

*Dysentery, Bacillary - prevention and control*



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*Bacterial vaccines*

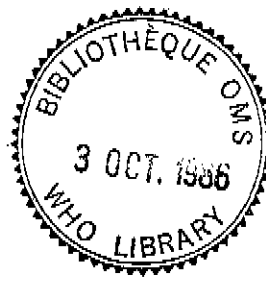
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DEVELOPMENT OF VACCINES AGAINST SHIGELLOSIS\* :

*recepteur*

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*multicell*

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## 1. INTRODUCTION

Dysentery and diarrhoea caused by Shigella are major public health problems in the developing countries. Shigella is one of the five most frequently identified pathogens in children with acute diarrhoea or dysentery (the others being rotavirus, enteropathogenic Escherichia coli, enterotoxigenic E. coli and Campylobacter jejuni). Infection with Shigella may lead to a number of serious complications and mortality rates can be high. Severe epidemics may be caused by S. dysenteriae 1 (Shiga's bacillus).

The development of vaccines for the prevention of shigellosis is particularly important since the measures currently used to control this infection, particularly those concerned with case management and the control of epidemics, are of limited efficacy. For example, strains of Shigella, especially epidemic strains of S. dysenteriae 1, may be resistant to most available antibiotics, and even appropriate antibiotics may not produce a rapid clinical improvement or prevent death when disease is severe. Oral rehydration therapy, also, is of little value for patients with dysentery, as dehydration is not necessarily an important feature of serious illness. Furthermore, treatment of serious episodes is expensive due to the need for specialized care, which may require the use of new and costly antibiotics. Measures besides vaccines that are of value in preventing shigellosis involve the interruption of transmission of the pathogen by improved personal hygiene and sanitation, and the provision of adequate quantities of water for household use. It is also probable that the incidence or severity of illness can be diminished by non-specific interventions such as measles immunization, measures to improve nutrition, and possibly vitamin A supplementation.

This report summarizes the present status of efforts to develop vaccines for shigellosis and lists recommended priority research topics. It is hoped that these recommendations will serve to stimulate further research which will ultimately lead to the development of efficacious anti-shigellosis vaccines. The report is the third of a series of reviews related to this subject issued by WHO since 1980<sup>1,2</sup>.

## 2. EPIDEMIOLOGY

### 2.1 Endemic shigellosis

Endemic shigellosis, due mostly to S. flexneri (in developing countries) and S. sonnei (in developed countries) is a worldwide problem. In most developing countries, shigellosis has a high morbidity rate, and in some there is also substantial mortality. Children between 1 and 5 years are especially affected. Transmission is primarily from person to person and is greatest when personal and domestic hygiene are very poor; food and water may also be sources of infection. Secondary infection rates within affected families may be as high as 30 to 50%. Natural animal hosts (excluding Rhesus monkeys in captivity) or environmental reservoirs have not been described.

### 2.2 Epidemic and pandemic shigellosis

A pandemic of shigellosis due to S. dysenteriae 1 began in 1969 in Central America and now encompasses a large area of Central Africa and countries of, and adjacent to, the Indian subcontinent. Plasmid analysis has shown that the pandemic is not due to the spread of a single clone of S. dysenteriae 1 and the reasons for the occurrence of large outbreaks at separate geographical sites are unclear. In each location, however, the strain has been resistant to multiple antibiotics, including those most frequently used to treat shigellosis; for example, the strain currently prevalent in Bangladesh is resistant to both trimethoprim-sulfamethoxazole and ampicillin.

<sup>1</sup> Enteric infections due to Campylobacter, Yersinia, Salmonella, and Shigella. Bulletin of the World Health Organization, 58: 519-537 (1980)

<sup>2</sup> The invasive diarrhoeas: A review of diarrhoeas due to Shigella species, Campylobacter jejuni, and enteroinvasive Escherichia coli. Unpublished WHO document CDD/BEI/82.4 (1982)

### 2.3 Control strategies

At present, the only strategies that can prevent infection are improved sanitation and increased water supply, which act by reducing faecal-oral transmission. Specific effective measures include handwashing with soap and water after defecating and before handling food, hygienic practices in the preparation and storage of food, and safe disposal of faeces. Breast-feeding also appears to be effective in reducing the severity of Shigella infections in infants and young children up to 3 years of age.

