

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization.

WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES

No. 414

CHOLERA IMMUNOLOGY

Report of a WHO Scientific Group

WORLD HEALTH ORGANIZATION

GENEVA

1969

© World Health Organization 1969

Publications of the World Health Organization enjoy copyright protection in accordance with the provisions of Protocol 2 of the Universal Copyright Convention. Nevertheless governmental agencies or learned and professional societies may reproduce data or excerpts or illustrations from them without requesting an authorization from the World Health Organization.

For rights of reproduction or translation of WHO publications *in toto*, application should be made to the Division of Editorial and Reference Services, World Health Organization, Geneva, Switzerland. The World Health Organization welcomes such applications.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the Director-General of the World Health Organization concerning the legal status of any country or territory or of its authorities, or concerning the delimitation of its frontiers.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature which are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

PRINTED IN FRANCE

CONTENTS

	Page
1. Recent concepts of immunity in cholera and enteric infections	5
2. Antigens and their fractions	10
3. Antibodies and their measurement	12
4. Relationship of antibodies to immunity	13
5. Vaccines	15
6. Recommendations for research	18

WHO SCIENTIFIC GROUP ON CHOLERA IMMUNOLOGY

Teheran, 14-19 September 1968

Members : *

- Dr K. Bhaskaran, Scientist, Central Drug Research Institute, Lucknow, India
(*Rapporteur*)
- Professor W. Burrows, Department of Microbiology, University of Chicago,
Ill., USA
- Dr G. Edsall, Superintendent, Massachusetts State Laboratory Institute, Boston,
Mass., USA
- Dr J. C. Feeley, Chief, Bacterial Vaccines Section, Division of Biologics Standards,
National Institutes of Health, Bethesda, Md., USA (*Rapporteur*)
- Dr O. Felsenfeld, Chief, Division of Communicable Diseases, Tulane University
Delta Primate Research Centre, Covington, La., USA (*Chairman*)
- Dr I. Joó, Deputy Technical Director, "Human" Serum and Vaccine Institute,
Budapest, Hungary (*Vice-Chairman*)
- Dr Y. Watanabe, Department of Microbiology, University of Texas Medical
Branch, Galveston, Texas, USA
- Professor R. G. White, Department of Bacteriology and Immunology, Western
Infirmary, Glasgow, Scotland

Secretariat :

- Dr B. Cvjetanović, Chief Medical Officer, Bacterial Diseases, WHO, Geneva
(*Co-Secretary*)
- Dr H. C. Goodman, Chief Medical Officer, Immunology, WHO, Geneva
(*Co-Secretary*)
- Dr W. H. Mosley, Chief, Epidemiology Section, Pakistan-SEATO Cholera
Research Laboratory, Institute of Public Health, Dacca, Pakistan (*Temporary
Adviser*)

* Unable to attend :

Professor Z. V. Ermol'eva, Department of Microbiology, Central Institute for
Advanced Medical Studies, Moscow, USSR.

Dr J. Sterzl, Head, Department of Immunology, Institute of Microbiology, Prague,
Czechoslovakia.

CHOLERA IMMUNOLOGY

Report of a WHO Scientific Group

The WHO Scientific Group on Cholera Immunology met in Teheran from 14 to 19 September 1968.

Dr A. H. Taba, Director, WHO Regional Office for the Eastern Mediterranean, opened the meeting and welcomed the members on behalf of the Director-General.

Dr O. Felsenfeld was elected Chairman and Dr I. Joó Vice-Chairman. Dr K. Bhaskaran and Dr J. C. Feeley were elected Rapporteurs.

This was the third WHO Scientific Group to discuss cholera research. However, whereas the earlier groups¹ discussed cholera research in general, the present Group confined its discussions specifically to cholera immunology, with special reference to the improvement of prophylactic vaccination.

1. RECENT CONCEPTS OF IMMUNITY IN CHOLERA AND ENTERIC INFECTIONS

The Group reviewed the current status of knowledge of immunology and its bearing on enteric infections and particularly on cholera.

The field trials of cholera vaccines that have been carried out in three countries in the last few years and the extensive information that has been accumulated regarding the epidemiology of endemic cholera clearly demonstrate that both natural infection and parenteral immunization are associated with the development of antibodies to cholera antigens and of relative resistance to the disease.

However, several findings during the past two decades have emphasized the importance of local intestinal factors in immunity to cholera. Besides failure to detect significant tissue damage or penetration of the tissues by the cholera vibrio, there is growing evidence of antibody formation in the tissue of the intestinal tract, especially under circumstances that most readily bring antigen into contact with these tissues. These findings can now be related to the knowledge of secretory "IgA"

¹ The reports of these two groups, which met in 1962 and 1964, have not been published.

antibody, which has been identified as the major antibody globulin found in various epithelial secretions.

Success in the immunological control of cholera would therefore appear to depend very largely on a clear understanding of the nature of local intestinal immune mechanisms and not merely on a knowledge of the serologic immunology of the disease.

The immune response should be considered in relation to each of the two relatively independent types of immune mechanism. In the first type, immunity is mediated by circulating antibodies; in the second type (specific cell-mediated immune response), it is mediated by sensitized lymphocytes. Little is known of the role of specific (or other) cell-mediated immune responses in cholera; studies have been concentrated on the more familiar relationship between resistance and circulating antibodies.

