency and lower cost. Not surprisingly, the enzyme immunoassays and immunoperoxidase methods are used currently in diagnosis of viral diseases. Reviews on this subject were published recently (Kurstak *et al.*, 1977, 1984; Voller and Bidwell, 1977; van Regenmortel, 1984).

The enzyme-linked immunosorbent assay (ELISA) is a highly specific serological tool in diagnostic virology, and immunoperoxidase methods are widely used for detection of viral antigens in infected cells, in both light microscopy and electron microscopy. In addition, titration of antisera with the immunoperoxidase method is currently performed by applying serial dilutions of the serum concerned on fixed infected cells (Kurstak *et al.*, 1978). The presence and quantity of antibodies are then detected with labeled antiglobulin or anticomplement antibodies.

The use of enzyme tracers is now widespread, not only in infectious diseases but also in many other disciplines (molecular biology, pathology, allergy, immunology, toxicology, endocrinology, and oncology). Essentially, two approaches can be taken in the case of enzyme immunoassays: the homogeneous and heterogeneous assays. In heterogeneous assays, which are not frequently used in the diagnosis of viral infections, a solid phase, to which one of the immunoreactants is attached, forms the basis of the test. Substrates which are converted by the enzyme in a colored product are commonly used, so that the degree of reaction can be monitored with the naked eye and be quantified with simple colorimeters. Colorimeters to read microplates are commercially available (Clem and Yolken, 1978).

An important application in enzyme immunoassays is the IgM serology, used to identify primary viral infections. This is based on the observations that primary infections result in the formation of antibodies mainly belonging to the IgM class whereas a recurrent infection by T-cell-dependent antigen (such as the viruses) results in the formation of IgG-type antibodies. However, the value of IgM serology for diagnostic virology cannot be overestimated. The design of enzyme immunoassays has been modified in order to decrease the interference with rheumatoid factors and to increase sensitivity. In the conventional methods IgM antibodies competed with their IgG counterparts and were detected by an indirect method. In the class-capture methods, anti-IgM antibodies adsorbed to the solid phase capture the IgM fraction from the patient's serum, which is subsequently tested with antigen for the presence of antibodies. Antigen adsorbed by IgM can then be detected by a variety of methods. The lack of competition between IgG and IgM offers important advantages for detecting IgM antibodies over previous methods (Chantler *et al.*, 1982).

In immunoassays an excess of coating antibodies may be detrimental to

the sensitivity of the assay. In our laboratory, we observed a strong decrease in sensitivity by overcoating with anti-herpes simplex virus antibodies, but less pronounced with an anti-parvovirus antiserum. This might be due to the tendency of the immunoglobulins to form weak interactions with other antibodies, so that a large fraction of apparently coated IgG risks being removed in subsequent washing steps, thereby removing virus adsorbed by these antibodies. The use of complete antisera instead of the IgG fraction for coating the solid phase may lead to other complications (for example, the competition between IgG and other proteins for the solid phase). Complete antisera gave a very pronounced optimum at a dilution of about 3000 times, though the sensitivity was lower than in the case where purified IgG was used.

Several factors may be responsible for poor sensitivity or nonspecific reactions. It is important to realize that, for peroxidase-mediated immunoassays, the concentration of H_2O_2 used is of utmost importance. The very small quantity of H_2O_2 needed makes the actual substrate concentration prone to strong fluctuations. Hydrogen peroxide is not only the substrate, but also an effective inhibitor for the enzyme. Therefore, increasing substrate concentrations will at first enhance enzyme activity, but at still higher concentrations strong enzyme inhibition will occur. In our laboratory we found that, in contrast to the standard composition in use (Voller and Bidwell, 1977), a 10,000–12,000 times dilution of a 30% solution gave optimal results. The presence of free antibodies in the conjugated fraction decreased considerably the sensitivity of the assay (Kurstak and Kurstak, 1974). It was found that free antibody reacted considerably faster than its conjugated counterpart. This has a direct impact on the sensitivity of the assay, and the presence of only 10% free antibody decreased the response by half.

