REGIONAL SYSTEM OF VACCINES FOR LATIN AMERICA AND THE CARIBBEAN (SIREVA)

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I. INTRODUCTION

In order for the Latin American and Caribbean countries to participate more fully in the process of developing new technologies and improved vaccines for use in preventing diseases in the Region, the Pan American Health Organization (PAHO) has developed a program for a Regional Vaccine System (SIREVA)(*).

In September 1991, Dr. Guillermo Soberón, Vice President of the PAHO Advisory Committee on Health Research (ACHR), made an official presentation of SIREVA to the ACHR. The committee expressed its congratulations and approval of the plan and pointed out the usefulness of SIREVA for providing low-cost high-quality vaccines to the social sectors which most need them.

In its strategy for implementation, SIREVA takes into account the existence of niches of well and experienced scientific and technological institutions and laboratories. Furthermore, it stresses the mechanism of technical cooperation among countries and existing research institutions geared toward efforts in the field of vaccine development in order to potentiate the results. It also emphasizes that the approaches selected should be able to attract the interest of the international scientific community, gain the political and economic support of the countries of the Region, attract the financial resources of international donors, and make possible the transfer of technology.

Similarly, in recognition of the fact that millions of children still die annually from diseases that could be prevented and in response to the Declaration of New York, in September 1990, the Children’s Vaccine Initiative (CVI) was launched by the World Health Organization (WHO), the United Nations Children’s Fund (UNICEF), the United Nations Development Program (UNDP), and The Rockefeller Foundation. In the World Summit for Children, a call was made for a global commitment to the production and delivery of "ideal children’s vaccines" that provide lasting protection against a wide range of diseases and are affordable and simple to administer.

This initiative represents a significant milestone in disease prevention. Accomplishment of its goals will require a substantial investment of money and time and effort on the part of the world's scientific community. Unfortunately, most of the capacity to participate in this scientific and technological revolution rests on the developed world, where existing laboratories are best equipped to turn their attention to this effort.

Notwithstanding, it is also necessary to strengthen the scientific and technological capacity in developing countries so that they too can be active participants in the vaccine development process and not just passive beneficiaries of the ultimate products.

Through SIREVA it would be possible to combine the CVI approach and the strengthening of regional institutions and laboratories in the Region. As a matter of fact, there are already some activities combining both efforts in the area of quality control and DPT vaccine production.

II. ACTIVITIES FOR EXPANSION OF SIREVA

Some distinctive approaches have been developed:

A) TECHNICAL AND SCIENTIFIC SUPPORT

- The Dutch-Nordic Consortium was approached because of its long tradition in the area of vaccine development and production activities and, as public laboratories, there was a possibility that technology transfer could be better arranged.

- The Food and Drug Administration (FDA) and National Institutes of Health (NIH), through Dr. Carl Frasch and Dr. John Robbins, are supporting technical and scientific development of the project related to the improvement of *N. meningitidis*, serogroup B and typhoid fever vaccines, respectively;

- The dengue vaccine (quadrivalent live attenuated virus) has the commitment of Dr. Nath Bhamaraprati of the Mahidol University, Thailand, a joint proposal with Brazil to develop further steps of scale-up technology.

- Presentation and discussion in the PAHO Advisory Subcommittee in Biotechnology of the ACHR, January 1992 in Mexico City.
B) DISSEMINATION OF THE SIREVA PROPOSAL

- The SIREVA proposal was disseminated in several meetings organized by CVI. Some of these meetings included: the Consultative Group in Seven Springs Conference Center in February 1991; the Preparatory meeting on the participation of public laboratories held at RIVM in June of 1991; the Management Committee in June of 1991; the Consultative Group at WHO, Geneva, in December 1991; the Task Force on Relations with Development Collaborators and Strategic Plans; Meetings at the Institute of Medicine and FDA.

- Through visiting health authorities of the Region.

C) FINANCIAL SUPPORT OF SIREVA

Although, several contact and discussions have been held with funding agencies, both governmental and non-governmental, for specific support of implementation activities of SIREVA, the results are well behind the needs.

The Canadian International Development Agency (CIDA) decided to support the project related to epidemiological surveillance of *S. pneumoniae*, for a period of two years.

The Swedish International Development Agency (SIDA) is supporting the Phase III field trials of cholera vaccine WC/rBS in the Region.

The Brazilian Ministry of Health very recently decided to fund, through the Self-Sufficiency program, the development of *N. meningitidis*, serogroup B.

The biggest challenge for the implementation of SIREVA continues to be medium and long-term continuous and steady funding.

d) POLITICAL SUPPORT OF SIREVA

There is favorable political willingness from the countries in the Region to support SIREVA judging from the manifestation of interest of several authorities to participate in the development of SIREVA. Mexico and Brazil have formally committed to the development of SIREVA, and the regional coordination structures called CENVAC-Cuernavaca and CENVAC-Rio de Janeiro are being organized. It is very important to establish some concrete activities in order to permit SIREVA to be visible and facilitate its development.
However, due to the noncontinuous and changing nature of administrations and health policies in the Region, (whenever a change of Minister of Health occurs, and it occurs rather frequently), it is very important to base the SIREVA activities in well known institutions in order to diminish the adverse political situation and favor continuity.

At the international level, SIREVA is gradually obtaining recognition, and its strategy of implementation is receiving approval. In cooperation with CVI, the first Regional Meeting of the Children's Vaccine Initiative, to be held the beginning of September 1993, is being organized. The objective is to develop a Regional Strategic Plan for the development and production of a high quality and improved DPT vaccine, and introduce appropriate combination vaccines based on DPT manufactured in the Region.

The close link with CVI activities and the back-up from them seems to be of great importance in obtaining not only the political support but also the financial support from the international agencies.

III. DEVELOPMENT OF MASTER PLAN

The development of a Master Plan for each vaccine was recommended by the PAHO Advisory Subcommittee in Biotechnology of the ACHR in a meeting held in January 1992, in Mexico City.

