INSTITUTE OF MEDICINE

SUMMARY OF A WORKSHOP

Temperature-stable Vaccines for Developing Countries

1989

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TEMPERATURE-STABLE VACCINES FOR DEVELOPING COUNTRIES: SIGNIFICANCE AND DEVELOPMENT STRATEGIES

Summary of a Workshop

INSTITUTE OF MEDICINE

Division of International Health

National Academy Press
Washington, D.C. 1989

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This activity was supported through PHS-OIH Contract No. 282-86-0113 with funds made available under an agreement between the U.S. Agency for International Development and the U.S. Public Health Service.

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CONTENTS

Summary 1

Background Papers

World Health Organization Minimum Requirements for the Main Vaccines of Interest to the Expanded Programme of Immunization, by Phillipe Sizaret 10

Economic and Logistic Impacts of Temperature-Sensitive Vaccines, by Donald S. Shepard, Charles S. M. Cameron, Jr., Mark L. Robbins, and Marjorie Pollack 36

Discussion of the Consequences of Vaccine Temperature Stability, by Stanley O. Foster 84

Immunologic Considerations in Achieving Long-Lasting Immunity with Vaccines, by Stanley M. Lemon 92

Discussant Paper for Immunologic Considerations in Achieving Long-Lasting Immunity with Vaccines, by Francis A. Ennis 107

Poliovirus: Structural Basis for Temperature-Dependent Loss of Immunogenicity, by James M. Hogle, Marie Chow, and Philip D. Minor 109

Molecular Approaches for Improvement of the Thermostability of the Oral Poliovaccines, by Olen M. Kew 125

New Technologies for Producing Antigens and Their Merits as Temperature-Stable Vaccines, by Fred Brown 132

Use of Antigens Produced by Recombinant DNA Technology with a Safe and Efficacious Adjuvant Formulation in a New Generation of Vaccines, by Anthony C. Allison and Noelene E. Byars 140

Stabilized Vaccines from the Practical Perspective, by Maurice R. Hilleman 151

Appendixes

A Agenda 158

B List of Participants 162

C Vaccine Storage Temperatures 166

SUMMARY

The Institute of Medicine convened this workshop April 13 and 14, 1987 at the request of the Office of International Health, U.S. Public Health Service, and with funding from the U.S. Agency for International Development.

The workshop was intended to assess (1) the relative importance of vaccine temperature sensitivity in hindering immunization efforts in developing countries, (2) what is known about the causes of temperature sensitivity, (3) new approaches to vaccines in relation to such sensitivity, (4) research and development strategies most likely to produce vaccines with the best characteristics for use in developing countries, and (5) the readiness of the field of vaccine stability for major investment.

Maintaining the immunization "cold chain" (i.e., ensuring that appropriate temperature storage requirements are met as vaccines move from production plants through central, regional, district, and local facilities) is a significant problem in developing countries. The cost of immunizing a child in developing countries is about US $10, and the cold chain accounts for about 10 percent of this. About 36 percent of cold chain costs are at the periphery of the vaccine distribution system.

Radical improvements in vaccine thermostability are not likely to be feasible in the near future because of gaps in understanding the underlying mechanism of thermal damage. Therefore, efforts in the near term should be targeted toward:

- further improvement of the existing cold chain, especially its reliability and management;

- ensuring that vaccines used in immunization efforts are the most thermostable that can be produced, through adopting stringent thermostability requirements (where these do not already exist) for vaccine procurement; and

- utilization of known stabilizers in all preparations used in developing countries, such as magnesium chloride for polio vaccine.

A strong program of activities directed toward improving temperature stability should include:

- a workshop conference on heat sensitivity of biological products to review existing knowledge, including biophysical information on temperature-related changes in proteins;

- investigation of the relationship of thermostability to immunogenicity and safety;

- investigations of the thermostability and effects of adjuvants;

- the fundamental cause of loss of potency due to heat or freezing; and

- studies of what specific structural or vaccine formulation characteristics confer heat stability, including study of differences in thermostability between strains of pathogens.

Vaccine development efforts should be encouraged in three areas because of their potential contribution to improving thermostability: acellular pertussis preparations, recombinant DNA technology as a means of antigen production, and conjugate vaccines with polysaccharide antigens.

Funding of such research toward more thermostable vaccines is adequately justified because of the high annual expenditures related to the cold chain. Responsibility for promoting research on issues related to vaccine thermostability rests with national and international organizations promoting immunization efforts in developing countries.

Relevant Aspects of Immunization Efforts in Developing Countries

Present immunization efforts in developing countries usually are conducted by national governments along guidelines suggested by, and with the financial support of, the World Health Organization's Expanded Programme on Immunization (WHO/EPI). These efforts concentrate on delivery of vaccines for six major childhood diseases--poliomyelitis, measles, diphtheria, pertussis, tetanus, and tuberculosis. A vaccine for yellow fever is also used in some countries. Storage requirements for these are shown in Annex 4. Oral live poliomyelitis vaccine (OPV) and measles vaccine are the least stable at temperatures encountered in developing countries; vaccines adsorbed on aluminum salts, such as diphtheria and tetanus toxoids or hepatitis B vaccines, loose their homogeneity in appearance and immunogenicity on freezing. Ensuring that the appropriate temperature storage requirements are met as vaccines move from production plants through central/national, regional, district, and local facilities is referred to as maintaining the "cold chain" (see papers by Shepard et al. and Foster in this volume). The "periphery" of the cold chain is the point in vaccine diffusion at which refrigeration becomes unavailable.

Some vaccines recently licensed (e.g., for hepatitis B) or nearing licensure (e.g., for rotavirus diarrhea) are candidates for inclusion in national immunization programs. Many other vaccine candidates are in development and might well be licensed in the next 10 years (New Vaccine Development, Volumes I and II, Institute of Medicine, 1984, 1986).

The World Health Organization's Expert Committee on Biological Standardization adopted minimum requirements for all EPI vaccines. Those requirements (see paper by Sizaret in this volume) cover potency and safety issues. Specific requirements for thermal stability exist only for BCG and measles vaccines. The stability requirements for live

poliovirus vaccines are to be formulated this year by the Expert Committee on Biological Standardization. Under current guidelines it may be possible for manufacturers to skirt the stability issue by producing a vaccine of such high initial potency that its rapid degradation is of little consequence unless time before use is protracted.

A large proportion of the vaccines used in national immunization programs are purchased by multilateral or bilateral health assistance agencies. These organizations generally supply vaccines meeting the WHO minimum requirements. UNICEF, a major purchaser and supplier of childhood vaccines worldwide, probably accounting for approximately two-thirds of the vaccines used in EPI programs, follows the WHO requirements. Procurement of vaccines by major donors has been pursued with an eye toward favorable pricing, taking into account WHO minimum requirements. As a result the cost of the six major vaccines is now on the order of $0.50 per child, sufficiently low to warrant investment in improved vaccines.

There is little information available on the variation in thermostability and immunogenicity in vaccines provided by various manufacturers. If two vaccines meet WHO minimum requirements, the one with greater thermostability, even if more expensive, might be more cost-effective at inducing adequate levels of immunity, because it would better withstand possible temperature abuse prior to delivery. Additionally, thermostability may make it possible to use the vaccine on the periphery of the cold chain.

Immunization Coverage

Participants in the workshop noted the expansion of immunization coverage and cold chain efficiency that had occurred in the past few years, largely as a result of EPI support to national efforts (see paper by Foster in this volume). Immunization efforts now deliver initial doses to about 60 to 70 percent of the targeted infants and children. However, the proportion fully immunized is approximately only 40 percent because the subjects failed to return for subsequent doses.

Immunization efforts to reach the 30 percent of children not yet contacted will be difficult, partially because they often live in rural areas. Extension of the cold chain to the unserved group would require a further major investment in capital equipment, such as refrigerators. Expanding immunization services to individuals not yet reached would probably be achieved in a more cost-effective fashion by improving vaccine thermostability.

Potency and Thermostability Requirements

The ultimate test of a vaccine's potency is its ability to generate protective immunity in recipients in the field when given in the recommended dose. Evaluating potency by natural challenge is only practicable in efficacy trials; therefore, surrogate methods are used

in the laboratory to assess potency of vaccine preparations and their safety. These include various tests for immunologic response to the vaccine preparation as well as the use of animal models to estimate potency and safety. With some vaccines too little was known until recently about the precise components of the immune response necessary for protection; furthermore, the measures predictive of safety are not always known. (With pertussis vaccine evidence is accumulating that antibodies to pertussis toxin and fumbrial hemagglutinin are protective.) The tests currently employed are primarily useful in standardizing vaccine preparations, but not enough is known about their predictive value relative to efficacy and safety in humans.

The participants in the workshop generally concurred that an important area for research was the development of better, rapid, simple indicators of vaccine potency. These might be used in assessing a preparation initially and later if a break in the cold chain was suspected.

Presently, thermostability guidelines for a vaccine merely prescribe a maximum permissible potency loss after a specified time/temperature exposure and/or the meeting of a minimum potency requirement to the expiration date for use. These do not provide sufficient information for deducing the kinetics of loss of potency, particularly if the vaccine contains active components with different thermodegradation kinetics. Hence the workshop participants also concluded that there was a need for development of guidelines for the description of the thermostability characteristics of vaccines, particularly those of interest to the WHO/EPI.

Desirable Thermostability for EPI Vaccines

The participants in the workshop discussed at length the feasibility and usefulness of stating desirable thermostability for vaccines in terms of temperature/time/potency-loss specifications.

In developing countries ambient daytime temperatures may routinely reach or exceed normal human body temperature (37°C). Inside vehicles, where vaccines may have to be transported without cooling, temperatures can considerably exceed 37°C for hours. Difficulties with the cold chain--equipment failures, malfunctions, or poor management procedures--could result in inappropriate storage temperatures for vaccines, including damage by freezing. At the periphery of the cold chain immunization workers without refrigeration equipment usually receive their vaccine supplies on a monthly basis.

Workshop participants agreed that no criteria for vaccine thermostability would be appropriate for all possible contingencies. However, they also thought that the capacity to withstand freezing and exposure to 37°C for 1 month and brief periods at temperatures up to 45°C without significant loss of potency could be a useful goal for thermostability improvement. There is a clear need for developing more detailed stability information on all EPI vaccines. (The kind of information needed is described below in "Immediate Research Needs.") Such information could serve as a guide for those concerned with

operational research issues, as well as for immunization program managers who are expected to decide what to do with a vaccine in the case of a break in the cold chain. Some participants considered that the goal stated above might be achievable for some, if not all, vaccines of the EPI in the not too distant future.

Most countries restock their central vaccine stores with supplies direct from manufacturers at least yearly. Therefore, the participants concurred that a long-term goal for thermostability (if the cold chain was ever to be substantially modified or eliminated) was 1 year at 37°C and the ability to withstand brief exposures up to 45°C.

Lack of Funding for Thermostability Studies

The participants in the workshop noted that tens of millions of dollars are spent annually on procurement of vaccines and on maintenance of the cold chain required for existing vaccines (see paper by Shepard et al. in this volume). However, vaccine acquisition is undertaken with little direct attention to considerations of thermostability, and virtually no funding has been directed to finding reasons for temperature-related loss of potency in existing vaccines, to enhancing thermostability, or to understanding the basic scientific process involved in temperature sensitivity of biological agents. Suggestions for work in these areas are made below.

Thermosensitivity of vaccines is an insignificant problem in developed countries. This is a result of near universal availability of reliable refrigerators and personnel trained to adhere to vaccine storage guidelines. Thus there is little motivation for U.S. domestic health agencies or vaccine manufacturers serving industrialized countries to invest in research for greater vaccine thermostability.

Since the need for more thermostable vaccines is mostly felt in developing countries, it seems logical that support for research on this topic should be made available by those agencies mandated to address health concerns in developing countries, such as development agencies or international organizations.

Potential Actions for Improvement

The cold chain represents about 10 to 13 percent of the total cost of delivering vaccines in developing countries, the bulk of this cost accruing at the periphery of the chain (see paper by Shepard et al. in this volume). Elimination of the cold chain at the periphery would save about 36 percent of total cold chain costs.* In the near future,

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*Elimination of the cold chain entirely, although not considered presently practical, would save about $40 million per year, because there are about 40 million children who receive immunization (40 percent of the birth cohort in developing countries of approximately 100 million). EPI cost per child is about $10, and the cold chain accounts for about 10 percent of this cost.

however, elimination of the cold chain is not likely to be feasible, even at the periphery, because radical improvements in vaccine thermostability are not yet in sight. Hence, immediate efforts should be targeted on (a) further improvement of the existing cold chain, especially its reliability and management (see below); (b) ensuring that the vaccines used in immunization efforts are the most thermostable that can be produced; and (c) incrementally improving vaccine thermostability.

Improving vaccine thermostability should (a) lower vaccine failure rates when temperature abuse is the cause of loss of potency and (b) expand the types of providers able to deliver vaccines, for example, to include traditional birth attendants. Increasing the number of properly immunized children would contribute to child survival efforts and raise the credibility of present and future immunization efforts.

Improving Cold Chain Efficiency

The biggest problem in immunization efforts is management of the delivery system, the cold chain being just part of it. Achieving heat stability in vaccines will not obviate management problems. Problems in the management of vaccine ordering, storage, and transportation, as well as problems in launching coherent campaigns and motivating mothers to make return visits for required multiple immunizations present critical long-term challenges. Operational research in these areas is greatly needed.

Efforts are also needed to maximize the efficiency of the existing cold chain. Approaches could include (a) developing better temperature-exposure indicators for more sensitive and accurate monitoring so vaccines can be taken beyond the present cold chain; (b) increasing the use of vaccines that do not depend on the cold chain for potency, such as tetanus toxoid; (c) utilizing local workers, such as traditional birth attendants, to increase coverage beyond the present chain; and (d) further improving cold chain technology, such as solar refrigerators.

Using the Best Vaccines in Immunization Efforts

The group reached consensus that more could be done immediately to ensure that the vaccines used in immunization efforts were those most likely to provide the highest levels of protection. Stringent requirements for efficacy, thermostability, etc., can ensure that vaccine lots are stable and are potent when used. The measures suggested below in regard to vaccine thermostability are likely to improve the cost-effectiveness of current investments in immunization.

The effectiveness of existing immunization efforts would be enhanced by promoting for each vaccine stringent requirements for thermostability that take into account cold chain capacity, field conditions, required immunogenicity, and currently available technology. An assessment should be undertaken of the characteristics

of future vaccines in relation to these standards. Manufacturers should be encouraged to improve the stability of existing vaccines; minimum requirements should be regularly reviewed to take account of the best current achievements in thermostability. These approaches should be considered by relevant agencies such as the WHO Division of Biologicals, the Expanded Programme on Immunization, and organizations purchasing vaccines for immunization programs such as UNICEF.

Subsequently, there should be monitoring of compliance with revised product requirements and the usefulness of incorporating temperature stability characteristics into them. WHO, in conjunction with major purchasers, could consider extending the system already established to independently monitor vaccines purchased for UNICEF to this purpose. The use of reputable testing laboratories and WHO reference vaccines, along with sample coding, was suggested as a way to achieve the most reliable monitoring of vaccine potency and thermal stability. Testing procedures should take into account the accidental damage most likely to occur under field conditions.

An economic commitment is required to support this expanded testing and monitoring. Funding for testing could be tied to the procurement process, because the purpose is to ensure the purchase of vaccines of the highest quality.

With improved thermostability EPI could save on the overall cost per properly immunized child. Therefore, research by manufacturers into product improvement, production of vaccines that exceed minimum stability requirements, and regular revision of requirements to reflect the state of the art should be encouraged.

For the present much better use can be made of available techniques to improve vaccine temperature stability. Currently, not all manufacturers of OPV use magnesium chloride ($MgCl_2$), an agent known to increase the heat stability of this vaccine. The use of $MgCl_2$ would help minimize the loss of OPV potency in the case of a break in the cold chain. A possible target for OPV would be stability for 3 days at 37°C with a maximum loss in potency of 0.5 logs or stability for 1 week at 37°C with a maximum loss in potency of 1 log. (International requirements for OPV are in preparation.)

Immediate Research Needs

Research funds and efforts must be allocated for the largest return. Vaccines that are already heat stable and potent, such as diphtheria and tetanus toxoids, may not need to be modified. (However, studies to decrease the number of doses needed with tetanus toxoid may be warranted later.)

A research conference on the heat sensitivity of biologicals is desirable. Because this is an interdisciplinary problem, such a conference may bring to light information that is already available in the fields of protein chemistry and biophysics that could be useful in solving the heat stability problem of vaccines. Part of the purpose of

such a conference would be to stimulate greater research interest in thermal stability.

A good data base on the thermostability of existing vaccines (including the effects of freezing) and the relationship of this to immunogenicity and other characteristics (e.g., safety) is needed.

The most urgently needed information involves the exact temperatures below which denaturation of vaccines occurs and their capacity to withstand exposure to ambient room temperature (e.g., 20°C), 37°C, and 45°C. The latter can be expressed in degradation rates (loss of potency per day), half-life, or number of days during which a vaccine will still retain the minimum acceptable potency.

Heat stability should be a high priority in the development of new vaccines. Research should be especially encouraged in three areas: acellular pertussis vaccine preparations; recombinant DNA technology as a method of antigen production; and polysaccharide or polysaccharide conjugate vaccines (e.g., for pneumococcus, meningococcus). Information is needed on the physical and chemical attributes of these agents that confer immunogenicity and on their potential heat stability. Research should emphasize dried vaccines whenever possible because existing lyophilized vaccines are relatively heat stable.

New vaccines under development for possible use in immunization programs in developing countries should be tested early for thermostability and modified, if possible, to improve their performance.

New adjuvants being proposed for use in vaccines and new designs of immunostimulatory complexes should also be tested for thermal stability. Such studies should include glycoside complexes (ISCOMs) and muramyl dipeptide/squalene/pluronic block formulations. Changes in adjuvant-containing vaccines due to lyophilization must also be examined because lyophilized vaccines must retain potency upon rehydration and adjuvant-antigen complexes may be disrupted by the process.

Research into protection against accidental freezing of vaccines sensitive to low temperatures is also important in maintaining vaccine potency. Studies on the reasons for loss of potency on freezing are therefore warranted. A better understanding of a vaccine's capacity to withstand lyophilization is also desirable.

Currently the most temperature-susceptible vaccine of frequent use is OPV, but its stability is not maximized in all existing products (see above). Immediate research into the reasons for temperature-related loss of immunogenicity of polio viruses (of all three types), particularly the apparently more temperature-sensitive strains, is warranted.

Pertussis is the weak member in the DTP vaccine. Improved acellular pertussis vaccines are being tested. The temperature sensitivity of all promising acellular pertussis vaccine preparations should be studied immediately.

Longer-Term Research Needs

Research should be pursued vigorously to understand (a) the fundamental causes of loss of potency of vaccines due to heat or freezing and (b) what specific structural or vaccine formulation characteristics confer heat stability. This research involves understanding the physical chemistry of the vaccine antigen itself, be it the intact live or killed virus or a protein, peptide, or recombinant substitute for the virus. Also needing investigation are the properties of stabilizers that will confer heat resistance. The goal is to develop a combination of antigen, adjuvant, and stabilizer that confers heat stability and provides effective and lasting immunogenicity. Particular attention needs to be given to purity because enzyme impurities in vaccines may accelerate biodegradation by enzyme action.

In the short run it may be most efficient to use currently available vaccines and put funds into developing new physical and chemical stabilizers that confer thermal stability. It is also desirable to promote research on technologies that indicate that temperature stress has overcome the stability of the vaccine. Simple indicators such as change in appearance of the preparation (e.g., flocculation or color) would be helpful in eliminating use of vaccine suspected of thermal damage. In the long run basic research into the structural biochemistry and physical chemistry of viral or other antigens is important because it may facilitate the development and production of heat-stable vaccines independent of specific stabilizers. New approaches may help elucidate the three-dimensional structure of the most immunologically effective antigen and allow modification of this structure so that it is more heat stable and still retains its potency.

Research into mutant viruses that have intrinsic heat stability and reduced neurovirulence is another approach to the OPV problem. However, how to conduct field testing of a new vaccine when a safe vaccine already exists would require careful consideration.

Research is also warranted into biologically compatible dispensers that allow appropriate timed release of vaccines, which could achieve full protection in only one contact with the health care system. Schedules requiring multiple immunizations result in decrease of vaccine coverage from between 60 to 70 percent for the first vaccine dose to 40 percent for the necessary boosters because of poor compliance in returning for subsequent doses. Research into one or two dose schedules is a high priority. Understanding the mechanism of immunologic memory is important because this desirable response is generally achieved only after a booster dose is given following the initial priming dose.

Development of simplified injection devices may also extend immunization coverage. Further study of the "Ezeject" system in this regard is warranted.

WORLD HEALTH ORGANIZATION MINIMUM REQUIREMENTS FOR THE MAIN VACCINES OF INTEREST TO THE EXPANDED PROGRAMME OF IMMUNIZATION

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SUMMARY

The main precautions that should be taken during the manufacturing and control process of vaccines for ensuring that such products are adequate form the basis of the World Health Organization's (WHO) requirements. The three main categories of tests, which deal with efficacy, stability, and safety, and the related requirements for each of seven vaccines against tuberculosis, diphtheria, pertussis, tetanus, measles, poliomyelitis, and yellow fever commonly used in humans are reviewed in this paper.

For inactivated vaccines, tests for relative potencies usually consist of challenge tests requiring rather large numbers of laboratory animals. The testing for potency of live vaccines (BCG, measles, oral poliomyelitis, and yellow fever vaccines) is done by ensuring that the actual number of live attenuated microorganisms per single human dose is not lower than a certain limit.

In regard to differences of strains used for the manufacture of certain attenuated vaccines, more data would be needed in order to compare the efficacy of BCG vaccines and of measles vaccines, especially when measles vaccines are injected into babies with antibodies of maternal origin. In the case of oral poliomyelitis (OPV), more data are also needed on the best relative proportions of the three polio types. The assurance for stability of vaccines is given by controlling the time validity and the capacity of products to sustain, to some extent, storage temperatures different from those recommended by manufacturers. In that respect one can generally say that inactivated vaccines pose fewer problems than live attenuated vaccines, and this is reflected by the fact that at the moment, WHO requirements include tests for thermal stability only for live vaccines.

As for tests on safety, they consist primarily, in the case of inactivated vaccines, of ensuring that inactivation or detoxification is complete. Even when this is the case, very rare serious accidents such as encephalopathy may occur (e.g., after immunization with bacterial vaccines containing a pertussis component); unfortunately, no test can predict such accidents, especially since they seem to be related to individual susceptibilities. The assurance for safety of live attenuated vaccines is obtained primarily by strictly adhering to protocols having led to the production of safe vaccines, and this includes using seed lot systems for relevant microorganisms and, if

applicable, for cell substrates as well. In addition, certain tests such as for OPV the neurovirulence test in monkeys and for BCG the injection of a large number of human doses to guinea pigs are mandatory.

INTRODUCTION

The major progresses in biotechnology that have occurred over the past few years by the use of recombinant DNA methods and polypeptide synthesis are likely to result in the production of vaccines giving better protection and fewer side effects and that can be administered under combined forms.

This paper is aimed at reviewing the main international requirements of seven vaccines in common use: diphtheria, tetanus, whooping cough (whole cell vaccine), tuberculosis, measles, poliomyelitis, and yellow fever.

The three main aims of the review will deal with protective efficacy, stability, and safety. In relation to problems of stability, the current WHO requirements on time validity are summarized in Table 1.

DIPHTHERIA AND TETANUS TOXOIDS (ADSORBED)

Because of the many analogies in manufacturing and testing procedures of diphtheria (D) and tetanus (T) toxoids, and because they are often used in a combined form, they will be considered together.

Several major articles on diphtheria (Relyveld and Raynaud, 1977; Alouf, 1982; Pappenheimer, 1984) and tetanus (Bizzini, 1984) have been published over the last few years.

The corresponding International WHO Minimum Requirements were published in 1979 (WHO Expert Committee on Biological Standardization [WHO-ECBS], 1979), and additions were made later (WHO-ECBS, 1981, 1982, 1984, 1985).

Efficacy, Potency

Diphtheria and tetanus toxoid vaccines are considered very efficacious, although it has not been possible thus far to formulate minimum requirements and methods of control of potency for diphtheria and tetanus toxoids acceptable to all WHO member states.

According to the international requirements, each single human dose of toxoids for primary immunization should not contain less than 30 IU for diphtheria and 40 IU for tetanus. If tetanus vaccine is used in combination with the pertussis component, the single human dose should not contain less than 60 IU of T as tested in mice. This additional amount of T toxoid is a precautionary measure, due to the fact that in

mice pertussis vaccines increase the immune response to tetanus toxoid. Precise methods of potency assays for D and T vaccines based on the determination of "effective doses 50 percent" (e.g., vaccine doses capable of protecting 50 percent of animals against challenge) and the subsequent comparison thereof have been thoroughly studied by Mussett and Sheffield (1973). Unfortunately, such methods are expensive because they are based on injecting with three dilutions each of test and standard vaccines.

National control authorities from some member states (USA, India) use simpler methods based on immunizing smaller numbers of animals with a single dilution of vaccine and then either challenging the animals or assaying the antitoxin content of their pool of sera. Shortcomings of such tests include possible important variations should only one of the test animals respond in an abnormal way. Furthermore, since no reference vaccine is used, variables such as strain, feed regimen, age, and health condition of laboratory animals may influence results.

The justifications for expressing potencies of D and T vaccines in international units of toxoids have been extensively examined by several authors. Following experiments in rhesus monkeys injected with two doses of toxoid vaccines exceeding the minimum number of international units required for humans, Hardegree and colleagues (1982) found that there was no relation between the amount of toxoid injected and the titer of antitoxin elicited. The same year Huet (1982) also concluded that using the international unit for immunogenicity had not resulted in improving the quality of toxoid vaccines. Relyveld (1985) observed that adding adjuvant to the diluting fluid of toxoid vaccines resulted in an increase in titer, especially for toxoids made from purified toxins. Nyerges et al. (1986) found no correlation between amounts of DT toxoid injected in humans and antitoxin levels. On the other hand, Van Ramshorst and colleagues (1973) found a close correlation between both such factors. One way to reconcile these observations would be to envisage that in several of the clinical studies the amounts of toxoids injected perhaps exceeded the optimum, so that a ceiling or even inhibitory effect on the immune response might have occurred.

One method of testing that might be acceptable to all national control authorities would consist of using in vitro methods to assess the antitoxin response of a small number of laboratory animals immunized with dilutions of both test vaccines and the reference vaccine. This can be done for diphtheria using antitoxin from mice (Kreeftenberg et al., 1986) and cell cultures. Although encouraging results have been reported in the assay of antitetanus toxin by in vitro methods such as RIA and Elisa, the possibility of such methods detecting antibodies other than antitoxins must be considered, especially for low-titer sera (Gentili et al., 1985; Simonsen et al., 1986).

Another way of simplifying current testing procedures is to ensure that potencies of test vaccines are above the minimum required, and this has been done by challenging small numbers of animals previously immunized with a single dilution of vaccines and/or reference toxoids (Knight and Roberts, 1987; Stainer et al., 1987).

Stability at Various Temperatures of Storage

Diphtheria and tetanus toxoids can withstand some deviation in storage temperatures recommended by manufacturers (WHO-Expanded Programme on Immunization [WHO-EPI], 1980a). For example, diphtheria and tetanus toxoids have been found to retain potency after 6 to 12 months at 20 to 25°C or after 2 to 6 months at 37°C. Problems are more likely to arise from storage temperatures below those that are recommended, which could, in the case of freezing, result in an alteration of the adsorbant-immunogen complex. There is however a certain safety margin since freezing points are often below -5°C (WHO-EPI, 1980b).

Safety

Several tests (injection of 500 Lf amounts of toxoids to guinea pigs, tests for irreversibility, tests for purity) are imposed by the WHO requirements for controlling the safety of diphtheria and tetanus toxoids.

Recipients of tetanus toxoid booster injections having high antitetanus toxin titers prior to the recall injection may experience Arthus-type reactions (Edsall et al., 1967; Fanet et al., 1972); adverse reactions may also occur in recipients of booster diphtheria toxoid injections (Relyveld et al., 1979), which explains why certain pharmacopoeias have set an upper limit for the toxoid content of diphtheria-adsorbed vaccines for adolescents and adults.

On the whole there is agreement that most of the rare serious incidents related to the use of DTP vaccines are caused by the pertussis component (Anonymous, 1979; Fenichel, 1982; Galazka et al., 1984).

PERTUSSIS VACCINE (WHOLE CELL)

Pertussis (P) vaccines are usually administered as mixtures containing diphtheria and tetanus toxoids in addition to the P component; the history of pertussis immunization has been extensively reviewed by Wardlaw and Parton (1983a,b) and Manclark and Cowell (1984).

Efficacy, Potency

Following several observations, pertussis vaccines containing at least 4 IU per single human dose are considered to be efficacious in humans. The testing for potency is done by intracerebral challenge of mice immunized 2 weeks earlier (WHO-ECBS, 1979). Unfortunately, the test is not precise, and the results, although expressed in relation to a reference that has been calibrated against the international standard of pertussis vaccine and is tested under conditions similar to test vaccines, may vary substantially among laboratories. It is for this

reason that since 1981 (WHO-ECBS, 1982) manufacturers may, in the case where a batch has failed the test for potency, repeat the test on the condition that they use as the final estimate of potency the geometric mean of all statistically valid assay results. One reason that potencies of pertussis vaccines tested by different laboratories can vary substantially may be due to individual variations in calibrating laboratory references against the international standard.

Attempts to prepare acellular pertussis vaccines over the past few years have been most useful in identifying the components likely to play a role in eliciting immunity. One of the key components seems to be the pertussis toxin; it has various pharmacological properties (Bergman and Munoz, 1977; Munoz and Bergman, 1978). Mice that are either passively or actively protected against the pertussis toxin resist intracerebral challenge (Oda et al., 1984; Sato and Sato, 1984; Sato et al., 1984). The protective role of the fimbriae hemagglutinin (FHA) in man, another product from pertussis bacteria capable of protecting mice against intranasal challenge, is less clear. Certain authors think that other antigens, such as the adenylate cyclase (Novotny et al., 1985) or agglutinogens (Preston, 1976), also contribute to active protection. The kinetics of pertussis toxin and FHA antibodies have been studied in man both after natural infection and after immunization with acellular pertussis vaccines (Sato and Sato, 1985). There is preliminary evidence suggesting that such a test based on the neutralization, by the serum of laboratory animals previously immunized, of the pertussis toxin action on cell cultures will play a major role in the routine testing of potency of acellular P vaccines. The suitability of such a test for controlling the potency of whole cell pertussis vaccines is worthy of examination.

Stability at Various Storage Temperatures

The effect of storage temperatures different from those recommended by manufacturers on the activity of DTP vaccines has been reviewed (WHO-EPI, 1980a). It appears that depending on products the stability of the pertussis component varies widely. This is not surprising if one thinks of the various factors that may intervene in the degradation process. Udayabhaskara et al. (1985) observed that the mean 50 percent titer fall of 6 batches of DTP vaccines exposed to 37°C was about 50 days, whereas according to another study (WHO-EPI, 1985) the half-life of the pertussis component in DTP vaccines from a different origin and similarly exposed was about 15 days. More studies on the stability of pertussis vaccines are needed; unfortunately the method for potency testing is not convenient or precise.

Like DT vaccines, DTP vaccines should not be frozen because of the deleterious effect of freezing on the pertussis component (WHO-EPI, 1980b).

Safety

The common side effects attributable to whooping cough vaccination are well known (American Medical Association, 1985). They usually

consist of local and general reactions such as fever, fretfulness, and sometimes persistent crying.