Nonspecific adsorption of proteins may also cause considerable background levels of enzyme activity. This might be due to contaminating enzymes. IgG or conjugates may also be adsorbed nonspecifically by coated proteins. This problem can be circumvented by an incubation step with 1% bovine serum.

The immune response has a significant impact on the quality of enzyme immunoassays. The variable regions of antibodies, i.e., the antigen binding sites, are generated in pre-B lymphocytes by a combinatorial assortment of a number of gene segments, each occurring several to many times in the germ-line DNA. This gene shuffling, called somatic DNA recombination, coupled with a process of somatic mutation, and recombination with the gene segments coding for the constant regions of the proteins of the different immunoglobulin classes followed by selective RNA transcription and splicing mechanisms, restrict the lymphocyte and its offspring (clone) to produce antibodies of only one specificity. BALB/c mice contain 200 mil-

lion B lymphocytes, of which only 10–40 million yield distinct clonotypes (Klinman and Press, 1975). Estimations indicate (Köhler, 1976) that 1000 to 7000 different antibodies in BALB/c mice may recognize the same antigenic site (epitope). The 5–10 different antibodies usually found against a single epitope are thus a very small selection among all possibilities. It was suggested (Briles and Davie, 1980) that this restriction does not result from affinity-dependent maturation, but that the lymphocytes of nonimmune individuals are idiosyncratic in their potential antiepitope response. This demonstrates why it is virtually impossible to produce the same antibodies in another animal. Even the monoclonal selection of antibody-producing lymphocytes by the hybridoma technique will not yield repeatable results if different animals are primed. To compound this complexity, a still poorly understood regulatory mechanism, in which other cells (macrophages, T-helper cells, I-suppressor cells) and other genes such as the Ir (immune response) genes play a dominant role, influences the quantity and nature of the antibody. The production of monoclonal antibodies by permanent cell lines has very important obvious advantages, but it also often has some less obvious disadvantages.

It is known that the antibodies of a polyclonal antiserum have differing affinities, which each possess a single affinity constant for a given determinant. The importance of this for immunoassays is obvious because the virus is measured only after its association with the antibody, and affinity will determine the fraction of antigen bound and given antigen and antibody concentrations according to the law of mass action (Ekins, 1980). It can be shown that low-affinity antibodies will bind poorly to added virus and that multiple washing steps may result in elution of the bound antigen (Pesce *et al.*, 1978). As a rule of thumb the negative logarithm of the molar concentrations exceeding the logarithm of the affinity (association) constant will result in poor binding. Therefore, high-affinity antibodies should be used to achieve high sensitivity. Although in the case of monoclonal antibodies populations with higher affinities may be selected, hybridoma techniques often yield low-affinity antibodies.

Two important problems with hyperimmune polyclonal antisera are the low fraction (often only 3%) of antibodies with respect to the total immunoglobulin content and the frequent occurrence of rheumatoid factors (Yolken *et al.*, 1977; Milan and Stigbrand, 1981). Both factors compound the problems with low-affinity proteins. Rheumatoid factors, mostly IgM, binding IgG and therefore lowering the antibody concentrations, can often be neutralized by the addition of an excess of normal IgG at 0.3 mg/ml in test conditions. Alternatively, mild treatment with a reducing agent such as 2-mercaptoethanol will dissociate the pentameric structure of these IgMs into monomers, resulting in a very significant lowering of its avidity. This

latter technique is not applicable to enveloped viruses (Yolken, 1982) or with fowl antisera.

Cross-reactivity, especially but not only with closely related viruses, e.g., herpes simplex virus type 1 and herpes simplex virus type 2, and nonspecificity are also frequently associated with polyclonal antisera. Although cross-reactivity may to some extent be removed by adsorption, this processing will generally also remove specific antibodies.