Following the original proposal contained in the SIREVA document, the Master Plan was conceived to update the available information and to elaborate a proposal for research leading to the development and production of a new generation of vaccines for use in children. It also encompasses various activities required for the development of vaccines in the Region. Therefore, it includes activities related to the epidemiological surveillance, research and scale-up of production, clinical studies and field trials, concepts of quality control and quality assurance, and the steps needed for registration and utilization of a vaccine. The following criteria was used to select a group of childhood diseases: epidemiological importance, social impact, scientific and technological feasibility, local and regional importance, and economic feasibility. Those diseases include: typhoid fever, meningococcal, dengue, and pneumococcal disease. More recently, the field trials of a cholera vaccine was also included in SIREVA due to the cholera epidemic which began in the Region in 1991.
The Steering Committee (SC) for each Master Plan was organized, and its participants would cover the different aspects required for vaccine development. A draft of a Master Plan was prepared previously to each meeting of the SC by a consultant specially assigned for that purpose.

The summary of the research proposals for each Master Plan and the actions taken for their implementation follow.

Upon request of the members of the ACHR, a copy of the publication will be provided.

A. MASTER PLAN FOR DEVELOPMENT OF S. PNEUMONIAE VACCINE

*S. pneumoniae* is the most common single bacterial etiological agent for respiratory tract infections, causing a spectrum of clinical conditions including pneumonia, meningitis, bacteremia, sinusitis, and otitis media. Approximately 5 million children die each year from pneumonia.

The extent of immunoprotection varies with age. Unfortunately, the currently available 23 valent pneumococcal capsular polysaccharide vaccines are poorly antigenic for children. The immune responses are particularly poor for serotypes frequently associated with pediatric disease, most notably serotypes 6, 14, and 23.

The development of a capsular polysaccharide based vaccine has been complicated by the existence of 84 serotypes of pneumococci. Since all serotypes are not equally prevalent, an effective vaccine needs to contain only those which are most commonly associated with invasive pneumococcal disease. Another complication is that pneumococcal infections are caused by several strains which have different capsular antigens. At least five to seven strains will need to be included in order to prevent the majority of pneumococcal infections.

The distribution of serotypes of *S. pneumoniae* associated with infection varies by age and region and may also change over time. Thus, data on serotype distribution among adults with pneumococcal disease cannot be used to reliably predict the serotype distribution in children. Existing data from Brazil on isolates from children with meningitis suggest that the serotype distribution is markedly different from that among children with bacteremia in North America and Papua, New Guinea.

Polysaccharides are T-cell independent antigens which usually induce increases in type-specific antibodies in older children and adults. Titers may be
relatively low and cannot be boosted by additional doses of polysaccharide vaccine. However, the most important drawback of these polysaccharide vaccines is their inability to induce protective immunity in infants and young children, the age group in which protection is most needed.

The immunogenicity and course of the immune response to polysaccharide antigens can, however, be dramatically altered by coupling polysaccharides or oligosaccharides to carrier proteins, transforming the T-cell independent carbohydrate into a T-cell dependent antigen. These antigens generate antibody responses to both the carbohydrate and protein antigenic determinants.

Different chemical strategies have been undertaken to create conjugates by covalently linking capsular polysaccharides to carrier proteins. Large molecular weight capsular polysaccharides as well as oligosaccharides of varying lengths have been used for preparing conjugate vaccines.

Although sixty years ago it was already known that the conjugation of polysaccharide antigens with protein carriers enhances their immunogenicity, it was only in 1980 that the first commercial \emph{H. influenzae}, type b vaccine became available. At the moment, four different conjugated protein-polysaccharide vaccines against \emph{H. influenzae}, type b, are commercially available. All of these are protected by patents except for the adipic hydrazide coupled vaccine.

A variety of proteins, including outer membrane proteins (OMP), excreted toxins of pathogenic bacteria and nontoxic cross-reacting materials (CRM), have been employed as carrier proteins. The most frequently used proteins are the readily available and accepted diphtheria and tetanus toxoids. Conjugation has also been used as a toxoiding process for a variety of carrier proteins such as diphtheria toxin, tetanus toxin, toxin A from \emph{Pseudomonas aeruginosa}, cholera toxin, and pertussis toxin.

As with other capsular polysaccharides, covalent binding of the pneumococcal polysaccharides both increases their immunogenicity and confers T-cell dependent properties. Several groups have been working on the development of conjugated pneumococcal polysaccharide vaccines.

**Proposed research activity**

The research activity should be centered around four areas:
1) The capsular polysaccharides. Initial research will be on type specific capsular polysaccharides already known to be common in Latin America and globally, including types 6B, 14, 19F and 23F. The final choice of capsular types will be made and refined based on ongoing epidemiological studies in Latin America. The new formulation will also take into account the extensive cross-reactivity of certain serotypes as well as greater stability of one serotype over the others.

2) Pneumolysin and pneumococcal surface proteins:

**Pneumolysin.** Pneumolysin is a powerful sulfhydryl-activated cytolysin which has a series of detrimental effects on components of the human immune system. Genetically toxoid derivatives have been produced whereby mutations have been generated that reduced the toxicity without impairing the immunogenicity of the pneumolysin derivative. It has also been suggested that the efficacy of the pneumococcal vaccine might be improved by supplementing it with inactivated pneumolysin in the form of pneumococcal capsular polysaccharides-protein conjugates.

**Surface Proteins.** Pneumococcal surface protein A (PspA) is a virulence factor on *S. pneumoniae* that retards the blood clearance of pneumococci and reduces virulence 10-1000 fold. Although PspA is serologically variable, the different PspAs are cross-reactive enough that immunization with a few PspAs would likely elicit protection against most or all pneumococci. Lyophilized PspA can elicit protective responses in mice in the absence of adjuvants.

**37 K proteins.** A surface protein (37K) has been described as appearing to be conserved in structure and to be present in all pneumococci. It has also been shown to be able to elicit protective antibody responses. Pneumococcal autolysin and neuraminidase also elicit protective responses, although they may be less effective than PspA, pneumolysin and possibly the 37K protein.