## 3. PATHOGENESIS AND CLINICAL FEATURES OF SHIGELLOSIS

### 3.1 Pathogenesis

Shigellosis is the most communicable of all bacterial enteric diseases: as few as 10 live organisms can cause disease in healthy adults. Little is known about the ways in which Shigella (which are acid-sensitive) survive in the environment of the stomach, evoke watery diarrhoea, which is a feature of some cases, or migrate from the lumen to the epithelial surface of the large bowel. It is certain, however, that to cause dysentery, Shigella must penetrate and multiply within the epithelial cells of the colon. This process leads to epithelial cell death, mucosal inflammation, and epithelial ulceration and haemorrhage, which are the pathological hallmarks of the disease.

### 3.2 Clinical features

The clinical manifestations of shigellosis generally include frequent passage of stools containing blood and mucus, fever, abdominal pain, and tenesmus. The illness usually starts with watery diarrhoea followed after 24 to 48 hours by the appearance of blood and mucus in the stools. Dehydration may occur, but only in a small proportion of cases. Various complications, which may occur in 15-30% of hospitalized cases, and especially in children, include prolonged anorexia, nutritional deterioration, protein-losing enteropathy, the haemolytic uraemic syndrome, leukemoid reaction, pneumonia, conjunctivitis, arthritis, paralytic ileus, toxic megacolon, colonic perforation, rectal prolapse, and persistent diarrhoea. Mortality rates in hospitalized cases due to S. dysenteriae 1 can exceed 10% despite treatment by recommended methods. The disease is most severe, and mortality highest, when it occurs following measles or there is pre-existing malnutrition.

## 4. IMMUNITY TO SHIGELLOSIS

### 4.1 Protection due to previous infection

Several lines of evidence indicate that shigellosis is an immunizing disease. These include epidemiological data, observations in volunteers, and studies in animals. Studies at a custodial institution in which infections with S. sonnei and S. flexneri 2a were endemic showed that children with very poor personal hygiene experienced a high attack rate of clinical shigellosis during the first 12-24 months after admission. Thereafter, attack rates fell sharply and remained low for the duration of stay in the institution despite continued frequent exposure to infected and ill children. This pattern seemed to indicate that after one or more clinical infections with Shigella the children became at least partially immune to reinfection. Likewise, the occurrence of shigellosis predominantly in young children in endemic areas suggests an age-related acquisition of immunity. Volunteer studies demonstrated that young adults who developed shigellosis after an initial experimental challenge were protected against illness when subsequently challenged with the homologous strain (S. flexneri 2a), the protective efficacy of prior infection being 64%. However, there was no difference between rechallenged and control volunteers in the excretion rate for Shigella; it is possible that more than one exposure to Shigella is needed to evoke high-level immunity. Finally, studies in both monkeys and rabbits have shown solid protection against homologous rechallenge 3 to 4 weeks after an initial virulent challenge.

### 4.2 Mechanisms of immunity to shigellosis

Immunity to shigellosis appears to depend largely or entirely upon local immune mechanisms. Parenteral immunization leads to high titres of circulating antibodies but is

not usually protective. Most examples of protection by immunization involve the presentation of antigens by the oral or enteric route. The exact mechanisms of protection are not known and may involve local antibodies, mucosal cell-mediated immunity, or both. It is likely that these interfere with multiplication of the small inoculum initially ingested, although it is not known at what site this effect occurs. Whether antibody to Shiga toxin plays a protective role, especially against infection due to S. dysenteriae 1, is not certain.

#### 4.3 Animal models for study of immune protection

Animal models that have been used to study protective immunity include the guinea-pig keratoconjunctivitis model (Séreny test), orally challenged Rhesus monkeys, and a recently developed oral challenge model in rabbits. The two oral challenge models appear to be preferable because they involve enteric infection. The monkey model has been used most extensively in vaccine studies. Its advantages include the similarity of the disease in monkeys to that seen in man, the fact that captive monkeys are highly susceptible to infection with Shigella, and the capacity of the model to demonstrate vaccine-induced protection. Disadvantages include the high cost of Rhesus monkeys, technical problems in handling them, and the possible confounding effect of previous unrecognized infection with Shigella.