Methods used for inactivating pertussis bacteria (treatment by formaldehyde, glutaraldehyde, heat, or prolonged storage, single or in combination) may influence the immunogenicity and residual toxicity of vaccines (Gupta et al., 1986). The safety tests of the WHO minimum requirements for pertussis vaccines are aimed at verifying that (a) no living bacteria are present, (b) a single human dose does not contain more than 20 IU of opacity, and (c) the product passes the "mouse weight gain test"; vaccines containing an abnormal level of pertussis toxin, endotoxin, and dermonecrotic toxin are likely to fail the test (Kurokawa, 1984).

Pertussis vaccines that meet the WHO requirements for safety may induce severe but rare major complications such as encephalopathy (Fenichel, 1982; WHO-EPI, 1982; Manclark and Cowell, 1984; Miller et al., 1985; Ehrengut, 1986). Although it has been mentioned, it is not clear whether such incidents are due to individual hypersensitivities to components such as LPF (Munoz and Mackay, 1984; Munoz et al., 1984; Miller, 1986). Examination of averages of extreme incidences of severe reactions recently reported (Anonymous, 1979) indicated that risks for permanent lesions or death are more than 3,000-fold greater after whooping cough than after pertussis immunization. As to encephalopathy and convulsions, relative risks exceed 1,000 and 400, respectively. Nevertheless, such accidents constitute a major problem to public health authorities and vaccine manufacturers (Boffey, 1984). In the United States the number of lawsuits filed for damages in relation to DTP immunizations went from 1 in 1978 to 73 in 1984, and claims per suit have risen from $10 million to $46.5 million (Hinman, 1986). Contraindications to pertussis immunization have been extensively reviewed (WHO-EPI, 1984).

Bacillus Calmette Guérin (BCG)

The WHO minimum requirements for dried BCG vaccines were published in 1966 (WHO-ECBS, 1966) and reviewed in 1978 and 1984 (WHO-ECBS, 1979, 1987).

Efficacy, Potency

In the first WHO requirements the use of a seed lot system was made mandatory; the main merit of the system is to reduce the chances of drifts in virulence and efficacy (Guld, 1974) by limiting to 12 the number of passages between the primary seed lot and the vaccine.

The efficacy of BCG vaccines has been studied extensively (Bjartveit and Waaler, 1965; Muggleton, 1971; Lugosi, 1972, 1985; Anonymous, 1979, 1983; Chaparas, 1982; Clemens et al., 1983; Collins, 1984; Curtis et al., 1984; Zilber et al., 1984; Muggleton et al., 1986; Ten Dam, 1986); it is extremely variable. Clinical trials are rendered complex by factors such as (a) need for long-term programs since

observation may need many years to be completed; (b) ethical problems, such as difficulty in having unimmunized controls (e.g., in countries where BCG vaccination is compulsory); (c) use of seed lots that may differ widely throughout the world, namely because of strain differences and many passages having occurred since initial BCG seeds were made available; according to Gheorghiu et al. (1978), BCG vaccines from seeds with weak residual virulence are less efficaciofs; (d) difficulty drawing firm conclusions from trials sometimes performed under conditions where there is uncertainty about the composition or even the quality of vaccines administered (e.g., differences in the live bacilli content of doses due to inadequate lyophilization or possible exposure of BCG microorganisms to UV light after reconstitution of the vaccine (WHO, 1987a), uncertainty about the correct route of administration, or uncertainty about the extent of natural protection due to previous exposure to various environmental mycobacterias.

It is therefore not surprising that conclusions on the efficacy of BCG vaccines differ so widely. Greatest efficacy seems to have been observed for the severe forms of tuberculosis such as meningitis. In collaboration with WHO, additional clinical efficacy studies on BCG vaccines have been organized and some of the recent results reported (Ten Dam, 1986; WHO-Tuberculosis Control Programme and EPI, 1986).

Efficacy studies in humans are indispensable and cannot be replaced by limited studies for ascertaining, for example, the capacity of BCG vaccines to induce tuberculin conversion.

According to the WHO requirements, another component of a BCG vaccine validation process consists of its characterization in a suitable animal model system. Laboratory animals commonly used include mice (Brandely et al., 1983) with intravenous challenge and guinea pigs (Smith, 1985) in which aerosol challenge can also be used. Some correlation has been observed in laboratory animals between protective efficacy and parameters such as BCG viability counts in spleen, spleen index, local granular formation, induction of delayed-type hypersensitivity reaction to tuberculin, and resistance to listeria monocytogenes challenge. Factors capable of influencing efficacy in animals include diet (McMurray et al., 1985), animal species, strain of BCG, and quantity of vaccine injected (Smith, 1985). The overall conclusion drawn by Smith (1985) from laboratory animal studies in which 10 BCG vaccines of different origin were examined was that 2 vaccines had no experimental protective value. As a matter of fact, the 2 nonprotective BCG vaccines were not for use in humans!

Another level of efficacy control of BCG vaccines imposed by the WHO requirements consists of determining for each vaccine lot the number of culturable particles, since living BCG organisms are indispensable for inducing protection. The test can be done by actual seeding of culture media or more rapidly by adenosine triphosphate determination (Gheorghiu and Lagranderie, 1979; Gheorghiu et al., 1984).

Thermostability

The test for stability, which consists of exposing samples of each batch of vaccine to 37°C for 4 weeks, has been made compulsory since 1978. Tests are considered successful if heat exposure does not decrease the number of culturable BCG particles by more than 80 percent. A collaborative study in 1977 by Sekhuis et al. (1977) on BCG vaccines from 10 producers has shown that after 1 month at 37°C the loss of potency was less than 75 percent in eight of the vaccines. The two vaccines for which a greater loss was observed had been lyophilized in rubber-stoppered vials, which suggests the importance of factors such as tightness, residual moisture, and nature of gas.

Safety

The tests for safety imposed by the WHO requirements include controls for the absence of virulent mycobacteria and determination of numbers of total and culturable particles per human dose.

The side effects that may be observed after BCG vaccination (Anonymous, 1979) include cutaneous lesions, which may occur even if vaccine organisms have lost their viability (Bunch-Christensen, 1981), and adenitis or lymphadenitis, the incidence of which varies with BCG strains, dose of live bacteria, and age of recipient (Guld et al., 1955; Lotte et al., 1984; Quast and Bijok, 1986).

In spite of the merits of BCG vaccines, better vaccines are needed for the successful active protection of individuals against tuberculosis, and WHO has launched a scientific research program for that purpose.

MEASLES VACCINES

In 1984 the global incidence of measles was about 50 per 100,000 persons, that is, about 30 times the incidence of tetanus and 83 times that of poliomyelitis (WHO, 1987b). The fatality rate of measles is important, and an estimated 1.86 million deaths due to measles occur each year in developing countries excluding China (WHO-EPI, 1988), which is respectively about 2.3 and 3.3 times the number of victims from tetanus and whooping cough.

Efficacy, Potency

All measles vaccines used today are of the live attenuated type and are produced on chicken embryo fibroblasts or human diploid cells. Efficacy of vaccines may vary in relation to parameters such as age of vaccinees, geographic location, state of development of countries, vaccine virus strain (Sassani et al., 1987), number of infectious viral particles per dose, etc. Wild strains and vaccine strains derived therefrom have been well documented by Hirayama (1983). Vaccine strains differ, among other things, by their degree of attenuation.

For vaccines derived from more attenuated virus strains, two injections are sometimes recommended (Mirchamsy et al., 1979). Passive immunity from maternal origin in babies several months old may hinder the establishment of active immunity (Black, 1982). In a study conducted in Gambia by Hull et al. (1983), the seroconversion rate in babies immunized from the sixth to the eighth month of age was 37 percent, whereas it was 89 percent with babies older than 9 months. Unfortunately, measles cases between 6 and 9 months are common in developing countries, especially when climates are hot. There is preliminary evidence suggesting that certain vaccine strains such as the Edmonston Zagreb strain, grown on human diploid cells, may yield vaccines capable of giving satisfactory seroconversion as early as 6 months of age (Sabin et al., 1983, 1984; Whittle et al., 1984; Tomkins et al., 1985; De Castro et al., 1986), and comparative field trials are conducted to verify this.

The minimum WHO requirements for measles vaccines (WHO-ECBS, 1966, 1982) are based on the principle of using the vaccine virus seed lot system; in the WHO document that has recently been revised, the maximum number of passages from virus seeds (used in the preparation of vaccines shown to be immunogenic and safe in man) to vaccines is limited to five. Potency tests of all batches consist of the assay of the number of viral particles capable of replication, and in the 1966 WHO requirements the number was set at 1,000 TCID 50 or PFU per human dose. However, the Japanese minimum requirements (Anonymous, 1986) have set the minimum potency of measles vaccines to 5,000 TCID 50 or PFU/dose.

Thermostability

In view of the importance of preventing measles and the difficulty of maintaining satisfactory temperature storage conditions for vaccines in developing countries with hot climates, it is desirable to use vaccines capable of withstanding short periods of adverse conditions of temperature. It therefore became obvious a few years ago that it was appropriate to define the conditions of a thermostability test for measles vaccines. Thermostability studies commonly performed derive from the pioneer work of Jerne and Perry (1956), who showed that provided certain conditions are met (such as degradation reactions being of a monomolecular type) there is, for a given product, a linear relationship between the degradation constant and the exposure temperatures. Jerne and Perry's conclusions were later confirmed and refined by Tydeman and Kirkwood (Kirkwood, 1984; Kirkwood and Tydeman, 1984; Tydeman and Kirkwood, 1984). Accelerated degradation tests were used as early as 1965 to examine the stability of measles vaccines (Grei and Rightsel, 1965). In 1979 a study of measles vaccines from six manufacturers was published by the EPI (WHO-EPI, 1979). An extensive study on the thermostability of measles vaccines was published by Allison et al. (1981); they observed that at exposure temperatures higher than 20°C there was an initial rapid loss of potency followed by a slower rate of degradation; this was also observed by Colinet and Peetermans (1982). Thermostability studies of measles vaccines eventually resulted in 1981 in a WHO requirement for

thermostability (WHO-ECBS, 1982). In keeping with the requirement, vials of measles vaccines that have been exposed 1 week at 37°C must (a) still contain the minimum number of living viral particles required (i.e., 1,000 TCID 50 or PFU per dose) and (b) exhibit a loss of potency not greater than 1 log. Results of a study conducted by WHO in collaboration with the London School of Hygiene and Tropical Medicine showed that out of 16 vaccine preparations examined six met both criteria six met neither of the two criteria, and four met only one criteria (WHO-EPI, 1981). The support by WHO of studies on the thermal stability of measles vaccines and the introduction in the minimum international requirements of a test for thermal stability have resulted in a marked improvement of the quality of such vaccines.

Safety

When human diploid cells are used as the substrate, the safety precautions considered by the WHO requirements for measles vaccines include testing for tumorigenicity and refraining from using cells beyond two-thirds of their normal life span. Regardless of which substrate is used, controls for the absence of extraneous agents in cell cultures, virus seed lots, animal sera used as growth factors, and trypsin are required. Since measles vaccines are produced in substrates from either well-characterized cell banks or closed monitored colonies of birds and from equally well-characterized virus seeds, the WHO requirements no longer demand for purposes of excluding contamination by extraneous agents, the inoculation of small laboratory animals with samples from each batch of vaccine. The absence of neurovirulence of the virus seed must be controlled by intracerebral inoculation of monkeys.

In addition to minor commonplace side effects such as fever and eruption, rare serious complications may occur (Modlin et al., 1977; Centers for Disease Control, 1982; Grattan-Smith et al., 1985). Comparing averages of extreme incidences of serious incidents (Fenichel, 1982) occurring either after the disease or vaccination indicates that many more serious adverse reactions are caused by the disease than by the vaccine; relative risks for encephalitis, panencephalitis, convulsions, and deaths are about 1,400, 14, 17, and 1,430, respectively. Major adverse reactions occurring after measles vaccination are unfortunately unpredictable; some relation between incidence of encephalopathies and residual virulence of vaccine strains may exist (Hirayama, 1983).

POLIOMYELITIS

Oral Polio Vaccine

The history of oral polio vaccines (OPV) has been reviewed by Sabin (1985).

Efficacy, Potency

The efficacy of OPV has been documented on several occasions (Cockburn and Drozdov, 1970; Grachev, 1984). Between 1955 and 1967 the incidence of poliomyelitis in 28 countries, including the United States and the USSR, in which oral polio vaccines were mainly used, decreased by 98.7 percent (Anonymous, 1969). For reasons that are not entirely clear, OPV's efficacy seems to be less satisfactory in developing countries, and, depending upon authors, factors such as bad storage, absence of control (Johnson et al., 1984; Hovi, 1986), presence of inhibitors in the gastroalimentary tract of babies (Dömök et al., 1974), or interferences by enteroviruses (Sabin, 1961) have been held responsible. In such countries the strategy advocated by Sabin (1982) is to organize annual mass campaigns, and this appears to be effective in eliminating wild polio strains from alimentary tracts.

The production and control of OPVs (WHO-ECBS, 1983) is based on the use of the seed lot system for vaccine strains and, when human diploid cells are used, for cells. Almost all of the OPVs produced throughout the world derive from the three strains that were isolated about 30 years ago and then made freely available by Sabin, through WHO, to vaccine manufacturers. Antigenic or pathogenic drifts of OPV vaccines are limited by strict temperature conditions (33 to 35°C) for in vitro multiplication, as well as by limiting the number of passages. Since the efficacy of vaccines depends on a number of variables, it is not possible to determine in an absolute manner the minimum number of infectious viral particles of each type that should be present in single human doses. For quality control purposes it is however important to decide on such numbers, and on the basis of experience the WHO-ECBS set such minimum numbers (in preparation). Another factor that might influence the efficacy of OPVs is the relative proportion of each type of vaccine virus, and, although in practice efficacious formulations are being used, it would be interesting to have more data gathered from different places. Changes to the existing requirements are subject to the finding of improved formulations, a problem that is currently under investigation by the WHO-EPI.

Thermostability

OPV is undoubtedly very sensitive to rises in temperature (WHO-EPI, 1980a). As might have been expected, OPV manufacturers have worked on improving OPV's stability. An unpublished study conducted a few years ago by WHO in collaboration with Dr. Mann, from the London School of Hygiene and Tropical Medicine, showed that half-lives at 8°C of nine trivalent oral polio vaccines containing various stabilizers ranged from 199 to 65 days, which represents about a threefold range of variation. In that study the most stable vaccines contained magnesium chloride as a stabilizer, as opposed to sorbitol or sucrose. The existing WHO requirements demand that all batches of OPVs be tested for loss of titer after 1 week at 37°C. However, the minimum performance stated in the requirements is not obligatory. Among the difficulties for testing for stability and potency, one can cite the discrepancies

of results of tests among laboratories due, for example, to the use of different cell substrates and of antisera cross-reacting with several polio types. However, the comparability of results is improved by using a common reference (Magrath and Seagroatt, 1985), but such a reference is not widely available and is furthermore unstable under nonfreezing storage temperatures.

From a practical point of view the loss of activity of vaccines after heat exposure could be determined either on the mixture of the three types or, on the other hand, on each type, after appropriate seroneutralization has been achieved. The first approach would be much simpler but would require prior demonstration that each of the three types is equally sensitive to temperature rises.

Safety

The preparation of safe OPVs depends on the strict observance of various precautions. Some of the controls are aimed at excluding the contamination by extraneous agents of vaccine virus seeds (WHO-ECBS, 1982), cell cultures, and animal sera used as growth factors. Classical viral contaminants that need to be excluded include hemadsorbing viruses, SV 40 virus, and Simian (herpes B) virus. Generally speaking, tests on cell culture systems for detecting viral contaminating agents are considered more sensitive than tests on small laboratory animals. Other safety tests include neurovirulence tests in monkeys, which need to be performed on each monovalent polio bulk. The absence of back shifts to neurovirulence is also examined by an in vitro test, the "rct 40 (replication at 40°C) test."

In spite of neurovirulence tests in monkeys being carried out systematically, very rare cases of paralytic poliomyelitis attributable to OPVs, especially type 3, do occur. Having examined 71 cases of paralytic poliomyelitis from vaccine origin, out of 76 million persons immunized within 20 years in Japan, Chino et al. (1984) concluded that there was a significant relationship between attenuation, as measured by the neurovirulence test in monkeys, and the incidence of vaccine paralytic cases. Unfortunately, those batches of vaccines that were less neurovirulent also induced lower seroconversion rates.

The incidence of paralytic cases of vaccine origin (Fenichel, 1982; Galazka et al., 1984) may be roughly estimated as less than one case per million, which may be considered a problem in countries where wild poliomyelitis incidence is disappearing (WHO Consultative Group, 1982; Anonymous, 1984; Kim-Farley et al., 1984). In such countries the small vaccination risk can be overcome by doing a first injection of inactivated polio vaccine before giving three courses of OPV or to use only inactivated polio vaccine.

Inactivated Polio Vaccine

The history of inactivated polio vaccine (IPV) has been reviewed by several authors, especially Salk (1983, 1984).

Efficacy, Potency

According to the WHO requirements (WHO-ECBS, 1982), the antigenic content of every batch of IPV must be determined in the final vaccine container for each of the three types; the requirements state that antigenic values of 40, 8, and 32D antigen units for types 1, 2, and 3 respectively are generally considered as capable, after a single injection, of inducing seroconversion rates of 85 to 95 percent and of 100 percent if immunological memory exists in vaccine recipients. The requirements also include a provision for an immunogenicity test on rats. However, there are no mandatory minimum values either for antigenic content or immunogenicity properties of IPVs. Recent studies have shown that when vaccines with antigenic contents greater than those mentioned in the requirements are used, high seroconversion rates can be induced only after two injections (Robertson et al., 1987; Anonymous, 1988). In view of the possibility of combining IPV with other vaccines, such observations might have important repercussions on future immunization programs, especially if campaigns in which IPV is used are not more costly than those using OPV. A recent episode of polio cases in Finland in individuals previously immunized with the required number of IPV doses has been found to be associated both with an antigenic modification of a type 3 wild strain and with insufficient titers of vaccine (Hovi, 1986).

Thermostability

IPVs are much more thermostable than OPVs. For example, IPVs may still be potent even after 4 weeks of storage at 37°C (WHO-EPI, 1980a); the fact that IPVs are more commonly used in developed countries where appropriate cold chain temperatures are easier to maintain probably explains why data on thermostability are scarce.

Safety

As for thermal stability, data on reactions attributable to IPV administration are scarce, which indirectly points to the safety of such products. An early preoccupation, following the occurrence in 1955 in the United States of 260 cases of polio having caused 10 deaths among persons immunized with IPV, was to verify that no living viral particle was left after inactivation. Tests on cell cultures are therefore mandatory for the routine control of inactivation of all batches of IPVs. Other mandatory tests are aimed at excluding the contamination of vaccines by extraneous agents and, in the case where human diploid cells are used, at excluding possible tumorigenic potentials.

Another important control test has to do with purity, and that test is even more important if continuous cell lines are used. Experimental studies aimed at ascertaining the degree of contamination of vaccines by residual cellular DNA have been conducted by Crainic et al. (1980a,b). Part D of the 1981 WHO Minimum Requirements on IPV was

replaced in 1985 (WHO-ECBS, 1987); the new document insists on a thorough characterization, including tests for retroviruses, of seeds of continuous cell lines used to produce substrates. The philosophy is that tests on final products should be related to the nature of likely contaminants, and this approach was further emphasized by a study group on continuous cell lines convened by WHO in November 1986 (WHO, 1987c). In the case of IPV derived from continuous cell lines, the former limit of 10 pg of residual cellular DNA per human dose has been maintained, although on the basis of the study group report, 100 pg may be suggested in the future. Regardless of which substrate is used, the former upper limit of 10 µg of nitrogen protein per human dose has been maintained. The fact that now even cells containing viral sequences (e.g., Namalwa cells) may be considered for the production of biological substances constitutes an important change in philosophy.

YELLOW FEVER VACCINE

Problems related to the prevention and control of yellow fever, especially in Africa, have been extensively reviewed in a recent WHO publication (1986). Current yellow fever vaccines are of the attenuated type and derive from the 17D vaccine strain. They are produced by inoculation of embronated hen's eggs and then preparation of embryo extracts.

Efficacy, Potency

A single injection of yellow fever vaccine gives long-term protection, and from a practical point of view reimmunization is recommended after 10 years (WHO, 1987c), although longer periods of protection have been observed (CDC, 1984).

The first WHO minimum requirements were formulated in 1958 and were revised in 1975 (WHO-ECBS, 1976). Further to a series of incidents such as failures of protection and encephalitis, a group of experts convened by WHO defined in 1981 the "procedure for approval by WHO of yellow fever vaccines in connection with the issue of International Vaccine Certificates" (WHO-ECBS, 1981). As for other attenuated vaccines, production and controls are based on the use of a virus seed lot fully controlled as regards safety and immunogenicity. Since 1985 an international master seed lot for the production of working seeds has been made available to WHO-authorized vaccine manufacturers through their national control authorities. The preparation of the WHO seed was undertaken in order to avoid incidents such as those observed in Latin America; they were due to the use of vaccine seeds that had been passaged too many times and resulted in serious adverse reactions. The essential test for the control of working seeds is the monkey intracerebral inoculation test, and it is used for demonstrating both immunogenicity and safety (WHO-ECBS, 1976). The potency control of routine batches of yellow fever vaccines is based on titration of the number of living viral particles. The minimum number required by the WHO requirements is 1,000 50 percent mouse lethal doses per human dose or its equivalent if the titration is performed on cell cultures.

Thermostability

Over the past few years several yellow fever vaccine manufacturers have been able to improve substantially the thermostability of their product, both by refining lyophilization techniques and by using vaccine stabilizers (Robin et al., 1971; Burfoot, 1977). Simple calculations made from results published by Robin et al. (1971) indicate that at that time yellow fever vaccines from three of eight manufacturers were no longer meeting the potency requirements after 2 weeks of exposure at 37°C. In addition, six out of the eight vaccines had lost more than 1 log of potency. In 1985 WHO organized a collaborative study on yellow fever vaccines from 12 WHO-approved producers; vaccines were assayed in collaboration with Barmes (Pasteur Institute Foundation) and Mann (London School of Hygiene and Tropical Medicine) (Anonymous, 1987). It appeared that after 2 weeks at 37°C, seven out of the 12 vaccines had titers still above the minimum required and that the loss was less than 1 log; most of the remaining vaccines were not meeting either of these two criteria. Although in both Robin's and WHO's studies results were expressed in MLD 50, titrations in the first study were done by intracerebral inoculation of mice, whereas in the other study they were done in cell cultures and results were then transformed in mouse lethal doses, the results of the two studies are therefore not strictly comparable. The WHO requirements on yellow fever vaccines were modified in 1987 (WHO-ECBS, in preparation) to the effect of advising the implementation of a stability test.

Safety

Following yellow fever vaccination, side effects are usually minimal; they consist of mild headache, myalgias, low-grade fever in 2 to 5 percent of vaccinees (CDC, 1984); hypersensitivity reactions due to egg proteins may also occur, and this is why a limit of 0.25 mg of nitrogen protein per dose, excluding stabilizers, has been set.

Encephalitis is a rare complication that may occur more frequently in babies, so babies less than 9 months old should not be immunized under normal circumstances (WHO, 1986).

The encephalitis cases associated with yellow fever immunization of children in Brazil (WHO-ECBS, 1981) have shown that it is important to control working vaccine seeds in monkeys. The use of human serum as a stabilizer is forbidden, due to the occurrence of hepatitis B cases in recipients of yellow fever vaccines containing contaminated human sera (WHO-ECBS, 1981).

Other mandatory safety controls are aimed at excluding the contamination of substrates by agents such as salmonella, mycobacterium avium, and fowl pox virus. On the other hand, the use of embryonated eggs from avian leucosis virus free flocks is recommended but not obligatory.

CONCLUSION

Using vaccines that are safe and efficacious is of critical importance to the success of active immunization; important improvements in stability, especially for live attenuated vaccines, have been achieved in the relatively recent past by using stabilizers and lyophilization. However, lyophilization is not suitable for certain vaccines such as OPV or IPV.

Increased stabilities are likely to be observed when vaccines are produced by techniques such as recombinant DNA or polypeptide synthesis; one reason for this is that such products will consist of purified antigens, so that it might no longer be necessary to maintain during storage the strict conditions of temperature that are otherwise required for the successful replication in vaccines of those living particles that are contained in live attenuated vaccines. Furthermore, future vaccines are likely to be purer and devoid of enzyme contaminants that may otherwise adversely affect stability.

Acknowledgments

I would like to express my thanks to Dr. J. Petricciani, Dr. A. Galazka, Dr. V. Grachev, and Dr. D. Magrath for critical review of this manuscript and to Ms. J. Brunt for having typed it.

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TABLE 1 Shelf Life of Seven Vaccines

Vaccines	Maximum Total Validity (From the Beginning of the Last Satisfactory Test for Potency)	Examples of Storage	
		By Manufacturers	After Issue
BCG	36 months[a]	24 months[a]	12 months[a]
Tetanus, Diphtheria-Tetanus	36 months	12 months	24 months
Diphtheria-Tetanus-Pertussis	30 months	6 months	24 months
Polio (oral)	12 months at -20°C plus 12 months at a temperature not higher than -5°C	12 months at -20°C	12 months at a temperature not higher than -5°C
Polio (inactivated)	18 months at 2 to 8°C 8°C	6 months at 2° to 8°C	12 months at 2° to
Measles	12 months[a]	Not mentioned	Not mentioned
Yellow Fever	18 months at -20°C	6 months	12 months

[a]Requirements allowing longer validity periods are undergoing editing.

Note: Unless specified, storage temperatures are those recommended by the manufacturer.

Source: WHO Requirements on Biological Substances published up to January 1988.

ECONOMIC AND LOGISTIC IMPACTS OF TEMPERATURE-SENSITIVE VACCINES

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INTRODUCTION

Childhood vaccinations have the potential to avert millions of deaths in children in developing countries each year. In 1974 the World Health Organization's (WHO) Expanded Programme on Immunization (EPI) was created with the formidable goal of immunizing all children in the world by 1990. Through the combined efforts of governments of developing countries, international donors, and private agencies, enormous progress has been made in many countries. By early 1986 it was estimated that 43 percent of children under 1 year of age in developing countries worldwide had received their third dose of DTP, one important indicator of vaccination coverage with newer assessments showing continual progress (EPI, 1986). Among the challenges in delivering vaccinations in a developing country are the temperature sensitivities of childhood vaccines. To remain potent these vaccines must be kept at specified temperatures from the time of manufacture to the point of use through the maintenance of an unbroken "cold chain."

This paper was requested by the Institute of Medicine to estimate the economic and logistic impacts of temperature-sensitive vaccines on vaccine delivery. This information is intended to assist policymakers in determining the priorities that should be accorded to research, development, and use of less temperature-sensitive vaccines and to compare this approach with other ways of strengthening EPI programs.

The next section describes the vaccines, cold chain requirements, and operational aspects of the cold chain at different levels of delivery. The following section presents the computer model used in the analysis. Two country studies of cold chain costs follow, based on Sahelian conditions. Subsequently, options for addressing constraints of the cold chain are examined and contrasted with other approaches to strengthening immunization programs. Finally, conclusions, limitations, and caveats are presented that summarize the analysis and note areas that are particularly sensitive to assumptions and data constraints.

THE COLD CHAIN

To maintain vaccine viability and antigenic potency, vaccines must be stored and transported at specified temperatures (Tables 1 and 2). The cold chain is the integrated linkage of refrigerator, freezer, and cold box storage at various sites, temperature-controlled transportation

between these sites, and management and personnel structures to maintain these temperatures. Cold chains include both the equipment and personnel to maintain the "cold" and also logistic, administrative, and supervisory skills required to ensure a continuous "chain" of refrigeration. This process must protect a vaccine from the moment it either enters a country or leaves a domestic factory to the time of patient vaccination. This chain may be broken and vaccine potency jeopardized by either brief severe temperature rises or successive moderate temperature elevations.

Temperature Stability of EPI Vaccines

Based on temperature stability, vaccines may be categorized into live and inactivated forms. Live viral vaccines have traditionally imposed more rigorous temperature requirements on the cold chain than killed forms. Measles and oral polio virus (OPV) are particularly heat and light sensitive, requiring freezing at -20°C for long-term storage. This requirement is in contrast to the majority of killed vaccines, which simply require refrigeration between 2°C and 8°C for long-term storage.

Among the killed vaccines, however, tetanus toxoid (TT) and combined diphtheria-tetanus-pertussis (DTP) vaccine may be inactivated by freezing. This fact imposes the additional requirement of maintaining these vaccines within the narrow temperature range of 2°C to 8°C. For short-term transport and storage, particularly at the more peripheral cold chain levels, all vaccines can be maintained at temperatures of 2°C to 8°C (Population Reports, 1986).

Description of a Representative Cold Chain

The organization of a country's cold chain depends on its transportation network, administrative structure, immunization strategy, and other factors. Generally, vaccines are distributed through several levels from a central store to health facilities and field sites. The actual organization depends on the vaccination schedule; infrastructure for health care, communication, and transportation; population densities; pattern of vaccine-preventable diseases; and other factors. Many countries still utilize "routine" immunization through fixed facilities combined with peripheral outreach programs. Other countries, such as Brazil and Ecuador, rely regularly on national campaigns. Described below is a representative cold chain for routine immunizations comprised of facilities at central, regional, district, health center, and outreach levels.

Cold chain requirements for temperature levels, staffing, equipment, and transportation differ significantly among these levels. At the central level, large quantities of vaccine are received and stored for up to 2 years. A critical, but often neglected, component at this level is the receipt and transfer or storage of vaccine from airport arrivals. Careful and timely transfers are crucial for the safe arrival of vaccines at the primary central facility. A cold room,

several freezers, and refrigerators are all part of the necessary equipment. Monitoring devices such as thermorecorders provide a continuous temperature record, while an alarm system signals sudden temperature rises. Electricity is the most common power supply at the central level and is more reliable than that available in the peripheral regions. Because the central store is the main store for the country and may contain vaccine worth up to $50,000, the presence of an automatically activated backup power source is vital.

Regional-level stores distribute the vaccine received from the central level to the district stores. Vaccine is generally shipped from the central facility either by refrigerated truck or cold box. Routine energy sources are less reliable at the regional level. Although a backup power source is recommended, it is frequently not available. Ideally, the monitoring equipment includes a temperature alarm system coupled with the use of vaccine indicator cards. Storage of vaccine is usually limited to a 3-month supply plus a 25 percent reserve stock.

District-level stores, situated in the larger towns, contain the vaccine supply for the immediate area and the surrounding health posts. Electricity, if available, is frequently unreliable as a power source at this level. This fact necessitates the use of electric ice-lined refrigerators and freezers or switching to alternative energy sources, such as gas or kerosene. Vaccine is generally stored at 4°C, limiting the maximum recommended storage time to 1 month. Cold boxes are the main form of transport both to and from this level. Each district store must have the capacity to freeze the ice packs required for cold box transport. If more than 20 ice packs per day are required, a special chest freezer or large ice-pack freezer would be advisable.

Health center stores hold a limited vaccine supply for local immunization and use in outreach programs. At this level certain facilities have only cold boxes, while others have a combined refrigerator/ice-pack freezer. Health centers that support outreach services have ice-pack freezing capacity for transport via vaccine carriers. With an adequate ice-pack lining, a single 0.6-liter vaccine carrier maintains suitable temperature for 2 days and contains approximately 300 doses of vaccine (Population Reports, 1986).

Maintaining the cold chain in a developing country often means importing equipment, spare parts, and sometimes fuel; training health and technical personnel in the use and maintenance of the equipment; and assuring unbroken function in regions with limited access. In view of these difficulties it is not surprising that reviews of the cold chain in a number of developing countries have identified several recurrent problems: electricity and other sources of energy for equipment are sometimes unavailable; dependable transportation among vaccination centers sometimes is lacking; and, most importantly, supervision and surveillance of the cold chain is sometimes inadequate (EPI, 1981, 1984).