3) C-polysaccharide. C-polysaccharide (C-PS) is a major component of the cell wall of the pneumococcus that is common to all pneumococcus serotypes. It is a vaccine candidate antigen that might supplement type-specific immunity and elicit protection against types not included in the vaccine. It is expected, however, that antibodies directed to the complete structure of the C-substance would be more effective in mouse protection studies than using anti-phosphocholine alone.

4) Protein carriers. Pending the results of additional research on the protective activity of pneumococcal proteins in animal models, one or more pneumococcal proteins may also be evaluated as carriers. If the resulting conjugates retain the ability to produce high levels of antibodies to the carrier protein and to the
polysaccharide. Pneumococcal protein carriers may be substituted for the more traditional carrier proteins.

5) Conjugation options. For the development of conjugation methodology, two strategies have been proposed:

- Utilization of a general conjugation technique which should be applicable to all of the type specific capsular polysaccharides identified on the basis of regional pneumococcal surveillance. One procedure that has been used successfully to prepare polysaccharide-protein conjugate vaccines is initial activation with CNBr, followed by attachment of a linker such as ADH or 6-aminohexanoic acid (6AH). Also, final carbodiimide conjugation can be used. For the carrier protein, one or more of the common bacterial toxoids can be used.

- Development of improved conjugation techniques in order to maximize the yields of the conjugation process in order to reduce the costs of the component polysaccharides and carrier proteins; develop a process with a minimum number of preparative steps in order to reduce the costs of process monitoring, equipment and the labor to facilitate scale-up; tailor the conjugation process for specific polysaccharides in order to avoid destroying labile antigenic determinants, as for acetyl groups, phosphoryl choline groups, or phosphodiester may be hydrolyzed by the alkaline pH utilized for CNBr activation; maximize the immunogenicity of the conjugate by controlling the size and structure (neoglycoprotein vs. cross-linked lattice) of the conjugate.

- Implementation of master plan

With financial support from CIDA, is the ongoing epidemiological study to determine the prevalence of subtypes of S. pneumoniae present in the Region. More detailed information is given on the specific topics, respectively, in the following sections.

B. MASTER PLAN FOR DEVELOPMENT OF TYPHOID FEVER VACCINE

Typhoid fever is a disease with wide distribution in developing countries. In the Region of the Americas, there are 595,000 cases every year of which 197,000 correspond to serious clinical cases. It is believed that the disease produces 10,000 deaths annually. Forty-two per cent of the cases are found in children under 14 years of age, of which 5 per cent are in children under 5 years of age.
Moreover, in recent years, several countries in the continent such as Chile, Mexico, and Peru have experienced several annual outbreaks, with thousands of cases. Also, cases of *Salmonella typhi* resistant to antibiotics have been responsible for many outbreaks of typhoid fever in the Americas, and they are becoming a problem in other geographic areas. This phenomenon complicates treatment and reemphasizes the need for preventive immunization.

Field trials have shown that effective vaccination is a cost-efficient method which rapidly lowers the incidence of disease to levels that interfere with natural transmission. This means that the development of a vaccine should be considered an effective tool to control the incidence of the disease.

The currently available whole-cell killed parenteral vaccines are not suitable for large-scale application because undesirable adverse reactions occur frequently and do not produce extended immunity.

Recently, a live oral vaccine based on the live *galE* attenuated Ty21a strain has been developed and licensed in many countries. The attenuation basis of this vaccine remains uncharacterized, its protective capacity and the duration of the protection it confers have not been demonstrated conclusively, and the feasibility of using this and other live vaccines as public health tools remains a subject of debate because several repeated doses must be administered to obtain effective and durable protection. It is therefore desirable to work on a suitable vaccine that does not have these shortcomings and disadvantages.

Through modern technology, an appropriate method of constructing new generation vaccines has been found, using subunit vaccines based on conjugated polysaccharides with proteins. These vaccines have been shown to be efficient and easy to administer to infant populations. The conjugation enhances the immunogenicity of polysaccharides and the conjugate appears to be free of adverse reactions.

- **Research proposal for improved vaccine**

It is proposed to develop and evaluate a conjugate vaccine for typhoid, based on the Vi antigen covalently linked to a "carrier protein" such as porin. It is expected that a vaccine made of Vi antigen conjugated to porins will protect adults and infants against typhoid. This latter age group is not currently covered by any available vaccines.
This approach is facilitated in typhoid fever because:

i) the disease is produced worldwide largely by a single bacterial clone exhibiting conserved antigenic properties;

ii) a capsular Vi antigen *Salmonella typhi* has already been shown to be effective in countries where typhoid is endemic, and;

iii) porins, a group of major outer membrane proteins (OMPs) of *S. typhi* have been shown to raise the humoral protective immune response in an animal model. The immunogenicity and the role as virulence determinants of the porins is also shared by other OMPs of typhoid and Gram-negative bacteria with potential role as carriers.

The concept of covalently linking the Vi antigen to a carrier protein would be an appropriate approach since successful vaccines to prevent *Haemophilus influenzae*, type b, have been shown to be efficacious in disease prevention in children. Bacterial capsular polysaccharides have been conjugated successfully to several proteins such as tetanus toxoid, diphtheria toxoid, and the meningococcal OMP. Porins of *S. typhi* have been used to raise protective humoral immune response in mice, and are also able to induce a cellular immune response in mice and humans. The sera of typhoid patients contains a high titer of antibodies against these proteins. These findings indicate that porins could be utilized to increase the immunogenicity of the Vi antigen by their T-cell stimulating ability, and simultaneously, to raise protective antibodies against *Salmonella typhi*.

- **Implementation of master plan: Joint development -- Mexico and Brazil**

The availability of the technology of Vi antigen production in Bio-Manguinhos/FIOCRUZ prompts the discussion on the possibility to develop this vaccine in steps as proposed in the Master Plan:

- **First step:** development of Vi vaccine. As a Brazil-Mexico joint effort, both countries would potentiate the existing technological capabilities and would abbreviate the outcome of the product;

- **Second step:** development of a conjugated vaccine. The technology to obtain the porins and conjugation technology is being worked on in Dr. Isibasi's laboratory.
As Chile also has manifested interest in taking part in the development of this vaccine, the Master Plan should be sent along with the SIREVA document to interested parties.