During recent years it has become apparent that serum antibodies belong to a complex family of related proteins, known collectively as the immunoglobulins. At the same time, there has been renewed interest in the antibodies of the gastrointestinal and other secretions in contact with mucous membranes, especially since the recent demonstration that IgA is the predominant immunoglobulin in parotid saliva, colostrum, tears, nasal and tracheobronchial secretions and small intestinal fluids. The "secretory" IgA differs from serum IgA in many respects. Serum IgA is a 7S monomer whereas at least 60% of colostrum and salivary IgA is a dimer containing an additional peptide chain with a molecular weight of 50 000. The additional peptide chain can be demonstrated in the cytoplasm of epithelial cells and has been termed "transport piece" on the assumption that it is added to IgA synthesized by submucosal lymphoid cells as it passes through the cells into the external secretions. There is, however, no direct evidence for this hypothesis.

Antibody activity has been demonstrated in IgA "secretory" immunoglobulins, and resistance to some viral infections in human volunteers has been correlated with the level of IgA antibody in nasopharyngeal secretions. Production of specific anti-influenza antibodies in respiratory secretions was shown to be better when influenza vaccine was administered locally as an aerosol than when it was injected, and incidence of illness during an epidemic was lower in the group who received the aerosol than in the group who received parenterally administered vaccine. Evidence also exists that antibody response in secretions of the vaginal tract is better after local administration of antigen than after parenteral administration.

Immunological memory is cell-mediated. While theories abound concerning its programming, it has been demonstrated that immunological memory for IgM formation is generally shorter than for IgG formation. This is of importance for the development of lasting immunity.

The antigen-antibody bond is weaker with IgM than with IgG antibodies. Avidity seems to vary according to the subclass of the Ig and increases with time and repetition of antigenic stimulus.

There is need for further knowledge of the mechanisms by which parenterally administered vaccines result in an effective antibody response against vibrios within the lumen of the intestine. The existence of an immunological system apparently unique for certain external secretions requires evaluation of its role in local intestinal immunity.

The pathogenesis of cholera appears to involve at least two fundamental processes: (1) the successful establishment of infection by *Vibrio cholerae* within the intestinal lumen; (2) the production by the infecting organism of a toxin that causes the diarrhoeal syndrome. Correspondingly, there appear to be separate immune mechanisms for each of these two processes, which may be referred to as antibacterial immunity and antitoxic immunity. These can be studied to some degree independently in various animals.

Antibacterial immunity

Since the infection is limited to the intestinal lumen, in all probability, the mechanism responsible for antibacterial immunity occurs within the lumen, rather than within the intestinal tissues. Although there are a number of theories concerning the nature of the immune mechanism, such as interference with bacterial adherence, phagocytosis, and bacteriolysis, there is no definite evidence to support any of these. There is a fundamental gap in understanding of the host-bacteria relationship in the human intestinal lumen; for example, it is not known whether the organism is able to multiply freely in the lumen or whether multiplication depends on a special relationship to the tips or the crypts of the villus. Studies on this point should be carried out during the incubation period of the disease, or perhaps during the recovery period, and not during the acute diarrhoeal syndrome when the relationship may be disrupted.

Antibacterial immunity can be successfully induced in man by parenteral vaccines containing killed whole cells or lipopolysaccharide, and the level of immunity has been well defined in several field trials. Also, the level of immunity has been correlated with the serum vibriocidal antibody titre. The vibriocidal titre is a measure of a complement-dependent reaction due to IgM and IgG and it is unknown whether these immunoglobulins play any role in intestinal immunity.

Antitoxic immunity

The site of action of the cholera toxin has not yet been established. Studies in the rabbit and dog suggest that it acts at the epithelial surface

of the intestine. In the dog, repeated intestinal challenges with toxin do not seem to induce effective immunity to subsequent toxin challenge, but parenteral administration of a toxin-containing vaccine results in protection of the loop from toxin challenge. A toxin-containing vaccine has not been tested in man. Humans develop serum-toxin neutralizing antibodies following clinical cholera, and these have been shown to be IgG. There are no studies relating this antibody to immunity in man. Thus, the relative importance of antitoxic and antibacterial immunity in man remains undefined.

Immunity following cholera infection

There is as yet no satisfactory way of measuring the level of immunity following cholera infection. Well-documented reports on second attacks of cholera are virtually non-existent, but the probability of such attacks occurring is also remote. This problem has an important bearing on the potential value of oral as opposed to parenteral immunization. The studies in the dog reported above suggest that antitoxic immunity might not be successfully induced by the oral route. Antibacterial immunity may possibly be more successfully induced by the oral rather than the parenteral route. However, two observations suggest that this may not necessarily be true. First, parenteral vaccine has been shown to produce a better and more sustained vibriocidal antibody response than that following clinical cholera. Secondly, recent epidemiological studies suggest that, in the endemic area, children may be reinfected year after year. If this is true, it follows that one oral immunization does not provide sufficient protection against another challenge. More data are necessary before the merits of oral versus parenteral immunization can be evaluated.

A critical factor in deciding on vaccines and routes of administration is understanding the mechanism of immunity within the lumen. If secretory IgA plays a major role, then oral immunization may be important.

Thus, there is a need to obtain more exact information about the mechanisms of immunity in the gut, the pathways by which antigen gains access to antibody-forming tissue in that area, and the patterns of the immune response following local as opposed to parenteral entry of the antigens involved. Interpretation of such studies, however, will be critically dependent on precise definition and characterization of these antigens and the roles that they play in both the pathogenesis of cholera and the development of immunity to the disease.