To put the challenges of the cold chain in perspective, their costs and logistical constraints must be related to those of the EPI program

as a whole. Although a variety of studies have examined the costs of EPI programs, only a minority have separately identified those costs directly attributable to the cold chain. Still fewer studies have expressed these costs on an annualized basis for each level of cold chain organization. To both ascertain and provide a standardized framework for examining specific cold chain costs, we have developed a computer model in conjunction with WHO, Geneva, for an idealized cold chain and performed a limited analysis of several countries.

Alternative Approaches to the Cold Chain

Some countries organize their vaccination program using predominantly mobile teams or national immunization campaigns. Mobile teams typically serve rural areas from a vehicle using either cold boxes or a mobile cold chain (freezer and refrigerator in the vehicle). If cold boxes are used, the length of the mobile team tour depends primarily on the availability of ice. With average frequency of opening (a couple of times per day), a good-quality cold box can generally maintain vaccines at proper temperature for 5 days with one filling of ice. Mobile teams are best suited to vaccinate people living in remote areas with limited access to health facilities.

National vaccination campaigns require a communitywide mobilization effort, in which susceptible children are vaccinated in the course of a few days. In pursuit of this goal, health workers are joined by people in government agencies, teachers, members of the military, and volunteers. Because a large number of doses are consumed at many sites within a few days, cold boxes, rather than permanent refrigeration, can be used to keep vaccine at the vaccination points for the few days of the campaign.

A MODEL FOR ASSESSING COSTS OF THE COLD CHAIN

Purposes

The previous sections described the requirements of the cold chain for the many levels associated with a national-level vaccination program. Ideally, it would be possible to collect information about a variety of country programs to assist in determination of the costs of cold chain programs. However, most WHO-based reviews of the cold chain have relied on qualitative statements with little quantification of impacts.

In response to this need, WHO funded development of a model, used in this study, to assist in:

- anticipating yearly routine costs as well as assisting planning of any expansions or improvements;

- delineating the critical data that must be collected to complete a detailed country-specific analysis;

- determining the proportion of total vaccination costs necessary to allocate for the cold chain; and

- facilitating cost-effectiveness analysis of the impacts of vaccine thermostabilizations and alterations in delivery schedules.

The model is described below.

The Model

To examine the economic impacts of the cold chain, a cost model was developed using spreadsheet software. Through discussions with staff in the EPI Office, WHO, Geneva, it was decided to develop a model for a hypothetical population of 10 million in West Africa. West African countries have widely scattered rural populations, a hot, harsh climate, and varying levels of success in management of the cold chain. Ambient temperatures were assumed to average 43°C, and operating costs were calculated on this basis.

Appendix A shows the model. The cover page contains demographic, capital cost, and other financially related data used throughout the analysis. The model corresponds to an idealized cold chain based on fixed centers. It represents an optimal delivery system for maintaining the integrity of a cold chain and effectively delivering vaccinations on a routine basis. Such a system, combined with good management, would help to ensure that the vaccines were properly temperature controlled. Recognizing the reality that some countries might design their systems differently, the model was developed to facilitate examination of many types of routine delivery systems.

Levels

Five levels of management are shown in the model: central, regional, district, health center, and outreach. It should be noted that management of EPI and the cold chain can be done using different configurations of these levels. For example, a vaccination program might be more heavily dependent on an outreach program, with few health centers in a country. Mobile teams are not included in this model but could easily be added. The types of equipment that would be required for the model country are briefly discussed below (EPI, 1985).

Central

The central level, as the repository of large volumes of vaccines, needs a cold room for keeping vaccines at +4°C. The smallest room typically available is 30 cubic meters of space, so considerable vacant space is available for ice. In addition, other needed equipment includes a freezer (440 liters) that chills to -20°C, an ice-pack freezer (234 liters) to freeze packs used in cold boxes, thermorecorders to monitor temperatures, and an alarm system to warn if temperatures vary. In addition, a standby generator is needed as backup to existing electrical power supplies. To transport vaccines to the regional level, either cold boxes or refrigerated vehicles are required.

Regional

The regional level is the main distribution point from the central level. It is a major storage depot for other levels. Each store at this level contained ice-lined refrigerators (250 liters), ice-lined freezers (250 liters), contact thermometers, alarm systems, and ice-pack freezers (234 liters). In addition, to transport vaccine to other levels, cold boxes (22 liters), ice packs, and a vehicle are needed.

District

The district level can serve as both a vaccination center and a distribution point for the health center and outreach teams. To keep vaccines and ice packs at proper temperatures, each store requires a refrigerator/freezer (262 liters), thermometers, and ice packs. Transportation of vaccines to health centers and outreach teams also requires ice packs, cold boxes (22 liters), and a vaccine carrier (2.8 liters).

Health Centers

Stores at this level use a refrigerator/ice-pack freezer (262 liters), thermometers, a cold box (8.5 liters), and ice packs. Since they would not likely store vaccines for an extended time, they would not need vaccine or ice-pack freezers.

Outreach

Outreach teams are mobile groups that carry a 1-day supply of vaccine into the field. As such, they need carriers and ice packs to transport the vaccines. In addition, they would need a vehicle for transportation, such as a bicycle or motorcycle.

Cost Analysis

The calculation of capital and operating costs and sources of key assumptions and data are described below.

Capital Costs

Costs, quantity of equipment needed per store, number of spares, number of stores, and useful life were compiled from data supplied by EPI, WHO, Geneva (discussions, data sheets, and some costs), and UNICEF, Copenhagen, Denmark (1987 costs by item, identification of individual model types). Capital costs for all equipment except standby generators and vehicles were supplied by UNICEF in 1987 dollars. Generators and vehicle costs were supplied by the EPI Office in 1987 dollars.

The model is used in a cost analysis for each level of cold chain organization and summarizes costs for comparison across and among levels. Different pieces of capital equipment have different useful lives. Capital equipment is usually purchased periodically in accordance with its useful life, whereas operating costs are incurred annually. In order to combine all costs in a cost analysis into a standardized form, the model calculates total annual costs.

Each of the five levels in Appendix A is presented on a page with three main parts: capital, operating, and combined annual costs. The capital cost section identifies each piece of equipment needed, computes annualized costs, and multiplies the result times the total number of pieces needed for that level. Annualization or amortization of costs is an economic concept that can be viewed as mortgaging the capital investments. As in purchasing an automobile, an annual payment schedule is calculated using the number of years of the loan (useful life) and an interest rate. In this analysis a real interest rate of 10 percent was used; however, rates can vary depending on the terms of finance.*

Operating and Maintenance Costs

Operating and maintenance costs represent the ongoing costs of running a cold chain. The analysis is segmented into four subcategories: labor, energy, repairs, and spare parts and tools. As expected, the type of labor and the percentage of time devoted to cold chain activities vary by level of delivery. Best expert judgment was used to identify categories of labor, such as EPI manager and cold chain clerk, and percentage of labor dedicated to cold chain activities. Specific labor categories by level are:

- central--EPI manager, cold chain manager, cold chain clerk, and cold chain technician;
- regional--regional director, supervisor, and driver/technician;
- district--head nurse, assistant nurse, and technician;
- health center--nurses; and
- outreach--nurses.

The types of personnel who make the cold chain function are managers, physicians, nurses, clerks, and repair technicians. The labor rate per hour is a composite based on researchers' experiences (Shepard et al., 1986) and represents typical wage rates in 1987 dollars.

Energy costs were calculated for each piece of equipment (excluding generators and vehicles) based on specifications of energy usage from

*The annualized cost of a piece of equipment with a useful life of $\underline{n}$ years at an interest rate of 10 percent is its capital cost divided by $1-(1.10)^{-\underline{n}}/0.10$.

WHO. In general, refrigeration equipment uses one or more types of fuel: electric power, kerosene, or propane. It was assumed that equipment at the central, regional, and district levels would use electricity for power and that a continuous source of power would be available from either electric utilities or standby generators. Energy costs at the health center level were analyzed for propane as the most likely source of fuel.

Unit energy costs were supplied from the Energy Assessment Office of the World Bank, Washington, D.C., and represent current estimates of prices in West Africa. Conversion from CFA francs, a widely used West African currency, to U.S. dollars was done at an exchange rate of 305 CFA per dollar based on an average 1987 rate.

Repair costs were estimated as 10 percent of total annualized capital costs: region-specific data were unavailable. Repair costs would be expected to vary dramatically from country to country, depending on such factors as climate, infrastructure, access to parts, and the way the equipment is handled.

Costs for spare parts and tools for cold chain-specific equipment were supplied by the Procurement Office, UNICEF, Copenhagen, and represent estimates of 1987 costs. Costs for spare parts for backup generators and vehicles were estimated by the EPI Office and are presented in 1987 dollars.

Total Costs

Combined annual costs are listed at the bottom of each level analysis. They are calculated as the sum of annualized capital and total annual operating and maintenance costs (energy, labor, repairs, and parts). The combined annual cost figures are the key financial measures used to compare among and across levels within a country.

Cost Results

Appendix A summarizes costs for each level, by capital and operating cost contribution, and sums them across levels to get a total national annual cost of the cold chain. In addition, three other types of summary financial components are given. First, percent distributions are shown for costs within each level and cumulative costs nationwide. In this way it is possible to determine how costs are distributed within and among levels. For example, while labor may be a relatively small percentage of costs at the central level, it is the dominant cost at health centers.

Second, cold chain costs as a percentage of EPI costs are shown. If data on total costs of EPI programs are available, the model will calculate cold chain costs as a percentage of total annual EPI costs. Since this cold chain study is performed in a hypothetical West African country, the EPI program's estimate of the average EPI cost of $10 per child was used, based on a review of available country studies

(Henderson, 1984), including preliminary results from West Africa (Shepard et al., 1986). Information about cold chain costs as a percentage of EPI costs would be useful to policymakers in identifying country-specific cost breakdowns and for comparing the financial and managerial status of cold chain operations across countries.

Third, a limited table on funding sources is presented that indicates the donor contributions as a percentage of both EPI and cold chain costs. This will identify the cost of the cold chain that would likely be assumed by health ministries.

Analysis of Results from the Model

Tables 3 through 6 summarize the cost analysis of the cold chain for a hypothetical West African country. The overall cost for a fixed-center cold chain system serving a population of 10 million people is approximately $334,000. Table 3 shows dollar amounts, and Table 4 shows the percentage distribution with each level.

Table 5 presents the cumulative percent distribution of costs across levels. It shows that the health centers, the level of which accounts for the largest proportion of national cost, account for 35 percent of total national costs. This result reflects the fact that there are 150 facilities assumed at this level, whereas for the district, regional, and central levels there are 50, 10, and 1, respectively. The regional and district levels represent 25 and 29 percent of total national costs, respectively, while the one facility at the central level accounts for 11 percent of all costs. Outreach accounts for less than 1 percent of costs.

When cost elements are examined, the cumulative distribution shows that operating costs are almost three-quarters of the total: labor and energy together are 63 percent of all costs. Much of the energy costs occur at the regional and district levels, where a combination of a need for freezers and the presence of many facilities boosts energy costs to 28 percent of national costs.

Table 4 contains the percent distribution of costs for each of the five levels. This clearly shows how costs vary by level. For example, in the central level there is only one facility, but it contains a large cold room, freezers, a standby generator, and refrigerated vehicle, all of which contribute to the capital costs: 46 percent of all costs at this level. Conversely, at the regional level there are fewer pieces of equipment and no refrigerated vehicle; however, the 10 facilities have a large number of personnel, so capital costs are only 24 percent, but labor costs are almost 60 percent. These types of differences highlight the importance of examining where and how costs are incurred.

Table 6 shows cold chain costs compared with total annual EPI costs, putting the costs in perspective. For this analysis it was assumed that the crude birthrate was 44 per 1,000 per year; the average of all low-income countries, excluding China and India (World Bank,

1984). It was assumed that 75 percent of these children, 330,000, would be fully vaccinated. The average cost per fully immunized child (FIC) was assumed to be $10, based on a review of EPI costing studies (Henderson, 1984). For the hypothetical model, cold chain costs represent 10 percent of EPI costs. This is slightly lower than reported for the immunization programs discussed in the next section, which average between 13 and 20 percent. It should be noted, however, that care must be taken when making cross-country comparisons since different types of immunization programs (routine versus mass campaigns) and different delivery channels (fixed, mobile, and outreach) can dramatically affect both cold chain and EPI costs.

Table 7 presents an analysis of donor contributions for both the EPI program and cold chain component on a dollar per FIC basis. Overall, for the hypothetical model, donors account for over 50 percent of EPI costs and over 60 percent of the cold chain program. This highlights the fact that a significant portion of donor contributions to EPI can be consumed in the cold chain. The table specifies the assumptions about the percentage of each cost element attributed to donor funding, a distribution derived from expert judgment and field experience. Obviously, determination of donor contributions is very sensitive to the percentage distribution by cost element. Assumptions in the model may not reflect funding patterns in other countries.

COLD CHAIN COSTS: COUNTRY EXAMPLES

In this section cold chain costs are presented for two African countries for which requisite data were available. Ambient temperatures in both countries are similar to the 43°C (109°F) assumed in the costing model. The cold chain costs as a percentage of total EPI costs are then compared with results from the hypothetical model. Several other countries are examined more briefly. In each country, the costs of the EPI program as a whole and the proportion attributed to the cold chain are computed.

Mauritania

Mauritania is a sparsely populated Sahelian country with about 2 million people for which a cost-effectiveness analysis has been conducted (Brenzel, 1986). To ascertain the financial requirements of the EPI program, the costs of vaccinations administered through fixed centers and mobile teams were combined. All monetary amounts were expressed in 1985 U.S. dollars. Capital costs were amortized over the useful life of an asset--25 years for buildings and 5 years for vehicles and equipment. The number of fully vaccinated children in the country was obtained through coverage surveys. This number was used to determine cost per completely vaccinated child.

Each cold chain-related expenditure was derived from EPI costs in Brenzel (1986) (see Table 7). These comprise the annualized cost of all cold chain equipment, fuel (butane gas), repairs for refrigerators, and personnel.

Personnel is typically the most complex resource to allocate. Three persons (two technicians and one inventory controller) were concerned exclusively with the repair and maintenance of cold chain equipment. Their salaries were attributed entirely to the cold chain and represented 1.9 percent of all EPI salaries. Other personnel involved in EPI have a number of duties related to the cold chain. These include checking and charting temperatures of refrigerators; performing minor maintenance and adjustments (e.g., changing wicks of kerosene refrigerators, defrosting, etc.); freezing ice packs and putting them inside cold boxes; arranging the pickup and delivery of vaccines within the time allowed by cold boxes; and scheduling outreach services. It was estimated that 10 percent of all EPI personnel time was related to these cold chain activities.

In total, 13 percent of immunization costs in the country (i.e., $1.69 out of $13.43 per fully vaccinated child) was due to the cold chain. This is in line with the 10 percent result estimated for the hypothetical model.

Large Sahelian Country

The second example is the projected cost of a planned EPI program in a large Sahelian country, which will rely heavily on mobile teams. Costs were expressed in 1985 prices. This country presently has a limited vaccination program with coverage surveys indicating that only about 15 percent of the children (primarily in urban areas) have been vaccinated. Its government has engaged in a detailed planning process with several international donors to initiate a strong EPI program through 111 health facilities. The program will be funded by international grants and loans and the government itself. The assessment of costs in the following analysis uses reports and insights from this planning process and is supplemented by data from EPI programs in other Sahelian countries.

Table 8 shows the projected annual costs of the anticipated EPI program. All capital items except buildings were assumed to have a 5-year useful life (the same life span as the donor-supported project). Buildings, whether new or existing, were amortized over 25 years. Local currencies were converted to U.S. dollars at the 1985 exchange rate based on World Bank figures.

The current EPI project design incorporates use of a recently developed inactivated polio virus (IPV) vaccine, rather than the oral polio virus (OPV). The IPV is less temperature sensitive and is thought to be able to confer adequate polio immunity with two doses plus a booster, rather than the three doses plus a booster required with OPV. Presently, however, IPV is 25 times more expensive than OPV.

To compute the cost per FIC, consultants estimated that the program would achieve a 65 percent coverage rate by the end of the proposed 5-year project. Altogether, the projected cost of the EPI program was estimated at $10.83 per FIC. Of this amount, $2.13, or 20 percent, was due to costs of the cold chain. The predominant parts of the cold

chain costs were the annualized costs of purchasing and operating cold chain equipment and specialized vehicles.

To equip about 100 health facilities, the total capital cost of cold rooms, freezers, and refrigerators was $759,000. Refrigerated and specially equipped vehicles were estimated to cost an additional $117,000. This investment is intended to establish routine immunizations at health facilities, thereby reducing the country's reliance on mobile teams. This strategy will require installing cold chain equipment and training personnel in most of these facilities.

As Tables 7 and 8 show, these country studies are consistent with previous estimates of cost per FIC ranging from $5 to $15; actual cold chain costs range from 13 to 20 percent of this amount.

VACCINE STABILIZATION AND OTHER APPROACHES TO IMPROVING VACCINATION COVERAGE

As discussed previously, cold chains impose financial and operational constraints that may limit immunization coverage. This section examines whether temperature stabilization of vaccines will improve vaccination coverage and compares the results with another approach--simplified vaccination scheduling. Both partial and complete vaccine thermostabilization will be considered.

Vaccine Thermostabilization

Current temperature sensitivity of vaccines could potentially limit vaccination coverage in two ways. First, it requires that resources be used to operate a cold chain, which increases the cost of vaccinations. A given amount of resources, therefore, would vaccinate fewer children than would otherwise occur. Second, the cold chain may impose resource constraints that the present vaccination programs simply cannot overcome. Some parts of the country may be reached infrequently or may be completely excluded from the vaccination program or children may be vaccinated with vaccines that are not fully potent due to cold chain problems.

The cold chain model was used to estimate the anticipated savings in cost from potential improvements in thermostabilization. Currently, measles and polio vaccines are kept frozen in the central store and during transport to the regional store to slow their deterioration (Table 2). Polio is the more heat sensitive of these vaccines. Indeed, a more stable polio vaccine (IPV), under the trademark Immovax, is being used in some developing countries. As discussed below, its high cost and other considerations offset some of its value.

As a first scenario, suppose that measles and a polio vaccine (IPV or OPV) would be partially temperature stabilized so as to remove the necessity for freezing. Then the -20°C freezers and ice-lined freezers could be eliminated from the cold chain at both the central and regional levels. To determine the effect on cold chain costs, we

eliminated the costs of capital, energy, and spare parts used by these freezers. We assumed in the most optimistic case that the refrigerators in the model cold chain had adequate space for these previously frozen vaccines. We assumed no change in labor since personnel would still be needed to maintain the remaining cold chain. The resulting cost savings were 2.5 percent of the cold chain costs, a modest savings. If refrigerator capacity would have had to be increased, the savings would have been even smaller. As the major impact of this scenario is on storage of vaccines at the central and regional levels, we would not anticipate a significant increase in vaccination coverage.

As a further improvement in thermostabilization, suppose that all EPI vaccines were thermostabilized to withstand temperatures of 45°C for 6 to 8 weeks. In that case it might be possible to eliminate the cold chain at the most peripheral levels in the vaccine delivery system (health centers and outreach). Using the results of the cost analysis of the model cold chain (Tables 3 and 5), this change would save 36 percent of the cold chain costs ($120,043 out of $333,727), a much more noticeable improvement.

In contrast to partial stabilization, if vaccines could be rendered completely cold chain independent, more resources could be saved. If the cost of vaccinations were a limiting constraint to coverage, we could indicate the magnitude of this limitation from the results of the previous examples. Mathematically, if the proportion of the cost of fully vaccinating a child due to the cold chain is f, then the proportion of additional children who could be vaccinated if those requirements were completely removed is $f/(1-f)$.* In the examples above the cold chain share f ranged from 10 percent for the model populations to 20 percent for the projection for the large Sahelian country. Entering this range into our formula, the number of children who could be vaccinated if money were the limiting constraint would increase from 11 to 25 percent. As Table 9 indicates, these are rather modest increases.

Money is probably not the limiting constraint for increasing coverage at present. Childhood vaccinations have been recognized by public health experts and donor organizations as enormously powerful and popular interventions for improving the health of children. As a result, donors have accorded a very high priority to funding this activity. As long as immunization programs remain a priority for donors, financing is probably not the binding constraint. If cold chain requirements were to be removed, it is likely that the donor funds saved would likely be reallocated to a lower-priority activity.

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*This result can be shown by supposing that the cost of vaccinating each child is X, and comparing the number of children who could be vaccinated for $1 million with $(1/X)$ and without $(1/[X(1-f)])$ the cold chain requirement. The difference in relation to $1/X$ is $f/(1-f)$.

In the longer run, however, donor funds will be less available, so that cold chain costs may become important. For example, over 12 African countries are receiving support for EPI from the U.S. Agency for International Development (AID) through the project for Combatting Childhood Communicable Diseases (CCCD). AID's agreement with these countries calls for their sustaining the operating costs of CCCD activities from resources within the country by the end of the project (initially set at 5 years).

While the total costs of the cold chain amount to only 10 percent of total EPI costs in the model country, their significance is somewhat higher than this percentage indicates. When costs are categorized into those usually paid by international donors versus national governments (Table 6), 62 percent of cold chain costs are typically donor supported, compared to 53 percent of all EPI costs. Thus, the financial burden of sustaining the cold chain is relatively greater than that for the EPI program as a whole. The distinction between donor and country financing also serves as a proxy for two other classifications: donor financing approximates (1) incremental cost (as contrasted with full costs) and (2) foreign exchange costs versus total costs (which include national currency costs). Donors typically pay the incremental costs of a vaccination program during its initial stages and typically meet foreign exchange costs. Using this approximation, the results in Table 6 show that incremental cold chain costs represent about 12 percent ($0.62 out of $5.27) of all incremental costs. A country that has trouble financing the incremental costs of the cold chain is likely to have trouble financing other incremental costs of its EPI program as well.

Logistic Constraints

The logistic constraints imposed by the cold chain vary by country and type of delivery system. In the countries examined as case studies, it appears that less rigorous cold chain requirements alone would have improved coverage very little. In general, most constraints in coverage are not related to the cold chain. These relate especially to management of the vaccination program and the motivation of mothers to bring their children for a complete set of vaccinations (Cameron, 1987).

To put in perspective the possible benefit of improving the heat stability of vaccines, it is instructive to compare coverage from vaccinations with that of oral rehydration therapy (ORT), which has no cold chain requirements. EPI estimated the worldwide coverage of children under 1 year of age in developing countries of the third dose of DTP to be about 43 percent as of early 1986 (EPI, 1986). For ORT, combining country-specific coverage data reported by the Programme for Control of Diarrheal Disease of WHO, it was estimated that 26 percent of episodes of diarrhea received ORT in 1985 (Shepard and Carrin, 1986). As both rates have been rising, more recent data can be expected to show higher rates. While the factors affecting the acceptance of both ORT and vaccinations are many, the comparison points out that even without cold chain requirements there are many other constraints to coverage.

A Simplification of Vaccination Scheduling

Immunization scheduling is a calculated compromise between waiting for the emergence of infant immunocompetence to vaccine antigens and achieving protection before the disease strikes. Recently, consideration of financial constraints, adverse seasonal climatic conditions, and patient compliance have assumed a more influential role in the design of immunization programs. Simplification of vaccination scheduling by reduction of the number of contacts may prove particularly useful in countries with the most limited health resources and dispersed populations. The recommended vaccination schedule in many countries requires five contacts, excluding boosters. This series begins with BCG at birth and is completed with measles vaccination at 9 to 12 months of age.

EPI costing studies have found that vaccines and vaccine supplies constitute only 22 percent of the cost of vaccinating a child. The personnel, training, equipment, fuel, maintenance, etc., required to operate a vaccination program constitute the remaining 78 percent (Henderson, 1984). If the number of vaccination contacts could be reduced, a substantial savings in these program costs could be expected. One simplified vaccination schedule has been used in Kolda, Kaya, and Kolokani areas of Senegal. Using this schedule, a child can be vaccinated with just two contacts (at about 3 and 9 months), rather than the traditional five contacts (Stoeckel, 1985). This two-contact schedule used inactivated polio vaccine (such as 40-8-32-D antigen unit), rather than OPV and incorporated four antigens (DTP and polio) into a single syringe.

Because there is typically a dropout rate of 20 to 30 percentage points between successive doses of a vaccine, fewer contacts should result in more fully immunized children. The two-contact schedule in Senegal is surrounded by considerable controversy. First, the actual coverage achieved in these areas of Senegal was only 49 percent of children having received the second dose--a rate comparable to that achieved with the traditional schedule (International Conference on IPV, Niamey, Niger, January 1987). The field efficacy of the four-antigen vaccine against polio also proved to be almost the same as that for OPV, based on preliminary analyses of outbreaks in The Gambia and Senegal (Jaiteh et al., 1987), despite high rates of seroconversion in controlled trials. Presently, there are other questions about the efficacy of the two-contact schedule. The pertussis vaccine is not yet fully protective with just two doses. Protection against DTP and polio is not completed as young as with the traditional five-contact plan; a polio booster may be essential, and antibody titers might decline more quickly after only two doses. Finally, as mentioned, the four-antigen vaccine costs about $2 per dose because the IPV complement costs about 25 times as much as OPV. While the ability to use only two doses is an advantage, the 6-month hiatus between doses may have caused families to lose interest in vaccinations.

Apart from the strengths and limitations of this particular vaccine, the concept of a simplified schedule is important. Suppose that an ideal two-dose vaccine could be developed with the same cost

per dose as existing vaccines and the same timing as the first two doses of DTP. This hypothetical vaccine would be expected to save about 60 percent of labor and vaccine costs (assuming that these are proportional to the number of contacts) and raises coverage. Based on West African and Sahelian coverage data, this schedule should achieve 73 percent coverage in rural areas--the coverage achieved by the second dose of DTP. The traditional schedule, which required a third dose of DTP, achieves about 50 percent coverage. Altogether, the modified plan would halve the cost per FIC. If vaccines could be developed to make a simplified plan as effective as the traditional schedule, the cost savings and potential increase in coverage could be substantial.

Other important research efforts include use of social marketing to increase the demand for vaccinations. This strategy has proved effective for stimulating demand for another primary health care intervention, ORT. In The Gambia, for example, it raised the use of ORT to 50 percent over a 2-year campaign (Shepard et al., 1985).

Mass media has been used as part of national vaccination campaigns in several countries and shows great promise (Cameron, 1986). For example, in Burkina Faso, Ecuador, and Brazil, mass communication was used to motivate people to receive immunizations. As mass media is used in other countries it is likely that EPI coverage will expand..

CONCLUSIONS

Costs of the Cold Chain

The total annualized cost of the cold chain for a hypothetical West African country serving 10 million people is estimated to be $334,000, which accounts for 10 percent of total EPI costs. That is, the total EPI program costs $10 per FIC, and the cold chain costs $1 per FIC. Country case studies showed cold chain costs as a percentage of EPI costs ranging from 13 to 20 percent.

Donors are estimated to contribute approximately 62 percent of model cold chain costs, the vast majority being amortization of capital expenditures. More than a third of annualized costs for the cold chain is associated with vaccine storage and delivery at the health center level, including propane gas to power refrigeration equipment.

There is a dearth of systematically collected and organized country-specific data on the costs of establishing and maintaining a cold chain. The model shows the types of data needed and anticipated magnitudes.

The hypothetical model is meant to approximate West African countries. It indicates the types of data required to apply the model to a specific country in any region of the world.

- Levels (central, regional, etc.) of vaccination delivery may vary by country.

- Vaccination strategies are not consistent across countries: fixed versus mobile teams, routine versus mass campaigns.

- Labor, unit energy, and repair costs used in the model represent a combination of best judgment and field experience. Future analyses should focus on refining these estimates.

- Number of stores by level were derived based on a compilation of West African models. Variation in this distribution could dramatically alter financial impacts.

- The real interest rate used in the model, 10 percent, might not reflect other countries' experience.

Effect on Coverage and Health

In general, the cold chain does not appear to be the most important constraint to increasing coverage of the EPI. If the cold chain could be eliminated altogether, we estimated costs would be reduced by 10 to 20 percent. If money were the binding limitation, coverage increased by modest amounts, ranging from 11 to 25 percent. Furthermore, analysis of proposed and operating EPI programs in the Sahelian country, Mauritania, Ecuador, and Swaziland suggests that in none of these countries is the cold chain a sole limiting factor to coverage. In all these countries strategies have been adapted and resources invested to provide coverage of at least 65 percent of children. Further increases in coverage are thought to depend more on program management and the willingness of mothers to bring children for vaccinations than on the cold chain. In the long run, when all children in accessible areas are being vaccinated, greater thermal stability will allow vaccines to reach the most remote areas where even a temporary cold chain is infeasible. Other factors may be important when national coverage is below 80 percent. The cold chain probably becomes important in the ability to increase coverage beyond 80 percent in a sparsely populated country.

Even as existing vaccines in the EPI program are made more temperature stable, new vaccines under development, such as malaria or rotavirus, will likely be included if and when they become effective and available. Furthermore, the extremes of heat in some developing countries mean that even a reasonably stable, freeze-dried vaccine requires a cold chain.

Another benefit of improved thermostabilization may be a more effective vaccine in the field, rather than better coverage. Field studies of vaccine effectiveness often show rates that are lower than rates of seroconversion. Many reasons are suspected to contribute--wrong dose, wrong age, contraindications for vaccination, as well as lapses in the cold chain. While it is difficult to assess the proportion of problems due to the cold chain alone, undoubtedly there will be some value. The range between field effectiveness rates and seroconversion rates indicates the potential magnitude for improvement.

The director of the WHO EPI wrote in 1984 that the two aspects of EPI that could contribute most to expanded coverage were the political will to make the necessary funds available for EPI and improved program

management (Henderson, 1984). Generous enthusiasm by many donors has eased financial constraints in many countries for the next few years. In the longer run, donor priorities might shift away from immunizations and a 10 to 20 percent savings in cost would become more important than it is now.

Social marketing (health communications) shows promise in increasing demand for vaccinations through mass media and other channels. Broad strengthening in program management and supervision has been repeatedly advocated, and development of vaccines that require fewer contacts would be quite valuable. Improved thermal stability of vaccines can contribute to a multifaceted strategy of strengthening vaccination programs. Millions of children's lives each year may be saved as a result.

In summary, this analysis shows the cold chain to be responsible for only a small part of the financial and logistical constraints for vaccination coverage. Efforts to improve vaccine thermostability will have the greatest payoff if they are addressed simultaneously with other improvements.

ACKNOWLEDGMENTS

This study of the financial and logistical constraints of temperature-sensitive vaccines was requested by the Institute of Medicine, National Academy of Sciences.

We would like to thank Dr. Pierre Claquin and Cynthia Dunn of the Resources for Child Health Project for their valuable comments and assistance in gathering data and reviewing material and Logan Brenzel for cost data on Mauritania. We would also like to thank Mr. Evans and Mrs. Anne Larsen, UNICEF, Copenhagen, for their assistance in determining capital and operating costs of the hypothetical cold chain used in the analysis. We are also indebted to Drs. Ciro de Quadros, Jean Marc Olive, Peter Carrasco, and others at the Pan American Health Organization for their assistance in providing materials and analyses of both the cold chain and EPI in Central and South American countries. Julia Walsh, M.D., of the Harvard School of Public Health, helped us with background on vaccinations, and Mr. Vanderplus of the World Bank estimated energy costs for the Sahelian countries.

We especially thank Mr. James Cheyne and Mr. John Lloyd of the Expanded Programme on Immunization, World Health Organization, Geneva, for their support in supplying data and expert information about the cold chain and its operation. The computer model referenced in this analysis was separately funded by EPI, which retains rights to its use and distribution.

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TABLE 1 Temperature Stability of EPI-Related Vaccines

Vaccine	Duration of Stability at 37°C (99°F)
Oral polio virus	1 day
Measles	1 week
BCG (freeze dried)	2 weeks
Inactivated polio virus[a]	4 weeks
Diphtheria, tetanus, pertussis	4-8 weeks
Tetanus toxid	8 weeks

[a]Though this form of polio vaccine is not universally approved by the WHO EPI Program, it is sometimes used in place of OPV.