An adult rabbit model has been developed for S. flexneri infection which involves pre-treatment of the animals with tetracycline, oral administration of tetracycline-resistant bacteria after neutralization of gastric acid, and a single intra-peritoneal dose of opium. With this model, small bowel colonization can be documented following inoculation with  $10^6$ - $10^9$  viable bacteria and death occurs following inoculation with  $10^{10}$  bacteria. An initial colonization provides solid protection against a subsequent colonizing or lethal challenge. This model should be useful for further studies of immunity to Shigella flexneri; it has not, however, proved suitable for studying immunity to S. dysenteriae 1, as the strains tested colonize poorly and do not cause illness.

#### 4.4 Serology of infection with Shigella

Shigellosis may evoke both humoral and cell-mediated immune responses and these may be either mucosal or systemic. So far, only systemic humoral responses have been studied in depth. Antibodies measured have been directed against the lipopolysaccharide (O-antigen) and Shiga toxin. Responses to outer membrane proteins (encoded by the 140 Mdal virulence plasmid) have been studied only to a limited extent, using the Western blot method.

##### 4.4.1 Assay of antibodies to Shigella LPS

Lipopolysaccharide (LPS) antigens can be prepared in pure form and used in enzyme immunoassays (EIA) for sensitive, class-specific antibody determinations. However, most Shigella O-antigens, except that of S. sonnei, cross react with those of certain E. coli, which reduces their specificity.

The O-antigen of S. dysenteriae 1 is related to E. coli O group 1, but has little known relationship with other E. coli or other enterobacteria. A sensitive and specific EIA has been developed which can be used for seroepidemiological purposes and to study the immune response to candidate vaccines.

S. flexneri have some O-antigenic relatedness with other enterobacteria, but there is also extensive cross reactivity between serotypes 1 to 5. Consequently, serotype-specific antibody assays based on LPS antigens are, with a few exceptions, not feasible. However, an S. flexneri EIA has been developed which is sensitive and readily detects antibody responses after natural infections. This assay may be useful for assessing the immunogenicity of candidate S. flexneri vaccines.

S. sonnei has a unique O-antigen which is so far known to be shared only by one serotype of Plesiomonas shigelloides. A sensitive and specific EIA exists which is suitable for assessing the immunogenicity of vaccine candidates and for seroepidemiological studies.

#### 4.4.2 Systemic and intestinal antibody responses

Studies of the Ig class of serum antibody to Shigella LPS in man show that the IgA response peaks after 2-3 weeks and remains elevated for about 2 months; the IgM response follows a similar pattern. In contrast, the IgG response peaks after 3-6 weeks and remains elevated for 6-12 months after the diarrhoeal episode.

Intestinal antibody responses to Shigella have not been extensively studied in man. Studies in rabbits, however, have shown that the inoculation of live S. flexneri or E. coli/S. flexneri hybrids into jejunal Thiry-Vella loops elicits a pronounced sIgA anti-LPS response. The observation that this response can be rapidly boosted by reinoculation after several months supports other evidence that memory exists in the intestinal sIgA system.

Few studies have been done on the Shiga antitoxin response following Shigella infections. Preliminary investigations using an EIA to detect Shiga antitoxin revealed antibody responses in the sera of patients infected with S. dysenteriae 1 but not in the sera of patients infected with S. flexneri or S. sonnei. However, another study using a sensitive and specific antitoxin assay, based on neutralization of toxin-induced damage to HeLa cell monolayers, detected toxin-neutralizing antibodies in the sera of patients convalescent from infections due to S. flexneri and S. sonnei. The reason for this discrepancy is not known and further studies are necessary to evaluate the usefulness of these different assays.

### 5. VIRULENCE DETERMINANTS OF POSSIBLE RELEVANCE TO VACCINE DEVELOPMENT

A detailed understanding of the virulence mechanisms of Shigella and of the specific antigens involved in virulence should facilitate the rational development of Shigella vaccines. Key events in the pathogenesis of shigellosis include epithelial cell invasion, intracellular multiplication of shigellae, epithelial death followed by bacterial spread within the mucosa, and finally, tissue destruction associated with locally severe inflammation. Current knowledge of these events and of the possible pathogenic role of Shiga toxin is summarized below.