In one study in an endemic area, it has been shown that infants exhibit a classical type of primary and booster response to injection of

cholera vaccine. With increasing age, the response to a single dose becomes more and more like a secondary response. Natural exposure to infection is clearly taking place during this period, and is manifested by a steady rise, at the rate of 5% a year, in the prevalence of vibriocidal antibodies. It seems, however, that this type of natural stimulus to cholera "immunity" need not depend on exposure to cholera vibrios alone, since adults in non-endemic areas (e.g., USA, Czechoslovakia, Hungary) show a low but significant prevalence of vibriocidal antibodies, and have been found to react with a secondary type response to injection of a single dose of cholera vaccine. Although such antibodies as are found in populations in cholera-free areas are often referred to as "natural" antibodies, it appears likely that they are not spontaneous in origin but arise from contact with antigenically related bacteria, such as *Brucella* and *Pseudomonas*.

Numerous other gaps in the understanding of cholera immunity exist; some persons develop cholera despite the presence of serum antibody levels generally associated with resistance to cholera. The most likely mechanism by which the cholera vibrio causes disease appears to be the production of toxins—either some or all of those already identified or others that may not yet have been identified. Nevertheless, vaccines that have proved relatively effective in field trials appear to lack toxin antigen.

Reinfection with cholera vibrios is common in endemic areas, but recurrent disease is rare. The early peak level and subsequent fall in antitoxic antibodies in children living in an endemic area raises questions regarding the nature of the antigenic stimuli and the host response resulting from repeated exposures to cholera infection.

Little information seems to be available regarding the factors, if any, that predispose to enhanced or depressed resistance to clinical cholera. Available data point to chronic malnutrition and to pregnancy as risk-enhancing factors, but the information on these and other comparable factors is meagre and precise studies on this question are still needed. Similarly, the role, if any, of passively introduced antibody in protecting against cholera is undetermined; the rarity of cholera in young infants precludes assessment of the role of maternal antibodies but current knowledge of such transfer suggests that it would probably not be effective in any case. Finally, the possibility that cholera immunity may be genetically determined needs to be carefully examined.

The concept of cellular immunity, mentioned above, appears unlikely to play a role in cholera. However, the present relative ignorance of the subject may make it seem less important than it is, and valid opportunities for its investigation should be encouraged.

2. ANTIGENS AND THEIR FRACTIONS

Bacterial antigens

Somatic antigens specific to the cholera vibrio are heat-stable. Heat-labile antigens are shared with many other vibrios. The specific antigens include the group antigen and the two serotype antigens, and possibly additional antigens could also be differentiated. It is this antigenic complex (including endotoxin) that induces the formation of mouse-protective antibody.

Antigenic structure, antigenicity and specificity of vibrio antigens

The predominant heat-stable antigens are designated by letters, A being the group-specific O antigen, B the Ogawa serotype, and C the Inaba serotype antigen. The Hikojima serotype contains both serotype antigens, as well as the group-specific antigen. Shift in serotype, from Ogawa to Inaba, has been observed to occur *in vivo* and *in vitro*. Quite recently other antigens of probable significance for the production of effective immunity to the disease have been described. One is the heat-labile choleric toxin, and the other is a protein containing a major antigen that gives identity reactions with highly purified lipopolysaccharide.

Immunochemistry of cholera antigens

The antigenic specificity of the serotype antigens is apparently determined by lipopolysaccharide moieties of high molecular weight — about 1×10^6 . Such lipopolysaccharide moieties have been isolated from both serotypes in a high state of purity and have been shown to induce the formation of mouse-protective and vibriocidal antibody. They are considered to represent endotoxin and are found in supernates of liquid cultures of cholera vibrio only when autolysis has taken place.

The antigens represented by the choleric toxin and the associated nontoxic protein occur in the intracellular substance of the vibrio and not in the cell wall. This complex diffuses freely into liquid culture media, and the toxic moiety may be considered to be an exotoxin. These two antigens are not separable by the molecular sieve Sephadexes, but may be resolved by ion-exchange chromatography. Because it carries a strong positive charge, the toxic antigen is the first to be eluted and has been designated Fraction I. Fraction II, eluted from DEAE¹ Sephadex in 0.5 M sodium chloride solution, is nontoxic. Both are lipoproteins, Fraction I containing 25–30% of lipid in the form of glycerides, and

¹ Diethylaminoethanol.

Fraction II 6-8% of serine-based lipid. Each contains about 3% of carbohydrate, the remainder being protein of more or less conventional amino acid composition. The toxic fraction can be further purified, — for example, by alcohol-ether precipitation in the cold — and has a molecular weight of about 10 000. In purified form, it contains two antigens demonstrable by gel diffusion. The nontoxic protein designated Fraction II, which has a molecular weight of 30-35 000, shows four precipitin lines on gel diffusion. The major line gives an identity reaction with highly purified lipopolysaccharide and induces the formation of vibriocidal antibody.

“ Whole-cell ” antigens

It seems probable that not all the antigens present in the bacterial cell are associated with the induction of an effective prophylactic immunity. Most vibrio strains are not highly toxigenic, and the vaccines at present in use produce antibacterial immunity, demonstrable *in vivo* by the mouse test and *in vitro* as vibriocidal antibody. Even in the case of vibrio strains of relatively high toxigenicity, the amount of toxin antigen in saline suspensions of agar-grown vibrios is small. It has been observed repeatedly that immunizations of experimental animals with living vibrios results in higher protective antibody titres against experimental enteric cholera than does immunization with killed vaccine, at least in some species.