C. MASTER PLAN FOR THE DEVELOPMENT OF IMPROVED *N. MENINGITIDIS*, SEROGROUP B VACCINE

Meningococcal diseases (MD) remain a significant health problem in many different countries, and prevention has been proven to be a cost-effective public health measure. There are 12 different serogroups within *Neisseria meningitidis*, but over 90% of MD is caused by groups A, B, and C. Protection against MD is associated with induction of bactericidal and probably opsonic antibodies. Although there is an effective polysaccharide vaccine for prevention of the disease due to serogroups A and C, the group B polysaccharide is poorly immunogenic, and antibodies to this polysaccharide do not appear to be protective. A number of alternative cell surface antigens have, therefore, been investigated as potential group B vaccine candidates. These include a chemically modified group B polysaccharide containing N-propionyl rather than N-acetyl groups conjugated to tetanus toxoid, the cross-reactive E. coli K92 polysaccharide conjugated to tetanus toxoid, cloned outer membrane proteins including class 1 incorporated into liposomes, and lipopolysaccharide-depleted outer membranes or outer membrane proteins.

The meningococcal outer membrane contains four to five major proteins. These have been identified as class 1 through 5, having molecular weights between about 46,000 and 25,000. The class 1, 2 and 3 proteins are porins, but the class 2 and 3 proteins are the major porin proteins and mutually exclusive. Antibodies to the class 1 protein appear to be more bactericidal than those to the class 2 and 3 proteins. For this reason, a number of investigators are developing purified class 1 protein vaccines for group B.

There is a large amount of antigenic diversity among group B meningococcal strains. There are approximately 20 different serotypes within group B based upon immunologic differences in the class 2 and class 3 major outer membrane proteins. The class 1 protein may be more important for induction of protective antibodies because there are fewer different class 1 proteins, and they may be more immunogenic than the class 2/3 proteins. In spite of the multiplicity of antigens, cross reactivity apparently exists between different strains, and preliminary results of efficacy trials and bactericidal assays suggest that cross protection will occur with carefully selected strains.
A number of efficacy trials with group B outer membrane protein vesicle vaccines have been conducted, the first of which was held in South Africa in the early 1980s using a serotype 2 outer membrane protein vaccine. Antibody studies showed the vaccine to be immunogenic in all age groups including infants. Although 4,400 children were enrolled and vaccinated, this was an insufficient number to obtain a statistical estimate of efficacy in this trial. Therefore, trials were subsequently conducted in Cuba, Chile, Norway and Brazil.

The Cuban group B meningococcal vaccine contains lipopolysaccharide-depleted outer membranes from a serotype 4 subtype P1.15 (B:4:P1.15) strain, group C meningococcal polysaccharide to control membrane solubility, a 65K protein (part or all of a "high molecular weight protein complex"), all combined with aluminum hydroxide. The vaccine contains per 0.5 ml dose, 50 ug protein and 50 ug C polysaccharide adsorbed to 2 mg aluminum hydroxide. It was administered in two doses 6 to 8 weeks apart. In a randomized double-blind study in school children, an efficacy of 83% was observed. Of major importance in the Cuban efficacy trial is the demonstration that antibodies induced to noncapsular surface antigens can protect against MD. The same vaccine was recently used in Brazil in 2.4 million children. A case control study demonstrated efficacy which varied by age. For children older than 48 months, efficacy was 74% (confidence interval from 16% to 92%), whereas in young children, no protective efficacy was shown.

The Norwegian group B protein vaccine contains lipopolysaccharide-depleted outer membranes from a serotype 15 subtype P1.16 strain, and 3% to 6% of high molecular weight proteins, but contains no meningococcal polysaccharide. The protein is stabilized in 3% sucrose, adsorbed to aluminum hydroxide with a protein to adjuvant ratio of 1:67. The vaccine contains class 1, 3, 4, and 5 proteins. It was formulated to contain 25 ug protein per dose and was administered as two injections 6 weeks apart. In a randomized, double-blind trial in 13 to 15 year olds, an efficacy of 57% was found.

A controlled, double-blind randomized efficacy trial of a meningococcal group B vaccine was conducted in Iquique, Chile, from 1987 to 1989. The experimental vaccine was prepared from a B:15:P1.3 strain and contained purified outer membrane proteins, essentially free of LPS, noncovalently complexed to an equal amount of group C polysaccharide. The complex was adsorbed to aluminum hydroxide (1 mg/dose) and given in two doses of 100 ug protein at 0 and 6 weeks. The study population of 40,000 volunteers included ages 1 to 21 years, and surveillance was continued for 20 months. The overall estimate of efficacy was 50%, but protection was found to be age-dependent. Efficacy was 70% in children 5 to 21 years of age, while no protection was seen in children 1 to 4 years of age.
The currently available candidate group B vaccines have several limitations: (1) efficacy is uncertain in young children who have the highest rates of disease; (2) there is little demonstrated efficacy after one dose in adults; and (3) maximum efficacy in any age group is in the range of 80%. Thus, additional development of vaccines for group B meningococcal disease is necessary.

The preceding review suggests that one very promising approach to the development of a more effective group B meningococcal vaccine is the use of lipopolysaccharide-depleted outer membrane protein vaccines containing proteins normally induced in vivo, and this approach is taken in the vaccine development program discussed in this document.

- Proposed research activity

There are relatively few laboratories in Latin America actively involved in studies relating to the characterization of meningococcal cell surface or the human immune response to vaccination and disease. Laboratories with such experience include those in São Paulo (Instituto Adolfo Lutz and Instituto Butantan) and Rio de Janeiro (Bio-Manguinhos/FIOCRUZ), Brazil and Havana, Cuba (Instituto Finlay).

