From the National Institute of Allergy and Infectious Diseases

Summary of the 21st United States–Japan Joint Cholera Conference

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The 21st Joint Conference on Cholera, sponsored by the United States–Japan Cooperative Medical Science Program and the Microbiology and Infectious Diseases Program of the National Institute of Allergy and Infectious Diseases, was held at the National Institutes of Health (Bethesda, Md) on 21–23 October 1985. This conference, which is held annually in either the United States or Japan, provides a forum for international communication and collaboration by investigators working on enterotoxigenic bacterial infections. More than 148 individuals from the United States and five foreign countries attended the conference. Four members of the Japanese Cholera Panel and 10 additional Japanese guests participated. Thirty-six papers were chosen for presentation from among 48 submitted abstracts. The program consisted of the following sections: epidemiology and clinical studies, bacteriology and virulence factors, toxinology and genetics, immunology and vaccine development, and pathophysiology. Selected papers from each of the program sections are summarized below.

Epidemiology and Clinical Studies

Dr. M. Santosham described prospective studies of the incidences and etiologies of diarrhea on the Fort Apache Indian Reservation in Arizona for a three-year period beginning in April 1981. Not unexpectedly, the highest attack rate of diarrhea was found in children less than two years old, with four to five episodes per year in children during this time—a rate similar to that in many developing countries. A viral pathogen, either rotavirus, coronavirus, or adenovirus, was most frequently found in infants less than one year of age. Rotavirus and *Shigella* were the most common pathogens found in hospitalized patients and in outpatients, respectively.

Participants in the conference included the following: P. Blake, Centers for Disease Control, Atlanta; J. D. Clemens, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh; J. D. Clements, Tulane University Medical Center, New Orleans; M. B. Cohen, University of Cincinnati Medical Center, Cincinnati; R. R. Colwell, University of Maryland, College Park, Maryland; R. Edelman, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland; R. A. Finkelstein, University of Missouri, Columbia, Missouri; A. Ghoda, Kitasato University, Tokyo; R. L. Guerrant, University of Virginia, Charlottesville, Virginia; T. Hirayama, University of Tokyo, Tokyo; K. Hisatsune, Josai University, Saitama, Japan; T. Honda, Osaka University, Osaka, Japan; T. Itoh, Juntendo University, Tokyo; M. Iwanaga, University of the Ryukyu, Okinawa, Japan; Y. Kudoh, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo; Y. M. Kupersztoch, University of Texas Health Science Center, Dallas; S. Kuwahara, Toho University, Tokyo; M. M. Levine, University of Maryland, Baltimore; J. J. Mathewson, University of Texas Health Science Center, Houston; J. J. Mekalanos, Harvard Medical School, Boston; H. Moon, National Animal Diseases Laboratory, Ames, Iowa; J. Moss, National Heart, Lung, and Blood Institute, Bethesda, Maryland; J. R. Murphy, Boston University Medical Center, Boston; J. P. Nataro, University of Maryland, Baltimore; J. Newland, Uniformed Services University of the Health Sciences, Bethesda, Maryland; N. Ohtomo, The Chemo-Sero-Therapeutics Research Institute, Kumamoto, Japan; S. H. Richardson, Wake Forest University, Winston-Salem, North Carolina; D. C. Robertson, University of Kansas, Lawrence, Kansas; R. B. Sack, Johns Hopkins University, Baltimore; E. Salazar-Lindo, Universidad Peruana Cayetano Heredia, Lima, Peru; M. Santosham, Johns Hopkins University, Whiteriver, Arizona; C. V. Sciortino, Veterans Administration, Charleston, South Carolina; T. Shimamura, Tokai University, Kanagawa, Japan; Y. Shimonishi, Osaka University, Osaka, Japan; N. Strockbine, Uniformed Services University of the Health Sciences, Bethesda, Maryland; Y. Takeda, University of Tokyo, Tokyo; M. R. Thompson, University of Cincinnati Medical Center, Cincinnati; M. Tomisato, University of the Ryukyu, Okinawa, Japan; H. Watanabe, National Institute of Health, Tokyo; C. S. Weikel, University of Virginia, Charlottesville, Virginia; S. C. Whipp, National Animal Disease Center, Ames, Iowa; T. Yokota, Juntendo University, Tokyo; and Y. Zinnaka, National Defense Medical College, Saitama, Japan.

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A 1984 cholera epidemic in Mali was reported by Dr. P. A. Blake. Multiple routes of transmission, including contaminated wells and stagnant millet broth, accounted for a mean cohort attack rate of 1.5%. Lack of use of rehydration therapy on the part of patients and health care personnel alike, together with maldistribution of oral rehydration solutions, accounted for a case-fatality rate of 30%.

J. J. Mathewson measured the prevalence of HEp-2 cell-adherent *Escherichia coli* (EAEC) among American travelers to Mexico. Only four of 42 EAEC strains belonged to traditional enteropathogenic *E. coli* (EPEC) O serogroups, and none secreted known enterotoxins. EAEC were recovered almost twice as often in patients with diarrhea as in well individuals. Overall, EAEC were associated with 30% of diarrheal episodes having no other identifiable etiologic cause. About half of the patients with EAEC had a serum antibody response to the strain isolated from their stool specimens. Some volunteers challenged orally with selected EAEC strains suffered bowel infection and diarrheal illness. Thus, EAEC are likely causes of some cases of travelers' diarrhea.