#### 5.1 Epithelial cell invasion

Available data indicate that a variety of plasmid and chromosomal genes mediate the ability of Shigella to invade, multiply in, and eventually destroy intestinal epithelial cells. A large plasmid (120-140 Mdal in size), referred to as the virulence plasmid, is essential to all Shigella serotypes for inducing their own phagocytosis by epithelial cells and for promoting the rapid intracellular growth of bacteria. The molecular mechanisms involved in the phagocytosis of Shigella by epithelial cells are not yet understood; however, several outer membrane proteins encoded by the virulence plasmid have been identified and may play key roles in the process. Recent evidence indicates that the same plasmid encodes a contact haemolytic activity which causes lysis of the membrane of the phagocytic vacuole, thus releasing bacteria into the cytoplasm where they are able to multiply rapidly and escape attack by lysosomal enzymes.

#### 5.2 Bacterial spread within the mucosa

The spread of shigellae within the mucosa requires that the bacteria survive and multiply within the lamina propria, a process which leads to acute inflammation and tissue destruction. Several chromosomal segments have been identified that specify bacterial functions thought to be involved in these processes. These regions have been associated with: (i) O-antigen formation, (ii) high-affinity iron uptake systems, or (iii) the capacity to provoke keratoconjunctivitis in guinea-pigs.

Two chromosomal loci specify complete O-antigen biosynthesis in S. flexneri. On the other hand, S. sonnei O-antigen is encoded by the large virulence plasmid. In S. dysenteriae 1, complete expression of O-antigen requires both a small 6 Mdal plasmid and a chromosomal locus. The role of Shigella O-antigen in bacterial virulence has not been fully defined, but smooth LPS may be important both for intraluminal survival of Shigella and for protection of the bacteria in tissue from the bacteriocidal activity of serum.

### 5.3 Shiga toxin

S. dysenteriae 1 tends to cause more severe dysentery than other serotypes of Shigella and also produces high levels of Shiga toxin; whether or not these two features of S. dysenteriae 1 are causally related is not known. Shiga toxin production has not been consistently demonstrated among other Shigella, and where it has been detected (some strains of S. flexneri 2a and S. sonnei) the level of toxin was 10 000- to 100 000-fold less than that measured with S. dysenteriae 1.

Shiga toxin produced by S. dysenteriae 1, which inhibits protein synthesis in eukaryotic cells, has been purified to homogeneity and partially characterized. It is composed of one A subunit containing the enzymatically active component and 5 or 6 copies of a receptor-binding B subunit, and has three biological activities: (i) cytotoxicity for certain eukaryotic cells, (ii) paralytic activity or lethality for various animal species, and (iii) enterotoxigenicity in the rabbit small intestine. These biological activities could explain the greater severity of disease caused by S. dysenteriae 1, the watery diarrhoea that may occur during shigellosis, and the lesions in the vascular endothelium of the kidney that characterize the haemolytic uraemic syndrome which sometimes complicates shigellosis.

## 6. SHIGELLA VACCINES

### 6.1 Background

In studies carried out in the 1940s and 1950s, parenteral killed whole-cell Shigella vaccines failed to provide significant protection either in experimental challenges of volunteers or in controlled field trials in endemic settings; a few attempts to immunize monkeys orally with killed Shigella also yielded equivocal results. Since the mid-1960s, research has focused largely on the development of live oral Shigella vaccines and several have been prepared that have proved to be safe in clinical studies; some of these also prevented shigellosis in experimental challenge studies in volunteers, in controlled field trials, or in both. However, none was ideal: too many doses were required, occasional genetic revertants arose, and in certain populations side effects (e.g., vomiting) were encountered.

The advent of recombinant DNA technology has brought the potential to analyse with precision important determinants of bacterial pathogenicity and immunogenicity at both the molecular and genetic levels. This should facilitate the construction of defined bacterial strains with the properties considered important in a vaccine for shigellosis, as well as safety, genetic stability, immunogenicity with a minimum number of doses, and amenability to large-scale production and lyophilization. An important requirement is that such strains efficiently colonize or invade the intestinal epithelium so that effective delivery of antigens to enteric lymphoid tissue is assured. Vaccine strains developed by these methods for other enteric diseases may also prove suitable as carriers for delivering the critical protective antigens of Shigella, thus generating bivalent or possibly multivalent vaccines. Currently, researchers are using genetic engineering techniques to develop several types of Shigella vaccines.

Previous and current live oral Shigella vaccine candidates can be divided into four broad classes:

- i) attenuated Shigella mutants;
- ii) "mutant hybrids" (Shigella attenuated by the incorporation of E. coli gene segments);
- iii) E. coli that contain introduced Shigella genes; and
- iv) other carrier bacteria (such as attenuated Salmonella typhi) that contain genes encoding synthesis of critical Shigella antigens.