The projects underway in Brazil are directed toward the development of an improved group B membrane vaccine containing iron regulated outer membrane proteins. However, additional research activities are needed for formulation, quality control, and clinical evaluation of these vaccines.

A. STRAIN SELECTION

Strains were selected based on their antigenic properties and their relationship to strains currently causing epidemic diseases. At the Adolfo Lutz Institute, thirty strains of *N. meningitidis*, representative of the important serotypes and subtypes in Brazil, were selected for vaccine evaluation. These included B:4:P1.15, B:8:P1.16, and C:2b:P1.3,6, as well as ten nontypable strains which were selected based on their outer membrane protein (OMP) patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Among 30 strains studied, four strains were selected for their growth characteristics in an iron deficient medium and for expression of important surface antigens.

B. CULTURAL CONDITIONS

Differences in the composition of bacterial culture mediums can induce changes in OMPs, for example, expression of the iron regulated proteins (IRPs), which
are induced under conditions of iron starvation. Based on the relative ability of the different meningococcal strains to grow in a chemically defined culture medium and from SDS-PAGE analysis of IRPs and other OMP expression, four representative strains were selected for vaccine evaluation. Growth experiments using the four strains were performed to determine the best culture conditions, including growth time, temperature, and concentrations of iron chelator and iron source. Two different iron chelators, desferal and EDDA were used.

C. IMMUNOASSAYS FOR MONITORING PROTEIN EXPRESSION

The expression of important meningococcal cell surface antigens should be monitored. This can be done using techniques such as ELISA, immunoblots, and colony blots using McAbs. Some of these McAbs are already available at Adolfo Lutz, but others are being made in cooperation with the Oswaldo Cruz Foundation (FIOCRUZ), in Rio de Janeiro. These McAbs will be used to determine the presence of important epitopes among disease causing strains of different serogroups, serotypes, and clones. They will also be used for vaccine quality control.

The bactericidal test will be used to provide information about the capacity of different meningococcal cell surface epitopes to induce protective antibodies. Those cellular surface antigens found to stimulate bactericidal antibodies are of greatest importance for vaccine development.

D. GENETIC ENGINEERING OF VACCINE STRAINS

It is desirable to obtain *N. meningitidis* polysaccharide B negative vaccine strains, since this polysaccharide need not be present in the final vaccine, and because nonencapsulated strains are much less pathogenic and, therefore, safer with which to work.

The desirability of including the class 4 protein in vaccines is unclear. At a meeting in September 1991, in Oslo, it was recommended that meningococcal vaccine strains not contain the class 4 protein because of concerns that it may induce blocking antibodies. Monoclonal antibodies against the class 4 protein are presently available at the Institute Adolfo Lutz to assist in these studies.

E. PURIFICATION OF OUTER MEMBRANE VESICLES

The growth medium used should contain no components known to cause sensitization, and for quality control and economic reasons, it should be a synthetic or defined medium. The Catlin meningococcal defined medium or modified Frantz
medium are suggested. The bacteria should be grown under controlled iron conditions using chelators to stimulate synthesis of IRPs. Outer membrane vesicles should be obtained either by extraction from the cells after 12 to 15 hours' growth, or from the culture medium after extended culture. By either method, the preparation of vesicles will contain near equivalent amounts of protein and lipopolysaccharides (LPS).

To be usable as a vaccine, the outer membranes must be detergent-extracted to remove most of the LPS. An appropriate detergent for this purpose is sodium deoxycholate, a bile metabolite naturally present in human tissue. It is essential not to remove all the LPS, otherwise the important membrane structure will be disrupted. Retention of vesicle structure should be confirmed by electron microscopy, and the retention of the major outer membrane proteins by SDS-PAGE. The detergent extracted membranes should be stabilized by a small amount of deoxycholate and 3% sucrose or lactose.

F. OTHER VACCINE STRATEGIES

Other methods of multivalent vaccine preparation using recombinant strains expressing multiple class 1 proteins are being pursued. If these studies are shown to produce vaccines that can induce bactericidal antibodies against multiple class 1 proteins, it would be very useful to negotiate technology transfers, and clinically compare such vaccines to the vesicle vaccines described here.

A class 5 related protein, named 5C by Achtman, was found in over 50% of meningococcal isolates examined, and anti-5C antibodies are bactericidal. The prevalence of this protein in group B and C strains in countries of the Region should be determined, and if prevalent, a 5C containing serotype 4 strain could be selected for vaccine studies.

Studies have shown that antibodies to gonococcal outer membrane proteins can be strongly enhanced by incorporation into liposomes, and studies with malarial protein vaccines have shown that the inclusion of LPS in the liposomes has a strong adjuvant effect. Such studies should also be considered using meningococcal outer membrane proteins.

Design of experimental vaccines

Studies will be performed to determine the best formulation of two or three strains differing in serotype, serosubtype, and iron regulated proteins to achieve the broadest cross-protection, as evidenced by induction of bactericidal antibodies. Lipopolysaccharide depleted outer membranes containing high molecular weight iron
regulated proteins will be prepared. These will be complexed with group C polysaccharide and alkaline detoxified meningococcal LPS. Because group C is an important cause of MD, the group C polysaccharide should be included to improve vaccine solubility. The detoxified LPS may provide additional protective antigens, and may help to restore the native conformation of the major surface proteins, increasing the quality of the vaccine induced immune response.

The vaccines should be formulated to contain 20 to 25 ug/dose of protein per strain, assuming 2 or 3 strains will be used with a maximum of about 70 to 80 ug protein per dose. The extracted membrane vesicles should also contain a small amount of residual nondetoxified LPS. The vaccine should also contain 50 ug of group C meningococcal polysaccharide and 25 ug of detoxified LPS per dose, all absorbed in 2 mg of aluminum hydroxide or aluminum phosphate.

- Implementation of the Master Plan for N. Meningitidis, serogroup B

In April 1993, a meeting was held in São Paulo to discuss the on-going activities of three institutions in which research is being conducted: Instituto Adolfo Lutz, Instituto Butantan, and Bio-Manguinhos/FIOCRUZ. A document of intention was signed by the directors of these institutions, defining the basis and mechanism of the joint work among the three laboratories. Also, the timetable of activities for each institution was established.