Toxins

With the demonstration, in various assay systems, of the elaboration by the cholera vibrio of a multiplicity of toxic substances, the term “ cholera toxin ” has become not only inexact but confusing. It has been suggested that the cholera toxins may be grouped in 3 types. Type 1 includes the mouse lethal factor and is probably conventional endotoxin. It is heat-stable, nondialysable, and occurs in liquid culture supernates only as a consequence of autolysis. Type 2 toxin is heat-labile, nondialysable and diffuses into liquid culture medium. This group includes the factors responsible for the toxin-induced net water and ion movement from the tissues into the lumen of the small bowel. Also in this group are the factor responsible for the toxic activity demonstrable by intradermal inoculation and designated “ permeability factor ”, and that demonstrable by cytopathic effects produced in cultured cells. There is some, but not definitive, evidence to suggest that the permeability factor and choleric toxin are not identical. The nature of the cytotoxic activity remains uncertain. Finally, type 3 toxin includes the

heat-stable, freely dialysable factors demonstrable by inhibition of active sodium transport in anuran epithelium, and by the inhibition of *p*-aminohippurate uptake by kidney tissue *in vitro*. There is some evidence, however, that the former activity may be due to the presence of ammonia; complete removal of ammonia also reduces the inhibition of PAH uptake by about one-third. Of these toxins, type 1 toxin is of interest largely because, in the form of highly purified lipopolysaccharide, it stimulates the production of vibriocidal antibody, which might be considered as responsible for antibacterial immunity. Another candidate for this role may be Fraction II described above. Purified type 2 toxin appears to be the most important of these antigens because its activity has been demonstrated in the small bowel of various animals, but it is not present in significant amounts in vaccines at present utilized.

Another pressing matter is the development of an *in vitro* serological test for the titration of toxin-neutralizing antibody. Of the serological methods, haemagglutination looks promising and quantitative complement fixation may also be used. Bentonite and latex agglutination appear less suitable. The development of a specific serological method is dependent, however, upon the further physical and chemical fractionation of the toxicity to give a product of antigenic homogeneity. The continuation of such chemical studies has, therefore, a high priority.

Toxoids

Some investigators have claimed that immunogenic potency is largely destroyed by treatment with formaldehyde; others who have used formalin-inactivated material have obtained inferior immune responses. In contrast, permeability factor toxin treated with formalin was more antigenic than untreated toxin. Doubts have been expressed, however, as to the necessity for "detoxification", since the purified preparations are innocuous when administered by parenteral routes.

3. ANTIBODIES AND THEIR MEASUREMENT

Methods for detection of agglutinating, vibriocidal, and toxin-neutralizing antibodies (both choleric toxin and permeability factor) appear to be reliable. The Group therefore concentrated mainly on a discussion of the nature of the antibodies. It was reported that at least two immunoglobulins (IgM and IgG) are involved in vibriocidal activity and that IgM usually predominates. Toxic antigens seem to behave like most other protein antigens. In view of the pathophysiology of cholera, attention was drawn to the importance of coproantibodies.

Little is known about the nature of these antibodies, although IgA and IgG (perhaps IgM) are found in the cholera stool and in the intestinal secretions of infected monkeys. However, the roles of these antibodies in intestinal immunity are not yet understood. Currently, all the toxin-neutralization tests are performed in animals, which makes it very difficult to examine many samples, particularly in serological surveys. The need for an *in vitro* method of antitoxin titration was stressed. It was also stated that the sera used by workers for the study of immunological responses to various antigens were derived from hyperimmunized animals, which makes it difficult to quantify the antigenicity of the various preparations.

4. RELATIONSHIP OF ANTIBODIES TO IMMUNITY

The part played by antibodies in killing and disposing of live bacteria has not been easy to evaluate. The phenomenon of bacteriolysis (Pfeiffer phenomenon) has been described in connexion with the activity of a serum antibody operating within serum or the environment of the peritoneal cavity, and it is doubtful how far this is relevant to an interaction within the gut lumen. Before reliable conclusions can be drawn concerning the significance of the level of antibody as estimated by any particular technique, the roles of the respective antigenic moieties in pathogenesis must be defined.

In a localized infection of the surface of the body, such as diphtheria, the production of disease is centered on the exotoxin; immunity can be related to the determination of serum antitoxin levels (measured in arbitrary units) in a neutralization test. Even in the relatively simple case of diphtheria, the relationship between antibody titre and immunity is not straightforward, since the process of accelerated production of antitoxin by a secondary response mechanism allows a person with a low initial serum level of antibody to manufacture additional supplies in time.

In an intestinal infection such as cholera, the crucial confrontation of cholera vibrios with antibody occurs within the environment of the gut lumen. The levels of any of the serum antibodies can bear only an indirect relation to the vibriocidal system within the gut. It is not possible at present to form a clear concept of the degree of involvement of vibriocidal antibody in this process, since vibriocidal activity is determined by tests on serum, which contains specific antibody, all the components of complement up to C'9, lysozyme and other factors, such as those absorbable on bentonite. It is highly doubtful whether specific antibody (IgM and IgG) requiring these components can function within the

intestinal lumen, although it is possible that a reaction involving some increase in permeability of the gut vessels (for example, a reaction dependent on antigen-antibody allergic interaction) might convey sufficient of all these components from the plasma into the gut lumen. Alternatively, immunity may be dependent entirely on that fraction of the IgA capable of exerting a vibriocidal effect in the presence of adequate lysozyme and in the almost total absence of complement factors. The significant level of this antibody fraction within the gut would not be expected to correlate simply with the level of IgA specific antibody in the serum, since variable but considerable amounts of IgA are manufactured locally by plasma cellular elements within the intestinal wall.

