Immunological Aspects of Cholera

The Technical Committee of the Pakistan-SEATO Cholera Research Laboratory of Dacca held a special meeting at the National Institutes of Health, Public Health Service, in February 1963 to discuss with members of its panel of experts and invited participants problems related to immunological aspects of cholera with particular reference to vaccine trials. The program was concerned with the immunological response of patients to Asiatic cholera, experimental cholera in animals, and methods by which cholera vaccine might be assayed in the laboratory and tested in the field for efficacy in man, and it also dealt with the important question of how these various aspects might be wed. The program was arranged by Dr. Joseph E. Smadel, chairman of the CRL Technical Committee. Dr. John C. Feeley and Dr. O. Ross McIntyre of the Division of Biologics Standards, National Institutes of Health, served as rapporteurs.

Report From Cholera Research Laboratory

Dr. Abram S. Benenson, director of the Pakistan-SEATO Cholera Research Laboratory, described the opening of hospital facilities at the laboratory in November 1962 and the treatment since that time of about 150 patients with cholera. In general, clinical observations made on many of these patients have substantiated those made by others in recent years, and rehydration procedures recommended by Phillips and associates have proved successful in their treatment. A significant number of patients, however, have manifested evidence of cardiac decompensation, electrocardiographic abnormalities, and failure to emerge promptly from the stage of vascular collapse. The patients in this category are not able to withstand full replacement of lost fluids but respond remarkably rapidly on a regimen in which thiamin and tetracycline (containing ascorbic acid) are added to the standard fluid therapy program. The implications of these findings were discussed as they relate to (*a*) extension of work on the role of acute nutritional deficiencies in certain of the cardiovascular manifestations of cholera, and (*b*) the importance of early termination of vibrio multiplication by antibiotic therapy.

Antibody Pattern in Man

Chairman: Dr. Robert Cruickshank, professor of bacteriology, University of Edinburgh Medical School, Edinburgh, Scotland

The recent introduction of reliable tests for demonstration of cholera agglutinating antibodies and of a simplified method for demonstration of vibriocidal antibodies has facilitated studies of the immunological response to cholera and cholera vaccination. Dr. Harry Smith, Jefferson Medical College, emphasized the importance of using selected live vibrios as antigens in the agglutination test in order to achieve a sensitive and reliable procedure. His studies on paired serums obtained from patients with cholera demonstrated a significant antibody rise in 67 of 73 bacteriologically proved cases. Absorption tests indicated that a rise in group antibody occurred in 88 percent and a rise in type-specific antibody in 67 percent of the specimens.

The titer of the convalescent specimens was higher than that observed after immunization of healthy United States Army and Marine recruits.

Dr. Kenneth Goodner, Jefferson Medical College, described work in which serum samples from several population groups were examined for cholera antibodies, with the same general techniques mentioned by Dr. Smith. In these agglutination tests, serial dilutions of unabsorbed serum were mixed with suspensions of living Ogawa and with suspensions of living Inaba organisms. The serologic data were further refined by absorbing a portion of each serum with Ogawa vibrios and another with Inaba vibrios after which the agglutinin titrations were repeated. The interpretation of the results and the designation of antibody patterns are given in the table.

Dr. Goodner found that about 1 percent of young adults in the United States had detectable cholera antibodies which fell into one of the patterns mentioned in the table. In contrast, 15 percent of Philippine blood bank donors (1961), 29 percent of Pakistani villagers (1961–62), 41 percent of persons from Hong Kong (1962), and 57 percent of those from Bangkok (1959) had demonstrable cholera antibodies. He also reported that administration of U.S. commercial vaccine elicited an antibody response in 417 of a group of 452 healthy American recruits (93 percent). The percentile distribution of the different patterns was as follows: ABC, 45; AB, 12; AC, 23; A, 14; B, 4; and C, 2. The findings indicate that cholera antibodies were induced with regularity by American vaccine. They also indicate that such antibodies are much less frequently encountered in populations in which cholera is endemic or epidemic and among whom vaccination is more or less widely employed. This observation, in turn, suggests that the vaccines were of low antigenicity or were not administered to large segments of the population.