Source: Adapted from WHO (1985).

TABLE 2 Current EPI Requirements for Vaccine Temperatures and Maximum Lengths of Storage

Location (Maximum Length of Storage)	Measles (°C)	OPV (°C)	BCG (°C)	DTP (°C)	Tetanus (°C)
Transport from manufacturer to central store (48 hours)	≤8	≤8	≤30	≤30	None
Storage in central store[a] (2 years)	-20	-20	0-8	0-8	0-8
Transport to region	-20 to 8	-20 to 8	0-8	0-8	0-8
Storage in regional store (3 months)	-20	-20	0-8	0-8	0-8
Transport to health center	-20 to 8	-20 to 8	0-8	0-8	0-8
Storage in health center (1 month)	-20 to 8	-20 to 8	0-8	0-8	0-8
Transport to vaccination location (1 week)	0-8	0-8	0-8	0-8	0-8

[a]Except maximum length is 8 months for BCG and 1.5 years for DTP and tetanus vaccines.

Source: Population Reports (1986).

TABLE 3 Cost of Model Cold Chain by Level (1987 U.S. Dollars)

Cost Elements	Central	Regional	District	Health Center	Outreach	Total
Annualized capital	16,729	19,442	20,890	31,133	1,389	89,583
Labor	8,162	47,740	46,606	14,157	0	116,665
Energy	8,721	7,848	21,900	58,145	0	96,613
Repairs	1,438	1,944	1,687	2,931	139	8,139
Spare parts	1,895	4,633	4,050	12,150	0	22,728
Totals	36,945	81,606	95,133	118,515	1,528	333,727

TABLE 4 Percentage Distribution of Costs of Model Cold Chain Within Each Level

Cost Elements	Central	Regional	District	Health Center	Outreach	Combined Levels
Annualized capital	46	24	22	27	91	27
Labor	22	59	49	12	0	35
Energy	23	9	22	48	0	28
Repairs	4	2	2	3	9	2
Spare parts	5	6	4	10	0	7
Total	100	100	100	100	100	100

TABLE 5 Cumulative Percentage Distribution of Costs of Model Cold Chain

Cost Elements	Central	Regional	District	Health Center	Outreach	Cumulative Percent
Annualized capital	5	6	6	9	0[a]	27
Labor	2	14	14	4	0	35
Energy	3	2	6	17	0	28
Repairs	0	1	1	1	0[a]	2
Spare parts	1	1	1	4	0	7
Total	11	25	29	35	0[a]	100

[a]Amounts are less than 0.5 percent, though not truly zero.

TABLE 6 Cold Chain Costs per Fully Immunized Child Compared with Total EPI Costs in a Hypothetical West African Country

Program	Annualized Cost (U.S. Dollars)	Percent of Cold Chain	Percent of EPI
Cold chain	1.01		10
Donor contribution	0.63	62	
National government	0.38	38	
EPI program	10.00		100
Donor contribution	5.27		
National government	4.73		

Source: Cold chain cost model (Appendix A).

TABLE 7 Costs of the Cold Chain in Mauritania in Relation to Costs of Routine Vaccinations

Category	EPI[a] (U.S. $)	Cold Chain (U.S. $)	Percent for Cold Chain
Salaries	190,050	22,245[b]	12
Vaccines	25,815	0	0
Transport	62,490	0	0
Training, management, supervision[c]	7,920	0	0
General	13,880	0	0
Supplies, energy	61,650	16,985[d]	28
Buildings[e]	18,420	0	0
Vehicles [e]	38,315	0	0
Equipment and spare parts	23,310	16,225	70
TOTAL	441,850	55,455	13
Per completely vaccinated child[a]	13.43	1.69	13

[a]Based on 32,901 children completely vaccinated.
[b]Based on $4,000 cost for 3 people involved full time in cold chain and 10 percent time for all other EPI personnel.
[c]Represents primarily supervision costs. Training could not be separated from management and supervision and did not relate specifically to the cold chain.
[d]Bottled propane.
[e]Annualized capital costs based on 25 years for buildings, 5 years for vehicles, and 5 years for equipment.

Source: Brenzel (1986).

TABLE 8 Annual Costs of the Cold Chain for a Planned EPI Program in a Larger Sahelian Country (in 1985 dollars)

Category	EPI Costs	Percent for Cold Chain	Cold Chain Costs
Recurrent Costs			
Salaries:			
Full-time cold chain	11,000	100	11,000
Full-time EPI	319,000	10	32,000
Health center staff (30% for EPI)	239,000	10	24,000
Salary supplements	3,000	10	0
Vaccines	1,135,000	0	0
Material	134,000	0	0
Vehicle operation	144,000	17	24,000
Travel (per diem)	143,000	10	14,000
Other operating	172,000	95	163,000
SUBTOTAL	2,299,000	11.7	269,000
Annualized Capital Costs			
Technical and cold chain equipment	200,000	100	200,000
Vehicles	181,000	17	31,000
Training	133,000	50	66,000
Technical assistance	80,000	50	40,000
Studies	40,000	25	10,000
Social mobilization	91,000	0	0
Administrative cost	13,000	0	0
Buildings	98,000	0	0
SUBTOTAL	836,000	41.6	347,000
TOTAL	3,134,000	19.7	616,000
COST PER CHILD	10.83	19.7	2.13

Source: Unpublished donor documents.

TABLE 9 Increased Coverage if the Cold Chain Were a Binding Constraint

Scenario	Percent of EPI Costs Due to Cold Chain	Percent Increase in Coverage Possible if Costs Were Constraint
Costing model	10	11
Small Sahelian country	13	15
Large Sahelian country	20	25
Summary estimate	15	18

APPENDIX A

COLD CHAIN MODEL--COST ANALYSIS

Expanded Programme on Immunization, World Health Organization

(Developed by Charles Cameron, Cambridge, Mass., February 1987)

Demographic, Coverage, and Cost Inputs

Country:	Hypothetical
Date:	February 28, 1987
Year of prices:	1987
Population (year):	10 million
Coverage (percent):	75
Fully immunized children:	330,000
Birthrate per 1,000:	44
EPI annualized costs:	$3,300,000

Item	Unit Cost (U.S. $)	Quantity Each Store	Spares per Unit/Year	Number of Stores	Number Years Life
Central-level vaccine storage					
+4°C cold room, 30 m^3	12,000.00	1	250.00	1	10.0
-20°C freezers, 8/500 liters	279.00	8	35.00	1	5.0
Ice-pack freezers	1,075.00	4	13.25	1	5.0
Thermorecorders	762.00	2	56.00	1	5.0
Alarm systems	0.00	0	0.00	1	5.0
Standby generator, 1/7 kVA	4,800.00	1	1,200.00	1	5.0
Central vaccine distribution to region					
Refrigerated vehicle	18,044.00	2	1,064.40	1	4.0
Regional vaccine storage					
Ice-lined refrigerators	1,197.00	2	150.00	10	5.0
Ice-lined freezers	1,197.00	1	150.00	10	5.0
Contact thermometers	98.00	3	0.00	10	5.0
Alarm system	145.00	1	0.00	10	5.0
Ice-pack freezers	1,075.00	1	13.25	10	5.0
Regional vaccine distribution to districts					
Cold boxes, 25 liter	360.00	6	0.00	10	5.0
Ice packs, 0.6 liter	0.35	300	0.00	10	5.0
District vaccine storage					
Refrigerator/freezer	649.00	1	81.00	50	5.0
Thermometer	98.00	1	0.00	50	5.0
Cold box, 25 liter	360.00	2	0.00	50	5.0
Ice packs, 0.6 liter	0.35	100	0.00	50	5.0
Vaccine carriers	19.00	4	0.00	50	5.0
Ice packs, 0.3 liter	0.29	20	0.00	50	5.0

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Item	Unit Cost (U.S. $)	Quantity Each Store	Spares per Unit/Year	Number of Stores	Number Years Life
Health center vaccine storage					
Refrigerator/ice-pack freezer	649.00	1	81.00	150	5.0
Thermometer	1.29	1	0.00	150	5.0
Cold box, 10 liter	120.00	1	0.00	150	5.0
Ice packs, 0.3 liter	0.33	50	0.00	150	5.0
Outreach immunization and vaccine collection					
Vaccine carrier	12.15	2	0.00	150	5.0
Ice packs, 0.3 liter	0.54	20	0.00	150	5.0

CENTRAL-LEVEL COST ANALYSIS

Capital Costs

Equipment	Useful Life (years)	Quantity Each Store	Annualization Factor	Annual Cost One Store	Annual Cost All Stores
Central-level vaccine storage					
+4°C cold room, 30 m^3	10.0	1	6.14	$1,953	$ 1,953
-20°C freezers, 8/500 liters	5.0	8	3.79	74	589
Ice-pack freezers	5.0	4	3.79	284	1,134
Thermorecorders	5.0	2	3.79	201	402
Alarm systems	5.0	0	3.79	0	0
Standby generator, 1/7 kVA	5.0	1	3.79	1,266	1,266
Distribution to the regions					
Refrigerated vehicle	4.0	2	3.17	5,692	11,385
TOTAL ANNUALIZED CAPITAL COSTS					16,729
Interest rate: 0.100					

Operating Costs

Labor Costs

	Fraction of Annual Labor	Labor Rate per Hour	Number of Hours/Year	Labor Cost per Store	Total Cost All Stores
EPI manager[a]	0.05	$9.00	88	$ 792	$ 792
Cold chain manager[a]	0.75	4.00	1,320	5,280	5,280
Cold chain clerk	0.25	0.75	440	330	330
Cold chain technician	1.00	1.00	1,760	1,760	1,760
TOTAL LABOR COST					$8,162

[a]Wages for EPI manager and cold chain manager are assumed to include base wage, benefits, and per diem.

Energy Costs

	Electricity ($0.25/kwh)	Kerosene ($0.57/L)	Gas ($1.77/kg)	Cost for Each Store
+4°C cold room, 30 m^3	1,277.50 kwh	0.00 L	0.00 kg	$ 319.38
-20°C freezers, 8/500 liters	1,277.50	0.00	0.00	2,555.00
Ice-pack freezers	1,058.50	0.00	0.00	1,058.50
Thermorecorders	0.00	0.00	0.00	0.00
Alarm systems	0.00	0.00	0.00	0.00
Standby generator, 1/7 kVA	0.00	2,400.00	0.00	1,368.00
Refrigerated vehicles	0.00	6,000.00	0.00	3,420.00
TOTAL ENERGY COSTS				$8,720.88

Spare Parts and Tools

	Cost per Unit	Quantity Each Store	Number of Stores	Annual Cost All Stores
+4°C cold room, 30 m^3	$ 250.00	1	1	$ 250
-20°C freezers, 8/500 liters	35.00	8	1	280
Ice-pack freezers	13.25	4	1	53
Thermorecorders	56.00	2	1	112
Alarm systems	0.00	0	1	0
Standby generator, 1/7 kVA	1,200.00	1	1	1,200
TOTAL COST OF SPARE PARTS				$1,895

OPERATING AND MAINTENANCE COSTS

Labor Costs	$ 8,162.00
Energy Costs	8,720.88
Repairs	1,438.00
Spare Parts	1,895.00
TOTAL	$20,216.00
Plus Capital Costs	16,729.00
COMBINED ANNUAL COSTS	$36,945.88

DISTRICT-LEVEL COST ANALYSIS

Capital Costs

Equipment	Useful Life (years)	Quantity Each Store	Annualization Factor	Annual Cost One Store	Annual Cost All Stores
Refrigerator/freezer	5.0	1	3.79	$ 171	$ 8,560
Thermometer	5.0	1	3.79	26	1,293
Cold box, 25 liter	5.0	2	3.79	190	9,497
Ice packs, 0.6 liter	5.0	100	3.79	9	462
Vaccine carriers	5.0	4	3.79	20	1,002
Ice packs, 0.3 liter	5.0	20	3.79	2	77
TOTAL ANNUALIZED CAPITAL COSTS Interest rate: 0.10					$20,890

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Operating Costs

Labor Costs

	Fraction of Annual Labor	Labor Rate per Hour	Number of Hours/Year	Labor Cost per Store	Total Cost All Stores
Nurse	0.06	$0.66	112	$ 74	$ 3,706
Technician/driver[a]	0.25	1.95	440	858	42,900
TOTAL LABOR COST					$46,606

[a]Drivers' labor rates reflect base wages, benefits, and a per diem.

Energy Costs

	Electricity ($0.25/kwh)	Kerosene ($0.54/L)	Gas ($1.77/kg)	Cost for Each Store
Refrigerator/freezer	1,752.00	0.00	0.00	$ 438
TOTAL ENERGY COSTS				$21,900

Spare Parts and Tools

	Cost per Unit	Quantity Each Store	Number of Stores	Annual Cost All Stores
Refrigerator/freezer	$81	1	50	$4,050
Thermometer	0	1	50	0
Cold box, 25 liter	0	2	50	0
Ice packs, 0.6 liter	0	100	50	0
Vaccine carriers	0	4	50	0
Ice packs, 0.3 liter	0	20	50	0
TOTAL COST OF SPARE PARTS				$4,050

OPERATING AND MAINTENANCE COSTS

Labor Costs	$46,606.00
Energy Costs	21,900.00
Repairs	1,687.00
Spare Parts	4,050.00
TOTAL	$74,243.00
Plus Capital Costs	20,890.00
COMBINED ANNUAL COSTS	$95,133.00

REGIONAL-LEVEL COST ANALYSIS

Capital Costs

Equipment	Useful Life (years)	Quantity Each Store	Annualization Factor	Annual Cost One Store	Annual Cost All Stores
Regional-level vaccine storage					
Ice-lined refrigerators	5.0	2	3.79	$632	$ 6,315
Ice-lined freezers	5.0	1	3.79	316	3,158
Contact thermometers	5.0	3	3.79	78	776
Alarm system	5.0	1	3.79	38	383
Ice-pack freezers	5.0	1	3.79	284	2,836
Regional distribution to districts					
Cold boxes, 25 liter	5.0	6	3.79	570	5,698
Ice packs, 0.6 liter	5.0	300	3.79	28	277
TOTAL ANNUALIZED CAPITAL COSTS					$19,442
Interest rate: 0.10					

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Operating Costs

Labor Costs[a]

	Fraction of Annual Labor	Labor Rate per Hour	Number of Hours/Year	Labor Cost per Store	Total Cost All Stores
Regional manager	0.20	$4.00	352	$1,408	$14,080
Supervisor	0.15	3.00	264	792	7,920
Driver/technician	0.75	1.95	1320	2,574	25,740
TOTAL LABOR COST					$47,740

[a]Labor categories are assumed to contain base wages, benefits, and per diem amounts.

Energy Costs

	Electricity ($0.25/kwh)	Kerosene ($0.57/L)	Gas ($1.77/kg)	Cost for Each Store
Ice-lined refrigerators	693.50	0.00	0.00	$ 346.75
Ice-lined freezers	693.50	0.00	0.00	173.38
Contact thermometers	0.00	0.00	0.00	0.00
Alarm system	0.00	0.00	0.00	0.00
Ice-pack freezers	1,058.50	0.00	0.00	264.63
TOTAL ENERGY COSTS				$7,848

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Spare Parts and Tools

	Cost per Unit	Quantity Each Store	Number of Stores	Annual Cost All Stores
Regional-level vaccine storage				
Ice-lined refrigerators	$150.00	2	10	$3,000
Ice-lined freezers	150.00	1	10	1,500
Contact thermometers	0.00	3	10	0
Alarm system	0.00	1	10	0
Ice-pack freezers	13.25	1	10	133
Regional distribution to districts				
Cold boxes, 25 liter	0.00	6	10	0
Ice packs, 0.6 liter	0.00	300	10	0
TOTAL COST OF SPARE PARTS				$4,633

OPERATING AND MAINTENANCE COSTS

Labor Costs	$47,740.00
Energy Costs	7,848.00
Repairs	1,944.00
Spare Parts	4,633.00
TOTAL	$62,164.00
Plus Capital Costs	19,442.00
COMBINED ANNUAL COSTS	$81,606.00

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HEALTH CENTER COST ANALYSIS

Capital Costs

Equipment	Useful Life (years)	Quantity Each Store	Annualization Factor	Annual Cost One Store	Annual Cost All Stores
Refrigerator/ice-pack freezer	5.0	1	3.79	$171	$25,681
Thermometer	5.0	1	3.79	0	51
Cold box, 10 liter	5.0	1	3.79	32	4,748
Ice packs, 0.3 liter	5.0	50	3.79	4	653
TOTAL ANNUALIZED CAPITAL COSTS Interest rate: 0.10					$31,133

Operating Costs

Labor Costs

	Fraction of Annual Labor	Labor Rate per Hour	Number of Hours/Year	Labor Cost per Store	Total Cost All Stores
Nurses	0.08	$0.66	143	$94	$14,157
TOTAL LABOR COST					$14,157

Energy Costs

	Electricity ($0.25/kwh)	Kerosene ($0.57/L)	Gas ($1.77/kg)	Cost for Each Store
Refrigerator/ice-pack freezer	0.00	0.00	219.00	$ 387.63
TOTAL ENERGY COSTS				$58,145.00

Spare Parts and Tools

	Cost per Unit	Quantity Each Store	Number of Stores	Annual Cost All Stores
Refrigerator/ice-pack freezer	$81.00	1	150	$12,150
Thermometer	0.00	1	150	0
Cold box, 10 liter	0.00	1	150	0
Ice packs, 0.3 liter	0.00	50	150	0
TOTAL COST OF SPARE PARTS				$12,150

OPERATING AND MAINTENANCE COSTS

Labor Costs	$ 14,157.00
Energy Costs	58,145.00
Repairs	2,931.00
Spare Parts	12,150.00
TOTAL	$ 87,383.00
Plus Capital Costs	31,133.00
COMBINED ANNUAL COSTS	$118,515.00

OUTREACH IMMUNIZATION AND VACCINE COLLECTION

Capital Costs

Equipment	Useful Life (years)	Quantity Each Store	Annualization Factor	Annual Cost One Store	Annual Cost All Stores
Vaccine carrier	5.0	2	3.79	$6	$ 962
Ice packs, 0.3 liter	5.0	20	3.79	3	427
TOTAL ANNUALIZED CAPITAL COSTS Interest rate: 0.10					$1,389

Operating Costs

Labor Costs

	Fraction of Annual Labor	Labor Rate per Hour	Number of Hours/Year	Labor Cost per Store	Total Cost All Stores
NONE					
TOTAL LABOR COST					$0

Energy Costs

	Electricity ($0.25/kwh)	Kerosene ($0.57/L)	Gas ($1.77/kg)	Cost for Each Store
NONE				
TOTAL ENERGY COSTS				$0

Spare Parts and Tools

	Cost per Unit	Quantity Each Store	Number of Stores	Annual Cost All Stores
Vaccine carrier	$0.00	2	150	$0
Ice packs, 0.3 liter	0.00	20	150	0
TOTAL COST OF SPARE PARTS				$0

OPERATING AND MAINTENANCE COSTS

Labor Costs	$ 0.00
Energy Costs	0.00
Repairs	139.00
Spare Parts	0.00
TOTAL	$ 139.00
Plus Capital Costs	1,389.00
COMBINED ANNUAL COSTS	$1,528.00

Total Distribution of Costs for Each Level

Cost Elements	Central	Regional	District	Health Center	Outreach	Total
Annualized capital	$16,729	$19,442	$20,890	$ 31,133	$1,389	$ 89,583
Labor	8,162	47,740	46,606	14,157	0	116,665
Energy	8,721	7,848	21,900	58,145	0	96,613
Repairs	1,438	1,944	1,687	2,931	139	8,139
Spare parts	1,895	4,633	4,050	12,150	0	22,728
Subtotal	36,945	81,606	95,133	118,515	1,528	
TOTAL ANNUALIZED COST						$333,727

Percent Distribution of Costs for Each Level

Cost Elements	Central	Regional	District	Health Center	Outreach	Combined Levels
Annualized capital	45	24	22	26	91	27
Labor	22	59	49	12	0	35
Energy	24	10	23	49	0	29
Repairs	4	2	2	2	9	2
Spare parts	5	6	4	10	0	7
Total	100	100	100	100	100	100

Cumulative Percent Distribution of Costs

Cost Elements	Central	Regional	District	Health Center	Outreach	Cumulative Percent
Annualized capital	5	6	6	9	0	27
Labor	2	14	14	4	0	35
Energy	3	2	7	17	0	29
Repairs	0	1	1	1	0	2
Spare parts	1	1	1	4	0	7
Total	11	24	29	36	0	100

Cold chain costs compared with total EPI costs

Program	Annualized Cost
Cold chain	$ 333,727
EPI program	$3,300,000

Percent of EPI costs: 10.1

Analysis of Donor Contributions ($/fully immunized child)

Cost Elements	Percent Donor	EPI Costs		Cold Chain Costs	
		Total/FIC	Donors	Total/FIC	Donors
Annualized capital	100	$ 1.70	$1.70	$0.27	$0.27
Labor	5	4.50	0.23	0.35	0.02
Energy	90	0.45	0.41	0.29	0.26
Repairs	20	0.20	0.04	0.02	0.00
Spare parts	100	0.45	0.45	0.07	0.07
Training	50	0.50	0.25	0.00	0.00
Vaccines	100	1.40	1.40	0.00	0.00
Vaccine supplies	100	0.80	0.80	0.00	0.00
Total		10.00	5.27	1.01	0.63
Percent of Total FIC Cost			53%		62%

DISCUSSION OF THE CONSEQUENCES OF VACCINE TEMPERATURE STABILITY

Stanley O. Foster, M.D., M.P.H.
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Introduction

In a discussion on vaccine research and development, Bill Foege was asked to describe vaccine technology in the year 2000. His reply was startling: a single injection administered at birth that will provide protection from multiple diseases. In the context of this meeting and other priorities in vaccine development, this ideal can be reformulated: a heat-stable vaccine administered as a single dose at birth with a nonreuseable injection device that protects against major childhood infectious diseases.

In preparing for this discussion I have chosen to interpret broadly the first objective of this meeting: "the relative importance of vaccine temperature sensitivity (and other factors) in hindering immunization efforts in developing countries." Thus in focusing on the need and opportunity for improving heat stability, it is important that we put heat sensitivity in the context of other constraints to immunization. Optimum use of research and development resources will require the identification of needs, an assessment of the feasibility of alternative approaches, and the establishment of priorities.

In the developing world 100 million children are born annually. Using the World Health Organization's (WHO) estimates of mortality in the absence of immunization (Table 1) and the current global estimates of vaccine coverage and efficacy (Table 2), it is possible to estimate the current global status of immunization, deaths prevented, and deaths not prevented (Figure 1).

TABLE 1 Morbidity and Mortality Risk in Unimmunized Children. Cases, Deaths, Disability per 100 Children

Disease	Cases	Deaths	Disability
Measles	95	3	20
Pertussis	80	2	10
Neonatal tetanus	2	1	0
Poliomyelitis	1	0.1	0.5

TABLE 2 Immunization Coverage and Vaccine Efficacy in Developing Countries

Vaccine	Estimated Coverage (%)	Estimated Efficacy (%)
Measles	35	85
Pertussis	44	80
Tetanus toxoid	16	98
Poliomyelitis	45	80
BCG	45	50

Current Obstacles to Mortality Reduction Through Immunization

Analyses of the obstacles preventing full immunization have identified three categories of constraints: political, operational, and technical. Political constraints relate to the priority given to immunization by national governments, Ministries of Health, health staff, and communities. Table 3 provides a few examples.

FIGURE I
DISEASES PREVENTABLE BY IMMUNIZATION
DEATHS PREVENTED AND NOT PREVENTED, 1986

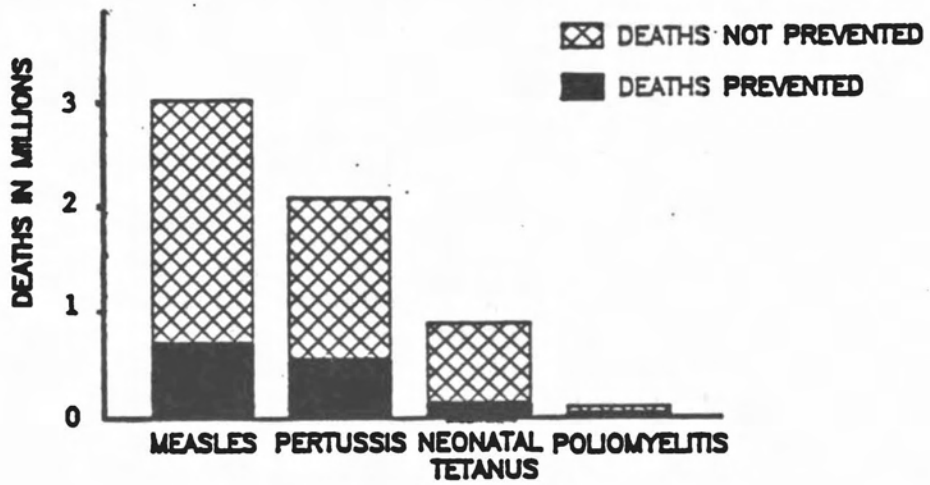

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TABLE 3 Political Constraints to Effective Immunization

Level	Example of Constraint
National government	Low-budget allocation to health
Ministry of Health	Allocation of resources to tertiary care
Health staff	Priority on curative services
Community	Nonparticipation in health services

Over the last few years, progress has been made in increasing the political priority for immunization as a key element of primary health care. The intervention of the executive director of UNICEF with heads of states (e.g., Colombia and Turkey) has increased knowledge of and commitment to immunization.

At the operational levels, constraints to immunization can be categories in terms of access to services (Table 4).

TABLE 4 Operational Constraints to Full Immunization by Level of Access

Level of Access	Constraint
Access	Staff understanding/commitment Immunization clinics 1 to 3 times per week Lack of screening/immunization of at-risk populations attending health facilities High dropout rates Nonparticipation of at-risk population
No access	Lack of facilities Lack of staff Lack of cold chain Lack of transport

With the increased international commitment to immunization, the Agency for International Development's Child Survival Initiative and UNICEF's GOBI strategy, almost all countries have access to sufficient quantities of cold chain equipment, vaccines, and supplies. For countries with severe economic problems, many of which are under IMF personnel restrictions, shortages of personnel and/or funds for operational costs limit their capability to utilize international resources and to expand services to populations without access.

In addition to the political and organizational constraints to immunization, technical barriers have been identified. Major technical issues limiting the achievement of effective universal immunization are summarized in Table 5.

In exploring opportunities to improve the heat stability of current and future vaccines, it is useful to review the functioning of the current cold chain system.

Current Cold Chain

In their excellent paper Shepard et al. (1987) describe the cold chain system: the movement of vaccine from manufacturer to air transport, to central storage, to regional storage, to facility storage, to outreach, and into the arms of children and fertile-aged women. Leadership of WHO and its cold chain unit has led to major improvements in the functioning of the cold chain system. Table 6 shows these changes for one west African country.

Inputs that have led to these improvements in the cold chain include use of WHO-tested and -approved equipment, provision of a thermometer for each refrigerator, designation of a single individual as responsible for operation and maintenance, training of that individual, daily recording of temperatures, and regular supervision.

TABLE 5 Current Technical Constraints to Effective Immunization

Constraint	Solution
Epidemiologic	Disease prior to age of immunization
Efficacy	Suboptimum vaccine efficacy (Table 2)
Injection device	Nonreuseable disposable injection device
Vaccine package	Single-dose self-contained unit
Heat stability	Heat-stable vaccines

TABLE 6 Cold Chain Improvements in a West African Country, 1978-1986

Indicator	1978	1982	1986
Number of refrigerators	18	20	30
Number functioning	3	18	22 of 23*
Percent functioning	16	90	96
Percent days temperature recorded	0	89	
Percent days temperature 0°C to 8°C	?	73	79

*Twenty-three of 30 checked in recent field assessment.

Improvements in Vaccine Efficacy

Improvements in the cold chain, the heat stability of measles vaccine, the use of cold diluent, and the more discriminate targeting of vaccine at the at-risk population have significantly increased the efficacy of immunization. Table 7 documents these improvements for measles vaccine.

Smallpox Vaccine--A Lesson from History

A significant factor in the achievement of global smallpox eradication was the development of a heat-stable freeze-dried vaccine. Together with the development of more effective mechanisms of vaccine administration, the efficacy of smallpox vaccination was increased to nearly 100 percent (Table 8).

TABLE 7 Improvements in Measles Vaccine Efficacy, 1972-1984

	1972*	1984
Target age group	6-36 months	9-23 months
Susceptibles	40%	80%
Vaccine efficacy	40%	85%
Protected per 100 doses	16	68

*McBean et al., 1976.

TABLE 8 Smallpox Major Reaction Rates by Vaccine Type and Method

Vaccination Technique	Heat-Labile Vaccine (%)	Heat-Stable Vaccine (%)
Scratch	30-50	70-80
Multiple pressure	50-70	70-95
Multiple puncture		95-100
Jet injector		95-100

Freeze-dried smallpox vaccine was stable at ambient temperature for 1 year. If immunization is to be expanded beyond the formal health system to traditional birth attendants and village health workers, a similar duration of stability will be required. Considering the current risk of HIV transmission through multiple use of needles and syringes, consideration should be given to a tandem research agenda of both a stable vaccine and a nonreuseable injection device.

Needs for Freeze-Dried Vaccine

In allocating resources to improving the heat stability of vaccines, it is important to identify the potential benefits of increasing heat stability: (1) extend vaccine delivery capability to health facilities without a cold chain; (2) extend vaccine delivery capability to health providers beyond the formal health system (e.g., traditional birth attendants); (3) facilitate the immunization of all at-risk populations coming in contact with the health system; (4) increase vaccine efficacy; and (5) lower costs and increase capability for national sustainability of immunization programs.

For the reasons stated above, increased heat stability would facilitate increases in coverage at health facilities currently delivering vaccines, at health facilities without operating cold chains, and by field personnel (traditional birth attendants and community health workers) capable of giving injections. Utilization of this capability would require simultaneous inputs in training, supplies, operational costs, and supervision. Increases in coverage in the at-risk populations will decrease disease-specific morbidity and mortality. Although the relationship between disease-specific reductions in morbidity and mortality and child survival have not been conclusively established, recent data from both Bangladesh and Senegal demonstrate a positive correlation between immunization and increased child survival.

Although recent improvements in the cold chain, training, management, and supervision have significantly increased the effectiveness of immunization, vaccine failures due to breaks in the

cold chain occur frequently, especially in remote rural areas with limited vaccine storage capability. Development of heat-stable vaccines would eliminate these failures.

Eliminating the need for a cold chain would reduce costs and contribute to national self-sufficiency in immunization. However, as demonstrated by Shepard et al. (1987), the cold chain represents only 10 to 20 percent of total EPI costs. Approximately one-third of these costs relate to the procurement of equipment and spare parts, funded for poorer countries by international donors. Another third relates to labor costs, most of which would still be needed for storage, distribution, and inventory control of heat-stable vaccines. The final third, energy and maintenance costs, would in large part be eliminated by the introduction of heat-stable vaccines.

From the economic perspective provided by Shepard et al., introduction of heat-stable vaccines would reduce the cost of fully immunizing one child from $10 to $9.30. Savings would be equally distributed between national governments and technical partners, 35 cents each. Due to donor reluctance to pay for operating costs, elimination of cold chain costs does not ensure that those resources would still be available for immunization.

Heat Stability of Current EPI Vaccines

Sizaret (1987) has provided a comprehensive review of current EPI vaccines, their potency, efficacy, stability, and safety. Data on stability at 37°C show a wide range among vaccines and manufacturers (Table 9).