The most noteworthy of these candidate vaccines are briefly reviewed below.

## 6.2 Attenuated Shigella mutants

### 6.2.1 T32 Istrati

This strain is a mutant developed in Romania by repeated passage of a S. flexneri 2a strain on agar; it lacks the plasmid that encodes invasiveness and appears to be genetically stable. Large controlled field trials in Romania and China using an immunization schedule involving 4 or 5 doses of up to  $3 \times 10^{11}$  live organisms per dose have shown this vaccine to be safe and effective in preventing clinical shigellosis; it is not known, however, whether the vaccine would be effective after lyophilization. In some field trials, significant protection was also observed against serotypes other than S. flexneri 2a, i.e., other serotypes of S. flexneri and S. sonnei. This attenuated mutant vaccine strain is in widespread use in Romania, where experience suggests that its efficacy can be prolonged with biannual booster doses. Its major shortcoming is the requirement for multiple large doses and frequent boosting.

### 6.2.2 Streptomycin-dependent mutants

Non-invasive streptomycin-dependent (SmD) strains of several S. flexneri and S. sonnei serotypes were developed in Yugoslavia and found to be safe and protective when used as oral vaccines in volunteers and in large-scale controlled field trials in that country. After 4 or 5 doses of up to  $5 \times 10^{10}$  live bacteria per dose, these vaccines evoked serotype-specific protection for at least 6 months, but less than one year; a single oral booster dose after one year maintained protection for an additional year. Limited field trials with SmD vaccines were also carried out in the USA in the early 1970s. In one trial involving institutionalized children who were intensely exposed to Shigella, the vaccines were ineffective. Moreover, in some trials, reversion to streptomycin independence occurred, especially with the S. sonnei strain. No further studies of these strains have been performed.

### 6.2.3 New attenuated mutants

New attenuated Shigella mutants are under development, including aromatic (aro<sup>-</sup>) auxotrophic mutants and galactose epimeraseless (gal E) mutants. These vaccines would differ from T32 and SmD mutants in being genetically defined and capable of invading epithelial cells. The basis for their attenuation would be their inability to sustain growth within host tissue. Another possible approach to preparing attenuated Shigella mutants would be to delete the genes encoding the production of Shiga toxin, high affinity iron uptake systems, or both, while retaining the plasmid that encodes epithelial invasion.

## 6.3 Mutant hybrid vaccines

The introduction of specific E. coli K-12 chromosome segments (e.g., the xylose-rhamnose region) into pathogenic Shigella drastically reduces their virulence. Transfer of these segments into attenuated colonial mutants of Shigella produced a series of relatively stable, non-invasive hybrid derivatives of S. flexneri 2a and S. dysenteriae 1 which protected monkeys against experimental shigellosis. The S. flexneri 2a hybrid was shown to be genetically stable and safe in both healthy adults and healthy institutionalized children. However, the extent of protection observed in volunteers did not reach statistical significance and it was noted that the strain did not proliferate well in vivo. The S. dysenteriae 1 hybrid, although genetically unstable in one of 145 volunteers, was notable in that it exhibited good intestinal colonization after a single oral dose. No field trials were undertaken to test the efficacy of these vaccine strains.

## 6.4 E. coli with genes encoding Shigella antigens

In the mid-1970s, loci specifying S. flexneri 2a group and type-specific somatic antigens were transferred into an E. coli O8 strain and the resultant genetically stable, non-invasive hybrid, which expressed the Shigella O-antigen, was tested as a live oral vaccine. Although protective in monkeys and safe in adults, this candidate vaccine did not protect adult volunteers in experimental challenge studies.

More recently, the 140 Mdal plasmid that encodes epithelial cell invasion has been transferred into E. coli K-12, together with chromosomal genes encoding the group and type-specific O-antigens of S. flexneri 2a. The resultant hybrid E. coli expresses smooth S. flexneri 2a O-antigen and invades epithelial cells, but does not cause fluid secretion in ligated segments of rabbit intestine. This vaccine is both safe and protective in monkeys. Clinical studies of vaccine safety and efficacy in healthy adult volunteers are under way. Analogous E. coli K-12 strains expressing other S. flexneri O-antigens or S. dysenteriae 1 O-antigen have also been prepared and studies in volunteers are planned.