Simplified scheme for determination of cholera antibody patterns

Raw serum tested with—		Absorbed with—	Absorbed serum tested with—		Pattern
Ogawa (AB)	Inaba (AC)		Ogawa	Inaba	
+	+	Ogawa	−	+	AC
		Inaba	−	−	
+	+	Ogawa	−	−	AB
		Inaba	+	−	
+	+	Ogawa	−	+	ABC (or BC) [1]
		Inaba	+	−	
+	+	Ogawa	−	−	A
		Inaba	−	−	
+	−	Ogawa	−	−	B
		Inaba	+	−	
−	+	Ogawa	−	+	C
		Inaba	−	−	

[1] Pattern BC cannot be determined since an organism having only A antigen has not been found.

These findings were substantiated by Dr. John C. Feeley, Division of Biologics Standards, using the more sensitive but more complex vibriocidal method in conjunction with agglutination tests in the titration of antibodies in paired serums from cholera patients in the Philippines and in single serums from normal subjects from various geographic areas. He also detected vibriocidal antibodies, usually of low titer but occasionally high, in most serums from healthy persons whether residents of the United States, the Philippine Republic, or Pakistan. Only occasionally were agglutinins demonstrated. The presence of vibriocidal antibody in many of these normal serums could not be readily explained by known artificial or natural exposure to antigens of *Vibrio comma*. Of interest was the demonstration that there was no difference in the mean levels of vibriocidal antibodies between a group of vaccinated and a group of nonvaccinated Pakistanis.

In discussing the relation of the results of serologic studies on patients and vaccinated persons to the antigenic composition of a "desirable" cholera vaccine, two points of view were evident. Some of the conference participants preferred a broad approach and wished for a vaccine that would elicit in normal persons an antibody response imitating that found in cholera patients. Others preferred a specific approach; they desired a vaccine that would induce in man those antibodies which protect experimental animals against *V. comma*. The question remains as to which antibodies, if any,

are truly protective in man. Will experimental disease in animals help decide whether resistance against a single bacterial antigen or an entire group of antigens is the goal for which to strive? There was no unanimity regarding which experimental disease in animals should serve as the test model for estimating protective antibodies. Would it be fatal infection in the mouse or the properly prepared guinea pig, or could it be the antibody that protected the baby rabbit against severe and fatal diarrhea induced by proper administration of *V. comma*? Proponents of both schools of thought continued to present their points of view throughout the conference.

Experimental Cholera in Animals

Chairman: Dr. Francis S. Cheever, dean, School of Medicine, University of Pittsburgh, Pittsburgh, Pa.

Research in many aspects of cholera has suffered for want of an entirely satisfactory animal model of the disease. The information summarized at this session indicated that definite progress has been made in developing animal models which duplicate at least certain of the features of the disease in humans.

Capt. Robert A. Phillips, U.S. Naval Medical Research Unit No. 2, related his experiences with infant rabbits infected by a modification of the Dutta technique. Disease was consistently produced in 12-day-old rabbits treated as follows: After gastric lavage with alkaline solutions administered by tube, *Vibrio comma* in mucin was introduced by tube into the stomach. Beginning 12–18 hours later, the animals rapidly lost fluid amounting to about 10 percent of their body weight either into the intestinal lumen or in the form of diarrheal stools. The animals were killed 1 hour after the onset of purging, at which time the intestinal contents had a chemical composition resembling that of the stool of the patient with cholera. If the animals were not killed, death occurred within several hours of the onset of purging and was attributed to the hypovolemic state. Captain Phillips also found, as have others, that inoculation of vibrios into ligated intestinal loops of adult rabbits and monkeys resulted in an outpouring of fluid into the loop. He reported that the composition of this fluid also resembled that of the human cholera stool if the contents were sampled prior to the onset of ischemic necrosis in the loop.