It was also defined that the technical discussions among the three Brazilian laboratories need to be improved. It is of the utmost importance to devise a joint protocol for vaccine development because of the existence of interface in the development of the research. In this initial phase, the direct participation of the director of each institution is required to establish greater confidence among the technical staff.

Chile, Argentina, and Cuba have already manifested their interest to participate in the development of this project. The Master Plan and the documentation originating from the meeting of the Brazilian institutions should be sent to these interested countries in order to explore the possibility of their participation.

Until now, the majority of financial support was made by the institutions involved. The Brazilian Ministry of Health, through PASNI, will transfer to these institutions up to US$500,000.00 and to CENVAC-Rio US$100,000.00 in order to expedite technical development;
In order to apply for international funding, a specific proposal will be prepared and sent throughout PAHO/SIREVA.

D. MASTER PLAN FOR DEVELOPMENT OF DENGUE VACCINE

The World Health Organization estimates that 40 million cases of dengue fever (DF) occur worldwide on an annual basis. It has been suggested that the global distribution of dengue coincides with the global distribution of the mosquito vector, *Aedes aegypti*. On the other hand, it is estimated that there were nearly 2 million cases of dengue hemorrhagic fever (DHF) over the last 25 years, of which 30,000 resulted in deaths. In the dengue endemic areas, more than 1.5 billion people are at risk, of which more than 600 million are children. Since the 1980s, epidemics have been reported from countries in Asia, Africa and the Americas, till then considered to be non-dengue areas.

The last decade has seen a marked increase in the incidence of dengue related infections in the Americas. Epidemics occurred in Brazil in 1982 and 1986, the former due to dengue 1 and 4 and the latter due to dengue 1. Between 1987 and 1990, epidemics due to type 1 were also reported from Bolivia, Paraguay, Ecuador, and Peru. It is significant to note that all four countries had either no history of dengue or absence of the condition for several decades. Dengue 3 is presently not reported in the Americas, but could be introduced from other parts of the world, where it is actively transmitted.

There is a similar increase in the incidence of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) epidemics. In terms of magnitude, the worst epidemic of DHF/DSS was reported from Cuba in 1981. The epidemic was associated with dengue 2 and involved more than 300,000 cases of which 158 resulted in death. In 1989-90, a second epidemic was reported from Venezuela involving 5,900 cases, including 73 deaths. Dengue 1, 2 and 4 were isolated from the Venezuelan epidemic.

The revision of the molecular epidemiology of dengue infections in the Americas shows distinct topotype patterns in the microevolution of the virus. In the Western hemisphere, Puerto Rican and Jamaican topotypes represent the two distinct topotypes of dengue 2. Similarly, the American topotypes of dengue 1 and 4 are unique and distinct for the Americas.

The earliest attempt to develop a vaccine against dengue was in 1945, when Sabin & Schlesinger (Science 101:640, 1945) attenuated dengue 1 and 2 by serial intracerebral passage in infant suckling mice or newly-weaned mice. Thereafter,
several groups have devoted efforts to develop attenuated virus strains, and attenuated strains for all four types have been tested.

The Walter Reed Army Institute of Research has recently reported the development of another attenuated dengue 4 vaccine, designated 341750 CARIB (Marchette et al, Am Trop Med Hyg 43:212, 1990). The parent dengue 4 strain was modified by serial passage in primary canine kidney cell cultures. Immunized animals were protected against lethal challenge with both homologous and heterologous dengue 4 strains. The results indicated that the vaccine was safe and reasonably immunogenic for humans.

Bhamarapravati and colleagues of the University of Mahidol, Thailand, have recently developed a live attenuated, quadrivalent vaccine. Again, the strategy for attenuation and vaccine development is similar to that of their earlier work on monovalent and bivalent dengue vaccines. The tetravalent vaccine is composed of a mixture of 4 attenuated viral strains, representing different dengue types. Dengue 1, 2 and 4 were attenuated by serial passage in primary dog kidney cells for 13, 53 and 33 passages, respectively. Dengue 3 was attenuated by 30 passages in primary monkey kidney cells, followed by 3 passages in fetal rhesus lung cells. All vaccine strains exhibit biological markers of attenuation and have been chosen based on their success as components of monovalent, bivalent (1/2, 1/4 and 2/4), and trivalent (1, 2, 4) preparations. The vaccine formulation contains 4000, 1000, 4000, and 1000 pfu of types 1 to 4, respectively.

The safety and immunogenicity of the quadrivalent vaccine was investigated in 16 adult males, of which 8 were flavivirus immune and 8 had no previous immunity to Japanese encephalitis (JE) or dengue. All vaccinees showed the development of neutralizing antibodies to all four serotypes within 30 days of vaccination. The stability of the attenuation was demonstrable as viruses isolated from vaccinees retained phenotypic characteristics of the vaccine strains. The potential of vaccinees to become focii for the spread of infection was studied by inoculating viruses isolated from volunteers into *Aedes aegypti* mosquitoes. The mosquitoes had a high threshold of infection, demonstrating the absence of risk in disseminating vaccine viruses to non-vaccinated individuals. Thus, the vaccine is a sound candidate for large scale evaluation and efficacy studies in children. Also, extended phase 3 trials are planned to provide conclusive evidence.

An alternative approach toward the development of a vaccine has been the adoption of modern biotechnologies and the use of viral proteins for the preparation of sub-unit vaccines. Active and passive protection experiments with E proteins and monoclonal antibodies directed to them, respectively, have demonstrated that anti-E
antibodies with high titer neutralizing activity protect mice against challenge with the homologous virus. It has also been reported that both active and passive immunization with the nonstructural NS1 protein or anti-NS1 antibodies, respectively, protected mice against challenge.