## 6.5 Other carrier bacteria that express Shigella antigens

### 6.5.1 Salmonella typhi hybrids that express Shigella antigens

Attenuated S. typhi strains developed as live oral vaccines for typhoid fever are potential carriers for the delivery of selected Shigella antigens to host lymphoid tissue; strains of this type might provide protection against both typhoid fever and shigellosis. Such attenuated S. typhi strains, which include Ty21a (a chemically-induced gal E mutant) and 541Ty (a genetically-defined aro<sup>-</sup>, pur<sup>-</sup> mutant), apparently reach intestinal lymphoid tissue where they stimulate cell-mediated immune mechanisms as well as, to a variable extent, circulating and local intestinal antibody responses.

One candidate vaccine (5076-IC) consists of Ty21a into which has been incorporated the 140 Mdal plasmid of S. sonnei and which expresses S. sonnei (as well as S. typhi) O-antigen. In volunteers, this bivalent vaccine is both safe and effective against challenge with S. sonnei. However, variability in the efficacy of different vaccine lots has delayed the initiation of field trials.

If other effective live oral bacterial vaccines are developed, for example, attenuated Vibrio cholerae, these might also prove useful as carriers of Shigella antigens.

### 6.5.2 General considerations regarding the effective expression of Shigella antigens by carrier organisms

As noted above, it is likely that only one or at most a few Shigella antigens, such as the O-antigen, specific outer membrane proteins, and perhaps a Shiga toxoid, may be required to evoke protection. It is, therefore, feasible to construct a series of hybrid plasmid "cassettes" or modules encoding these antigens which could be inserted into selected "antigen delivery systems" (e.g., E. coli or heterologous live bacterial vaccines). For example, the S. dysenteriae 1 chromosomal and plasmid genes encoding O-antigen synthesis have been cloned and combined into a single hybrid plasmid, thus creating a convenient vehicle for transfer of the determinants of this antigen to a variety of carrier bacteria.

Further research will be required, however, to define the requirements for efficient expression of these antigens in an optimally immunogenic form. For example, transcriptional and translational problems may be encountered, which may require that the relevant genetic regulatory mechanisms be analysed and modified. Similarly, carrier organisms may not assemble and present heterologous antigens in an optimally immunogenic form. In particular, the assembly of functional heterologous O-antigen may require additional genetic manipulations that provide an alternative LPS core upon which the new O-side chain can be built. It may also be appropriate to eliminate or modify the expression of homologous O-antigen so that it does not compete with the heterologous moiety for attachment to the core. A better understanding of the interactions of proteins with other components of the bacterial membrane, such as LPS and peptidoglycan, is required, as these interactions may have a considerable influence on the ways in which such antigens are assembled at the bacterial surface and interact with the mucosal immune system.

## 7. SHIGELLA VACCINE FIELD TRIALS

Field trials are being contemplated for candidate Shigella vaccines to determine their efficacy under probable conditions of vaccine use. The general concepts of clinical trials (e.g., the need for a sufficiently large sample, an adequate control population, and unbiased case detection mechanisms) apply to these trials as to any others. Additional features of

particular importance for a Shigella vaccine trial must also be included. Some of these general and specific features are described below.

### 7.1 Field trial population

The field trial should involve populations similar to those in which the vaccine would eventually be used. The immunized and unimmunized groups should be comparable with regard to age, sex, nutritional status, and incidence of previous infection with Shigella. The population should be defined with regard to general demographic characteristics and the presence of risk factors particularly pertinent to shigellosis (e.g., malnutrition, measles, and possibly vitamin A deficiency).

### 7.2 Randomization

Generally, random assignment of vaccine or placebo to individuals is preferred; however, some live Shigella vaccines may be excreted in faeces and transmitted to others in the family. By this means, members of a vaccinee's family may be unintentionally immunized. For potentially transmissible vaccine strains, randomization should be by household and the same preparation should be given to all eligible members of a household.

### 7.3 Outcome events

Shigella vaccines are intended to protect individuals from clinical shigellosis; however, the possibility that immunization might diminish the incidence of asymptomatic infection should also be considered. Among outcome events, diarrhoea or dysentery associated with a positive stool culture for Shigella is the most important, although the incidence of asymptomatic infection should also be determined.