Priorities for Development of Heat-Stable Vaccines

Currently, most EPI are using five vaccines: BCG, DTP, poliomyelitis, measles, and tetanus toxoid. In the next few years addition of hepatitis B and rotavirus is projected. Although in the long term it would be desirable for all vaccines to be heat stable, consideration needs to be given to establishing criteria for the allocation of the following research priorities: (1) high disease mortality; (2) potential for increased coverage; (3) heat sensitivity of current vaccine; (4) lack of replacement vaccine in field-testing stage (e.g., pertussis); (5) technical feasibility for improving heat stability; and (6) projected cost.

Recognizing there may not be uniform agreement in the above criteria, or in my assessment of vaccines for priority research attention to improve heat stability, I will, for the purpose of discussion, identify three priority vaccines for improved heat stability: measles vaccine, tetanus toxoid, and oral poliomyelitis vaccine.

TABLE 9 EPI Vaccine Stability at 37°C

Vaccine	Duration of Heat Stability According to WHO Standards	
	Shortest	Longest
Diphtheria tetanus	2 months	6 months
Pertussis	4 weeks	2-4 months
BCG	?	?
Measles	2-6 days	1-4 weeks
Poliomyelitis oral	1-3 days	1 week
Poliomyelitis injectable		4 weeks

Source: WHO WER 1980:252-253.

If this presentation is successful in generating discussion, it will have achieved its purpose. Thank you.

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IMMUNOLOGIC CONSIDERATIONS IN ACHIEVING LONG-LASTING IMMUNITY WITH VACCINES

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INTRODUCTION

Although immunization has been highly successful in reducing the incidence of many infectious diseases in well-developed regions of the world, the overall effectiveness of immunization programs has been disappointing in many developing nations. While failure to achieve adequate delivery of vaccine may be of overriding importance, many other factors may be responsible for the limited impact of immunization in these areas. This paper will focus on immunologic aspects of immunization against infectious agents, with emphasis on two of the infections targeted for prevention under the World Health Organization's Expanded Programme on Immunization (EPI): poliomyelitis and measles. Current concepts of immunity against these infections will be addressed, as will factors involved in serum and secretory immunoglobulin responses to vaccines. Given the breadth of this topic and limitations on space, this review can be no more than a rather cursory survey; the references cited are only illustrative and in no way complete.

IMMUNITY TO POLIOVIRUS AND MEASLES VIRUS

Immunity to Poliovirus

The presence of poliovirus type-specific serum-neutralizing antibody generally has been assumed to provide protection from paralytic poliomyelitis. Data directly supporting this assumption are relatively scarce, however, and it may be questioned (Fox, 1984). Direct clinical studies of immunity to paralytic poliomyelitis have been hindered by the fact that paralytic disease occurs relatively rarely following natural infection with wild-type virus. Thus, more is known of immunity to poliovirus infection per se.

Prospective studies carried out in Louisiana prior to the introduction of vaccine demonstrated quite convincingly that reinfection frequently follows exposure of previously "immune" persons to virus in a household setting (Gelfand et al., 1957). Following exposure to children undergoing primary poliovirus infection, approximately 20 percent of household contacts with preexisting homotypic serum-neutralizing antibody demonstrated fourfold or greater increases in serum-neutralizing antibody. Moreover, virus was found in the feces of an additional 3 percent of exposed persons. Given the methodology involved in these studies, these figures most likely underestimate the actual frequency of reinfection. Viral replication

is probably limited to the gastrointestinal tract, and reinfection is not associated with clinical illness. Virus shedding appears to be of much briefer duration than that observed in children undergoing primary infection. Thus, the risk of virus transmission is probably much less than with primary infection.

This study is important in that it indicates that mucosal immunity is far from complete after natural infection with wild-type poliovirus, but it does provide the first barrier to invasion by limiting replication in the gastrointestinal tract. However, this study sheds no light on the relationship between serum antibody and protection from paralytic disease. The level of protection from paralysis conferred by preexisting serum antibody probably depends largely on the route(s) by which poliovirus invades the central nervous system from its site of primary replication in the gut (Fox, 1984). Preexisting neutralizing antibody should prevent passage of virus to the central nervous system if this occurs by way of the blood. Poliovirus-specific antibody, passively administered to nonhuman primates, did confer protection against paralytic infection in experiments carried out several decades ago (Bodian, 1952). Poliovirus has also been shown capable of invading the central nervous system and inducing paralysis in orally infected cynomolgus monkeys with preexisting homotypic serum-neutralizing antibody (Craig and Brown, 1959). These apparently conflicting results may be related to differences in the titer or the virulence of the challenge inocula. However, the latter observation suggests that virus might gain access to the central nervous system by routes not involving the bloodstream, perhaps along nerve fiber pathways. We should also note that viremia is common early in the course of primary poliovirus infection, before neutralizing antibody develops, yet symptomatic invasion of the central nervous system occurs only with relatively low frequency.

Intestinal immunity may be of primary importance in prevention of paralysis, then, either because virus replication in the gut is reduced, significant viremia is made less likely, or an alternative route of access to the system is protected. There are substantial differences in the mucosal immune response to orally administered, attenuated poliovirus vaccine and parenterally administered, inactivated poliovirus vaccine, which will be addressed in greater detail below.

Immunity to Measles

As with poliovirus, serum-neutralizing antibody has generally been considered to be of primary importance in immunity to measles virus. Proof of the importance of serum antibody in immunity against measles derives in part from early clinical studies documenting the efficacy of passively administered pooled human serum globulin in preventing or modifying the clinical course of measles virus infection. In prospective studies the presence of serum-neutralizing antibody to measles virus has been shown to be highly predictive of protection against disease on natural exposure to virus during epidemics (Neumann et al., 1985). Other simpler tests for serum antibody, such as

solid-phase immunoassays, an antihemolysin test, and the hemagglutination inhibition (HAI) test may provide similar, although somewhat less sensitive, measures of immunity.

Mucosal immunity to measles virus may also be important in prevention of measles virus infection. Infants with titers of maternally derived, serum-neutralizing antibody ranging from 1:25 to 1:110 were universally infected following aerosol exposure to attenuated measles virus vaccine, as judged by subsequent rises in serum antibody titers (Sabin, 1983). Infants in developing nations remain susceptible to measles despite the presence of maternally derived antibody. Such levels of serum antibody usually prevent takes of subcutaneously administered vaccine but not natural measles. This observation indicates that serum antibody by itself does not provide complete protection from measles virus infection.

Immunologic Memory

Immunologic memory may be defined as the ability to undergo a booster antibody response following reexposure to a previously recognized antigenic stimulus. The presence of immunologic memory does not require the existence of detectable antibody, although this may largely reflect methodological difficulties in detecting small quantities of antibody. While the molecular basis of immunologic memory is poorly understood, the phenomenon may be of great importance in determining the duration of immunity to agents such as poliovirus or measles virus. In theory the antibody boost observed during reinfection with poliovirus, for example, could play a substantial role in protecting the central nervous system from virus invasion. The location of memory is also relevant. Recall in intestinal immunity may provide local protection and reduce the amount of replication in the gut, thereby preventing viremia. In the absence of intestinal immunity, viremia may occur before there is an opportunity for serum antibody responses to be boosted.

Herd Immunity

While immunization with either oral poliovirus vaccine or attenuated measles vaccine should limit the risk of virus transmission following reexposure to virus, it has not been possible to demonstrate effective "herd immunity" to either measles or poliomyelitis, even in highly immunized populations (Fox, 1983). Person-to-person transmission of virus continues to occur even when greater than 85 percent of individuals are seropositive. The most important factor determining the absence of herd immunity may be the clustering of susceptible individuals because of religious or socioeconomic factors (Fox, 1983).

Attenuated poliovirus vaccine virus is transmitted to close contacts. Vaccine virus is readily isolated during virus surveillance programs carried out in well-immunized populations. Thus, use of attenuated virus vaccines may have broader consequences for a

population than administration of inactivated virus, as vaccine virus may be transmitted to persons not reached through formal vaccine programs. However, type 3 poliovirus shed from vaccine recipients has a high frequency of back mutation at some nucleotide base locations (Minor et al., 1986). Paralytic disease may occur in both vaccine recipients and their contacts, at rates approximating 1 case per 2.6 million doses distributed (Nkowane et al., 1987). Such cases may result from phenotypic reversion of virus but may also reflect unknown host factors. Even with wild-type virus, paralysis occurs in only 1 out of approximately every 1,000 infected.

MOLECULAR DETERMINANTS OF IMMUNITY

Recent studies have focused on the molecular structures present on the surface of poliovirus type 1 that react with murine monoclonal-neutralizing antibodies. These "neutralization-immunogenic sites" have been well characterized through the combined application of classical virology, molecular biology, and x-ray crystallography (Hogle et al., 1985). Similar data have also been generated recently for human rhinovirus type 14 and the influenza hemagglutinin molecule. The specific molecular structures identified in these studies as binding neutralizing antibodies are important B-lymphocyte determinants. Similar studies using human antibodies and examining human B-cell determinants will be of critical importance for the eventual design of effective synthetic or recombinant vaccines. There are almost certainly T-cell determinants of equal importance in the evolution and maintenance of protective immunity but about which much less is known.

Many, if not all, sites present on the surface of a protein structure may be potentially immunogenic. However, some antibodies may not provide protection against infection or disease because they are not directed against relevant epitopes. Thus some monoclonal antibodies may react specifically with a virus yet fail to effectively neutralize its infectivity. Neutralization of poliovirus, one of the simplest of viruses, is a complex and potentially reversible phenomenon and may have multiple mechanisms. While some neutralized virus-antibody complexes are still capable of attachment and penetration of cells in vitro, uncoating of virus and further replication events appear to be blocked. Available data suggest that neutralization of poliovirus may be dependent on the bivalent attachment of antibodies to the virion (perhaps as few as four immunoglobulin molecules per virus particle) (Emini et al., 1983). Bivalent binding of antibody may lead to reversible conformational shifts in the viral capsid that prevent uncoating of the viral RNA.

One danger that should not be overlooked in the design of recombinant or synthetic virus vaccines is that an incomplete antibody response to antigen(s) representing only part of the immunogenic complement of some viruses may be harmful rather than beneficial. This fact was realized only by hard clinical experience with respect to early formalinized, inactivated measles and respiratory syncytial virus vaccines. Formalin inactivation of measles virus present in an early inactivated vaccine led to a relative loss of the immunogenicity of the

fusion protein "F," while the immunogenicity of the hemagglutinin moiety was relatively preserved (Choppin et al., 1981). Infection with wild-type virus subsequent to immunization with this vaccine led to severe clinical manifestations characterized as "atypical measles"--bizarre rashes, high fever, and pneumonitis. The enhanced disease manifestations probably represented a hypersensitivity response to measles virus antigens in immunized persons who lacked anti-F antibodies. Anti-F antibodies act normally to prevent cell-to-cell spread of virus, and thus measles virus could continue to replicate in the presence of existing antibodies. Cell-mediated immune responses recognized infected cells, may have contributed to disease manifestations, but were unable to eliminate infection until anti-F antibodies were formed. For somewhat similar reasons, intramuscular administration of an early formalin-inactivated respiratory syncytial virus vaccine led to exaggerated disease upon subsequent natural infection, a phenomenon that has been reproduced recently in the cotton rat model (Prince et al., 1986). Immunity against infectious agents is usually quite complex. We must be more careful as we begin to look at subunit antigens comprising a relatively small portion of the immunogenic mass of a virus or bacterium. Special attention must also be paid to the effect of stabilizers added to vaccine preparations in order to ensure that they do not have differential effects on selected antigenic components of an inactivated vaccine.

IMMUNE RESPONSE TO VACCINE ANTIGENS

Antigen Presentation

For most soluble protein antigens, the T-helper (T_h) lymphocyte plays a critical role in the induction of antibody responses and the establishment of immunologic memory. Initial activation of the T_h cell requires the presentation of antigen in close association with Ia histocompatibility molecules on the surface of specialized antigen-presenting cells (Shevach, 1984). These Ia-bearing cells include macrophages, epithelial Langerhans cells, dendritic cells, and B-lymphocytes. Macrophages and other antigen-presenting cells "process" antigen prior to its presentation on the cell surface. Processing may involve ingestion of the antigen and usually results in its fragmentation. The processing of antigen by the presenting cell produces antigenic fragments capable of interaction with specific Ia molecules present on the cell surface. It has been suggested that amphipathicity (i.e., the presence of hydrophobic and hydrophilic faces on opposing ends of the antigen fragment) may be an important property of antigen fragments capable of stimulating T cells; the hydrophobic face of the antigen fragment presumably interacts with the Ia molecule or other structures on the cell membrane, while the hydrophilic face may interact with the T_h-cell receptor (Berzofsky et al., 1986). Because the antigen fragment is presented in close association with the Ia molecule, individual variation in the latter may define which antigen fragments may be presented. Thus the types of Ia molecules present may profoundly influence the antibody repertoire of the individual, and the structure of T-cell determinants may vary from individual to individual.

B-cell Versus T-cell Determinants

T-cell antigenic determinants are usually distinct from B-cell antigenic determinants, the latter representing the molecular structures (epitopes) specifically binding to the variable region of immunoglobulin molecules (idiotypes). The fact that antigen processing usually leads to fragmentation of the antigen suggests that T-cell determinants may be largely sequential in nature (i.e., defined by continguous amino acids within the primary structure of a protein) (Berzofsky et al., 1986). Experimental observations suggest that this is indeed the case, although there are few data addressing the T-cell determinants of most important vaccine immunogens. In contrast, B-cell determinants are often conformational or assembled determinants, structures formed by discontiguous amino acids and dependent on tertiary or quaternary protein structure. This is the case with poliovirus (Hogle et al., 1985).

Implications for Vaccine Development

The foregoing has important implications for development of synthetic oligopeptide vaccines or vaccines based on recombinant DNA technology. Presumably, a successful vaccine must contain both B-cell and T-cell determinants if it is to induce good antibody responses and establish good immunologic memory. However, B-cell determinants may be difficult to reconstruct through synthetic means if they are assembled and conformationally dependent, as exemplified by poliovirus (Hogle et al., 1985). Nonetheless, our current appreciation of the three-dimensional structure of immunogenic sites on poliovirus should open the way to synthetic reconstruction of these sites. In addition, the inclusion of specific T-cell determinants, as such determinants are characterized for antigens such as poliovirus, should increase the chances of success for synthetic vaccines of the future. It may be necessary for an oligopeptide vaccine to contain a mixture of potential T-cell determinants, if these vary significantly from person to person.

Taken together, these considerations seem to bode well for the future of synthetic vaccines, as we may expect further knowledge to be gained concerning the cellular events underlying initiation and maintenance of antibody responses and the molecular structure of critical immunologic determinants. In the context of this workshop, oligopeptide vaccines could have much to contribute to development of thermostable vaccine preparations.

VACCINE VARIABLES ASSOCIATED WITH IMMUNOGENICITY

Vaccine Dose

The immunogenic response to inactivated protein antigens (e.g., poliovirus and hepatitis B virus vaccines) is dose dependent, with larger amounts of antigen prompting earlier antibody responses of greater magnitude. As the duration of a protective antibody response can be related generally to the magnitude of the initial (or boosted)

response, it seems reasonable to assume that larger doses of most inactivated antigens would produce more long-lasting immunity. This of course is not the case with replicating antigens, where the total immunogenic mass delivered to the host depends largely on the efficiency of replication.

Replicating Versus Nonreplicating Antigens

Advantages of live virus vaccines include (1) ease of administration (a single dose in most cases except for poliovirus, where three doses of a trivalent vaccine are given to ensure that infection occurs with all three vaccine serotypes); (2) immunity that may more closely mimic natural immunity following infection with wild-type virus (in the case of oral poliovirus vaccine, secretory immunity is induced and enteric infection with wild-type strains prevented to a greater extent than with inactivated vaccine) (Ogra et al., 1980); and (3) usually longer duration of immunity (administration of yellow fever virus, measles, mumps, and rubella virus live attenuated vaccines apparently leading to very long immunity). In contrast, immunity following inactivated influenza vaccine is relatively brief, requiring annual reimmunization, while that achieved with inactivated rabies virus and hepatitis B virus vaccine is probably measurable in terms of 5 to 10 years. The role played by immunologic memory in prolonging the effective duration of immunity is uncertain. However, infections with long incubation periods (e.g., hepatitis B) may provide sufficient time for protective anamnestic responses to develop.

It is possible that inactivated poliovirus vaccine may represent an exception to this latter distinction between inactivated and replicating antigens. Darrell Salk (1980) has argued that the duration of immunity obtained with inactivated vaccine may not be much different from that obtained with live vaccine and that immunity may result from the presence of immunologic memory. This of course would be dependent upon the timing of viremia and the anamnestic response in serum antibodies. There are no data suggesting that inactivated vaccine will induce immunologic memory capable of influencing replication of virus in the gastrointestinal tract. Newer inactivated poliovirus vaccines have enhanced potency relative to earlier preparations. A recent study compared the antibody response to attenuated poliovirus vaccine and a high-potency inactivated vaccine (McBean et al., 1984). An initial series of two vaccine doses (either attenuated or inactivated) was followed by a booster dose of the same vaccine 14 months later. A substantially greater booster effect occurred for neutralizing antibody to types 1 and 3 viruses in recipients of inactivated vaccine, compared with recipients of the attenuated virus. Twenty months after initiation of the immunization series the serum antibody titers in the two study groups were equivalent with respect to type 2 poliovirus, while the geometric mean antibody titers for types 1 and 3 virus were 2.4- and 4.3-fold greater respectively in the group receiving inactivated vaccine. Given that the duration of measurable protective antibody levels is related to the magnitude of the initial (or boosted) antibody response, this study suggests that the duration of detectable

serum antibody following immunization with high-potency inactivated poliovirus vaccine is at least comparable to that obtained with attenuated virus. However, the real issue is the length of protection afforded the individual. Oral poliovirus vaccine may result in more durable immunity because of its ability to stimulate mucosal immunity (see below). Other factors favoring use of attenuated vaccine may include the induction of humoral as well as mucosal immunologic "memory" and frequent boosts due to circulating vaccine virus.

The remarkable length of protection that can be achieved with a single dose of an attenuated replicating virus vaccine is exemplified by measles virus. Successful immunization with attenuated measles vaccine appears to result in immunity similar to the lifelong protection that follows natural infection with wild-type virus. A 16-year prospective study of the immunogenicity of further-attenuated measles virus vaccine compared antibody responses following immunization and natural measles virus infection (Krugman, 1983). Antibody responses were measured in terms of HAI activity and thus underestimated the duration of neutralizing antibody. Geometric mean titers in the natural infection and vaccine groups were 1:410 and 1:333 respectively 1 month after onset of disease or immunization and 1:22 and 1:6 16 years later. At the end of 16 years, HAI antibody could not be detected in 13 percent of the immunized group (HAI titer < 1:2) but was present in all naturally infected persons (although in 15 percent the HAI titer was ≤ 1:4). However, given the relative insensitivity of the HAI test, neutralizing antibody was probably present in most if not all of these low-titer or HAI-negative late sera (Neumann et al., 1985; Krugman, 1983).

Adjuvants

Although many different approaches to adjuvancy of vaccine antigens have been examined experimentally, only adsorption or precipitation with alum has enjoyed wide use and universal acceptance. The mechanisms of action of adjuvants in general and alum in particular are incompletely understood and clearly beyond the scope of this discussion. As reviewed by Edelman (1980), several mechanisms have been proposed to explain the adjuvant action of alum. These include a "depot effect" resulting in sustained release of antigen over a period of time and a local inflammatory effect leading to the production of local granulomas and the recruitment to the site of injection of immune cells involved in antigen processing and antibody production. Alum adsorption has been shown to augment the immunogenicity of many but not all antigens and has a generally excellent safety record. Its effect in general is greatest on the antibody response to primary immunization, as alum adsorption apparently has little effect on the response to booster doses of diphtheria and tetanus toxoid vaccines or to booster doses of inactivated influenza vaccine (Edelman, 1980). Many other vaccine adjuvants have been studied, but none has the proven safety of alum.

It can be reasonably argued that any adjuvant that enhances the magnitude of a primary antibody response will probably also favorably

influence the duration of the antibody response, but whether this alters duration of immunity will need to be determined. Prospective studies of antibody responses in recipients of new vaccines will be needed to confirm the longevity of the antibody response and its relation to protection from disease.

Route of Administration

Site of injection may profoundly influence the immunogenic response to replicating or nonreplicating vaccine antigens. With respect to nonreplicating antigens, this influence presumably derives from factors favorably or negatively affecting the interaction of antigen with cells involved in one or more of the steps in antibody production. Although both inactivated rabies virus and hepatitis B virus vaccines are usually administered by intramuscular injection, intradermal injection of as little as one-tenth the normal intramuscular vaccine dose may lead to acceptable antibody responses to these extremely expensive vaccines (Bernard et al., 1982; Halsey et al., 1986). Intradermal vaccine administration is thus a recommended procedure for preexposure (but not postexposure) rabies prophylaxis. Presumably, intradermal injection provides for efficient uptake and processing of antigen by Langerhans cells within the epidermis, thereby enhancing the immunogenic response relative to that seen following intramuscular injection. While a potentially useful approach, it should be realized that correct intradermal injection may be difficult to achieve in mass immunization programs. Also, intradermal administration of alum-adjuvanted vaccines may be associated with occasional nodule formation at the injection site. An "intradermal" head has been developed for the jet injection apparatus, but there is relatively little experience with its use for this purpose.

While intradermal injection of vaccine may offer advantages over intramuscular injection in terms of immunogenicity, subcutaneous administration of vaccine may be relatively less efficient (Bernard et al., 1982). This has been well demonstrated for the plasma-derived hepatitis B vaccine, which was tested in field trials and intended for administration by intramuscular injection into the deltoid muscle. After licensing, the antibody response to the vaccine was found to be unexpectedly poor in some medical centers, with seroconversion rates as low as 50 to 70 percent compared with the 85 to 95 percent rates achieved in controlled trials (Centers for Disease Control, 1985). Centers reporting poor immunogenic responses to the vaccine were found to have been more likely to have given the vaccine by gluteal, rather than deltoid, injection (Centers for Disease Control, 1985), and poor responses among individuals in several centers giving vaccine by gluteal injection were found to correlate with increasing obesity and female sex (Weber et al., 1985). It is probable that vaccine intended for intramuscular injection frequently was deposited in subcutaneous tissues following attempted gluteal injection, particularly in females and obese persons. Interestingly, we also have found a negative correlation between obesity (or weight-height index, a surrogate measure of obesity) and antibody response in persons receiving plasma-derived hepatitis B vaccine by intramuscular deltoid injection. We have no explanation for this finding at present.

Mucosal Immunization

Mucosal immunity, consisting of secretory 11s IgA, variable amounts of other immunoglobulins (particularly IgM), and immunoreactive lymphocytes and phagocytic cells, is a complex phenomenon that to a large extent develops independently of systemic immunity. Populations of antigen-reactive B-lymphocytes, particularly IgA-committed cells, are found in both the respiratory and gastrointestinal tracts (Ogra et al., 1980). Following mucosal exposure to antigen, these cells undergo proliferation and enter the systemic lymphoid circulation, homing eventually to mucosal sites elsewhere in the body (Ogra et al., 1980; Cebra et al., 1986). For example, intestinal immunization of women with an avirulent strain of E. coli has been shown to result in secretory IgA antibodies in colostrum and milk, a result of the clonal expansion, systemic circulation, and eventual migration of immunoglobulin-secreting cells from the gut to breast tissue (Goldblum et al., 1975).

The independence of what has been termed the common mucosal immune system is demonstrated by the observation that intranasal or intestinal administration of sufficient amounts of inactivated poliovirus vaccine may induce a specific (albeit short-lived) nasopharyngeal or colonic secretory antibody response with low or absent serum antibody, while the same antigen administered parenterally leads to negligible levels of secretory antibody but good levels of serum antibody (Ogra et al., 1980).

Mucosal defenses also appear to function independently of systemic immunity. Following intestinal immunization with attenuated poliovirus vaccine (or natural poliovirus infection), local IgA antibody develops and a relatively high level of intestinal immunity exists, as evidenced by less frequent "takes" of live virus on subsequent oral challenge (Ogra and Karzon, 1971; Ogra et al., 1980). Protection is not complete, however, even after natural infection as detailed above (Gelfand et al., 1957). On the other hand, much less resistance to intestinal challenge with poliovirus is evident following parenteral administration of inactivated poliovirus, despite high levels of serum antibody. Serum IgA levels are similar to those found after attenuated vaccine administration (Ogra and Karzon, 1971; Ogra et al., 1980). Effective intestinal immunity may be of substantial importance in prevention of paralytic disease. In support of this hypothesis, Craig and Brown (1959) reported that 6 of 16 cynomolgus monkeys with preexisting serum-neutralizing antibody induced by parenterally administered inactivated vaccine became paralyzed after oral challenge with virulent poliovirus, compared with only 1 of 10 monkeys with antibody induced during previous live virus infection.

In addition to preventing paralysis by limiting viral replication in the gastrointestinal tract or preventing infection altogether, effective intestinal immunity diminishes the potential for transmission of virus. Poliovirus transmission may follow shedding of virus in nasopharyngeal secretions or in feces. While parenteral immunization with inactivated vaccine does appear to reduce or prevent nasopharyngeal shedding of poliovirus upon subsequent challenge,

nasopharyngeal shedding may depend on spread of virus from the gut to the nasopharynx by way of the blood (Ogra and Karzon, 1971). Thus prevention of nasopharyngeal shedding may be more dependent on serum than secretory immunity. However, the relative contributions of virus shed from the nasopharynx and virus shed in feces to endemic spread of poliovirus is not entirely clear and may vary according to socioeconomic status and overall hygienic conditions.

Although inactivated poliovirus vaccine may be less effective than oral poliovirus vaccine in inducing solid intestinal immunity, widespread use of strictly inactivated vaccine has led to the control and disappearance for a number of years of wild-type poliovirus from highly developed countries such as Finland. Nonetheless, the relative absence of effective intestinal immunity may have played a role in the recent extensive dissemination of an atypical variant of poliovirus type 3 within the highly immunized Finnish population (Hovi et al., 1986).

HOST-ASSOCIATED VARIABLES

Age and Sex

Age may profoundly affect the response to immunization for several different reasons. Young infants and children appear to respond relatively well to protein antigens such as that in the plasma-derived hepatitis B vaccine (Milne et al., 1986). Their response may be significantly better than older individuals. On the other hand, bacterial polysaccharide antigens generally do not elicit good antibody responses in children under 18 months of age, possibly reflecting host immunologic immaturity with respect to T-cell-independent antigens.

For replicating antigens such as measles vaccine, age may have a striking influence on vaccine efficacy due to the presence of maternally derived neutralizing antibodies (Ministries of Health of Brazil, Chile, Costa Rica, and Ecuador and PAHO, 1983). These antibodies may partially or completely prevent infection and appropriate immunologic responses to attenuated measles vaccine up to and even beyond 12 months of age. Those younger children who respond do so with lower titers of antibody. This probably reflects impaired replication of vaccine virus and a reduced total immunogenic mass. Immunization with attenuated measles vaccine thus needs to be delayed until maternally derived antibodies have declined to levels permitting effective immunization (in practice 9 to 15 months of age, depending on the infant's risk of acquiring wild-type measles infection during the interim). For similar reasons, "booster" responses to attenuated oral poliovirus vaccine may be much less impressive than booster responses to inactivated vaccine (McBean et al., 1984). However, one can argue that boosters may not be necessary if the individual is already capable of limiting viral replication.

Sex has notably less influence on antibody responses to vaccines, but sex-related differences have been noted, particularly with the plasma-derived hepatitis B vaccine (Weber et al., 1985). Other factors

possibly influencing the immune response to vaccination include malnutrition and interference of attenuated vaccine virus replication (i.e., poliovirus) by other viruses resident within the gut.

SUMMARY

For many inactivated vaccines the duration of effective immunity is a critical limiting factor. In contrast, live attenuated vaccines, such as attenuated measles virus vaccines, generally provide more long-lasting protection because of the potentially greater antigenic mass provided for immunization during replication of the vaccine agent in the host. However, the duration of the serum antibody response following immunization with high-potency inactivated poliovirus vaccine may rival that induced by attenuated poliovirus vaccines. With either type of vaccine the duration of detectable serum-neutralizing antibody generally correlates with the magnitude of the initial (or boosted) antibody response. Nonetheless, protection may extend beyond the period of detectable antibody if immunologic memory has been established. In this respect the presence of mucosal immunity and immunologic memory may be critical. With poliovirus the serum-neutralizing antibody may not be sufficient to prevent paralytic disease as virus may reach the central nervous system by neuronal spread. Mucosal immunity may therefore be of great importance in prevention of poliomyelitis by limiting the replication of virus at the primary site of invasion. Despite this there is good evidence that intestinal protection is not complete, as measured by virus shedding, even after natural poliovirus infections. Thus, while empirical correlates of immunity are known for poliovirus and measles virus, abundant questions remain.

The neutralization-immunogenic sites of poliovirus and some other viruses have recently been characterized at the atomic level. Current efforts directed at gaining a better understanding of antibody induction and immunologic memory, and detailed studies of the molecular structures comprising the immunologic determinants of other viruses and bacteria, may point the way toward development of new vaccines with improved immunogenicity and thermostability.

Acknowledgment

I am indebted to Catherine M. Wilfert, M.D., for her many helpful comments and suggestions concerning this manuscript.

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DISCUSSANT PAPER FOR IMMUNOLOGIC CONSIDERATIONS IN ACHIEVING LONG-LASTING IMMUNITY WITH VACCINES

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Dr. Lemon has provided a good review of topics related to the immune responses to antigens in vaccines, focusing on vaccines against poliomyelitis and measles. As he indicated, vaccines should induce immune responses that will protect against clinical illness, but they need not prevent subclinical infections. The immunity acquired against poliomyelitis by natural infection may be accompanied by subsequent gastrointestinal infection by poliovirus and shedding of virus to others, but clinical illness is not observed. Similarly, oral polio vaccine virus-induced immunity will protect against paralysis, but virus infection of the gastrointestinal tract can still occur. A higher degree of intestinal infection and shedding of polioviruses is more likely to occur in virus-exposed recipients of killed polio vaccines, since mucosal immunity is not induced by the parenteral vaccine, but protection of the vaccine recipient against paralysis still occurs.

The situation with current live measles vaccine can be juxtaposed to that which occurs with current live polio vaccines. Measles vaccines are attenuated and given by injection. The administered virus thus bypasses the respiratory mucosal lymphoid tissues, thought to be a site of primary virus replication, and more directly enters the systemic circulation. This explains why the attenuated vaccine is rapidly cleared and is not antigenic in infants with residual maternal antibody. There have been efforts to administer measles virus by aerosol in order to allow localized virus replication in the respiratory tract and to avoid the rapid clearance of parenterally administered attenuated vaccine virus. There are major logistical problems with an aerosol approach, so concern persists about the best way to protect the infant who has waning levels of maternal antibody, which interfere with successful vaccination by parenteral live attenuated vaccine, but who will get natural measles if exposed. Thus, these vaccines illustrate the importance of the natural or the vaccine route of infection and the fact that infection, in the absence of clinical disease, may occur after successful vaccination. This is likely to be a common event after administration of the peptide vaccines of the future.

Another point I want to emphasize concerning immunity to viral vaccines is that the presence of antibody, particularly neutralizing antibodies, generally correlates with protection against clinical illness, but this does not mean that the antibodies are the sole mechanism of protection. For example, it is likely that limited replication of virus occurs at mucosal sites; after successful vaccination, for example, influenza, measles, and clinical disease are prevented because (a) the amount of infectious input virus is reduced

if there is adequate local antibody and (b) the cells that become infected with virus are destroyed early during their infectious process by virus-specific MHC-restricted cytotoxic T lymphocytes (CTL).

I want to briefly mention some important differences between antiviral antibodies and virus-specific CTL: the specificity of recognition, the mechanism of action, and the immune responses to viral antigens by neutralizing antibodies versus CTL.