Since vibriocidal activity may be the crucial factor in immunity to cholera, it is doubtful whether much significance can be attached to estimations of the serum antitoxin responsible for neutralizing choleric toxin in, for example, an isolated loop of rabbit intestine or a skin test. Theoretically, the demonstration of high levels of choleric antitoxin could indicate immunity to clinically manifest cholera but not to the multiplication of virulent vibrios within the lumen of the gut. However, in the presence of adequate vibriocidal antibody, a low level of antitoxin would still be consistent with a state of immunity to cholera. Indeed, the possession of an adequate vibriocidal mechanism could reduce the immunogenicity of an otherwise effective dose of cholera vibrios to ineffective levels.

The determination of vibriocidal and agglutinating antibodies and of skin permeability factor (PF) by neutralization tests is now possible using microtechniques, making these tests applicable to mass surveys. The vibriocidal antibody titre among populations in cholera endemic areas has been demonstrated to correlate closely with protection from cholera. This serological test may therefore be useful (1) for preliminary tests of vaccine potency; (2) in interpreting the results of vaccine field trials; and (3) in evaluating the effectiveness of vaccine programmes and the level of immunity to cholera in various populations.

The vibriocidal and PF neutralization test are also useful in epidemiological studies for detecting inapparent infection with *V. cholerae*. However, PF neutralizing titres have not been correlated with immunity.

There is a definite need to develop a test for measuring antibodies to "diarrhoea toxin" (choleric) that will be suitable for large-scale field surveys in order to determine what role these antibodies play in immunity to cholera.

Because of the vast amount of added information that is gained by serological studies, it is essential that serological surveys be incorporated as a basic feature of all future cholera vaccine field trials. In order to understand the significance of these serological tests, the immune mechanism operating in the intestinal tract must be defined, and the relationship

of the serum antibodies measured by these serological tests must be correlated with the antibodies operating in the intestine.

5. VACCINES

The results of the several field trials conducted in East Pakistan (1963, 1964 and 1966), the Philippines (1964 and 1966), and India (Calcutta) (1964 and 1965) were reviewed by the Group. The general trend of experience has been similar in all the trials, except those with oil-adjuvant vaccine (Philippines, 1964). In most instances, the maximum observed protection was in the range 30-80% with significant immunity enduring for only 3-6 months. The oil-adjuvant vaccine gave longer protection, but its use was accompanied by an unacceptable incidence of severe local reactions. Protection has been poorest in the young age groups (<10 years of age), especially in the highly endemic area in East Pakistan. It was agreed that the accumulated evidence to date suggests that the fluid vaccines at present available can be expected to produce neither a high degree of immunity nor protection of long duration in populations at risk.

Attempts to correlate the outcome of field trials with laboratory assays of vaccine potency have not been entirely successful. International collaborative assays of the vaccines employed in the 1964-65 Calcutta field trials were arranged by WHO. However, owing to a lack of statistically significant differences in the field performance of these vaccines, it was not possible to be certain that the laboratory assay results (based on mouse-protection and other tests) reflected the efficacy of the vaccines in the field. The fluid El Tor vaccine (Philippines, 1964) was reported to be slightly, but not significantly, more potent for both man and mouse than the fluid "classical" vaccine used in this trial. Comparison with the field behaviour of the vaccine used in East Pakistan seems to indicate that the vaccine of higher mouse potency was more protective in the field. It was noted also that the mouse-protection assay appeared capable of ranking vaccines in approximately the same order of antigenicity as experiments on vibriocidal antibody response in young children in East Pakistan.

With reference to the production of whole-cell killed cholera vaccines, the Group stressed the importance of using stable smooth antigenic strains and considered that properly maintained stock strains of known characteristics were preferable to fresh isolates for vaccine production. No particular significance was attached to the differences between "El Tor" and "classic" biotypes so far as vaccine production was concerned, since antigenically they are almost identical. "Classic" strains appear

perfectly adequate and have been shown to protect against El Tor infection. As regards the choice of media, growth conditions and the use of killing agents or preservatives, the Group agreed that the field and laboratory data at present available do not permit any one procedure to be specified as superior to others.¹

The Group also discussed the possible use of vaccines containing living avirulent vibrios. There is considerable interest in this approach to oral vaccination, and certain studies are in progress in India. The theoretical advantages of an effective live attenuated oral vaccine are obvious, but the problems are great. Safety must be a prime consideration. Candidate strains for oral vaccines must be able to multiply in the human gut without causing disease, must have a high degree of stability and no tendency to revert to virulence, and must possess several suitable genetic markers to allow their precise identification. In addition to the selection of a suitable strain, methods of administration and regimens for use present major problems. While the investigation of live attenuated oral vaccines is recognized as a promising approach, considerable time will be needed to perfect such vaccines to the point of readiness for extensive human trials. Hence, serious efforts must be initiated soon. Meanwhile, progress may be made towards more readily attainable goals, such as the production of combined bacterial and toxin-toxoid vaccines or the development of vaccines containing adjuvants. Contemporaneously with studies on live oral vaccine, similar studies should be made on killed oral vaccine.

The possible use of live parenteral vaccine should also be considered, since a number of animal studies indicate the antigenic superiority of living vibrio cells.

The use of such highly purified antigens as the Ogawa lipopolysaccharide and the Inaba polysaccharide-proteolipid complex offers the theoretical possibility of administering larger doses of effective antigen containing less extraneous reactive material. In a field trial in East Pakistan, the Ogawa lipopolysaccharide induced protection only in adults; however, the antigen is highly specific for the Ogawa serotype, whereas most infections in this trial were due to the Inaba serotype. The purified Inaba fraction will be tested in the field in the autumn of 1968. Although these products have a high mouse-protective activity, the antibody response to Ogawa antigen in animals and man appears to be somewhat inferior to that of whole-cell vaccine.