The use of synthetic peptides may also represent a viable option for dengue subunit vaccines. Detailed analyses of the sequence, structure, and immunology of E glycoprotein have identified at least two to three immunogenic domains. Type-specific, neutralizing, and protective monoclonal antibodies have identified protective epitopes in the disulfide bridge stabilized amino-terminal R1 region of the E protein.

The use of viral protective antigens produced by recombinant techniques has been documented for a number of diseases, most notably in the development of the vaccine against hepatitis B produced in yeast. Most of the interest has been focused on the E protein and the NS1 non-structural protein.

The construction of chimeric dengue viruses by the substitution of structural proteins on a dengue 4 cDNA backbone with those from dengue 1 or 2 was reported. The use of chimeras as vaccines can be made possible by engineering mutations conferring attenuation. The technique also paves the way to the development of hybrid flavivirus vaccines, eg., JE-dengue chimeras.

- **Proposed development of quadrivalent vaccine**

In view of the progress achieved by Dr. Bhamaraprati in the attenuation of virus strains for the four types of dengue virus, it has been suggested to explore the possibility of establishing a collaborative relationship with his group in order to shorten the period required for research.

- **Implementation of the Master Plan**

Dr. Nath Bhamaraprati from the University of Mahidol, Thailand, participated in the dengue meeting convened at PAHO Headquarters in 1991 and in São Paulo in November of 1992. There was preliminary agreement that the establishment of technical cooperation between the University of Mahidol and the Oswaldo Cruz Foundation would be very important. After the São Paulo meeting, Dr. Bhamarapravati visited the Oswaldo Cruz Foundation for a preliminary discussion. Further steps must be pursued in order to fully explore the possibilities of technical cooperation.
IV. SIREVA AND CENVAC

The Mexican government has already assigned a Coordinator and an advisor for CENVAC. A specific grant has been assigned by PAHO to the Mexican Foundation for Health, with the purpose to organize the CENVAC-Cuernavaca. The Brazilian Ministry of Health has appointed a group of professionals to organize the CENVAC in Brazil.

The recent First Joint Meeting of SIREVA and CENVACs was held in São Paulo, Brazil, from 6-8 June 1993. During the meeting, the organization and functions of CENVAC, the implementation mechanisms of projects such as the S. pneumoniae epidemiological study, the cholera vaccine field trials, and several other technical matters were discussed in more detail.

It was proposed and agreed to change the denomination of CENVAC-MEXICO and CENVAC-BRAZIL to Cuernavaca and Rio de Janeiro, respectively, in order to confer the improved image of the nature of each CENVAC.

- Organization, structure, and functions of CENVAC

The principal purpose of SIREVA is to develop, under the institutional umbrella of PAHO, a capability for research and development of vaccines in Latin America and the Caribbean.

The Regional Vaccine Centers (CENVACs) will be organized to accomplish the general purposes of SIREVA and to coordinate the projects for development of the proposed vaccines. In addition, they will be responsible for identifying other purposes to whose ends they may wish to contribute.

It is envisaged that these two Centers be organized at the local level, with streamlined, efficient structures, in order to directly coordinate the actions required to integrate the activities among institutions.

Each CENVAC will have a full-time coordinator designated by PAHO and a Technical and Scientific Council (TSC) consisting of well known professionals in the areas of epidemiology, vaccine development, and production and representatives of the affiliated laboratories and epidemiological units.
The TSC will have the following functions:

- To examine the scientific and technological alternatives that can best be worked on for the development of each vaccine in the region, so as to identify the alternative in which a scientific and technological capability can be developed. The TSC will also discuss other related matters pertaining to the selection and review of projects, selection of research, and epidemiological unit participants. It will have a mandate of two years.

- The administration of the projects, charged with the coordination of the CENVACs, must coordinate and facilitate the preparation of the projects, administer the projects in the executing laboratories, promote articulation among all the units participating in a project in the execution of the work, and evaluation of the results.

The CENVAC will also develop other specific functions such as: monitoring and analyzing changes in the epidemiological profiles of diseases in the Region and identifying new subjects for work or collaboration; identifying installed capacity in the Region that could be brought into projects in progress or into new programs; identifying needs for manpower training, technical advisory services, and collaboration between research and production facilities; and integrating with organs of the State and health ministries for the established purposes. Any technical and financial support that may be needed must be provided to these units under the approved projects.

Therefore, as proposed, the CENVACs are structures for the coordination of a network of affiliated laboratories and epidemiological units already in place in the countries of the Region. These countries will be participating in projects for the development of the vaccines previously identified as priorities.

In addition, SIREVA/CENVAC must actively seek integration with the already existing structures, as follows: articulation between the health ministries and project-executing institutions; articulation of projects with the state health agencies through the affiliated laboratories and epidemiological units; articulation through PAHO with international agencies for the various aspects of technical cooperation, technology transfer, and funding.

CENVAC-Rio de Janeiro will be located at the Oswaldo Cruz Foundation, an organ of the Ministry of Health of Brazil. CENVAC-Cuernavaca will be part of the Institute of Public Health, of the Department of Health, in Mexico.
Though placed at those locations, these Centers would be part of the SIREVA structure and, as such, units of the Pan American Health Organization.

The affiliated laboratories and epidemiological units to be selected must be proposed and recommended by each CENVAC.

This council must also decide on the execution of projects, the need for resources and their priorities, and the monitoring and periodic evaluation of the work.

These two Centers will be related to each other and to the different institutions and affiliated laboratories. There will be no geographic limits for each Center, and a project might be executed by both Centers.

The proposed Management Advisory Committee (MAC) in the office of the director of SIREVA, consisting of: the Technical Coordinator, the Directors of the Centers in Brazil and Mexico, representatives of the network of affiliated laboratories, and of the donors, will formalize relations between the Centers for purposes of integration.

The purpose of establishing a technological capability for vaccine development in Latin America and the Caribbean must contend with the historical obstacles to integration between the different countries of the Region and between the different institutions within a single country.