- The viral antigenic sites (epitopes) that induce CTL are different from those that induce neutralizing antibodies. They are more likely to be conserved, such as on nucleoprotein peptides-segments, or on the anchored hydrophobic region of the hemagglutinin HA2 subunit of influenza A virus, unlike the domains on the hydrophilic tip of the HA1 subunit that react with neutralizing antibodies.

- CTL do not "see" or neutralize extracellular virus. They recognize and kill virus-infected cells that express the specific viral antigenic determinant in conjunction with an MHC determinant (usually Class I) on the membrane of virus-infected cells.

- CTL responses to specific antigenic determinants on viral peptides demonstrate strong MHC haplotype preference. For example, influenza nucleoprotein-specific peptide CTL responses are observed in individuals with the HLA B37 haplotype; CTL responses to the influenza HA_2 peptide epitope occur in individuals with the HLA B5 haplotypes.

If these CTL responses to peptide are restricted to certain individual MHC haplotypes, what does this mean for vaccine development?

- The antigenic peptides that induce these responses are much more conserved than those that induce neutralizing antibodies. For example, an influenza vaccine that contains the epitope on the HA_2 subunit will protect certain strains of mice against all virus strains of the influenza H1 subtype of 1934 to 1986. Thus, this peptide should provide truly cross-reactive protection within a subtype.

- Another major consideration of these peptide-induced CTL responses concerns the haplotype restriction of peptide-induced CTL responses. Mice of the $H\text{-}2^d$ haplotype, but not $H\text{-}2^b$ haplotypes or $H\text{-}2^k$ haplotypes, would be protected; similarly, humans with HLA B35 would probably be protected, but others may not be protected against H1-subtype viruses using this peptide vaccine.

Therefore, it seems important that future peptide vaccines should be developed with the possibility in mind that multiple peptides might be necessary to protect, such as peptides that induce neutralizing antibody and peptides that induce CTL. Early in peptide vaccine development preclinical studies (e.g., in inbred mice) and clinical studies in the HLA-typed individuals should be performed to ascertain whether important differences in immune responses to specific viral peptides may be related to MHC haplotypes.

It would appear best to plan to develop peptide vaccines that contain peptides that will be potent inducers of both neutralizing antibody and HLA-restricted CTL.

POLIOVIRUS: STRUCTURAL BASIS FOR TEMPERATURE-DEPENDENT LOSS OF IMMUNOGENICITY

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Poliovirus provides an excellent system for investigation of the structural and chemical basis for temperature-dependent loss of immunogenicity. The three-dimensional structure of two poliovirus strains, the Mahoney strain of type 1 poliovirus (PM1; Hogle et al., 1985) and the Sabin strain of type 3 (PS3; Filman et al., 1989) have been determined by x-ray crystallographic methods. The antigenic sites of all three serotypes of poliovirus and their location in the three-dimensional structure of the virus have been described. The virus has been shown to undergo a temperature-dependent conformational change, and the effects of this conformational change on recognition of specific antigenic sites by antiviral antibodies have been characterized. Finally, the Sabin vaccine strains are temperature sensitive. For the Sabin strain of type 3 poliovirus, the temperature sensitivity of the vaccine has been shown to map a single amino acid substitution in the capsid protein that renders the virion thermolabile, and several ts(r) revertants have been isolated and sequenced. In this paper we will briefly describe the structure of the virus and the location and structure of its major antigenic sites. The effect of conformational changes on antibodies with known specificities will be discussed as a model of the general effect of temperature-dependent structural change on antigenicity. The ts/ts(r) phenotype of the Sabin 3 strain will be discussed as a model for temperature-dependent loss of immunogenicity in live virus vaccines.

Poliovirus

Poliovirus is a member of the Picornavirus family, which also includes foot and mouth disease virus, hepatitis A virus, the rhinoviruses, and the coxsackieviruses. The poliovirion is approximately 310 A in diameter, with a molecular weight of 8.5 million. The virus capsid is comprised of 60 copies each of 4-coat

protein subunits: VP1 (Mwt 35,000), VP2 (Mwt 30,000), VP3 (Mwt 26,000), and VP4 (Mwt 7,500) arranged on a T=1 icosahedral shell. The capsid encloses a unique single-stranded molecule of positive sense RNA (Mwt 2.5 million). The RNA is polyadenylated at its 3' end and linked via a phosphotyrosine bond to a small (22 amino acid) protein, VPg, at its 5' end. In infected cells the RNA genome is translated in a single open reading frame yielding a large polyprotein (Mwt 220,000). The viral proteins are produced from the polyprotein by posttranslational cleavages catalyzed by virally encoded proteases. One of the early cleavages yields a protein P1 (Mwt 100,000) that contains all of the capsid sequences. P1 is processed to VP3 and VP1, and an immature precursor VP0 to yield VP2 and VP4 occurs late in assembly, apparently after the encapsidation of the viral RNA (Rueckert, 1985).

Poliovirus Structure

The three large capsid proteins are similar in structure. Each consists of a conserved core with variable connecting loops and terminal extensions (Figure 1). Four of the strands (B, I, D, and G) form a highly twisted beta sheet that forms the front and bottom surfaces of the barrel, and the remaining four strands (C, H, E, and F) make up a shorter flatter beta sheet that forms the back surface of the barrel. The strands comprising the front and back surfaces are joined at one end by four short loops, giving the barrel the shape of a triangular wedge. Similar core structures have also been described in other animal viruses (Rossman et al., 1985; Luo et al., 1987) and in several plant viruses (Harrison et al., 1978; Abad-Zapatero et al., 1980; Liljas et al., 1982; Hogle et al., 1986). In contrast to the conserved cores, the three proteins have dissimilar connecting loops and amino terminal extensions.

In the virion the cores pack to form the closed shell of the icosahedral particle. VP1 is located with the narrow end of its core near the particle fivefold axes. VP2 and VP3 alternate around the threefold axes with the narrow ends of their cores pointing toward the threefold axes. The tilts of the cores give the particle the shape of the solid shown in Figure 2, with prominent protrusions at the particle fivefold and threefold axes. Similar packing of cores has been seen in rhinovirus 14 (Rossmann et al., 1985) and Mengo virus (Luo et al., 1987) and in the S domains of several T=3 plant viruses (Harrison et al., 1978; Abad-Zapatero et al., 1980; Hogle et al., 1986).

The aminoterminal extensions of the coat proteins form a network of interactions on the internal surface of the virions. The protein VP4 (which is totally internal and is covalently linked to VP2 until late in the assembly of the virus particle) behaves very much like the detached aminoterminal extension of VP2. The network of interactions is particularly extensive between fivefold-related protomers (where the term <u>protomer</u> refers to those subunits derived from cleavage of the same P1 molecule). This is consistent with biochemical evidence that suggesting that a pentamer of protomers is a major intermediate in the assembly of the virions. One especially striking feature of the internal network is the structure formed by the interaction of five

aminotermini of VP3 at the particle fivefold axes, making a twisted tube with parallel beta interactions. This tube is flanked by five copies of a short two-stranded antiparallel beta sheet at the amino terminus of VP4. The amino terminus of VP4 has been shown to be modified by myristylation, and the interaction between the beta tube of VP3 and the beta sheets of the five copies of VP4 is mediated by the myristate moiety (Chow et al., 1987).

The loops and carboxyterminal extensions of VP1, VP2, and VP3 decorate the outer surface of the virus. The short loops connecting the top three strands of the beta barrel of VP1 accentuate the protrusion at the particle fivefold axes. Similarly, the top two loops of the cores of VP2 and VP3 are exposed at the threefold axes. Large loops from the more open ends of VP1 (residues 207-237) and VP2 (residues 127-185) together with a loop near the carboxyterminus of VP1 (residues 270-290) form a very large protrusion near the particle twofold axes. A small loop in the middle of the first (B) strand of the core of VP3 (residues 50-70) forms a smaller protrusion near the particle threefold axes.

Location and Structure of the Antigenic Sites

The antigenic sites of all three serotypes of poliovirus have been investigated by sequencing mutants that are resistant to neutralization by specific monoclonal antibodies (Minor et al., 1983, 1985, 1986; Diamond et al., 1985; Blondel et al., 1986; Page et al., 1988). The mutations (summarized in Table 1) have been located in the three-dimensional structure of the virion. In interpreting the distribution of the monoclonal release mutations we sought to identify clusters of mutations that might represent portions of the same antigenic site. The antigen-binding site of an antibody molecule is quite large (30 A in diameter), and recent crystallographic studies of antigen-FAB complexes have shown that the entire binding surface of the antibody is involved in interactions with the antigen (Amit et al., 1986). We therefore reasoned that mutations located within 30 A of each other on the surface of the virus would fall within the "footprint" of the antigen-binding site of an antibody and would be likely to constitute parts of the same antigenic site.

Based on these spatial criteria alone we have identified three major antigenic regions. Region 1 includes residues from the top loop of VP1 (site 1A), the third loop of VP1 (site 1B), and the loop connecting the E strand of VP1 with the back helix (site 1C). This region is located near the fivefold axes of the particle. Region 2 includes residues from the large loop connecting the E strand to the back helix of VP2 (site 2A), the large loop connecting the G and H strands of VP1 (site 2B), and the carboxyterminus of VP2 (site 2C) and is located near the twofold axes. Region 3 includes residues from the loop near the carboxyterminus of VP1 (site 3A), from the small insertion in the B strand of VP3 (site 3B), from the top loop of VP3 (site 3C), and from the top loop of VP2 (site 3D) and is located near the threefold axes. Note that all three regions contain residues that are separated in the linear sequence but close in the three-dimensional

structure. Indeed, sites 2 and 3 include contributions from more than one polypeptide chain. Thus, these sites conform to the classical definition of a three-dimensional or conformational antigenic site.

The reactivity of these mutants with panels of monoclonal antibodies has provided evidence that the grouping of sites, originally defined by these spatial considerations, is correct. Thus, there are examples of an individual monoclonal antibody that fails to neutralize strains with mutations in sites 1A, 1B, or 1C; an individual antibody that fails to neutralize strains with mutations in sites 2A or 2B; an individual monoclonal antibody that fails to neutralize strains with mutations in sites 3A or 3B; and an individual monoclonal antibody that selects for and fails to neutralize strains with mutations in sites 3C and 3D. To date, no antibody has been identified which similarly links site 3AB to site 3CD. Failure to observe this link may indicate that these two regions, presently identified as separate subsites of site 3, actually constitute separate sites. Alternatively, failure to link the sites may merely reflect the limited number of monoclonal antibodies (approximately 30 anti-type 1, 7 anti-type 2, and 50 anti-type 3 monoclonals) studied in the panels.

All of the regions contributing to the antigenic sites are in the loops or carboxyterminal extensions of the subunits that are exposed on the surface of the virus and therefore accessible to antibody binding. These sites are also sufficiently flexible to accommodate mutations locally without affecting the global structure of the coat proteins or the virus. With few exceptions the specific residues involved in the monoclonal antibody-selected mutations are accessible to a probe the size of an antigen recognition site of an antibody and are presumably part of the physical binding site between virus and antibody. The few exceptions are partially buried residues that appear to function in stabilizing a local loop or stabilizing the interactions between two loops that are part of the same major site. Thus, all of the mutations observed to date in poliovirus appear to confer resistance to neutralization through local changes. The decoration of the outer surface with exposed flexible loops that are capable of accommodating mutations is also seen in rhinovirus 14 (Rossmann et al., 1985), in mengovirus (Luo et al., 1987), and in the hemagglutinin (Wiley et al., 1981) and neuraminidase (Colman et al., 1983) surface proteins of influenza virus, suggesting that the presentation of external flexible loops may be a general mechanism by which animal viruses escape immune surveillance.

To this point we have considered information from all three serotypes to construct a consensus map of the antigenic structure of poliovirus. There are, in fact, differences in the apparent immunogenicity of the several major sites and differences in the linkage between the subsites within each major site. The vast majority of monoclonal antibodies to both type 2 and type 3 poliovirus are specific for site 1, whereas monoclonal antibodies directed against site 1 in type 1 poliovirus are rare. Indeed, with the exception of a single Sabin 3 specific monoclonal antibody that is specific for site 3A, antibodies to sites 2 and 3 in type 3 poliovirus are obtained only when trypsin cleaved virus or a serotypically unusual type 3 strain

from the recent Finnish epidemic are used to immunize (Minor et al., 1986). Conversely, site 1 antibodies to type 1 poliovirus have been obtained only by immunizing with heat-denatured virus (Blondel et al., 1986) or by direct intrasplenic immunization (Minor et al., 1986).

We have identified a trypsin cleavage site located in the top loop of VP1 (Fricks et al., 1985). Trypsin cleavage does not affect the infectivity of the virus or the ability of antibodies specific for sites 2 or 3 to neutralize the virus. Trypsin cleavage does, however, abolish the ability of all site 1-specific antibodies to bind or neutralize the virus. Thus, the ratio of the binding titer to trypsin cleaved virus versus the binding titer to native virus may be used to estimate the percentage of antibodies in sera that are directed against site 1. Using this technique, Icenogle et al. (1986) have shown that the dominance of site 1 is dependent on the serotype and perhaps the strain of virus used to immunize, the species and strain of animal immunized, and the route of administration.

Conformational Rearrangements of Poliovirus

The poliovirion is known to undergo conformational rearrangements (reviewed in Koch and Koch, 1985; Rueckert, 1985). Some of the previously described conformationally altered forms of the virus and their properties are summarized in Table 2. We have used a variety of probes, including proteolysis, synthetic peptide antibodies, chemical modifications, and monoclonal antibodies, to further characterize these altered forms of the virus. In this paper we will focus on two forms--the heated virus and the eluted or cell released form of the virus--as models for investigating the effects of conformational rearrangements on the antigenic sites of the virus.

The heated virus (or H antigen) is obtained by exposing the virus briefly to elevated temperatures. For the common laboratory strains of poliovirus (such as the Mahoney strain of type 1 poliovirus), the transition occurs after several minutes' exposure at between 50°C and 55°C. Several mutant strains have been described that undergo the transition at much lower temperatures. A similar if not identical transition can be induced upon exposure of virus to mercurials or UV radiation, and empty particles isolated from infected cells may be converted to a similar form by exposure to slightly alkaline pH.

The eluted or cell-released form of the virus is produced when virus is exposed to susceptible cells. Typically the virus is attached to cells in the cold, shifted to 37°C, and the cells pelleted. For several poliovirus strains, approximately 10 to 30 percent of the attached virus is eluted from the cells as the altered form with the properties shown in Table 2. The transition may also be induced by treatment with extracts from membranes of susceptible cells. This altered form of the virus is reported to be the predominant population found within infected cells. Antiviral agents such as arildone and its analogues, which interfere with early events in polio infection (subsequent to attachment), are known to inhibit this transition. We believe that this form of the virus may provide a model for early

transitions in the process of penetration and uncoating, and we are actively attempting to produce enough of this altered particle for crystallographic studies. Like heat-treated virus, the eluted virus particle has a much higher reactivity with several antipeptide antibodies, including several antibodies to peptides from the normally internal aminoterminal extension of VP1.

Effect of Conformational Rearrangements of Antigenicity

We have characterized several representative anti-type 1 monoclonal antibodies with known specificities for their abilities to bind to native, heat-treated, and eluted virus. The results are summarized in Table 3. Antibodies that recognize antigenic site 3 seem to be particularly sensitive to conformational rearrangements. Monoclonal 9, which recognizes antigenic site 3B, shows a two- to three-log drop in binding titer upon heat treatment of the virus and a much larger drop upon cell attachment. Monoclonals 10 and 13, which recognize antigenic site 3CD, have an undetectable binding titer to either altered conformational form of the virus. Antibodies that recognize site 2 appear to have intermediate sensitivity to conformational change. Monoclonal 4, which recognizes both sites 2A and 2B, has undetectable titer to eluted virus and a two- to three-log drop in titer upon heat treatment. Monoclonals 6 and 7, which recognize site 2B, show a more modest (one- to two-log) drop in titers against the altered forms. The single site 1-specific anti-type 1 antibody (C3) is insensitive to the structural changes associated with heat treatment and cell attachment. Some care must be taken with the interpretation of this observation, however, since this antibody was produced by immunization with heat-treated virus. Extension of the analysis to type 3 poliovirus provides 25 additional examples of site 1-specific monoclonal antibodies. Of the 25 antibodies, 16 are specific for native virus, 8 are equally reactive (less than one log difference in binding titer) with native and heated virus, and 1 is specific for heated virus. The reactivities of these antibodies with eluted virus have not yet been tested. The results with the type 3 antibodies confirm that a substantial fraction of antibodies with site 1 specificity are relatively insensitive to conformational changes.

The differences in the sensitivities of the antibodies to conformational changes of the virus are consistent with differences in the structures of the antigenic sites. Monoclonals 10 and 13 each selected and are sensitive to mutations in both sites 3C (VP3 76) and 3D (VP2 72). An examination of the structure shows that in the virion the two component subsites are contributed by subunits in adjacent pentamers (i.e., the closest approach between VP3 76 and VP2 72 occurs between a copy of VP3 76 from a protomer within one pentamer and VP2 72 from an adjacent pentamer). Thus, juxtaposition of the two component subsites would be especially sensitive to conformational rearrangement. Similarly, monoclonal 4 recognizes a site with contributions from site 2A (the major loop in VP2) and site 2B (the large loop in VP1). Rearrangements that produce a change in the relative orientation of VP1 and VP2 or even in the relative orientation of the two component loops themselves would be expected to have a

profound effect on this site. In contrast, the sites recognized by monoclonals 6 (site 2A) and C3 (site 1A) appear to consist predominantly of a loop from a single-coat protein subunit and would be expected to be less sensitive to conformational changes in the virion. The relatively small sensitivity of monoclonals 6 and 7 may reflect partial disruption of the loop in VP2 itself. Site 1 antibodies in type 3 poliovirus seem to fall into at least two groups, one of which is sensitive to conformational rearrangements and the other which behaves more like the C3 antibody in type 1. The site 1 antibodies that are sensitive to conformational change may recognize portions of site 1A in more than one fivefold-related subunit. The relatively high sensitivity of the site 3B-specific monoclonal 9 may reflect an as yet undetected multiple subunit structure in this site. In fact, several monoclonal antibodies to type 3 poliovirus show a specificity for site 3AB (site 3A is VP1 280-290, site 3B is VP3 50-70). On the other hand, monoclonal 9 has been shown to be capable of bivalent attachment (i.e., both antigen-binding sites on an antibody molecule can attach to a single virus particle [Icenogle et al., 1983]). The large decrease in titer may be ascribed to a conformational change that leaves the individual binding site intact but that changes the spatial arrangements between two such sites in the virus such that bivalent attachment is no longer possible.

Although these observations provide considerable insight into the range of possible effects of structural rearrangements on antigenicity, it must be noted that they do not directly address the more complex and more relevant question of the effect of conformational changes on immunogenicity. Indeed, despite the apparent insensitivity of several of the neutralizing monoclonal antibodies to conformational change, it has been known for some time that heat-treated virus, many types of chemically modified or killed virus, and disrupted virus (e.g., subunits) produce very low neutralizing titers when used as immunogens. On the other hand, our own data show that several recent human oral polio vaccinees have very high titers for the eluted form of the virus (Fricks and Hogle, unpublished observations). This suggests that conformational changes induced by permissive hosts may expose new antigenic sites. The possibility that these new sites may play a role in protection against poliovirus infections is currently being investigated.

Causes of Thermolability in Live Vaccines

In live virus vaccines the immunogenicity is also dependent on the ability to amplify the administered dose via replication in the vaccinee. In such instances the immunogenicity is dependent both on the integrity of the antigenic sites and on the viability of the virus. The Sabin vaccine strains of poliovirus are temperature sensitive, growing well at 37°C but not at 39°C. The molecular basis for the temperature sensitivity of the Sabin strains of both type 1 and type 3 poliovirus have been investigated by constructing hybrids of the Sabin strains and the respective neurovirulent and nontemperature-sensitive parent by recombinant DNA methods.

In Sabin type 1 the temperature sensitivity appears to be pleiotrophic, with contributions from both capsid and noncapsid coding regions of the genome (Omata et al., 1986). In Sabin 3, however, the temperature sensitivity of the vaccine strain has been mapped to a substitution of a phenylalanine in Sabin 3 in place of a serine in the parental Leon 3 at position 91 of the capsid protein VP3 (Minor et al., 1989).

A number of ts(r) revertant strains of Sabin 3 have been isolated and sequenced (Minor et al., 1989). Many of these ts(r) strains are genotypic revertants (i.e., they revert to serine at position 91 of VP3). Several of the ts(r) strains, however, appear to revert through second site suppressor mutations. We have located the serine to phenylalanine change associated with the ts phenotype of Sabin 3 and the relevant sequence changes in the second site revertants in the three-dimensional structure of the virion. A comparison of the structure of PM1 (which has a serine at VP3 91) with PS3 shows very few structure changes in the region of VP3 91 due to the substitution of phenylalanine. In fact, the most significant difference in the PS3 structures is that the phenylalanine side chain is forced to occupy a position such that the aromatic ring is exposed to the solvent on the surface of the virion. Presumably this destabilizes the virus by promoting rearrangements that allow the phenyl group to be buried. The second site substitutions in the revertant strains generally occupy a position in the interfaces between fivefold-related protomers and may confer thermostability by stabilizing these interfaces against conformational changes. We have undertaken a program of combined crystallographic and computer simulation studies of the ts(r) strains in order to better understand the factors controlling thermostability of the poliovirion. We hope that this information will provide an opportunity to design a vaccine with improved thermostability.

This work was supported by NIH Grant No. AI20566 (JMH) and the World Health Organization. This is publication no. 5907-MB of the Research Institute of Scripps Clinic.

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FIGURE 1

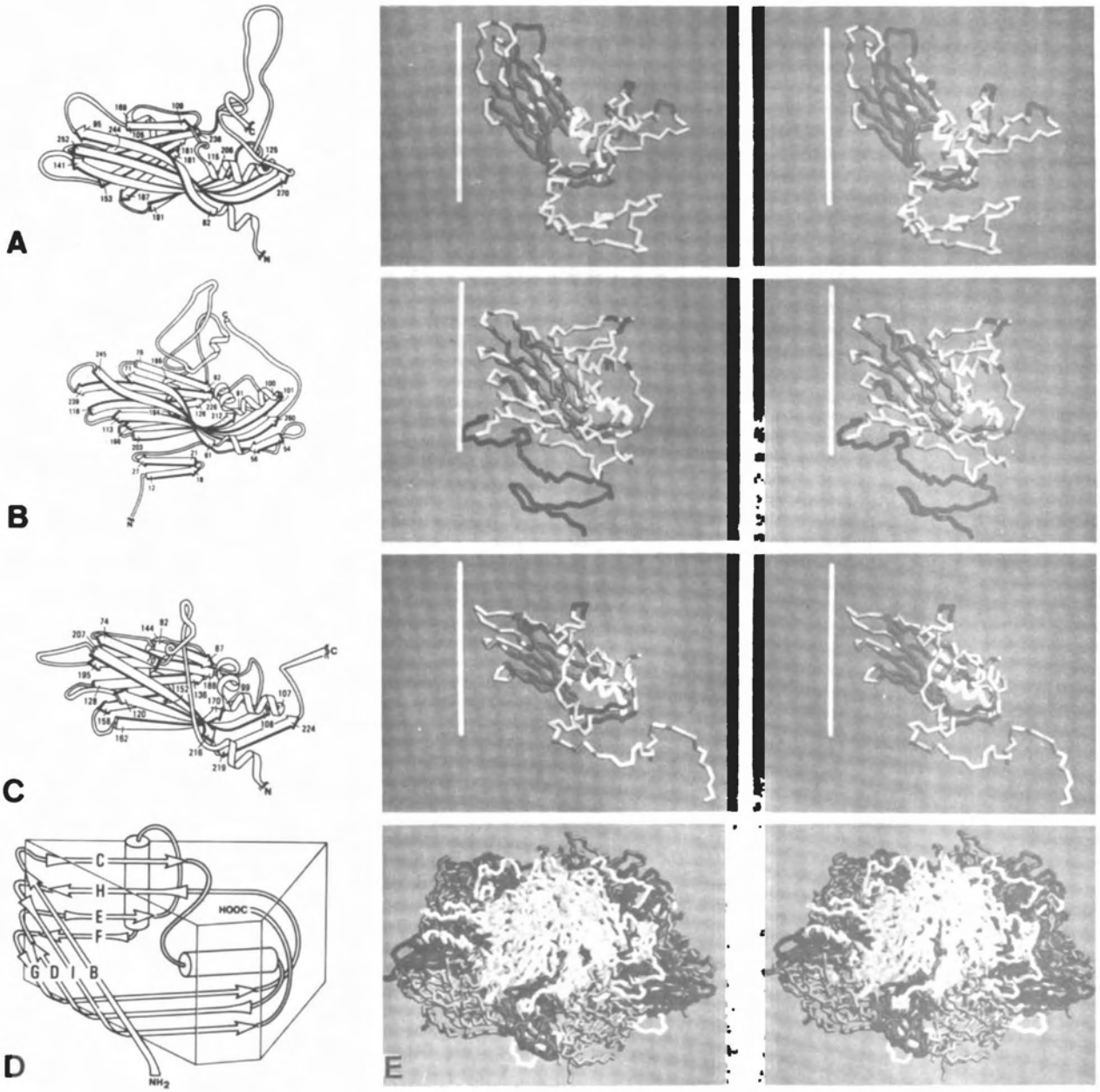

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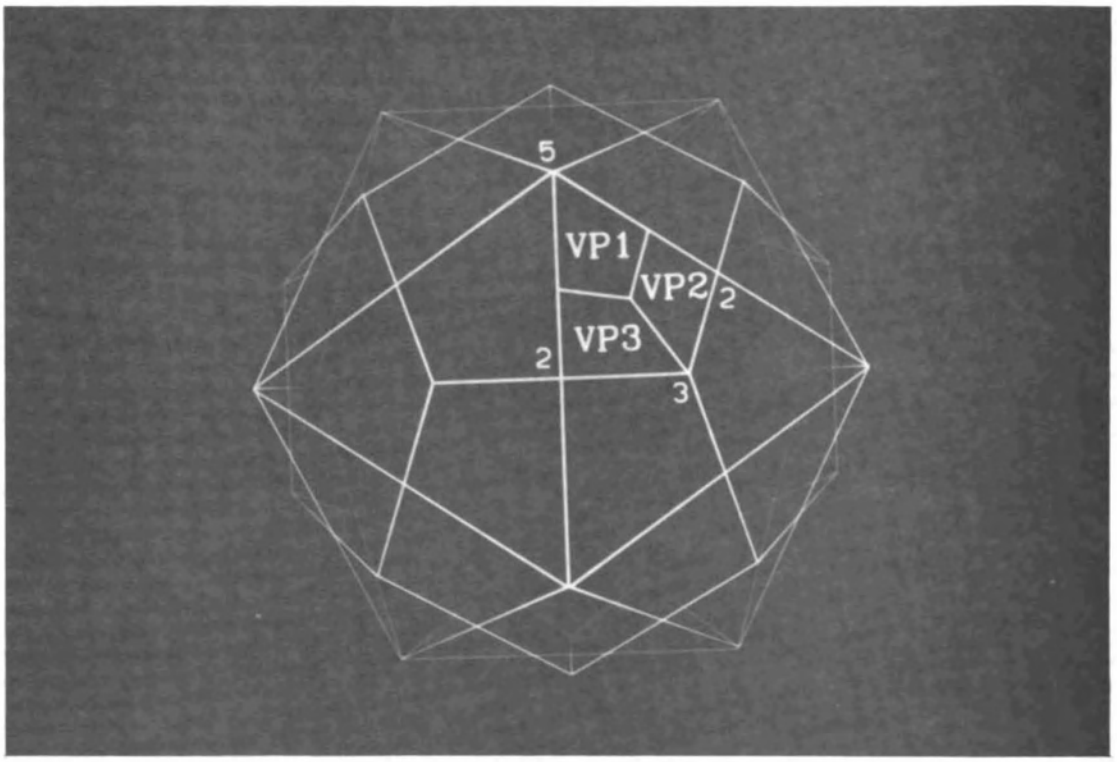
FIGURE 2



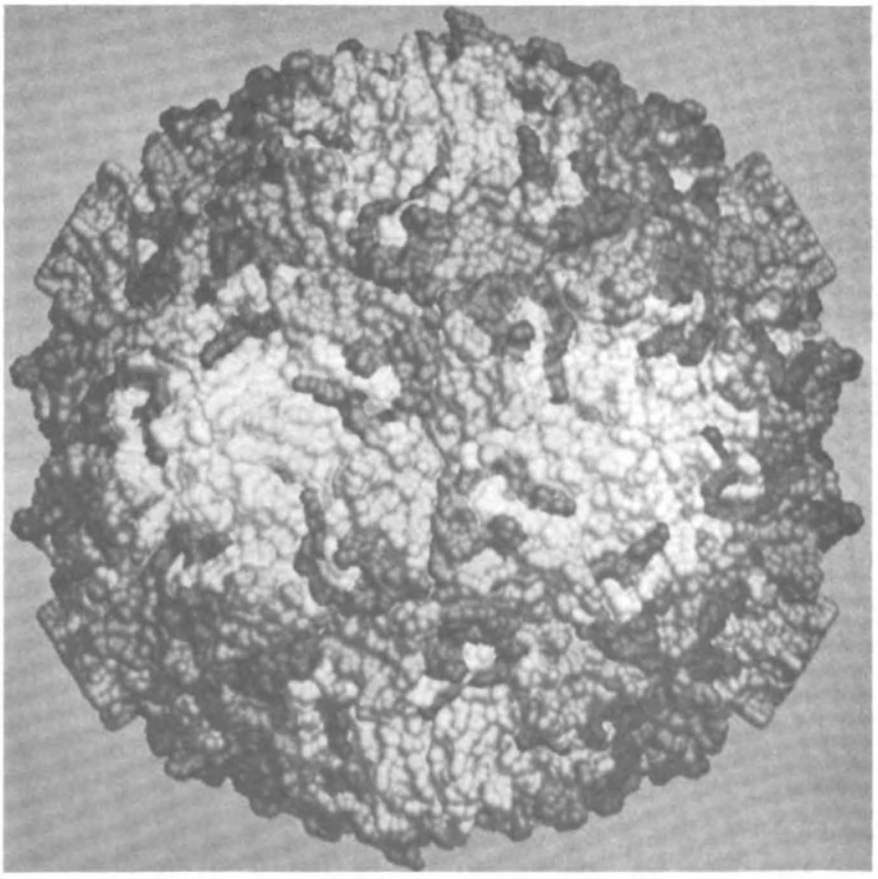


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FIGURE 1. Structure of poliovirus coat proteins: (a) VP1--at the left is a schematic "ribbon plot" of the protein with residue numbers for reference. At the right is a stereo representation of the alpha carbon model. The core is shown in dark gray, the nonconserved loops and terminal extensions are light, and the residues corresponding to antigenic sites as defined by monoclonal release mutations are shown in black. The bar represents a particle fivefold axis. In general, the bottom of the figure corresponds to the portion of the protein that is exposed on the inside surface of the particle; the top of the figure corresponds to the outside of the particle. (b) VP2--the long black polypeptide chain is VP4. The bar represents a particle threefold axis. (c) VP3--the bar represents a particle threefold axis. (d) A schematic representation of the core with the strands of the beta barrel designate b-i. (e) A stereo representation of a pentamer of capsid proteins. VP1 is light, VP2 is gray, and VP3 is dark gray.

FIGURE 2. The poliovirus particle. Top--a geometric figure (one of the stellated icosadodecahedra) that has the general shape of the poliovirus particle. The positions occupied by the subunits VP1, VP2, and VP3 that form a protomer (i.e., those subunits derived from a single molecule of P1) are shown. One of the 12 particle fivefold axes, 1 of the 20 particle threefold axes, and 2 of the 30 particle twofold axes are labeled as reference points. Bottom--a space-filling representation of the poliovirus particle. VP1 is light, VP2 is gray, and VP3 is dark gray (the colors appear darker toward the perimeter of the particle due to depth cueing). The orientation is similar to that of the schematic figure at the top.