The Group also considered the possible significance of the choleric toxin antigen as an immunizing agent and noted that studies in rabbits and dogs suggest that antitoxic immunity may be an important

¹ On the basis of recent field and laboratory experience, however, the Requirements for Cholera Vaccine (Requirements for Biological Substances No. 4), published by WHO, have recently been revised (*Wld Hlth Org. techn. Rep. Ser.*, 1969, No. 413, Annex 1).

component of the immune mechanism. Vaccines at present available have little or no ability to induce antitoxin formation. It was generally agreed that the toxin antigen (or possibly the toxoid) would be most useful in combination with antigens capable of stimulating antibacterial immunity. Several problems surround the production of a purified toxin. Purified ileal loop toxin is labile, rapidly losing both toxigenicity and immunogenicity. Production of a satisfactory toxoid from the ileal loop toxin has not been achieved, and reports on human responses to the products so far obtained were considered unimpressive. In contrast, an experimental crude toxoid of the permeability factor toxin appeared to be more antigenic than its parent toxin in animals. While a highly purified toxin immunizing agent would be of academic interest as a tool in investigating the mechanism or mechanisms of immunity, the manufacture of such a product is unlikely to be economical. In general, it was felt that the best and most immediate prospect for enhancing the efficacy of cholera vaccine lay in adding the toxin (or toxoid) antigen to the antigens already present in cholera vaccines that are known to stimulate a significant degree of immunity.

Enthusiasm for the use of antitoxic immunizing agents must be tempered somewhat by the evidence from studies in dogs and rabbits, which indicates that it is essential to neutralize the toxin within the lumen of the gut before it becomes attached to reactive sites. This clearly implies the need for a highly functional immune response that provides adequate and continuous amounts of antitoxic antibody in intestinal secretions. The situation would seem to allow little time for the development of an anamnestic response, such as occurs in some other antitoxic immunity systems (e.g., in tetanus).

It is known that cholera vaccine can be combined successfully with a variety of other antigens (e.g., those of typhoid and tetanus). The routine use of a cholera vaccine combined with tetanus (or perhaps diphtheria) toxoid would no doubt be of great public health value in developing countries, since both tetanus and diphtheria are highly prevalent in cholera endemic areas.

In view of the relatively brief duration of the immunity induced by present vaccines, the possible use of adjuvants to enhance the response and extend this period is attractive. Aluminium compounds are recognized as the safest adjuvants and their use is based on vast experience with other vaccines; accordingly, their use with cholera vaccine is an immediate possibility. Evidence for their ability to enhance the immune response to cholera vaccine is already available. Although oil adjuvants are more effective than aluminium compounds, there are many unsolved problems involved in their use, particularly with respect to safety, and they have been associated with unexpectedly severe reactions when added to tetanus toxoid, cholera vaccine and typhoid vaccine. On the other

hand, they have achieved an impressive record of safety with influenza vaccine. In view of the superior adjuvant effect of oil emulsions, their continued study, including the use of metabolizable oils and emulsifiers other than "Arlacel A", is to be encouraged.

Untoward reactions to cholera vaccine have not usually been a major problem when proper control over antigen content has been exercised and care has been taken to ensure freedom from reactive substances derived from the culture media. The reasons for the reactions to the oil-adjuvant vaccine observed in the Philippines are not well understood. One possible reason was the use of subcutaneous rather than intramuscular injection in the field trial. Some evidence suggests that enzymatic hydrolysis of Arlacel may have been partly responsible for several such experiences with this adjuvant. Aluminium compound adjuvants might be expected to be less toxic.

Hypersensitivity reactions to cholera vaccine have sometimes been a problem. In view of the prevailing practice of repeated vaccination and the possible development of more potent antigens and adjuvants, the Group agreed that caution should be exercised and that the possible appearance of untoward immunologic phenomena should always be borne in mind.

6. RECOMMENDATIONS FOR RESEARCH

The Group recommended that further research on cholera should have the following objectives :

A. General immunology and pathogenesis

1. Extension of general knowledge about infection and immunity, especially in the intestinal tract.
2. Characterization of the mechanism and the dynamics of local immunity in the intestine.
3. Definition of the site of cholera infection and elucidation of the kinetics.
4. Clarification of the possible role of cellular immunity in cholera.
5. Identification of accessory or non-specific factors — nutritional, genetic, or other — that influence resistance or susceptibility to cholera.
6. Improved understanding of the kinetics of antigen-antibody reactions in cholera.
7. Determination of the role of serum antibodies in the gut.

8. Clarification of the relation between serum IgA and intestinal IgA.

9. Investigation of the possible usefulness of as yet untested adjuvants, such as vegetable oils and vitamin A — including the possibility of enhancing oral immunization by this means.

10. Clarification of the relationship between “ permeability factor ” and “ diarrhoea factor ”.

In view of the fact that dependable treatment of cholera is already available, the use of human volunteers may be contemplated in solving those problems that cannot be resolved by animal or field studies, provided that all necessary precautions are observed. Objectives 2, 3, 4 and 7 above are examples of problems to which useful answers may be obtainable only by direct studies in human volunteers.

B. Specific immunological mechanisms

1. Correlation of vibriocidal titres, antitoxin titres and such other serological indices as may be developed with susceptibility or immunity to cholera.

2. Development of further tests for assessing cholera immunity, especially tests suitable for application to serological epidemiology.

3. Improved understanding of immune responses in cholera; studies should be made using all applicable modern techniques, both in man and in animals.