Moreover, the magnitude of the resources and efforts wasted in isolated endeavors is making the solution of shared problems more difficult with every passing day. The recent and foreseen advances in scientific knowledge and technological proficiency for the development and production of vaccines are of such magnitude, and are being generated at such speed, that it is becoming impossible for any single institution or even a single country in the Region to keep abreast and operate satisfactorily and competitively. Therefore, the purpose and strategy of integration must be accompanied by mechanisms for the implementation of integrated projects. It will also be necessary to identify and develop the existing potential capacities of human resources in the Region.

Integration among institutions (in one country or many) must therefore be a fundamental SIREVA strategy, and as such, an object of special effort by the CENVACs. This integration can arise or be promoted in different ways or by different devices, such as: the prioritization of shared objectives and the complementarity of projects; a division of activities or tasks in the same project, clearly explained and periodically evaluated in their execution; the identification of each institute’s
strengths, or the apportionment of tasks that make the most of its strongest area of specialization; and the investment of financial resources so as to increase the technological strength of the unit or institute in its area of specialization; and the exchange of human resources (via seminars, meetings, and trainee programs).

Another strategy involving the creation of specific mechanisms for maintaining this relationship is the capability or potential for articulation of CENVAC with institutions outside the system or in other countries.

Some possible arrangements in this regard would be: the identification of needs for the support of projects in progress; external financial contributions; the implementation of consultancies, training, and diverse exchanges; an external evaluation of projects, or of stages of them; and publication of the work done, as considered appropriate.

CENVAC will establish itself not just as an intermediary, but as an essential and effective component of the system, on the strength of its promptness to respond in contrast to the traditional inefficiency of government bureaucracies in the Region. Also, it will serve as a component of an international organization unencumbered by local impediments and seeking simple, smooth-functioning management and coordination procedures.

V. IMPLEMENTATION OF OTHER ACTIVITIES UNDER SIREVA

A. S. PNEUMONIAE SUBTYPES PREVALENCE STUDY

The Canadian International Development Agency (CIDA) was approached and gave their support to the epidemiological surveillance of S. pneumoniae, with special emphasis on a serotype prevalence for the design of a multivalent pneumococcal capsular polysaccharide-protein conjugated vaccine. The design of the study will be elaborated in such a way that it will be able to provide:

1. prevalence of serotypes in the Region;
2. difference in prevalence according to geographic or climatic region;
3. a representative study.

In order to achieve this, standardized methodology will be developed and each participating center should proceed according to the established guidelines.
The criteria for selection of the countries will take into consideration the epidemiological importance, the geographic distribution of the study, and the existence of technical feasibility. An expert group will make site visits for the technical assessment of the country studies.

Among the many activities to be accomplished are: the selection of the Technical Advisory Committee, an evaluation of the National Research Protocols, and preparation of Technical Service Agreements with the countries before the study can start. The Timetable was defined and the studies are scheduled to start by next August or September.

B. CVI'S MEETING ON REGIONAL DPT VACCINE PRODUCTION

After reviewing the current status of DPT vaccine manufacturing in the world and considering the rapid technological changes occurring with regard to DPT and DPT-based combination vaccines, the Task Force on Strategic Plans of the Children's Vaccine Initiative considered the importance of strengthening DPT vaccine manufacturing capability in developing countries and recommended undertaking a major program to ensure the supply of high quality DPT vaccines in the developing world and to introduce appropriate combination vaccines based on DPT manufactured in developing countries.

The strategy proposed by the CVI Task Force on Strategic Plans requires coordinated planning at national, regional and international levels. The advanced status of vaccine research and manufacturing in several countries in the Region of the Americas and the existence of the Regional System of Vaccines/SIREVA prompted a request by the CVI Standing Committee to the PAHO Director to organize a meeting to prepare a regional plan for producing improved DPT and DPT-based combination vaccines in the Region of the Americas.

The CVI Standing Committee Meeting will be held the first week of September, at PAHO Headquarters. The Chairman will begin by discussing the meeting on DPT vaccines which will be held at the end of June, at WHO/Geneva. The prospects of improving DPT vaccines and DPT-combination vaccines will be brought up-to-date.

The DPT Director and Chief of Production of each laboratory in the Region will be invited to participate and to present a short paper on the situation of DPT production.
The review of DPT vaccine production in the Region will be presented by PAHO staff. This review should be comprehensive and give an overview, diagnosing the situation, needs, and requirements in order to participate in the production of improved DPT and DPT-combined vaccines.

In order to gather all the required information for the presentation, two groups will be organized and will visit each of the laboratories in the Region during the following 30 days. A comprehensive questionnaire will be prepared to assess the actual situation and the potentiality of each laboratory.

The last session will deal with discussion and possibly the development of a Regional strategic plan for DPT vaccine production.

C. NETWORK OF VACCINE QUALITY CONTROL LABORATORIES

The existence of several quality control laboratories in the Region that perform relatively well the activities of vaccine quality control prompted PAHO to develop a project to organize a Network of Vaccine Quality Control laboratories. This project was prepared after a meeting at PAHO Headquarters, in May 1991, with participants from several quality control laboratories.

The following are the objectives:

1. Guarantee the potency and safety of the vaccines used in Latin America at every step: acquisition, receipt, storage, distribution, and immunization;

2. Actively support the vaccine quality control programs in the different countries and encourage the development of official quality control laboratories;

3. Strengthen the quality control laboratories of the countries so they can control the quality of all the vaccines used in their immunization programs.

A specific project is already prepared and has been presented to several international funding agencies for support.

D. FOUR SWEDISH WC/RBS CHOLERA VACCINE TRIALS

The final report of the Phase II Study conducted in Barranquilla, Colombia, has been completed.
The protocol for the Phase III trial was also finished, and site visits to Barranquilla and Buenaventura were conducted. However, due to a decrease in the number of cholera cases in these localities, the expert group that made the site visit recommended that, since Colombia is no longer suitable for this project, another country should be selected.

The PAHO Cholera Task Force, by analyzing the cholera epidemiological data and taking into consideration the up-to-date information, will make the preliminary selection of cities and localities with potentialities for field trials. Thereafter, a consultant will be hired to accomplish and finalize the details for the field trials.