Hybridoma antibodies display many of the most desired attributes of polyclonal mixtures while eliminating most of the problems, but they have also some less perfect traits. The preparation of hybridoma clones is so time consuming that one must be really convinced that this elegant methodology is the best way to achieve the desired result.

Low avidity of the monoclonal antibodies can be a problem in diagnostic virology and probably accounts for the often lower sensitivity of enzyme immunoassays using monoclonal antibodies when compared with the corresponding assays with polyclonal antisera. This is so despite the fact that polyclonal may have only a few percent of antibody. The added bonus of avidity of a mixture of antibodies with relatively low intrinsic affinities can, however, be obtained by pooling antibodies from different clones, although in preliminary studies (Yolken, 1982) adverse effects by pooling monoclonal antibodies directed against influenza virus neuraminidase are observed.

Monoclonal antibodies monospecificity can, depending on circumstances, be a downright disadvantage or an advantage. Viral antigens often show natural variation, e.g., influenza virus, which may significantly lower reactivity, making it imperative to use entire panels of antibodies. Additionally, noncompetitive sandwich assays may not be feasible or may have a very low sensitivity if only one of a very few of the target determinants are present on the antigen.

VIII. CONCLUSIONS

The in-house preparation of reagents for enzyme immunoassays in virology is important for several reasons: (1) conjugates can be prepared for all purposes and are not limited to the relatively few commercially offered, (2) quality of the conjugate is generally considerably improved, e.g., enzyme activities 10 times higher than of those offered commercially; (3) costs are reduced, often by a factor of 5–10; and (4) enzyme immunoassays are much more versatile by preparations adapted to particular situations. A slight disadvantage is that personnel need to be trained and be made aware of the intricacies of these, nonetheless simple, techniques.

IX. APPENDIX

A. Determination of Purity, Activity, and Concentration of Peroxidase and Immunoglobulins

Materials Needed

Hydrogen peroxide (H_2O_2)

Sodium phosphate buffer, 0.1 *M*, pH 6.8: 50 m*M* dibasic and 50 m*M* monobasic sodium phosphate

3-Dimethylaminobenzoic acid (DMAB): 330 mg/100 ml in above buffer

3-Methyl-2-benzothiazolinone hydrazone (MBTH): 20 mg per 100 ml of above buffer

Spectrophotometer (UV and visible) and cuvettes

Methods

Purity and concentration of IgG

Dilute IgG to about 0.5 mg/ml in PBS.

Measure optical density at 278 and 251 nm.

Ratio A_{278}/A_{251} should exceed 2.5 for pure enzyme.

Concentration of IgG equals about $A_{278}/1.4$ mg/ml.

Concentration and purity of peroxidase

Dilute peroxidase to about 0.25 mg/ml.

Measure optical density at 401 and 275 nm.

Concentration of peroxidase equals $A_{401}/2.25$.

"Degree of purity = RZ" equals A_{401}/A_{275}. For peroxidase isozyme C, about 3.4–3.5 is commonly obtained.

Activity of peroxidase

Mix 1 volume of hydrogen peroxide (0.03%) with 6 volumes of DMAB and 3 volumes of MBTH.

Add peroxidase.

Measure increase per minute in optical density at 590 nm.

Convert volume activity to specific activity: the operational molar extinction coefficient of the product is 47,600.

B. Enzyme Immunoassay (EIA) Procedures

Time and temperature are optimal for the system used. These parameters may have to be changed slightly for other systems.

Four different manipulations are performed:

1. Coating: either with antibody (1 μg/ml) or virus (10 μg/ml) in carbonate buffer

2. Washing: 3 times for 5 min with PBS-Tween
3. Incubation (for immunologic reactions) with immunoreactants diluted in PBS-Tween
4. Enzyme revelation: with the solution containing the hydrogen donor supplemented with 0.003% hydrogen peroxide

Details of procedures

a. Direct sandwich: (i) coating with antibody-solution for 60 min at 37°C; (ii) washing; (iii) incubation with serial dilution of virus solution for 100 min at 37°C; (iv) washing; (v) incubation with conjugate (5 μg/ml, 4% PEG) for 30 min at 37°C; and (vi) washing and revelation.