4. The fractionation, purification and characterization of the choleric toxin. This should be studied intensively with a view to :

- (a) ascertaining the nature of the toxic moiety;
- (b) developing a specific serological reaction allowing the titration of neutralizing antibody *in vitro*; and
- (c) developing a stable, immunogenic antigen.

5. Identification of possible immunopathological mechanisms arising from cholera infection or immunization. Particular attention should be paid to the possible role of the organism in inducing kidney disease or “ amyloid ” changes. Hyper-immunized subjects may be especially suitable for such investigations.

C. Development and testing of vaccines

1. The development of live attenuated vaccines, and their characterization by genetic markers, stability, and relevant immunological

criteria. Preliminary studies on the use of such vaccines should be carried out in small groups of human subjects to obtain basic information on their reactivity, acceptability and immunogenic potency.

2. Assessment of the potential usefulness of live attenuated vaccines in parenteral immunization.

3. Evaluation of the efficacy of oral vaccination with either live or dead vaccines.

4. Assessment of the usefulness of combined oral and parenteral vaccination schedules.

5. Assessment, by means of field trials, of the value of mineral-carrier cholera vaccine.

6. The collection of basic information on the reactivity and immunogenic potency of

(a) cholorigenic toxin; and

(b) Fraction II protein as an inducer of vibriocidal antibody formation.

For this purpose, preliminary studies with partially purified material should be carried out in small series of human subjects.

7. The development of improved parenteral vaccines, especially vaccines that will give more lasting protection.

8. The development of effective stabilizing mechanisms for cholera toxin and toxoids.

9. Preparation of combined toxin antigen and bacterial vaccine, with field trials if preliminary studies (as in 6 above) warrant this step.

10. Expansion of the use of serological tests, and detection of inapparent infection as well as clinical cholera in field trials.

11. Establishment of optimal vaccine schedules, doses and routes, with special attention to the possibility of extending the interval between boosters or reimmunization.

12. Construction of alternative mathematical models applicable to the design and interpretation of vaccine essays in man.

13. Assessment by field trials of other candidate vaccines that meet the criteria of safety, potency and acceptability indicated elsewhere in the report.

14. Investigation of the possibility of combining cholera vaccine for basic immunization with tetanus toxoid.

15. Development of improved laboratory models for assaying cholera vaccines, in conjunction with field trials to determine their correlation with protection in man.

The Group also recommended that co-operative international studies on the measurement of antibody titres, testing of antigens, etc. should be organized and that the necessary reference sera, vibrio strains, etc. should be made available. It was hoped that it would be possible for WHO to facilitate and promote further research on the fundamental immunology of cholera.

ACKNOWLEDGEMENT

The Group acknowledged the special contributions to its discussions made by Dr D. Barua, Bacterial Diseases, WHO.

**WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES**

<i>Recent reports:</i>		Price		
No.		s.d.	\$	Sw. fr.
369	(1967) Arboviruses and Human Disease Report of a WHO Scientific Group (83 pages)	6/8	1.25	4.—
370	(1967) Pesticide Residues in Food Joint report of the FAO Working Party on Pesticide Residues and the WHO Expert Committee on Pesticide Residues (19 pages)	3/6	0.60	2.—
371	(1967) Research in Psychopharmacology Report of a WHO Scientific Group (39 pages)	5/-	1.00	3.—
372	(1967) Epidemiology and Control of Schistosomiasis Report of a WHO Expert Committee (35 pages)	5/-	1.00	3.—
373	(1967) Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Emulsifiers and Stabilizers and Certain Other Substances Tenth report of the Joint FAO/WHO Expert Committee on Food Additives (47 pages)	5/-	1.00	3.—
374	(1967) Prevention of the Re-Introduction of Malaria Report of a WHO Meeting (32 pages)	3/6	0.60	2.—
375	(1967) Chemotherapy of Malaria Report of a WHO Scientific Group (91 pages)	8/-	1.25	4.—
376	(1967) The Education of Engineers in Environmental Health Report of a WHO Expert Committee (26 pages)	4/-	0.60	2.—
377	(1967) Joint FAO/WHO Expert Committee on Nutrition Seventh Report (84 pages)	8/-	1.25	4.—
378	(1967) Joint FAO/WHO Expert Committee on Zoonoses Third Report (127 pages)	10/-	1.75	5.—
379	(1967) Control of Ascariasis Report of a WHO Expert Committee (47 pages)	6/-	1.00	3.—
380	(1967) Current Problems in Leptospirosis Research Report of a WHO Expert Group (32 pages)	4/-	0.60	2.—
381	(1968) Neurophysiological and Behavioural Research in Psychiatry Report of a WHO Scientific Group (33 pages)	6/-	1.00	3.—
382	(1968) WHO Expert Committee on Malaria Fourteenth Report (50 pages)	6/-	1.00	3.—
383	(1968) Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Flavouring Substances and Non-Nutritive Sweetening Agents Eleventh Report of the Joint FAO/WHO Expert Committee on Food Additives (18 pages)	4/-	0.60	2.—
384	(1968) WHO Expert Committee on Biological Standardization Twentieth Report (100 pages)	10/-	1.75	5.—
385	(1968) Training of Medical Assistants and Similar Personnel Seventeenth Report of the WHO Expert Committee on Professional and Technical Education of Medical and Auxiliary Personnel (26 pages)	4/-	0.60	2.—
386	(1968) Hormonal Steroids in Contraception Report of a WHO Scientific Group (28 pages)	4/-	0.60	2.—
387	(1968) Research on Human Population Genetics Report of a WHO Scientific Group (32 pages)	4/-	0.60	2.—
388	(1968) Exercise Tests in Relation to Cardiovascular Function Report of a WHO Meeting (30 pages)	4/-	0.60	2.—