TABLE 1 Antigenic Sites of Poliovirus

Site	Type 1	Type 2	Type 3
1 A	VP1 100[1a]	VP1 96-100[2]	VP1 91-102[3]
1 B		VP1 169[2]	VP1 168[3]
1 C			VP1 254[3]
2 A	VP2 164-170[3,4,5]		VP2 165-173[3b]
2 B	VP1 220-226[3,4,5]		
2 C	VP2 270[4,5]		
3 A			VP1 287-290[2,3]
3 B	VP3 58-60[4,5], VP373[4]		VP3 58-60[3c]
3 C	VP3 76[5]		VP3 73-79[3c]
3 D	VP2 72[4,5]		

1. Blondel et al., 1986.
2. Minor et al., 1986.
3. Minor et al., 1985.
4. Diamond et al., 1985.
5. Page et al., 1988.

[a]Observed only with antibody obtained by immunizing with heat-treated virus.
[b]Observed only with monoclonals obtained by immunizing with Finnish strain or trypsin-treated virus.
[c]Observed only with monoclonals obtained by immunizing with trypsin-treated virus.

TABLE 2 Properties of Virus and Related Particles

Treated	Native	Eluted	Heat
Antigenicity	N[a]	H	H[a]
RNA	+	+	-
VP4	+	-	-
pI	7.0	4.5	4.5
Trypsin sensitive	+/-	++	+++
Able to attach to cells	+	-	-
N-terminus of VP1[b]	inside	outside	outside

[a]By definition.
[b]As determined by reactivity to antipeptide antibodies and protease sensitivity.

TABLE 3 Titers of Type 1 Monoclonals to Virus and Related Particles

		Titer[a]		
Antibody	Site	Native	Eluted Virus	Heat-Treated Virus
C3	1A	6.7	6.5	6.7
4	2AB	6.8	4.2	< 2
6	2A	7.3	6.2	6.3
7	2A	6.4	4.4	5.1
9	3B	6.8	3.3	4
10	3CD	7.1	< 2	< 2
13	3CD	7.1	< 2	< 2

[a]Expressed as log (H^3-cpm) precipitated/μl of acites.

MOLECULAR APPROACHES FOR IMPROVEMENT OF THE THERMOSTABILITY OF THE ORAL POLIO VACCINES

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Introduction

Studies on polio vaccines have undergone a renaissance in recent years. A formidable array of new and powerful technologies has allowed virologists to probe the genetic and structural aspects of poliovirus function with unprecedented precision. Through the convergence of rapid nucleic acid sequencing, monoclonal antibody production, peptide synthesis, development of infectious recombinant cDNA clones of poliovirus genomes, and reverse genetics through site-directed mutagenesis, it has been possible to identify the sequence changes associated with attenuation (Nomoto et al., 1982; Stanway et al., 1984) and to systematically evaluate their relative contributions to the phenotype of reduced neurovirulence (Omata et al., 1986), as well as to identify the genetic determinants of antigenicity (Minor et al., 1986). Solution of the x-ray diffraction crystal structure of type 1 and type 3 (Hogle et al., 1985, 1987) poliovirions represents a milestone in our understanding of virion function. For the first time, capsid-associated primary structure information can now be evaluated in three-dimensional terms, and mutations altering capsid function and stability can be precisely mapped to the crystal structure. Thus we can now visualize the poliovirus antigenic sites as structures in three-dimensional space and can envision how mutational or conformational changes can alter their functions.

Armed with these new insights and scientific capabilities, prospects are excellent for application of these powerful technologies to the development of new polio vaccines and the improvement of existing vaccines. The paper by Hogle et al. (1987) illustrates how recent basic research information can contribute to the improvement of vaccine programs. In this brief discussion I wish to place the molecular findings within the context of the information currently available and evaluate the lessons and opportunities provided by the many diverse findings for the improvement of vaccine stability.

Overview of the Problem

Paralytic poliomyelitis remains a major public health problem in developing countries, where up to 400,000 cases occur annually (Assaad and Ljungars-Esteves, 1984). Outbreaks of poliomyelitis follow deficiencies in immunization. While insufficient immunity can occur for many reasons, the causes potentially most damaging to public confidence in immunization programs are breakdowns in the cold chain,

allowing children who had received multiple doses of vaccine to remain susceptible to poliomyelitis.

As pointed out by Sizaret (1987), the thermolability of the live attenuated oral polio vaccines makes them the weak link among the vaccines distributed through the Expanded Programme of Immunization. Although direct heat inactivation of the vaccine virus is presumably the most frequent cause of cold chain breakdown, other indirect mechanisms may play a role. The presence of 1 M $MgCl_2$ as a stabilizer in vaccine preparations depresses the freezing point of the vaccine virus suspensions. Freezers frequently used for vaccine storage (rated for -20°C) may, when operating in tropical conditions, cycle above the melting points of the vaccine preparations, thereby subjecting the virus to strongly inactivating freeze-thaw conditions.

The differential sensitivities of the vaccine strains to thermal inactivation (Sabin 1 $\geq$ Sabin 3 > Sabin 2; Mauler and Gruschkau, 1978) present an additional complicating factor. The trivalent polio vaccines are formulated at infectivity ratios (Sabin 1 > Sabin 3 > Sabin 2) that partially compensate for growth rate differences. Partial heat inactivation of a trivalent preparation generally enrich for the more rapidly growing Sabin 2 component, thereby increasing its potential to interfere with the replication of types 1 and 3 components during intestinal replication. As a result, many vaccinees may produce neutralizing antibodies preferentially or exclusively to type 2 polioviruses, the serotype least frequently associated with paralytic poliomyelitis in developing countries.

New Insights Offered by Crystallographic, Immunochemical, and Molecular Genetic Studies

The accompanying paper by Hogle et al. (1987) very elegantly describes the importance of conformation to the structure of poliovirus neutralization epitopes and the effects of conformational rearrangements upon antigenicity. I will briefly touch on their main points.

1. The poliovirus neutralization epitopes defined by mutations conferring resistance to monoclonal antibodies map to the surface of the crystal structure. The spatial relationships among mutants within each resistance class are consistent with the view that the crystal structure represents the native conformation of the virion to which neutralizing antibodies attach.

2. Three primary antigenic sites for binding neutralizing antibodies reside on the virion surface. The antigenic sites differ in their immunogenicities in different species and in their sensitivities to degradation under the physiological conditions found in the intestine. The binding of neutralizing antibodies to any one of the three major sites is sufficient to neutralize virus infectivity in vitro. Natural variation within the sites accounts for the observed antigenic differences among polioviruses.

3. More than one capsid protein contributes residues to the primary antigenic sites. As a consequence, poliovirus neutralization antigens are largely conformational in nature. Sites 2 and 3, more than site 1, are formed by noncontiguous intervals along the primary sequence brought together in three-dimensional space by polypeptide chain folding. The conformational nature of poliovirus epitopes explains the generally poor immunogenicity of synthetic peptides mimicking continguous intervals along the polypeptide chain.

4. Poliovirions undergo substantial conformational rearrangements. Various conditions of heat, extremes of pH, chemical denaturants, attachment to cellular receptors, and antibody binding can shift the equilibrium away from the native conformation.

5. Conformational rearrangements largely destroy native antigenicity. Conformational rearrangements alter the spatial relationships among the residues of the neutralization antigenic sites in as yet undefined ways, such that the majority of neutralizing antibodies cannot bind the virus. The immune system is presented altered antigenic structures that do not stimulate the formation of neutralizing antibodies. Some (presumably nonconformational) subsites may be conserved during conformational rearrangements. The immune response to these determinants may be insufficiently strong or broad to confer protective immunity.

6. Conditions stabilizing the native conformation conserve native antigenicity. An important stabilizing condition from the standpoint of the oral polio vaccines would be the alteration of virion structure to confer increased thermostability of virions. Revertants of the temperature-sensitive phenotype of the vaccine strains were found to be thermoresistant. The nature of the stabilizing mutations could be localized within the virion crystal structure and the effects interpreted in terms of alterations in hydration of a specific amino acid residue.

Approaches to Improve the Thermostability of the Oral Polio Vaccines

Several different strategies currently or potentially exist to enhance the thermostabilities of the polioviruses. It appears likely that integration of the lessons of many diverse observations and approaches can effect immediate practical improvements, while continued basic investigations promise very substantial forthcoming benefits.

Chemical

The observations of Wallis and Melnick (1962) on the protective effects of molar concentrations of $MgCl_2$ found early and wide application in the polio vaccine cold chain. Knowledge of the crystal structure of the poliovirion should enable us to better understand the precise molecular contributions of divalent cations to virion stability. Noting that the thermostability of the polio vaccines peaks

sharply at pH values near neutrality, Mauler and Gruschkau (1978) developed an alternative inorganic chemical stabilizer containing 300 mM sodium phosphate, pH 6.7, allowing storage of live polio vaccines at 0°C to 6°C for up to 12 months.

Molecular modeling of the interactions of organic compounds with the crystal structure of picornavirions opens the way for systematic design of stabilizers. For example, antiviral drugs such as arildone and WIN 51711 stabilize picornavirions against alkaline and thermal denaturation by binding stoichiometrically to VP1 (Smith et al., 1986). These drugs specifically bind to a pocket within VP1, locking the virions into a conformational state that allows attachment to cells but prevents uncoating. While the inhibitory effects of these agents in vitro and in vivo (McKinlay and Steinberg, 1986) would appear to preclude their use as stabilizers for live polio vaccines, detailed understanding of their mechanism of action may permit the design of second-generation stabilizers whose effects might be reversed under the physiological conditions encountered by the polio vaccines (e.g., acidic pH).

Genetic

Thermoresistant mutants of polioviruses have been studied for over 30 years (Youngner, 1957). When virion preparations are incubated at 50°C, heat-stable variants are easily selected from populations of all three poliovirus serotypes. The proportion of thermostable variants that are preexistent in unselected virus populations (approximately 10^{-5}) suggests that the resistant phenotype is conferred by one or at most two substitution mutations. While both heat and chemical denaturants such as urea destabilize the conformation of native antigenicity, thermal resistance and urea resistance apparently involve separate capsid mutations (Hallum and Youngner, 1966).

It would be very interesting to reexamine the thermoresistant mutants described by Youngner and others using the rapid methods of primer-extension genomic sequencing. It would be of particular interest to learn if the mutations selected at 50°C cluster within the same region of the crystal structure as the mutations described by Hogle et al. (1987) in thermoresistant revertants of the temperature sensitivity phenotype selected by growth at 39°C to 40°C. It would also be important to apply our newer molecular tools to critically evaluate the structural requirements for long-term stability at ambient temperatures (up to 37°C) as opposed to capsid alterations conferring thermostability at 50°C and replication at higher temperatures.

The classical approaches may not be entirely suitable for the development of polioviruses that have very high thermal resistances. Apparently acquisition of higher thermal resistance requires more radical structural changes than could be obtained by stepwise selection of spontaneous mutants (Hallum and Youngner, 1966).

A potential concern is the effect of mutations for heat resistance upon the phenotype of attenuation for neurovirulence. For both the

type 1 (Nomoto et al., 1982) and type 3 (Stanway et al., 1984) Sabin polio vaccine strains, changes in attenuation are associated with capsid alterations, which at least for the type 1 vaccine contribute to the attenuation phenotype (Omata et al., 1986). In this context it is relevant to recall the observations of Wallis and Melnick (1963), whose heat-resistant mutants of poliovirus type 1 Mahoney were found to be attenuated for neurovirulence in monkeys.

Protein Engineering

The advent of protein engineering provides systematic strategy for design and production of macromolecules with desirable properties. Here x-ray crystallographic data are indispensable for interpretation of initial observations and modeling for modification of protein function. Clues for the stabilization of protein and nucleoprotein structures are available from many diverse studies, a few examples of which are briefly described.

Hepatitis A Virus The virions of hepatitis A virus (HAV), a picornavirus distantly related to enteroviruses, are very heat stable. Thermal inactivation curves for HAV are displaced approximately 20°C higher than those of enteroviruses. In the presence of 1 M $MgCl_2$, HAV infectivity is maintained during heating at 80°C (Siegl et al., 1984). Comparisons of the crystal structures of poliovirus and HAV (studies are under way) should illuminate the molecular factors contributing to increased interprotomeric bond strengths, and it may be possible to engineer similar bonding domains within capsid structures having poliovirus antigenicities.

Plant Viruses The crystal structures of some highly thermostable (Francki, 1980) icosahedral RNA viruses of plants have been determined (Harrison et al., 1978). Their architecture is assembled from the presumably evolutionarily homologous eight-stranded beta barrel structures that also constitute poliovirions. While structural details and conformational dynamics are quite different from picornavirions, it may be instructive to critically analyze the number and nature of stabilizing bonds in these virions.

Other Thermostable Proteins. The structural bases of protein stability have been extensively investigated for small proteins and enzymes (reviewed by Privalov, 1982). Understanding the mechanisms of virus stability is complicated by the complexities of subunit cooperativity and the relative contributions of nucleic acids and other components to virion stabilities. Nonetheless, the properties of extremely thermostable proteins (e.g., those from organisms capable of growth at temperatures exceeding 80°C) offer some lessons to virologists armed with the crystal structure of a virus and may assist in the interpretation of results. Certain classes of amino acid substitutions are preferred in very thermostable proteins (Argos et al., 1979), which strengthen interactions without causing significant alterations of polypeptide conformation. Further, extreme thermostability involves multiple stabilizing factors: increased hydrogen bonding, additional hydrophobic interactions, and increased ion pairing (Walker et al., 1980).

Site-Directed Mutagenesis. The availability of infectious recombinant DNA clones of complete poliovirus genomes (Racaniello and Baltimore, 1981) opens the way for engineering of the capsid proteins through site-directed mutagenesis. Studies of this kind are under way in several laboratories, with the crystal structure of the poliovirion serving as a central point of reference. The feasibility of this approach to enhance certain biological functions has been repeatedly demonstrated with enzyme proteins (Ackers and Smith, 1985).

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NEW TECHNOLOGIES FOR PRODUCING ANTIGENS AND THEIR MERITS AS TEMPERATURE-STABLE VACCINES

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Wellcome Biotechnology Limited

Introduction

The availability of dry vaccines that would overcome the storage and transport problems inherent in many of the vaccines in use at the present time would provide considerable advantages in vaccination programs and in the day-to-day use of vaccines. These advantages were clearly demonstrated in the smallpox eradication campaign when freeze-dried vaccinia virus was used, and the success of the campaign can be attributed in no small measure to the availability of a vaccine that was as active at the time it was inoculated as when it left the laboratory where it was manufactured.

One of the criteria placed on the acceptability of the vaccine was its stability at 37°C over 1 month, conditions that are likely to be more severe than those normally met in the field. However, most viruses are not as robust as vaccinia virus, and with few exceptions the majority of attenuated vaccines are stored and transported "wet." This in turn involves a cold chain to maintain their viability, an imposition that is both costly and cumbersome. Similar considerations also apply to inactivated vaccines, which depend on the intactness of the virus particle for their potency (e.g., the Salk polio vaccine and foot-and-mouth disease vaccine).

With the advent of recombinant DNA technology, methods for sequencing nucleic acids, and the capability of synthesizing genes, the production of large amounts of proteins is now largely a matter for bioengineering rather than research. The immunogenic proteins of many viruses have now been identified and synthesized by the application of these techniques. Almost without exception however, these proteins, like their counterparts derived from the virus particles, have low immunogenic activity. Recent advances in the presentation of these proteins, notably in the form of immunostimulating complexes (ISCOMs), are likely to overcome the difficulties of low potency. Moreover, ISCOMs have the added advantages of not requiring an adjuvant and of being fully active after freeze drying, thus putting within our grasp at the same time increased potency and the possibility of a dry vaccine.

The objective of this paper is to provide a summary of the new methods for producing antigens. Because of their simplicity in structural terms relative to other microorganisms, emphasis will be placed on viruses and the lessons that have been learned from their study.

The Concept of Subunit Vaccines

Three important advances in the study of viruses that took place about 30 years ago form the basis of our present knowledge on virus antigens. The first was the growth of viruses in large amounts in tissue culture, arising from the need to provide vaccines against such diseases as poliomyelitis and foot-and-mouth disease. This in turn allowed the viruses to be purified and examined chemically, thus providing the opportunity to relate structure to function. The third was the visualization of viruses in the electron microscope, resulting largely from the introduction of the negative staining technique. Coinciding with these advances was the growing use of radioactive isotopes to label specific components, with the consequent streamlining of their chemical analysis.

From these early experiments it soon became apparent that protein subunits of viruses would evoke a protective response in experimental animals, albeit at a lower level than that provided by the intact virus particle. In most instances the examples were provided by those viruses containing a lipid envelope because these could be dissected readily by the use of mild detergents. Nevertheless, the so-called naked viruses could also be dissected with the retention of some immunogenic activity.

These experiments established the concept that the immunogenic activity of viruses was largely confined to an individual protein, although it left unsolved the problem of enhancing their activity to the level they possessed when forming part of the virus particle. In the 1960s little attention was being paid to the relationship between configuration and biological activity. However, as a result of the need to make the best use of genetically engineered proteins, it has become a topic of increasing interest, not only to those involved in vaccination but also to physical chemists. Once the chemistry underlying these relationships is solved, we can expect to see major advances in the use of genetically engineered proteins as vaccines.

The Concept of Peptide Vaccines

Anderer showed more than 20 years ago that short fragments from the C terminus of the coat protein of tobacco mosaic virus would elicit antibody that neutralized the infectivity of the virus. This observation was followed by the demonstration that a synthetic peptide with the same amino acid sequence would also elicit neutralizing antibody. This pioneering observation was followed by Sela's more extensive experiments on synthetic antigens, which led to the realization that it was possible to produce completely synthetic antigens by means of organic chemistry. With the development of nucleic acid sequencing methods and the cloning of DNA, the amino acid sequences of many immunologically important proteins became available in the late 1970s and early 1980s and led to an upsurge in interest in the possibility of producing synthetic immunogens. This was demonstrated very clearly by Lerner and his group who, in collaboration with my group, synthesized a peptide that protected experimental animals against infection with foot-and-mouth disease virus.

In this approach it is necessary to identify the immunogenic site of the relevant protein. Several approaches have been made to this problem (Table 1), and a combination of these methods has resulted in the identification of synthetic peptides for foot-and-mouth disease, hepatitis B, influenza, and polioviruses. This approach has many commendable features, the principal of which is the indefinite stability of the product if kept dry and the fact that it can be delivered by a delayed-release mechanism under conditions that allow its biological activity to be retained. Moreover, the flexibility of the peptide approach, allowing the substitution of amino acids at positions on the sequence that confer distinct immunological properties, has much to commend it.

The Concept of Anti-idiotypes

Idiotypes are unique epitopes within the variable regions of immunoglobulin molecules induced by inoculation of antigens. The basis for using anti-idiotypes to induce an immune response is the demonstration that some anti-idiotypic antibodies can represent an internal image of the antigen that was used to elicit the original antibody response. Thus, immunization with the anti-idiotypic antibodies can lead to antibodies that react with the original antigen.

A number of observations indicate that the injection of anti-idiotypic antibodies can prime B-cells and enhance their response to a subsequent exposure to the corresponding antigen. Moreover, in some systems such as tobacco mosaic virus, a specific immune response can be obtained without injection of the corresponding antigen. Although the mechanisms involved are still obscure, the potential of anti-idiotype vaccines must be considered seriously when it is necessary to (1) induce an immune response when the corresponding antigen is not easily available, (2) enhance by priming when the corresponding antigen is a poor immunogen, and (3) avoid the use of adjuvants.

Production of Immunogenic Proteins and Peptides

Several methods are now available for producing an immunogenic protein. The first step is identification of the gene coding for it. With DNA viruses the appropriate sequence can be used directly. With RNA viruses the sequence must first be transcribed into the complementary DNA before insertion into the appropriate expression system.

Antigenic proteins can be expressed either in vitro or in vivo. Viral proteins have been produced in vitro in bacterial, yeast, and mammalian cells and in bacterial and insect viruses. *E. coli* cells were the first to be used for this purpose because their biochemical genetics were so well understood. In some instances the yield of foreign protein expressed accounts for as much as 20 percent of the total cell protein. However, the bacterial cell system suffers from the fact that the expressed proteins are not glycosylated, a severe

handicap with surface projections of lipid-containing viruses that are invariably glycosylated. Recently, yeast and animal cell systems have received more attention because they overcome this difficulty. Bacterial viruses (e.g., phage) and the baculovirus Autographa Californica nuclear polyhedrosis virus have also received considerable attention, and the latter system is currently being studied extensively because of the reported extremely high yields of the product of the inserted gene. The baculovirus system has the added advantage that the virus grows well in caterpillars, so that a tissue culture system is not required.

In all these methods the expressed protein has to be separated from the cellular material. This can be done, even on a large scale, by the use of either specific antibody or affinity columns. Clearly this step, although technically feasible, adds to the cost of the process.

The in vivo approach to the production of immunogens has also attracted considerable attention during the last few years. This method depends on the creation of recombinant infectious agents by inserting foreign genes into such organisms as Salmonella typhimurium (Ty 21a) and adeno, herpes, and vaccinia viruses. Although a considerable amount of work has been done with S. typhimurium, by far the greatest amount has been done with vaccinia virus for several very good reasons: (1) it was a highly successful and relatively innocuous vaccine; (2) it was easily prepared, very cheap, and could be made by the extremely simple technology of growing it in calf lymph; (3) it did not require medical personnel for its administration; (4) a single injection gave long-lasting immunity; and (5) it is a highly stable vaccine when freeze dried, losing little or no activity when kept at 37°C over long periods. In the context of this meeting this last point is extremely important.

Production of Peptides

Peptides can be produced by three main methods that depend on (1) organic chemistry, (2) enzyme chemistry, and (3) recombinant DNA technology. The first method was devised by Bergmann in the 1930s and is based on the stepwise addition of amino acids to each other, isolating the correct intermediate at each step in the synthesis. Originally, this type of synthesis was performed in solution, and the intermediates were isolated by crystallization, but the introduction of solid-phase synthesis by Merrifield in the early 1960s, with the consequent elimination of the need to isolate the growing chain at each step, has not only made peptide synthesis a much easier procedure but has also rendered it amenable to automation.

The second method relies on the use of enzymes to link the amino acids in a stepwise manner. This method avoids the use of the protected animo acids that are required in the chemical synthesis, but it is still at the experimental stage. The bottleneck in the use of this method is the need to work out the conditions necessary for each step of the synthesis that will be different for each peptide. In contrast, chemical synthesis relies on a general method of attachment of each amino acid to the growing chain.

Synthesis by recombinant DNA technology is dependent on the availability of the DNA coding for the amino acid sequence. With the development of methods for chemically synthesizing genes, together with the means to express these sequences in E. coli cells, for example, the microbiological synthesis of peptides can be achieved in the same way that proteins are produced. There are already several examples in which peptides have been synthesized as part of a fusion protein (e.g., with ß-galactosidase), but there is no reason the peptide cannot be synthesized with appropriate cleavage sites to allow it to be hydrolyzed free from the carrier protein. Isolation could then be achieved with an antibody column.

Presentation of Immunogenic Proteins and Peptides

Antigenic proteins and peptides can now be produced in large amounts by a variety of biochemical, microbiological, and chemical methods. In most instances, however, these proteins are not immunogenic and their activity in eliciting protective antibodies is much less than that elicited by the protein when it forms part of the virus particle. It is clearly necessary, therefore, to overcome this problem if we are to gain the full benefit of the sophisticated recombinant DNA technology that has been developed.

With proteins several approaches to the problem have been made, but by far the most successful and reproducible is the ISCOM described by Morein and his colleagues. The cage-like structure obtained by reacting the viral proteins with the plant glycoside saponin are as active as the virus particles themselves. The added advantages of stability to freeze drying and activity without an adjuvant make ISCOMs the best candidates so far described for the presentation of antigens to the immune system.

So far the success with the glycosylated proteins has not been demonstrated with proteins that are not glycosylated. Nevertheless, it would seem likely that this problem is not insurmountable.

When a protein forms part of a structure that is rigid, such as poliovirus or foot-and-mouth disease virus, release of the immunogenic proteins from the constraints imposed by the architecture of the virus results in a major alteration in its configuration. The released proteins have extremely low immunogenic activity compared with the virus particle, and so far no methods have been devised for increasing this activity.

Because peptides are much smaller and therefore more likely to be amenable to manipulation, the possibility of using them as immunogens has been explored with several viruses during the last 5 years. With foot-and-mouth disease it has been shown that a carrier protein is not necessary, and the dried peptide can be used in a delayed-release mechanism without loss of activity. In some experiments the implanted peptide, released into the muscle 28 days after the primary injection, was as effective as a similar amount delivered by syringe, providing a clear demonstration of its stability at body temperature and its value for booster vaccinations.

The in vivo presentation of foreign antigens as products of recombinant vaccinia virus replication is clearly a desirable goal. However, the question of configuration of the expressed antigens in this context has not been discussed. It seems to the author that this is an outstanding issue that requires further study.

Prospects for Dry Vaccines

The problems involved in producing antigenic proteins and peptides in large amounts have now been solved. The outstanding problem that remains in converting them into immunogenic products is their presentation to the host. With the exception of the surface protein of hepatitis B virus, the proteins of other viruses, whether isolated from virus particles or produced by genetic engineering, have been disappointing in their immunogenic activity. It is clear that the conformation of the proteins is critical in achieving the desired response. This problem appears to have been solved fortuitously for the surface projection proteins of several lipid-containing viruses by incorporating them into ISCOMs by the use of the plant glycoside saponin. Moreover, these cage-like structures can be freeze dried without loss of activity and do not require added adjuvant. It still remains to be shown that the corresponding genetically engineered proteins behave similarly.

Peptides present somewhat different problems. Although it has been shown that small quantities of uncoupled peptides will evoke a protective immune response, an adjuvant has been necessary to elicit this response. However, it should not be difficult to incorporate a synthetic peptide into an ISCOM, which will enable the necessary level of immunity to be achieved. Moreover, peptides can be biosynthesized as part of a fusion protein with, for example, ß-galactosidase. In our experience these constructs have a dramatically enhanced activity and are still as active after freeze drying. By incorporating such constructs into ISCOMs, it should be possible to prepare highly active vaccines.

The use of vaccinia virus as a vector for the expression of foreign antigens has been the subject of much scientific and lay attention since it was first described 5 years ago. Since vaccinia virus itself can be freeze dried and stored at temperatures as high as any likely to be encountered in the field without any loss of activity, this seems to be an obvious route for the provision of dry vaccines. The major difficulty is the possible side effects of the recombinant viruses. Despite reassuring scientific predictions it will be necessary to conduct carefully controlled trials before such vaccines are acceptable. Since the proportion of recipients suffering side effects following "normal vaccination" is so small, and the predictions are that the recombinant viruses will cause even fewer problems, it is difficult to see how such a trial could be conducted that would be statistically meaningful. Nevertheless, such an approach is vital if we are to make use of such a potentially powerful weapon in the control of a variety of diseases.

Even with vaccinia virus as a vector, however, one is still left with the problem of configuration. As far as I am aware this limitation has not been addressed. Recombinant vaccinia viruses are essentially producing foreign antigen in vivo rather than in vitro. The site of replication may well be of great importance and need not necessarily be the same for different antigens.

Despite these shortcomings there is a strong probability that dry vaccines will be available in the foreseeable future. Coupled with the possibility of delayed-release mechanisms becoming available, one can conjure up the vision of one injection incorporating booster shots, thus obviating the need for several visits to the clinic. This in itself would be no small achievement.

TABLE 1 Identification of Immunogenic Sites on Proteins

1. Measure biological activity of fragments.
2. Identify regions accessible to water.
3. Interpret the secondary structure (e.g., helical regions, amphipathic regions, flexibility).
4. Relate antigenic variation to amino acid sequence variation in naturally occurring and laboratory-derived variants.
5. Measure the antigenic activity of proteins expressed from fragments of the coding gene.
6. Map synthetic fragments of the protein with neutralizing antibody.

TABLE 2 Advantages of a Peptide Vaccine

1. Product chemically defined.
2. Stable indefinitely.
3. No infectious agent present.
4. No large-scale production plant required.
5. No downstream processing required.
6. Can be designed to stimulate appropriate immune responses.
7. Provides opportunity to use delayed-release mechanisms.

USE OF ANTIGENS PRODUCED BY RECOMBINANT DNA TECHNOLOGY WITH A SAFE AND EFFICACIOUS ADJUVANT FORMULATION IN A NEW GENERATION OF VACCINES

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Live attenuated viruses and bacteria have produced useful vaccines but with the risk of severe infections in recipients whose immune responses are deficient. The same is likely to be true if vaccinia virus is applied on a wide scale as a vector for antigens of other organisms. A safer strategy is to use inactivated viruses or bacteria in vaccines or preferably major antigens produced by recombinant DNA technology, together with an efficacious adjuvant formulation. The development of an adjuvant formulation comprising a synthetic analog of muramyl dipeptide in an emulsion of squalane and a Pluronic block copolymer is described. Various protein and glycoprotein antigens administered in this adjuvant formulation elicit cell-mediated immunity and the formation of antibodies of protective isotypes. Used with inactivated viruses or viral antigens produced by recombinant DNA technology, it has elicited protective immunity against feline leukemia virus, simian acquired immune deficiency syndrome (AIDS) virus, and herpes simplex virus in guinea pigs. In experimental animals the adjuvant formulation markedly augments immune responses to inactivated influenza virus antigens, as well as hepatitis B virus (HBV) and human immunodeficiency virus (HIV) surface and core antigens produced by recombinant DNA technology. Using the monoclonal immunoglobulin product of a murine B lymphoma with the adjuvant formulation, protective immunity against the tumor has been elicited. If the adjuvant formulation proves to be as safe and efficacious in humans as already shown in experimental animals, it could become a component of a new generation of vaccines against viruses and other infectious agents and some malignancies.

Introduction

In 1987 the World Health Organization (WHO) celebrated the tenth anniversary of global eradication of smallpox. That dramatic achievement illustrates the power of vaccination against a disease for which no effective chemotherapy was available. Other successes of vaccination, in some cases reinforced by public health measures, include marked reduction in morbidity and mortality due to yellow fever virus in Africa and Central America; greater than 98 percent reduction in morbidity due to six of the seven principal diseases for which vaccines are used in the United States (diphtheria, measles, mumps, pertussis, paralytic poliomyelitis, and rubella, including congenital rubella syndrome); and 88 percent reduction in the incidence of the seventh, tetanus (Hilleman, 1985).

A major current aim of WHO is extension of such an immunization program to developing countries. Yet the need for new and improved vaccines remains as great as ever. Now that the magnitude of AIDS as a global problem is emerging, the great potential value of a vaccine against the human immunodeficiency virus type 1 (HIV-1) that causes AIDS is evident. The spread in many tropical countries of strains of malaria parasites resistant to available drugs has intensified the search for a malaria vaccine. The ability of immune responses to control the growth of virus-induced tumors (Allison, 1980) and some other tumors (Kripke, 1984), the definition of tumor-specific antigens and growth factors, and the feasibility of producing them by biotechnology raise the hope that vaccines may also be useful to prevent certain types of malignant disease and, conceivably, as a modality of cancer therapy.