Using the infant rabbit model, Phillips and associates have carried out passive protection studies. Varying doses of antiserum were administered to the rabbits intraperitoneally at 72, 24, or 4 hours prior to infection. Four of 14 animals given antiserum 24 hours prior to challenge failed to develop diarrhea, whereas 37 of the 39 rabbits given normal serum, no serum, or antiserum 4 hours prior to challenge developed typical experimental cholera.

Dr. O. Ross McIntyre and Dr. John C. Feeley, Division of Biologics Standards, presented preliminary findings from passive protection experiments similar to those described by Captain Phillips. They attributed the greater degree of protection they achieved to use of a more potent antiserum and a lower challenge dose of vibrios. In their studies, approximately 400 viable Ogawa organisms suspended in physiological saline containing 0.1 percent trypticase were injected into the small intestine at laparotomy. All infant rabbits were protected against experimental cholera when 2.0 ml. of hyperimmune Ogawa rabbit antiserum was administered intraperitoneally 24 hours prior to challenge. Only borderline protection, evidenced by a delay in death, was produced by 0.02 ml. of this serum, and no protection was offered by 0.0002 ml. No evidence of protection was seen in animals given 2.0 ml. of normal adult rabbit serum. Rabbits that were protected had in their circulating blood agglutinating antibody levels of 1 : 1,280 and vibriocidal antibody levels of 1 : 32,000. Those in which death was delayed had agglutinating titers of 1 : 40 and vibriocidal titers of 1 : 640. The Ogawa hyperimmune serum was prepared by immunizing rabbits repeatedly over a period of a month with live vibrio cultures of the challenge strain. Whether the protection in the baby rabbits was elicited by the antibodies detected in vitro or by other immune substances remains to be determined.

Limitations of the rabbit technique were amplified in the discussion following these papers. The most significant is the inability to infect rabbits older than 3 weeks. The short interval during which the animal is susceptible undoubtedly hampers studies of active immunization. The possibility that maturation of the

rabbit gut might bring changes in permeability was suggested by Captain Phillips as an explanation for the development of resistance.

Experiences of his group with various models of experimental infection with *V. comma* were outlined by Dr. Samuel B. Formal, Walter Reed Army Institute of Research. The enhancing effect of purines on the virulence of purine dependent vibrios injected intraperitoneally into mice was discussed. Dr. Formal also discussed the fatal enteric infection of the starved, opium-treated guinea pig induced with *V. comma* or *Shigella* organisms. Virulence of *Shigella*, but not of *V. comma*, was enhanced for the guinea pig by subcutaneous injection of carbon tetrachloride. The histopathological changes in the intestine of the guinea pig with cholera resembled those seen in human infection, that is, blunted villi with intact epithelium. Desquamation of the epithelium in experimental cholera in the guinea pig, as in cholera in man, appears to be a postmortem artifact. Immunofluorescent studies of tissue sections of guinea pig gut by Dr. Eugene H. LaBrec, also at Walter Reed, showed that the cholera vibrio grows only on the epithelial surface. *Shigella*, on the other hand, invades the mucosal layers and produces micro-ulcers.

The report of Jenkin and Rowley implicating lactic acid as the principal agent responsible for increased permeability and tissue damage in the ligated rabbit gut segment was also investigated. Dr. Formal and co-workers found instead that lactic acid accumulates only after ischemic necrosis sets in. Stool material which accumulates prior to the onset of hemorrhagic necrosis is similar to that in the human disease, as noted also by Captain Phillips. Histopathological findings show an intact epithelium at this time.

Recent studies at Walter Reed by Dr. Formal in collaboration with Dr. N. K. Dutta, using infant rabbits, showed that a sonic-disrupted preparation of *V. comma* containing no viable cells was capable of inducing fatal experimental cholera. After heating the preparation at 60° C. for 1 hour, its ability to induce diarrhea was lost.