No.		Price		
		s.d.	\$	Sw. fr.
389	(1968) Morbidity Statistics Twelfth Report of the WHO Expert Committee on Health Statistics (29 pages)	4/-	0.60	2.—
390	(1968) Medical Radiation Physics Report of a Joint IAEA/WHO Expert Committee (19 pages)	4/-	0.60	2.—
391	(1968) Pesticide Residues Report of the 1967 Joint Meeting of the FAO Working Party and the WHO Expert Committee (47 pages)	6/-	1.00	3.—
392	(1968) Organization of Services for the Mentally Retarded Fifteenth Report of the WHO Expert Committee on Mental Health (55 pages)	6/-	1.00	3.—
393	(1968) Smallpox Eradication Report of a WHO Scientific Group (52 pages)	6/-	1.00	3.—
394	(1968) Streptococcal and Staphylococcal Infections Report of a WHO Expert Committee (56 pages)	6/-	1.00	3.—
395	(1968) Hospital Administration Report of a WHO Expert Committee (29 pages)	4/-	0.60	2.—
396	(1968) Immunology of Malaria Report of a WHO Scientific Group (50 pages)	6/-	1.00	3.—
397	(1968) Intra-Uterine Devices : Physiological and Clinical Aspects Report of a WHO Scientific Group (32 pages)	4/-	0.60	2.—
398	(1968) Cytogenetics of Vectors of Disease of Man Report of a WHO Scientific Group (41 pages)	6/-	1.00	3.—
399	(1968) Microbiological Aspects of Food Hygiene Report of a WHO Expert Committee with the Participation of FAO (64 pages) -	8/-	1.25	4.—
400	(1968) Paediatric Research Report of a WHO Scientific Group (26 pages)	4/-	0.60	2.—
401	(1968) Screening for Inborn Errors of Metabolism Report of a WHO Scientific Group (57 pages)	8/-	1.25	4.—
402	(1968) Genetics of the Immune Response Report of a WHO Scientific Group (52 pages)	6/-	1.00	3.—
403	(1968) Principles for the Clinical Evaluation of Drugs Report of a WHO Scientific Group (32 pages)	6/-	1.00	3.—
404	(1968) Water Pollution Control in Developing Countries Report of a WHO Expert Committee (38 pages)	6/-	1.00	3.—
405	(1968) Nutritional Anaemias Report of a WHO Scientific Group (37 pages)	6/-	1.00	3.—
406	(1968) Research into Environmental Pollution Report of Five WHO Scientific Groups (83 pages)	8/-	1.25	4.—
407	(1968) WHO Expert Committee on Drug Dependence Sixteenth Report (28 pages)	4/-	0.60	2.—
408	(1969) Respiratory Viruses Report of a WHO Scientific Group (100 pages)	10/-	1,75	5.—
409	(1969) Planning and Evaluation of Health Education Services Report of a WHO Expert Committee (32 pages)	6/-	1.00	3.—
410	(1969) Urban Air Pollution, with Particular Reference to Motor Vehicles Report of a WHO Expert Committee (53 pages)	6/-	1.00	3.—

WHO PUBLICATIONS

SUBSCRIPTIONS AND PRICES 1969

Global Subscription

The global subscription covers all WHO publications, i.e., the combined subscription "C" and, in addition, the *Weekly Epidemiological Record*, *Monograph Series* and any other occasional publications.

£45 \$150.00 Sw. fr. 450.—

Combined Subscriptions

Special prices are offered for combined subscriptions to certain publications, as follows:

Subscription

A <i>Bulletin, Chronicle, Technical Report Series, and Public Health Papers</i>	}	£15	\$50.00	Sw. fr. 150.—
B <i>World Health Statistics Report and World Health Statistics Annual</i>				
C <i>Bulletin, Chronicle, Technical Report Series, Public Health Papers, Official Records, International Digest of Health Legislation, World Health Statistics Report, World Health Statistics Annual, and World Health</i>	}	£33	\$110.00	Sw. fr. 330.—

WHO Distribution and Sales Unit will be pleased to submit a quotation for any other type of combined subscription desired.

Individual Subscriptions

<i>Bulletin</i> , vol. 40 and 41 (12 numbers)	£9	\$30.00	Sw. fr. 90.—
<i>Chronicle</i> , vol. 23 (12 numbers)	£1 4s.	\$ 4.00	Sw. fr. 12.—
<i>International Digest of Health Legislation</i> , vol. 20 (4 numbers)	£3 10s.	\$12.00	Sw. fr. 35.—
<i>Technical Report Series</i>	£5	\$16.00	Sw. fr. 50.—
<i>Official Records</i>	£5	\$16.00	Sw. fr. 50.—
<i>World Health Statistics Report</i> , vol. 22 (12 numbers)	£5	\$16.00	Sw. fr. 50.—
<i>Weekly Epidemiological Record</i> , 44rd year (52 numbers)	£5	\$16.00	Sw. fr. 50.—
<i>Vaccination Certificate Requirements for International Travel</i>	£1	\$ 3.00	Sw. fr. 10.—
<i>World Health</i> , vol. 22	£1 4s.	\$ 5.00	Sw. fr. 12.—

Subscriptions can be obtained from WHO sales agents for the calendar year only (*January to December*).

* * *

*Specimen numbers of periodicals and a catalogue
will be sent free of charge on request.*