b. Indirect sandwich: (i) coating, washing, and incubation with virus as in procedure a, steps i, ii, iii, and iv; (ii) incubation (4% PEG) with antiserum from other species or modified antibodies; (iii) washing; (iv) conjugate incubation and revelation as in procedure a, steps v and vi.

c. Direct immobilized antigen method: (i) coating with viral solution for 30 min at 37°C; (ii) washing; (iii) incubation with conjugate at 37°C for 90 min; (iv) washing and revelation.

d. Indirect immobilized antigen method: (i) coating as in procedure c, step i; (ii) washing; (iii) incubation with serial dilution of antiserum; (iv) washing; (v) incubation with conjugate as in procedure a, step v; and (vi) washing and revelation.

Solid-phase variation

Polystyrene wells: use 0.2 ml of reactant.

Nitrocellulose membranes: use less than 1 μl.

Activated beads (Sepharose, polyacrylamide, cellulose): use in small tubes.

C. Preparation of Hydrogen Donors

5-Aminosalicylic acid: Dissolve 1 g in 1 liter of 0.01 *M* sodium phosphate buffer, pH 6.0, containing 0.1 m*M* EDTA. Store at −20°C (stable for years).

O-Phenylenediamine: Dissolve 0.4 mg/ml in 0.1 *M* phosphate-citrate buffer, pH 5.0.

Chloronaphthol: Dissolve 0.40 mg in about 0.3 ml of absolute ethanol and add to 100 ml of 0.05 *M* Tris-HCl, pH 7.6.

MBTH and DMAB: Prepare phosphate-citrate buffer, pH 7.0, by adding 120 ml of 0.1 *M* citric acid and 500 ml of 0.2 *M* dibasic sodium phosphate to 1 liter. Prepare 3×10^{-2} *M* DMAB and 6.0×10^{-4} *M* MTBH and 1.0 *M* sodium azide in this buffer (separate stock solutions). Mix

256 ml of buffer with 22 ml of MBTH, 22 ml of DMAB, and 2 ml of azide stock solutions and mix gently.

3,3′ Diaminobenzidine tetrahydrochloride: Dissolve 80 mg/100 ml in 0.05 *M* Tris-HCl, pH 7.6. Filter before use.

ACKNOWLEDGMENTS

This work was supported by grants to Professor E. Kurstak from the Natural Sciences and Engineering Research Council of Canada and the Medical Research Council of Canada.

REFERENCES

Barman, T. E. (1974). "Enzyme Handbook," Vol. 2. Springer-Verlag, Berlin and New York.

Berkson, J. (1944). *J. Am. Stat. Assoc.* **41,** 70.

Briles, D. E., and Davie, J. M. (1980). *J. Exp. Med.* **152,** 151.

Carlier, Y., Bout, D., and Capron, A. (1981). *Bull. Inst. Pasteur (Paris)* **79,** 313.

Chantler, S., Evans, C. J., Mortimer, P. P., Cradock, J., Watson, J. E., and Ridehalgh, M. K. S. (1982). *J. Virol. Methods* **4,** 305.

Clem, T. R., and Yolken, R. H. (1978). *J. Clin. Microbiol.* **7,** 55.

Dietzler, D. N., Weidman, N., and Tieber, V. L. (1980). *Clin. Chim. Acta* **101,** 163.

Ekins, R. (1980). *Nature (London)* **284,** 14.

Engvall, E., and Perlmann, O. (1971). *Immunochemistry* **8,** 871.

Felgner, P. (1978). *Zentralbl. Bakteriol. Parasitank. Infektionskr. Hyg. Abt. 1: Orig. Reike A* **242,** 100.

Klinman, N., and Press, J. (1975). *Transplant. Rev.* **24,** 41.

Köhler, G. (1976). *Eur. J. Immunol.* **6,** 340.

Kurstak, E. (1971). *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.) Vol. 5, pp. 423–444. Academic Press, New York.