Dr. Rolf Freter, Jefferson Medical College, also described studies of fatal enteric cholera in the guinea pig. In his procedure, the guinea pig must first be prepared by starvation, opium administration, and inhibition of the normal flora by streptomycin. This technique permits induction of experimental cholera with an infective dose sufficiently small to demonstrate active and passive immunity. Following oral administration of streptomycin-resistant cholera vibrios, leakage of fluid into the intestinal lumen was of sufficient magnitude to account for the death of the animal. In guinea pigs, the fatal outcome of experimental cholera could be prevented by administration of a streptomycin-resistant *Escherichia coli*. The physiological and bacteriological changes accompanying starvation and administration of opium and streptomycin allow speculation that the poorly nourished human with altered intestinal mobility and bacteriological flora may be the person susceptible to cholera.

Of considerable interest were the experiments cited by Dr. Freter indicating that protection against enteric cholera infection in guinea pigs was due to copro-antibody, while serum antibody had no effect. Dr. Freter pointed out that Jenkins and Rowley found copro-antibody to be protective also in the other models, the rabbit loop and the suckling rabbit. The original studies of Burrows had shown an anti-bacterial action of copro-antibody in guinea pigs. In Dr. Freter's model, copro-antibody was protective without reducing the number of vibrios in the intestine. He noted that the guinea pig, like man, was completely insensitive to oral administration of endotoxin.

It was recognized in the discussion following the above papers that a number of experimental models of cholera infection are now available which bear similarities to the disease in man. The conference participants generally agreed that further investigations should be pursued in relation to assay of cholera vaccines and studies on the pathogenesis of the disease, and they expressed hope that work in different laboratories could be correlated by use of common serums and cultures. There was general agreement also that an animal model closely resembling human cholera is desirable for studies on pathogenesis. On the other hand, some participants thought such a model is not essential for vaccine assays. This schism found the clinically oriented participants interested in an immunological ap-

proach to prevention of an experimental diarrhea in animals, while the laboratory-oriented participants were more interested in a simple lethal test in a small animal such as a mouse. It seems clear, at this point, that the real significance of any vaccine assay procedure will not be determined until results with it are correlated with those of a controlled field trial of the vaccine in a cholera area.

Experimental Vaccines and Assays

Chairman: Dr. Joseph E. Smadel, Division of Biologics Standards, National Institutes of Health, Bethesda, Md.

Cholera vaccines prepared by several different methods have been used for a number of years. Although the most widely employed product at the present time is phenolized vaccine, formalin-killed, heat-killed, and living vaccines have been used. In some quarters at least, doubt exists concerning the value of immunization as presently practiced and the duration of whatever immunity is conferred. Another problem in the use of cholera vaccine is a fairly high incidence of untoward reactions. Furthermore, the capacity of parenterally administered vaccine to protect against an infection limited to the lumen of the gut has been challenged. The papers presented focused on some of these problems.

A communication from Dr. J. Gallut, Institut Pasteur, which was read to the conference, reviewed available immunochemical information concerning the various antigens of *Vibrio comma*. He concluded that the lipopolysaccharide complex constituting the main part of the cell wall is responsible for the greater part, if not all, of the animal-protective activity of cholera vaccine.

Dr. Ataur Rahman, Jefferson Medical College, on leave from the Institute of Public Health, Dacca, presented his work on the antigenicity of cholera vaccines injected subcutaneously in rabbits. He found that phenolized vaccines were less antigenic than vaccines containing live vibrios. Differences in antigenicity among strains were noted, and recently isolated strains were not always the best antigens. It appears that careful selection of strains and particular attention to maintenance of cultures, methods of cultivation, and killing agents employed might significantly improve the vaccine used at the present time. Dr. Rahman also presented data indicating that the antibody response of rabbits given one dose of vaccine was about equal to that obtained with two doses.