Cases may be detected among patients attending hospitals or clinics, or by frequent (e.g., twice or thrice weekly) active surveillance in the community. Cases detected by active surveillance are likely to be relatively mild, whereas those detected in hospitals and clinics are likely to be more severe. The possibility that immunization may be most effective in reducing the incidence of severe, rather than mild or asymptomatic, infection should be considered. This may require that case detection methods emphasize surveillance of patients presenting to clinics and hospitals. On the other hand, active, community-based surveillance will be especially important when evaluating a S. sonnei vaccine, as disease caused by this serotype tends to be mild. Asymptomatic infections should be detected by periodic culturing of a random sample of healthy vaccinees and controls.

Case definitions must be developed that are precise, generally applicable, and can be easily used by field workers in an unbiased way. Specifically, definitions are needed for the following: "a vaccinated individual", "diarrhoea", "dysentery", "shigellosis", "mild, moderate, and severe disease", "persistent diarrhoea" and "asymptomatic infection". Definitions may combine both clinical and laboratory findings and should distinguish cases in which Shigella is the only isolated pathogen from mixed infections due to Shigella and another enteric pathogen. Sample size calculations should be based on established rates of specific outcome events in the community under study (e.g., dysentery or diarrhoea due to the Shigella serotype present in the vaccine).

### 7.4 Laboratory methods

#### 7.4.1 Tests for faecal leukocytes and blood

To differentiate dysentery from diarrhoea, standardized procedures should be used to document the presence of faecal leukocytes and blood. A chemical test for blood plus a microscopic examination for faecal leukocytes on each specimen may be required.

#### 7.4.2 Microbiological methods

Whenever possible, stools or rectal swab specimens should be plated onto solid media immediately after collection. When this is not practical, specimens should be transported to the laboratory within 24 hours (preferably less) using buffered glycerol saline (chilled, if

possible) as the transport medium. Cary-Blair medium is not ideal for the transport of Shigella but should be used when other enteric pathogens will be sought.

Primary isolation should include at least two culture media. The choice depends somewhat on the serotypes being sought; however, xylose-lysine-desoxycholate (XLD) agar would usually be one choice, the second being either MacConkey or SS agar. A detailed description of methods for isolating and identifying Shigella is provided in the Manual for Laboratory Investigations of Acute Enteric Infections (Unpublished WHO document CDD/83.3 Rev. 1, 1986).

## 8. RESEARCH RECOMMENDATIONS

### 8.1 Animal models

8.1.1 A convenient small animal model is required that would be suitable for detailed, quantitative studies on the pathogenesis of infection with live Shigella (including S. sonnei, S. flexneri and S. dysenteriae 1), the efficacy of candidate vaccines, and mechanisms of immunity to infection. Where possible, isogenic strains of Shigella with specific genetic modifications or deletions should be used for studies of mechanisms of pathogenesis and immunity.

### 8.2 Virulence and immune mechanisms

8.2.1 The cell surface antigens of Shigella should be more extensively characterized and their role in pathogenesis and immunity defined. Minor and as yet unidentified antigens should also be investigated, including those expressed under conditions prevalent in the bowel. Monoclonal antibodies to such antigens will probably be required.

8.2.2 The role of Shiga toxin in the virulence of S. dysenteriae 1 (and other shigellae) should be determined and the protective role of mucosal or systemic antitoxin, if any, defined. The generation of toxin-negative mutants of S. dysenteriae 1 is critical to this analysis. Such strains should be evaluated by standardized methods, preferably at a single reference laboratory, to confirm the toxin-negative phenotype (or genotype). Sequellae of shigellosis that may be toxin-mediated, such as the haemolytic-uraemic syndrome, should be included in these studies.

8.2.3 The process and mechanism of mucosal colonization by Shigella should be defined, and the antigens involved in this process identified. The uptake and fate of Shigella in epithelial M cells should be investigated and correlated with the immunogenicity of individual strains. Isogenic virulent and avirulent mutants should be used in these studies.

### 8.3 Immunity

8.3.1 The extent to which protection against shigellosis induced by prior infection is serotype-specific, or extends to heterologous serotypes, should be evaluated in animal models, volunteer studies, longitudinal population-based studies, and vaccine field trials. Attention should be given to possible cross protection between S. flexneri serotypes, and between S. flexneri and S. dysenteriae or S. sonnei.