Kurstak, E., and Kurstak, C. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), pp. 3–30. Academic Press, New York.

Kurstak, E., Tijssen, P., and Kurstak, C. (1977). *In* "Comparative Diagnosis of Viral Diseases" (E. Kurstak and C. Kurstak, eds.), Vol. 2, pp. 403–448. Academic Press, New York.

Kurstak, E., de Thé, G., van den Hurk, 1., Charpentier, G., Kurstak, C., Tijssen, P., and Morisset, R. (1978). *J. Med. Virol.* **2,** 189.

Kurstak, E., Kurstak, C., and Morisset, R. (1982). *In* "Standardization of Immunological Procedures" (*Int. Congr. Herpes Virus of Man and Animal, 17th, 1981*). *Dev. Biol. Stand.* **52,** 187.

Kurstak, E., Tijssen, P., and Kurstak, C. (1984). *In* "Control of Virus Diseases" (E. Kurstak and R. G. Marusyk, eds.), pp. 477–500. Dekker, New York.

Langone, J. J. (1982). *Adv. Immunol.* **32,** 157.

Leinikki, P. O., and Passila, S. (1977). *J. Infect. Dis.* **136,** 294.

Lethonen, O. P., and Viljanen, M. K. (1982). *Int. J. Bio-Med. Comput.* **13,** 471.

Marschner, 1., Erhart, F., and Scriba, P. C. (1974). *In* "Radioimmunoassay and Related Techniques in Medicine," Vol. 1, p. 111. International Atomic Energy Agency, Vienna.

Milan, J. L., and Stigbrand, T. (1981). *Clin. Chem.* **27,** 2014.

Oellerich, M. (1980). *J. Clin. Chem. Clin. Biochem.* **18,** 197.
O'Sullivan, M. J., Bridges, J. W., and Marks, M. (1979). *Ann. Clin. Biochem.* **16,** 221.
Pesce, A. J., Ford, D. J., and Gizutis, A. (1978). *Scand. J. Immunol.* **8,** 1.
Rubenstein, K. E., Schneider, R. S., and Ullman, E. F. (1972). *Biochem. Biophys. Res. Commun.* **47,** 846.
Scharpe, S. L., Koodreman, W. M., Blomme, W. J., and Laekeman, G. M. (1976). *Clin. Chem.* **22,** 733.
Schuurs, A. H. W. M., and van Weemen, B. K. (1977). *Clin. Chem.* **81,** 1.
Soula, A., Laurent N. Tixier, G., and Moreau, Y. (1982). *In* "Standardization of Immunological Procedures." *(Int. Congr. Herpes Virus of Man and Animal; 17th, 1981). Dev. Biol. Stand.* **52,** 147.
Tijssen, P., and Kurstak, E. (1983). *Anal. Biochem.* **128,** 26.
Tijssen, P., and Kurstak, E. (1984). *Anal. Biochem.* **136,** 451.
Tijssen, P., Su, D.-M., and Kurstak, E. (1982). *Arch. Virol.* **74,** 277.
Van Regenmortel, M. H. V. (1984). *In* "Control of Virus Diseases" (E. Kurstak and R. G. Marusyk, eds.), pp. 405–422. Dekker, New York.
Van Weemen, B. K., and Schuurs, A. H. W. M. (1971). *FEBS Lett.* **15,** 232.
Voller, A., and Bidwell, D. E. (1977). *In* "Comparative Diagnosis of Viral Diseases" (E. Kurstak and C. Kurstak, eds.), Vol. 2, pp. 449–457. Academic Press, New York.
Wisdom, G. B. (1976). *Clin. Chem.* **22,** 1243.
Yolken, R. H. (1982). *Rev. Infect. Dis.* **4,** 35.
Yolken, R. H., Kim, H. W., Clem, T., Wigatt, R. G., Kalica, A. R., Chanock, R. M., and Kapikian, A. Z. (1977). *Lancet 2,* 263.

Index

S