Studies directed toward isolation and purification of a protective antigen from an Ogawa El Tor vibrio were presented by Dr. Y. Watanabe, University of Texas Medical Branch. A highly purified lipopolysaccharide antigen has been prepared which forms a single band in ultracentrifugal and immunodiffusion analyses. On a dry-weight basis, this antigen accounts for a major portion of the mouse-protective activity of the whole vibrio cell. A slightly less purified "practical antigen" can be prepared in sufficient yield to make it feasible for use in man. Results of a small clinical trial in volunteers indicated that the practical antigen produced milder reactions than commercial cholera vaccine and induced a serologic response consisting of vibriocidal and mouse-protective antibodies. Unfortunately, this antigen prepared from an Ogawa strain elicits little cross-protection in mice against Inaba strains, and methods used thus far have failed to yield a satisfactory antigen from Inaba cultures with similar properties. Other chemical fractionation methods are now being pursued to prepare an Inaba antigen to broaden the immunological coverage.

Current immunization schedules for cholera vaccine embody the tacit assumption that immunity is of short duration and specify that reinforcing injections should be given at 6-month intervals. While this concept is not supported by solid scientific evidence, if immunity is truly of short duration or of poor quality, one approach might be the use of various adjuvant materials in the vaccine. This problem was discussed by Dr. Feeley, who reported that the agglutinin response of the guinea pig could be dramatically enhanced by the use of a mineral oil emulsion adjuvant. A significant but less dramatic enhancement was achieved with alum-precipitated vaccine. Dr. Feeley felt that use of adjuvants in man might be considered if field studies with non-adjuvant vaccines reveal immunity to be of poor quality or of short duration.

In view of the apparent importance of coproantibody in the protection of guinea pigs

against experimental enteric cholera, Dr. Freter reopened the question of possible oral immunization of man. Such an approach had been employed previously, but most existing reports are discouraging, showing highly erratic production of copro-antibody by human volunteers. Using Farr's isotopic assay procedure, which appears well suited for titration of antibody in fecal material, Dr. Freter demonstrated that 5 percent to 80 percent of orally administered antibody could be recognized in stool material of human volunteers, provided that diarrhea was induced in the subject with magnesium sulfate. Conventional methods of antibody titration were unsatisfactory when applied to human stools, a finding which explains the negative results of earlier investigators. After parenteral immunization, 47 percent of persons tested had demonstrable copro-antibody but excreted it for only 2 to 3 weeks. The copro-antibody level decreased more rapidly than serum antibody. Oral administration of large amounts of heat-killed cholera vibrios daily for 4 weeks elicited copro-antibody in 77 percent of persons tested, and this could be readily maintained by one weekly dose of antigen. Oral vaccine was heat stable and tasteless and it gave no untoward reactions. Dr Freter reasoned that oral immunization would be the only feasible means of maintaining immunity in man, if the importance of copro-antibody in human cholera is similar to that found in the existing experimental models.

Dr. Margaret Pittman, Division of Biologics Standards, in a review of the various types of vaccines and potency assays, stressed the importance of the manufacturing procedures. At this time, agar- and broth-grown whole cell and the new fractionated cell vaccines are the most promising immunizing agents, and a quantitative active mouse protection test offers the greatest promise for potency assay. Dr. Pittman emphasized the importance of fully characterizing vaccines used in field trials by as many in vitro and in vivo tests as possible in hopes of learning which of the laboratory-determined characteristics of these materials can be related to protection of man.

The importance of stable forms of cholera vaccines for field trials and for reference purposes is obvious. Dr. Joseph Lowenthal, Walter Reed Army Institute of Research, discussed his work leading to a lyophilized vaccine of good potency for use in coming WHO field studies. Several different preparations were made and their potency was assessed by mouse protection tests. Acetone-killed and dried vaccine was unsatisfactory. The presence of phenol during the drying process was deleterious. The best results were obtained with a formalin-killed lyophilized vaccine. Standardization of antigen content, a frequent problem in cholera vaccine production, was most conveniently approached by determination of the nitrogen content of the preparation under study.