Mechanisms of Protective Immunity

The advances made in immunology during the last few decades allow greater understanding of the principal mechanisms by which host animals are protected against different types of infectious agents. In many cases it is sufficient to elicit the formation of antibodies, for example, against diphtheria or tetanus toxoids, the capsular polysaccharides of bacteria, and viruses such as poliomyelitis. However, antibodies of isotypes that activate complement and act synergistically with antibody-dependent effector cells (IgG2a in the mouse and rat, IgG1 and IgG3 in the human) often confer better protection than antibodies of other isotypes (Wechsler and Kongshavn, 1986). For some virus infections, such as herpes viruses and tumor-inducing viruses, cell-mediated immunity plays a major role in host defense. Sensitized T-lymphocytes can lyse virus-infected or virus-transformed autologous cells and protect the host in other ways. To vaccinate effectively against this type of virus or against tumor antigens, it is necessary to elicit cell-mediated immunity. The same appears to be true of immunity to bacteria that can multiply inside cells, such as *Mycobacterium tuberculosis* and *M. leprae*.

Strategies for Vaccine Development

Live Attenuated Organisms

Live attenuated viruses and bacteria elicit both cell-mediated and humoral immune responses. Their efficacy is established in several important vaccines (vaccinia virus, yellow fever virus, oral poliomyelitis virus, measles virus, rubella virus, and *M. bovis* BCG). However, there is a risk of severe infections in persons with congenital or acquired immunodeficiency. Inherited immunodeficiency can be general (children with defective T-lymphocyte function have severe, often lethal, vaccinia or BCG infections) or inability to respond well to certain antigens while responding well to others, as discussed below for hepatitis B virus surface antigen. Children in developing countries often have defective T-lymphocyte function because

of malnutrition and parasitic infections (Dowd and Heatley, 1984). Measles is a more severe disease in African than in North American or European children (Hull et al., 1983), and even vaccine strains of measles virus can produce severe infections in African children.

It has been suggested that a second type of HIV (HTLV-IV or HIV-2) isolated from West Africans is avirulent (Kanki et al., 1986). This isolate has been considered as an AIDS vaccine, but a closely related virus can be associated with immunodeficiency in some individuals (Clavel et al., 1986). Establishing that an attenuated HIV is completely safe, and would not in some recipients produce immunodeficiency or central nervous system infection, is close to impossible.

During the past few years there has been interest in vaccinia virus as a vector for antigens of other organisms, including HIV. However, we believe that regulatory authorities will be reluctant to use vaccinia vectors in humans in North America, Europe, and Japan and that having a different moral standard for highly developed and less developed nations is undesirable. Moreover, widespread use of vaccinia vectors in developing countries, where immunodeficiency is common, will produce some severe infections. If that is the only way to prevent a disaster such as an epidemic of AIDS, the benefit may well outweigh the risk. However, if efficacious vaccines can be developed in other ways, the benefit can be obtained without the risk.

Antigens Produced by Peptide Synthesis or Recombinant DNA Technology

An alternative strategy is to use inactivated viruses, subunits provided by recombinant DNA technology, and/or antigens produced by peptide synthesis in vaccines. In view of the possible biological effects of even fragments of nucleic acids, the use of antigens free of nucleic acid contamination is obviously desirable. While antibodies against peptides are valuable research tools, it is difficult with peptides to reproduce conformational determinants, and cooperative binding of antibodies to several determinants on a protein may be required for optimal protection. The importance of three-dimensional structure is illustrated by conversion of the core antigen of hepatitis B virus into the e antigen by treatment with sodium dodecyl sulfate (MacKay et al., 1981). Despite much research on peptides as potential vaccines and a great deal of discussion of the subject (Porter and Whelan, 1986), we are not aware of a single peptide vaccine in use or even in development.

In contrast, vaccines using recombinant viral subunits have demonstrated efficacy and are licensed for use in vaccines. The prototype is hepatitis B virus surface antigen (HBsAg) cloned and expressed in yeast in a form physically and antigenically resembling the 22-nm particles in serum. This was shown to immunize chimpanzees against infection with HBV, and, following trials in several groups of patients (Hilleman, 1985; Scolnick et al., 1984), vaccines based on such preparations of recombinant HBsAg have been approved for use in

the United States. In some cases glycosylation may be necessary for recombinant proteins to attain conformation comparable to that in intact virions and on infected cells. Expression in mammalian cells ensures the correct type of glycosylation, as in the case of glycoprotein D of herpes simplex virus (Berman et al., 1985).

Adjuvants

Although it is possible in principle to produce many microbial antigens by recombinant DNA technology, their full promise in vaccines will not be realized unless they are used with an effective adjuvant. The only adjuvants authorized for human use are aluminum hydroxide and aluminum phosphate. Although these are sufficient for many vaccines, comparative studies show that Freund's complete adjuvant (FCA) is often more efficacious in eliciting CMI in experimental animals and also protection against challenge with viruses and other infectious agents. The objective of our research was to develop an adjuvant formulation with efficacy comparable to FCA but without unacceptable side effects. These include muscle damage or granulomatous reactions at injection sites, pyrogenicity, anterior uveitis, arthritis, and vasculitis (Allison et al., 1986).

The adjuvant-active component of the mycobacterial cell wall in FCA is muramyl dipeptide N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) (Berman et al., 1985), which is pyrogenic and has other undesirable effects (Allison et al., 1986). Of more than 130 synthetic analogues of MDP tested, we found two that were highly efficacious adjuvants and showed good separation from side effects. One of the these, N-acetylmuramyl-L-threonyl-D-isoglutamine, was selected (Allison and Byars, 1986). It is a potent inducer of the formation of interleukin-1 by monocytes and macrophages, and we believe that its mode of action is to induce the release of a cascade of lymphocyte growth factors.

The second requirement was for a formulation that would optimize the interaction of antigens with the surface of antigen-presenting cells, in the lymph nodes of the drainage chain from injection sites, including interdigitating cells (which present antigens to T-lymphocytes) and follicular-dendritic cells (which present antigens to B-lymphocytes). This was achieved by formulating a nonionic detergent with unusual properties, Pluronic L121 triblock polymer, with squalane and a small proportion of an emulsifying detergent, Tween 80. L121 consists of a central block of polyoxypropylene, which is hydrophobic, flanked by two small blocks of polyoxyethylene, which is hydrophilic because of the hydrogen-bonding capacity of its oxonium ions. Electron micrographs show that the formulation consists of spherules on the surface of which antigens are retained. We postulate that such retention occurs because of the amphipathic character of many antigens (balance of hydrophilic and lipophilic groups) and their capacity to form hydrogen bonds with polyoxyethylene. L121 activates complement by the alternative pathway, and C3b on the surface of the spherules facilitates binding to follicular dendritic cells and interdigitating cells, following which antigens are transferred to the surfaces of those cells.

Both the MDP analog and Pluronic formulation are required for optimal adjuvant activity: the combination is termed Syntex Adjuvant Formulation-1 or SAF-1 (Allison and Byars, 1986). This formulation does not produce tissue damage at injection sites (unlike saponin, which is cytolytic) or elicit a granulomatous reaction at those sites (unlike aluminum salts), and no systemic reaction is demonstrable. A variety of viral subunits, monoclonal immunoglobulins, and other antigens, when administered intramuscularly or subcutaneously in SAF-1, have been found to elicit high titers of antibodies and CMI. The antibodies are predominantly of isotypes that interact with complement and antibody-dependent effector cells (e.g., IgG2a in the mouse), which are required for protection against many microorganisms and tumors (Wechsler and Kongshavn, 1986). CMI is also elicited, as shown not only by delayed-type hypersensitivity but also by proliferative responses of T-cells to antigen and production of interleukin-2, as well as specific genetically restricted lysis of target cells bearing the immunizing antigen.

Examples of the Use of SAF-1 to Elicit Immunity to Viruses

Our first practical success was the development of an efficacious vaccine against feline leukemia virus (FeLV) (Braemer et al., 1984). When formalin-inactivated virus was administered to 32 cats in SAF-1, persistent viremia following challenge was seen in only 6 percent, in contrast to 75 percent of controls receiving the same challenge. Only animals with persistent viremia develop lymphocarcoma and other late manifestations of the disease. The same antigen administered in conventional adjuvants (alum/Quil A) elicited significantly less protection. This enabled us to develop the first effective commercial vaccine against a retrovirus.

The same protocol was used in collaborative experiments carried out by Marx et al. (1986) with the simian AIDS oncovirus in rhesus monkeys. Again, excellent protection was observed: five monkeys immunized with antigen in the adjuvant formulation had no viremia following challenge and one had transient low viremia; all six remain healthy. In contrast, all six control animals receiving the adjuvant formulation with no antigen became viremic and three have died. A possibly analogous human situation would be in immunization against HTLV-1, which is prevalent in parts of southern Japan, Africa, and other countries and is associated with the pathogenesis of adult T-cell leukemia and a progressive neuropathy (Gessain et al., 1985). It seems likely that vaccination with cloned viral surface and core antigens in our adjuvant formulation would protect against this oncovirus, which is now beginning to spread in Europe and North America.

In contrast, an attempt to vaccinate rhesus monkeys against HTLV-III, using a lower dose of inactivated virus antigen in the adjuvant formulation, did not protect the animals from viremia, although high levels of antibodies were produced (Letvin et al., 1987). Further experiments are in progress using higher concentrations of better defined antigens. We are especially interested in the possibility that cell-mediated immune responses to core antigens

(products of the gag gene on the surface of infected cells) may be protective, since these antigens are better conserved in different isolates.

Among the conventional virus vaccines that we have attempted to improve with adjuvant formulation is that against influenza virus. The inactivated virus vaccine elicits satisfactory responses in most adults but not in persons over 65, who are especially susceptible to the effects of the virus. We find that in mice and guinea pigs submicrogram doses of influenza vaccine in SAF-1 elicit optimal antibody responses (Allison and Byars, 1986), and these are seen in 3-week-old and aged animals. We hope that these responses, unequivocally superior to those observed with conventional vaccine, will translate into more economical use of antigen in humans and, especially, better protection of elderly people.

We have also collaborated with colleagues outside Syntex in attempts to improve vaccination against hepatitis B virus (HBV). The first-generation vaccine included antigen derived from serum (Hilleman, 1985), and the second-generation vaccine is surface antigen (HBsAg) produced by recombinant DNA in yeast (Scolnick et al., 1984). Three doses of these vaccines elicit levels of antibodies against HBsAg believed to be protective in about 95 percent of healthy North Americans and Europeans. In susceptible persons (drug addicts, promiscuous homosexuals, and infants) responses are lower. This may be due partly to inherited low responsiveness to HBsAg determinants, which has been shown in experimental animals (Milich and Chisari, 1982), and partly to suboptimal immune responses of those persons to many antigens. Experimental animals with low responses to HBsAg determinants can respond well to pre-S determinants (Milich et al., 1985), so the first problem might be overcome by expanding the repertoire of antigenic determinants by including pre-S, which has been expressed with HBsAg in yeast, and core (HBcAg), which has been produced by recombinant DNA technology in *E. coli* (Murray et al., 1984). The core antigen is expressed on the surface of infected human hepatocytes and is the principal target of cytolytic T-lymphocytes (Mondelli et al., 1982). Nonspecific low response might be avoided by the use of a more efficacious adjuvant. We and K. Murray have found that in experimental animals humoral and cellular responses to recombinant HBsAg and HBcAg in SAF-1 are comparable to those with FCA and superior to those with alum. Thus a third-generation vaccine can be envisaged using S+, pre-S, and/or C antigens administered in the type of adjuvant formulation we describe.

In principle HBV can now be controlled in industrialized countries, but the main need is in less developed countries (e.g., Southeast Asia and Africa), where persistent infections are still highly prevalent and strongly associated with chronic liver disease (cirrhosis) and cancer (hepatocellular carcinoma) (Beasley and Hwang, 1984). In Taiwan immunization of infants can prevent acquisition of HBV from mothers after birth, which is otherwise common and usually results in persistent infection (Beasley et al., 1983). Widespread use of a third-generation HBV vaccine could prevent one of the world's most common cancers.

Other highly prevalent virus-associated cancers are nasopharyngeal carcinomas and Burkitt lymphomas in Southeast Asia and Africa. M. A. Epstein and A. J. Morgan have used a purified surface antigen from the herpes virus in question, the Epstein-Barr virus, in FCA to immunize susceptible subhuman primates. They have found the same antigen in SAF-1 to elicit high levels of antibodies in mice and are currently investigating whether such a vaccine is protective in cottontop tamarins. If that proves to be the case, a vaccine against these malignancies may be feasible. Applying it on the necessary large scale and demonstrating efficacy will present logistic difficulties and take a long time, but it is likely that the major scientific problem will have been solved.

The same may already be true of herpes simplex virus (HSV). Among several candidate vaccines we shall discuss only one, of which we have personal experience. P. Berman et al. (1985) of Genentech has cloned in CHO cells a major surface antigen common to HSV-1 and HSV-2 (glycoprotein D or gpD). They found that guinea pigs immunized with gpD in FCA were strongly protected against vaginal infection with HSV-2. In a single experiment so far carried out with gpD in SAF-1, we have obtained similar results: protection against challenge was greater than that obtained with a potent antiviral drug, and in the immunized animals the virus did not spread to spinal ganglia, the source of recurrent infection (Table 1). These exciting results raise the possibility that gpD should be included in a first vaccine package for young children to protect against HSV-1 and as a booster in a second vaccine package for late teenagers to protect against HSV-2. The vaccines should reduce virus multiplication in the oral and genital sites of these two primary infections and the probability of spinal ganglion infection and recurrence, which produces substantial morbidity and is a major factor in herpes virus transmission in the community.

Vaccination Against Malignancies

Together with R. Levy of Stanford and his colleagues, we are investigating the possibility that SAF-1 can also be used with nonviral antigens of malignant cells. The monoclonal immunoglobulin (McIg) products of B-lymphomas have been used as antigens. Mice immunized with the McIg coupled to a carrier in SAF-1 proved resistant to tumor challenge, and tumor-bearing mice could still be immunized, so that recoveries were observed after chemotherapy under conditions when the latter alone did not cure. Using a similar protocol human McIgs elicited anti-idiotypic cellular and humoral immune responses in cynomolgus monkeys.

If approval of regulatory authorities is obtained, it is planned to extend these studies to humans during the next few months. Toxicology studies of the adjuvant formulation in laboratory animals, including subhuman primates, have not revealed unacceptable side effects. The projected studies should establish whether the adjuvant formulation is safe and efficacious in humans. We are currently also evaluating whether immunization of mice with TGF- , a product of many solid tumors that is present on their plasma membranes and acts as an autocrine

growth factor, can protect them against tumor transplants. If these experiments prove successful, other defined tumor antigens will be tried, and a new approach to cancer therapy may be feasible.

Formulation and Stability

We have developed a convenient method to produce the Pluronic/squalane component of the adjuvant formulation on a large scale at pH 7.0 in preparations that show no detectable change when maintained at 4°C or 20°C for 1 month. Studies at high temperatures are ongoing. The MDP analog is very stable at pH 5.0 and fairly stable at pH 7.0. We plan to have two components of the vaccine, which can easily be mixed in a syringe just before injection. The components selected will depend on the stability of the antigen: if the antigen is stable in solution at pH 5.0, the antigen and MDP analog in solution will be one component. Otherwise the antigen and MDP analog can be freeze dried together. We are also ascertaining whether the Pluronic/squalane at pH 5.0 is stable, in which case a single-component vaccine can be prepared with stable antigens. Although this developmental work is ongoing, the results obtained so far are encouraging enough for us to believe that it will be possible, without much more elaboration, to produce the adjuvant formulation in vaccines that are sufficiently stable to survive all but the most extreme conditions.

Acknowledgments

We are indebted to the chemists who synthesized MDP analogs (J. Moffatt, J. Nestor, G. Jones, M. Venuti), to R. Cohn for the electron micrograph, to G. Nakano and M. Welch for technical assistance, to collaborators mentioned in the text and references, and to T. Matthews and E. Fraser-Smith, who collaborated in the experiments with herpes simplex virus.

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TABLE 1 Efficacy of Vaccination of Guinea Pigs with Recombinant Glycoprotein D of Herpes Simplex (HSV-2) Against Genital Infection with HSV-2

Group	Number of Animals	Percent Dead	Percent with Genital Lesions	Mean Lesion Score	Percent Ganglion Infection
Untreated	40	30	93	2.5	30[a]
Vaccinated[b]	10	0	40	0.08[c]	0

[a]This is an underestimate because it does not include animals that died, presumably from central nervous system infections. The probable infection rate of nerve ganglia was 60 percent.
[b]30 μg glycoprotein D was given subcutaneously per animal once at 5 weeks and once at 7 weeks of age.
[c]Lesion scores are a measure of the severity of the infection calculated from the overall mean from the daily mean lesion scores over a 14-day period. The difference of the vaccinated from the control group was statistically significant ($p < .05$, Mann Whitney U test).

SOURCE: Unpublished observations of A. Allison, P. Berman, N. Byars, E. Fraser-Smith, and T. Matthews.

STABILIZED VACCINES FROM THE PRACTICAL PERSPECTIVE

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The Expanded Programme on Immunization (EPI) has established a target of immunizing all children in the developing world against six diseases by 1990. These diseases are measles, poliomyelitis, diphtheria, pertussis, tetanus, and tuberculosis. Most recently, a seventh vaccine has been added, that against hepatitis B. Three of the seven vaccines--measles, poliomyelitis, and tuberculosis--are made of live organisms, and four consist of subunits, toxoids, or killed whole organisms: diphtheria, pertussis, tetanus, and hepatitis B. Additional vaccines might be included in the EPI in the future. The central limitation for delivering these vaccines is the need or desire for improving temperature stability, so as to limit or eliminate the requirement for the cold chain. This imposes a wide diversity of problems for which there can be no single answer and, above all else, no simple solution.

Fred Brown has presented an excellent review of newer technologies that are providing and will continue to provide the basis for new or improved vaccines. His central idea, I believe, is that (a) most, if not all, future vaccines may well be immunogenic proteins or peptides or even anti-idiotypic antibodies that could offer the chance for stabilization by drying and that (b) the immunogenicity of such substances can be enhanced by incorporation into optimal presentation forms such as immunostimulatory complexes (ISCOMs), which can also be lyophilized.

Anthony Allison also has made a most excellent presentation covering new approaches to enhancing immunogenicity of antigens through application of appropriate antigen-presenting substances and adjuvants. All these have merit in offering means to enhance immune responsiveness, but they do not of themselves provide a solution to temperature preservation of antigens. Of special note was Dr. Allison's description of his squalene-pluronic polymer-MDP-T adjuvant, SAF-1, which I believe may be a giant step forward in the problem of immunoenhancement and adjuvantation.

Future solutions aside, the most urgent problem or hope for today is how to deliver vaccines for the EPI that would expose them to elevated temperatures of variable and undefined height and duration. This hope for temperature stabilization suffers from two problems. First, there is the desire to take the product beyond the confines of even physiologically normal temperatures. Second, there is the assumption of a technical data base that simply does not exist.

The simplest way to look at a vaccine, perhaps, is a collection of immunologic determinants or epitopes that are presented to the

recipient in live and nonliving form. Such biologicals, whether live, killed, or subunits of organisms, are made of organic polymers that inherently are not stable. To preserve their function and activity, these organic chemicals must retain not only their primary structure but also their more subtle secondary and tertiary structures and interrelationships if their appropriate activities are to be retained. The rate for loss of biologic integrity and activity is largely temperature dependent, especially when enzymes are present that break covalent bonds. As a general rule, subunit structures are more stable than whole organisms, which in turn are more stable than living agents, but there can be striking exceptions.

The principles applied to the preservation of existing vaccines are based on empiricism backed up by historic precedents that, in practice, are no more than rules of thumb, influenced here and there by a spark of intuition. The available information on lyophilization, stabilization, and preservation of biologicals fills rows of shelves in libraries. Based on review of the available literature, one can prepare a general table of biologics' likes and dislikes (Table 1). Review of these attributes provides no real insights other than to emphasize a few points that are pretty much conventional wisdom.

The overriding truth is that biologicals are highly structured chemicals, and the objective is to allow them either to retain or at least to regain their original shapes and interrelationships if altered, as by drying. Excessive removal of water on lyophilization, for example, might destabilize their structures and prevent their assuming their original shapes on rehydration. Preservation of biologics' functions depends not only on covalent bonding but also on secondary and tertiary structures of chemicals that are folded and weakly held together by hydrogen bonding. Retention of glycosyl groups may be essential in some instances to assure appropriate folding that in turn may be needed to assure appropriate presentation of immunologic determinants (epitopes). Formaldehyde can help stabilize secondary structure of proteins by cross-linking free amino groups. Oxygen and oxidants destabilize proteins by their action on the sulfhydryl group of cysteine, causing cross-linking and distortion. Methionine contains sulfur in a thioether linkage; on oxidation to a sulfoxide, the hydrophobic residue becomes hydrophilic, causing distortion of the secondary structure of a protein. All of these adverse effects are promoted by increase in temperature. These are the problems, challenges, and guidelines that need to be addressed in attempts to resolve the problems of temperature stability.

It is perhaps tempting to leapfrog the current problems with the idea of creating new products by new technologies that might be hoped to obviate the need for resolving present needs for old vaccines. My belief is that future technologies will provide new and greater problems for EPI that are already unaffordable in terms of costs. This will be greatly amplified if and when a vaccine against acquired immune deficiency syndrome (AIDS) is developed, with its inevitable imperative for universal immunization.

It is worthwhile to take a brief overview of certain practical realities for useful application of some of these new technologies

(Table 2). It is basic that vaccines, no matter how their epitopes are presented, must be safe, must protect against disease, and must afford a reasonably long-lasting immunity that, hopefully, will involve both T- and B-cell responses and that will induce immunologic memory.

Recombinant viral vector vaccines, particularly vaccinia, have problems, first, of significant virulence for man of the vector itself; second, of host immune response to the vector that limits its reuse for follow-up injections; and, third, of possible altered tissue tropism whereby the new determinants may allow access of the vector to cells it would not ordinarily infect, with adverse consequences on the host. The potential problems of altered tissue and organ tropism may make viral vector vaccines untestable in man.

The epitopes of most antigens are not simply linear epitopes but, instead, may depend upon the appropriate folding of a globular polypeptide. The principal usefulness of synthetic oligopeptides may be for locating and defining individual epitopes. They are typically of poor immunizing capability and require coupling to protein carriers. Further, their use is limited to continuous epitopes, which greatly limits their prospects for being practical as agents for routine immunization.

Subunit antigens made by recombinant technology offer far greater chance for success since they do reproduce the total length of the antigen and may fold appropriately. Remarkable success has been achieved with hepatitis B vaccine produced using antigen made in ordinary baker's yeast transfected with a gene for surface antigen. Successes may be more readily achieved with globular proteins that are embedded in the lipid bilayer of enveloped viruses than with nonenveloped agents such as picornavirus antigens that appear to depend upon structural relationships with each other as well as their internal components to achieve appropriate epitope configuration.

Although glycosylation appears unimportant to date with hepatitis B and with HIV (AIDS) surface antigen, this may not always be the case. Some workers believe that mammalian cells may afford more appropriate glycosylation than yeast, even though there is heterogeneity in glycosylation in all cell types, and there is no example to establish that antigen produced in mammalian cells is superior to any other. Furthermore, antigen production in transformed mammalian cells is hampered by the need for total removal of all contaminating DNA originating from integrated or vector promoter sequences derived from oncogenic viruses. Indigenous retroviral contaminants may also be present. Transfected yeast cells do not have these safety problems.

Anti-idiotype antibodies that are produced by immunization against the variable region of specific antibody and that may present an image of the original antigen have been considered for vaccine application. In the practical sense, however, there is no present example of a successful anti-idiotype vaccine, just as there is no example of an adequate synthetic vaccine.

In tackling the EPI dilemma one is faced with difficult if not insuperable problems. In the absence of a brilliant technical

breakthrough, it is perhaps more realistic to ask what can be done to optimize and improve the current situation with respect to temperature stability within the limits of current knowledge.

Ultimate resolution may come from a vastly improved data base, but the time for resolution is long and the costs will be large. Shorter-term improvements may be possible, however, by casting aside the ideals of ultimate perfection and settling for what is good enough. But what might be done?

1. I think Dr. Brown's emphasis on dried vaccines has merit, and this may become attainable for DTP once the acellular pertussis antigen has been perfected.

2. Some of the vaccines are already quite good and need only to be better defined in their use. Hepatitis B recombinant antigen, for example, is remarkably stable to increased temperature, but this needs to be delineated more precisely. The stabilized live measles virus vaccine that was developed in our laboratories to meet WHO concepts of need in tropical distribution, may be adequately employed with limited refrigeration.

3. Specifications for products purchased for the EPI need to be tightened and the products tested for compliance. Cheapest price for unregulated product almost certainly guarantees exclusion of quality.

4. Incentives need to be offered to manufacturers to optimize the quality of their products. One means to promote this is by making volume purchases large enough to permit research expenditures, and another means is by offering a price bonus for products with stabilities exceeding a specified target level.

5. Above all, there is a real need to fund basic research in academia to establish new principles and technologies that can be applied to stabilizing biologics.

The overwhelming message, I believe, is that there is a need to create a means and an incentive for meaningful product improvement. This means money, and it seems critical to weigh the costs for improving the stability of vaccines against the costs for establishing an effective cold chain. It might be that maintenance of an acceptable cold chain is far cheaper and more doable than achieving stability by exploring new frontiers.

TABLE 1 Favorable Conditions for Biologics

Lyophilization, with optimal residual moisture, and with a matrix structure provided by excipients such as gelatin.
Freezing, but with maintenance of constant temperature to prevent disruption by shifts in ice crystals.
Purity, especially with freedom from destructive enzymes (proteinase, lipase, amylase).
Freedom from natural or added surfactants.
Freedom from atmospheric oxygen and oxidants.
Addition of "protective" substances such as proteins, polypeptides, amino acids, sugars, glycerides, etc.
Optimal pH.
Optimal cationic environment--especially in relation to enzymic activities (chelators or added salts).
Cross-linking of amino groups by formaldehyde to stabilize protein configurations.
Darkness and freedom from photoactive substances.

TABLE 2 Problems with Selected Epitope Delivery Systems

	Cautions
Live	
Recombinant viral vectors	Virulence, immune response to vector, altered tissue tropism.
Nonliving	
Subunit	
Synthetic	Poor antigenicity; carrier coupling problems; limitation to continuous polypeptide epitopes.
Recombinant	
Transformed mammalian cell	Residual DNA; oncogenic transforming virus, oncogenic viral promoters, retroviral contamination.
Yeast	Practical and proved. Scale-up is doable. Possible questions if glycosylation is needed.
Anti-idiotype	Futuristic. No practical examples.

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APPENDIX A

INSTITUTE OF MEDICINE

WORKSHOP ON TEMPERATURE-STABLE VACCINES FOR DEVELOPING COUNTRIES: SIGNIFICANCE AND DEVELOPMENT STRATEGIES

April 13-14, 1987

2101 Constitution Avenue, N.W.
Room 150
Washington, D.C.

AGENDA

Monday, April 13

9:00 - 9:10	Welcome	Samuel Thier, President Institute of Medicine
9:10 - 9:20	Objectives of the Meeting	Roy Widdus, Director, Division of International Health George Curlin, Office of International Health, Public Health Service
9:20 - 9:30	Review of Agenda and Meeting Procedures	Neal Halsey, Workshop Cochair, Johns Hopkins School of Hygiene and Public Health Joseph Melnick, Workshop Cochair, Baylor College of Medicine

CONSEQUENCES OF VACCINE TEMPERATURE STABILITY

9:30 - 9:45	WHO Minimum Requirements for the Main Vaccines of Interest to the Expanded Programme of Immunization	Phillipe Sizaret, World Health Organization
9:45 - 10:15	Economic and Logistic Impacts of Temperature-Sensitive Vaccines	Donald Shepard, Charles Cameron, Mark Robbins, Harvard School of Public Health
10:15 - 10:30	Discussant	Stanley Foster, Centers for Disease Control
10:30 - 10:45	Break	

10:45 - 12:00	Group Discussion: Significance of the Requirement for a Cold Chain o What is the relative impact of suboptimal temperature stability and other factors on present immunization efforts? (Program operations, vaccine efficacy, costs) o What are the potential improvements in program efficiency and effective immunization coverage if temperature-stable or otherwise improved vaccines were available?	
12:00 - 1:00	Lunch	

ENSURING PROTECTION: IMMUNOLOGIC AND CHEMICAL CONSIDERATIONS

1:00 - 1:20	Immunologic Considerations in Achieving Long-Lasting Immunity with Vaccines	Stanley Lemon, University of North Carolina, Chapel Hill
1:20 - 1:30	Discussant	Francis Ennis, University of Massachusetts Medical Center
1:30 - 1:50	Poliovirus: Structural Basis for Temperature-Dependent Loss of Immunogenicity	Marie Chow, Massachusetts Institute of Technology
1:50 - 2:00	Discussant	Olen Kew, Centers for Disease Control
2:00 - 3:30	Group Discussion: Immunologic and Chemical Considerations for Temperature-Stable Vaccine Development o What are the lessons to be learned for temperature-stable vaccine development from our immunologic and chemical knowledge?	
3:30 - 3:45	Break	

STABILIZING EXISTING VACCINES

3:45 - 4:05	Experience in Stabilizing Vaccines	Julian Peeterman, Smith Kline - RIT, Belgium
4:05 - 4:15	Experience in Stabilizing Poliomyelitis Vaccines	Joseph Melnick, Baylor College of Medicine
4:15 - 5:00	Group Discussion: Can We Further Improve the Stability of Existing Vaccines?	
6:00 - 6:30	Cocktails	
6:30	Dinner	

<u>Tuesday, April 14</u>

NEW TECHNOLOGIES FOR IMPROVED VACCINE STABILITY

8:30 - 9:00	New Technologies for Producing Antigens and Their Merits as Temperature-Stable Vaccines	Fred Brown, Wellcome Biotechnology
9:00 - 9:15	Use of Antigens Produced by Recombinant DNA Technology with a Safe and Efficacious Adjuvant Formulation in a New Generation of Vaccines	Anthony Allison, Syntex Research
9:15 - 9:30	Discussant	Maurice Hilleman, Merck Institute for Therapeutic Research
9:30 - 11:00	Group Discussion: What Are the Most Promising Strategies for Development of Vaccines with Improved Temperature Stability (Specific vaccines, technologies) o Which new technologies offer the best prospects for improving temperature stability and/or other desirable vaccine characteristics? (Specific EPI vaccines, new vaccines, vector approaches)	

11:00 - 12:00	Overall Conclusions, Strategies, and Policy Implications o What are the priorities for the development of new or improved vaccines (specific vaccines, improved stability, few doses, immunogenicity, etc.) for developing country immunization programs? o What are the important gaps in knowledge related to temperature stability? How can they best be filled? o Overall priorities among all areas: present practicable improvement, near-term directions, mid-term directions.	
12:00 - 1:00	Lunch	
1:00 - 3:45	Overall Conclusions, Strategies, and Policy Implications (continued)	
3:45 - 4:00	Summary of Conclusions	Neal Halsey, Joseph Melnick
4:00	Adjourn	

APPENDIX B

INSTITUTE OF MEDICINE

WORKSHOP ON TEMPERATURE-STABLE VACCINES FOR DEVELOPING COUNTRIES: SIGNIFICANCE AND DEVELOPMENT STRATEGIES

April 13-14, 1987

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APPENDIX C

GENERAL INFORMATION

- VACCINE STORAGE TEMPERATURES

VACCINE LEVEL: MAXIMUM STORAGE TIME	CENTRAL STORE up to 8 months	REGIONAL up to 3 months	HEALTH CENTRE up to 1 month	TRANSPORT up to 1 week
MEASLES YELLOW FEVER ORAL POLIO	-15°C to -25°C			
DPT TETANUS TOXOID DT BCG			0°C to +8°C	

Note:

- **Never freeze DPT or TETANUS (which both freeze at temperatures below -3°C).**
- **Storage times are recommended maximum figures. Remember to check expiry dates.**

Source: EPI Technical Series. The Cold Chain Product Information Sheets. 1986/87, No. 1. WHO/UNICEF EPI Technical Series Document No. WHO/UNICEF/EPI.TS/86.1.

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