8.3.2 The roles of humoral and cell-mediated immune mechanisms, especially those operative within the bowel mucosa or at its surface, should be defined. The antigens (or antigen combinations) responsible for these responses should be identified.

8.3.3 It should be determined whether antibodies in milk or colostrum provide protection against shigellosis; if so, the antigens and antibodies involved, and the extent of this protection, should be defined.

8.3.4 The systemic and mucosal immune responses in shigellosis should be defined. These include responses to O-antigens, outer membrane proteins, Shiga toxin, and other antigens of possible pathogenic importance.

#### 8.4 Vaccine development and evaluation

8.4.1 For public health use in developing countries, research efforts should focus on the development of vaccines for S. dysenteriae 1 and the most prevalent serotypes of S. flexneri (1b, 2a, 3a, 4a). Polyvalent vaccines may be required.

8.4.2 Vaccine development should focus on live vaccines for oral use, including both avirulent mutants of Shigella and hybrid strains (heterologous vectors expressing Shigella antigens).

8.4.3 Optimal carriers for hybrid vaccines should be defined. These may include live S. typhi vaccine strains, avirulent E. coli, and live, avirulent V. cholerae vaccines. The features that determine the efficacy of a carrier should be defined.

8.4.4 Optimal methods for the preparation and administration of live oral Shigella vaccines should be defined. This would involve studies to determine means of formulation that maximise bacterial recovery and growth in an immunogenic form when reconstituted. It should also be determined whether or not the inoculum needs to be protected from gastric acid.

8.4.5 Shigella isolated from field trial participants should be carefully preserved to permit their subsequent evaluation for possible antigenic differences between isolates from vaccinees and controls.

8.4.6 Vaccine efficacy should be assessed with regard to duration of protection, efficacy of booster immunizations, age of vaccinees (especially below the age of 3 years), and effect upon disease severity, and in relation to the nutritional status of the vaccinees.

8.4.7 Standard criteria should be developed for the evaluation of Shigella vaccine efficacy. These should include definitions of Shigella diarrhoea and criteria for assessing the severity of illness. Standard laboratory diagnostic methods should also be recommended.

8.4.8 Vaccine development should seek a product that would be effective and safe in infants below 1 year of age and could be incorporated into the delivery system of national Expanded Programmes on Immunization.

#### LIST OF PARTICIPANTS

Dr C. Ferreccio, Department of Program Support, Ministry of Health, Santiago  
Chile

Dr S. Formal, Chief, Department of Bacterial Diseases, Walter Reed Army Institute of  
Research, Walter Reed Army Medical Center, Washington, D.C., USA

Professor M.M. Levine, Director, Center for Vaccine Development, University of Maryland  
School of Medicine, Baltimore, MD, USA

Professor A.A. Lindberg, Head, Department of Clinical Bacteriology, Karolinska Institute,  
Huddinge University Hospital, Huddinge, Sweden (Chairman)

Dr P.A. Manning, Department of Microbiology and Immunology, University of Adelaide, Adelaide,  
SA, Australia

Dr T. Meitert, Cantacuzino Institute, Bucharest, Romania

Professor A.D. O'Brien, Department of Microbiology, Uniformed Services University of the  
Health Sciences, Bethesda, MD, USA

Dr S.C. Pal, Director, National Institute of Cholera and Enteric Diseases, Calcutta, India

Dr David Sack, Associate Director, Disease Transmission, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh (Rapporteur)

Dr P. Sansonetti, Service des Entérobactéries, Institut Pasteur, Unité 199 INSERM, Paris, France

Professor Y. Takeda, Department of Bacterial Infections, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Dr D. Taylor, United States Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Professor K. Timmis, Département de Biochimie médicale, Centre médical universitaire, Geneva, Switzerland

Observer:

Dr F. Tron, Director of Clinical Research, Pasteur Vaccins, Marnes-la-Coquette, France

International Centre for Diarrhoeal Disease Research, Bangladesh:

Dr M. Bennis, International Research Associate, Pathogenesis and Therapy

Dr I. Ciznar, Associate Director, Host Defense

Dr A. Salam, Bangladesh Medical Officer

Secretariat:

Dr B.B. Gaitonde, WHO Regional Office for South-East Asia

Dr N.F. Pierce, Research Coordinator, WHO Diarrhoeal Diseases Control Programme

(Secretary)

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