The roundtable discussion following the papers centered chiefly on the fact that, although numerous assay procedures are of potential value in assessing the potency of vaccine, a definitive appraisal of these procedures must await completion of controlled field trials. In the meantime, it was suggested that coordinated studies be pursued on the relative protective value of various cholera antigens in the experimental models currently available.

Plans for Field Trials

Chairman: Dr. Roderick Murray, director, Division of Biologics Standards, National Institutes of Health, Bethesda, Md.

A trial of vaccine in an endemic cholera area avoids some of the rapidly varying extraneous factors inevitable in a study carried out during an epidemic in a previously cholera-free area. The Pakistan-SEATO Cholera Research Laboratory is ideally placed for conducting such field studies. Dr. Benenson, director of the laboratory, pointed out, however, that many problems face a full-fledged field trial of cholera vaccine in East Pakistan at the present time, including difficulties imposed by the terrain, limited medical staff, and deficient epidemiologic intelligence. He proposed that while the laboratory is developing to the point where it can launch a vaccine study in a population on which adequate baseline information has been collected, efforts be devoted to groups having a high risk of cholera, such as the organized workers in certain industries. He also suggested studies on family contacts of index cases. The extremely high incidence of cholera in such

families makes it desirable to undertake protection studies in this group, though the short incubation period of the disease may preclude an entirely successful investigation.

Dr. Paul Joseph, Communicable Disease Center, Public Health Service, indicated the possible importance of carriers in the dissemination of the disease in the Philippines and the desirability of undertaking further studies on this subject in East Pakistan.

The necessary epidemiologic, immunological, administrative, statistical, and medical groundwork needed before a successful cholera vaccine field trial can be mounted was described by Dr. Thomas Francis, Jr., University of Michigan. In particular, the necessity of adhering to the fundamental principles of a well-thought-out protocol was emphasized as essential for the achievement of a satisfactory study.

Dr. Kenneth Goodner, Jefferson Medical College, and Dr. Theodore E. Woodward, University of Maryland School of Medicine, described the bacteriological, serologic, and clinical support required for mounting a successful field trial. Both stressed the need for adequate numbers of flexible, intelligent personnel capable of carrying out their respective tasks.

The question of when a full-fledged field trial of vaccine can be undertaken was left undecided. All observers felt that effort should be concentrated on completing as rapidly as possible the groundwork needed for such a trial. It was believed that valuable information can be obtained from limited studies on population groups at high risk, but only if well manned and well organized. It was generally agreed that vaccines having a high degree of protection against the disease in animal models are the logical ones to test in such a trial, but caution was also expressed against relying too much on the results of animal testing. It was pointed out that protection against Vi antigen is important in mouse typhoid protection but that this factor may have little or nothing to do with human protection.

The group also thoroughly debated the need and the justification for including a noncholera vaccine in the trial. The majority attending the conference advocated inclusion of another vaccine (tetanus toxoid and typhoid vaccine were considered) in a double-blind study. However, inclusion of a placebo vaccine assumes that appropriate medical measures are taken to assure the best and earliest medical treatment to all members of the study group. The need for smoothly operating reporting systems and transport systems for delivery of cholera cases to the hospital was also stressed.

Defense Training for Health Personnel

A 1-week course for health and medical personnel on chemical and biological defense techniques will be given August 19–24, October 14–19, and December 9–14 in 1963, and February 10–15 and April 6–11 in 1964. One 2-week session, with the second week devoted to radiological defense, will be held June 8–20, 1964. The Public Health Service will conduct the classes at Fort McClellan, Ala., in cooperation with the U.S. Army Chemical School.

Representatives of State and local health departments, the Veterans Administration, the Public Health Service, faculty members of affiliated schools in the Medical Education for National Defense Program, and other persons with responsibility in chemical, biological, and radiological defense may apply for the courses.

Enrollment forms are available from the Deputy Chief, Training Branch, Division of Health Mobilization, Office of the Surgeon General, Public Health Service, Washington, D.C.