Dr. Y. Kudoh reported an outbreak of acute enteritis in an elementary school in Tokyo that was caused by *E. coli* strain O145:H$^-$. This strain produced a poorly characterized toxin that was biologically similar to Shiga toxin but immunologically distinct from Shiga toxin, LT (labile toxin), and ST (stable toxin).

Dr. R. Colwell grew *Vibrio cholerae* O1 in microcosms simulating conditions of the estuarine environment. Dr. Colwell discovered that under these in vitro conditions, the bacteria can enter into and persist in a dormant state, refractory to culture by routine laboratory methods. The bacteria, however, were recovered by animal passage, and they retained the potential for virulence. Dr. Colwell suggested that such cryptic *V. cholerae* may exist in nature.

Dr. E. Salazar-Lindo reported a double-blind, placebo-controlled trial of erythromycin treatment of dysentery in Peruvian children aged three to 60 months. Treatment was started immediately, before culture results were known, and always within five days of onset of dysentery. In campylobacter disease, erythromycin resulted in a significant shortening of both clinical illness and excretion of *C. jejuni* compared with placebo; however, erythromycin treatment failed to affect the illness caused by *Shigella* organisms.

Bacteriology and Virulence Factors

Monoclonal antibodies have helped to identify and purify virulence antigens of *V. cholerae*. The identification of 16 separate outer-membrane antigens of *V. chloerae* has been accomplished by C. V. Sciortino by using a library of 66 monoclonal antibodies. One monoclonal antibody, combined with an outer-membrane protein identified as an iron-regulated porin, killed *V. cholerae* in the absence of complement. Using a monoclonal antibody, M. Tomisato has identified an antigenically distinct pili-like adhesin on *V. cholerae* Ogawa strains, but not on Inaba strains.

Dr. H. Hisatsune analyzed the sugars of the polysaccharide portions of LPS contained in non-O1 group *V. cholerae*, *Vibrio fluvialis*, and *Vibrio vulnificus* and related these sugars to the chemotaxonomy of these organisms. T. Ito reported that cyclic AMP added to cultures of *V. cholerae* modulated the growth and physiology of 1940 and 1982 isolates differently. Drs. M. Iwanaga and T. Shimamura modulated production of cholera toxin in vitro by changing bicarbonate and atmospheric CO_2 concentrations, by adding or deleting thiol compounds to the growth media, and by immobilizing culture flasks for several hours before shaking them.

Dr. J. Moss reported experiments establishing that intestinal epithelial cells contain an ADP-ribosylarginine cleavage enzyme. This discovery raises the possibility that such cells may have the ability to reverse the cholera toxin–catalyzed ADP-ribosylation reaction and, thus, the metabolic derangements secondary to adenylate cyclase activation. The ADP-ribosylarginine cleavage enzyme could thus assist in recovery from cholera.

Dr. D. C. Robertson summarized recent results of his long-term studies of the synthesis and cellular distribution of heat-labile enterotoxigenic *E. coli* (ETEC) enterotoxins. LT subunits A and B are localized in the periplasmic space of ETEC, whereas cholera toxin is actively exported from *V. cholerae*. The question of how LT leaves ETEC is yet unanswered; possibilities include passive leakage or lysis of the cell. Dr. J. Mekalanos, by using a recently developed set of fusion vectors for genetic analysis of extracellularly secreted proteins of *V. cholerae*, postulated that the A and B subunits of cholera toxin are assembled in the periplasmic space before they are secreted into the extracellular environment.

Toxinology and Genetics

Dr. N. A. Strockbine discovered that *E. coli* strain O157:H7, which causes epidemic hemorrhagic colitis, harbors two different cytotoxin-converting phages. Each of these phages contain a gene coding for a cytotoxin with Shiga-like biologic properties but with different antigenic properties. Shiga antitoxin neutralizes one toxin, but not the other. Characterization of the new, non-Shiga toxin is underway. Cloning and DNA sequencing of the Shiga-like toxin gene to determine the primary structure of its product was reported by Dr. J. W. Newland.

Dr. J. P. Nataro described genetic analyses of EPEC virulence plasmids. He discovered that full virulence of many EPEC serotypes appears to be dependent upon a 55–65-megadalton plasmid that encodes adherence to HEp-2 cells, a 94-kilodalton protein, and possibly other functional proteins. Early results from volunteer studies reveal the 94-kilodalton protein is immunogenic, is associated with EPEC virulence, and, possibly, is an intestinal adhesion factor for EPEC.

Detailed genetic analysis of the cyclic AMP-mediated transcriptional regulation of the ST-1 enterotoxin of *E. coli* strains was reported by Y. M. Kupersztoch. He postulated the existence of a protease that nick translates ST in the periplasmic space, an occurence allowing the ST to be exported from the cell.

A 120–140-megadalton plasmid of *Shigella* spp. encodes functions necessary for invasion of epithelial cells. Dr. H. Watanabe devised a method to transfer these nonconjugative plasmids from virulent *Shigella* into nonvirulent *E. coli* and *Shigella*. He examined the restoration of invasiveness in the strain that received the plasmid or plasmid fragments. A 4.1-kilobase DNA sequence, conserved among *Shigella* spp., is necessary for invasions.

Microbial toxins act through cyclic AMP, cyclic GMP, or poorly understood calcium-dependent, cyclic nucleotide–independent pathways to cause intestinal secretion. Dr. C. S. Weikel described studies of the latter pathway that involved activation of protein kinase C (PKC) in piglet jejunum. Dr. Weikel showed that phorbol esters, which directly activate PKC, cause net water and electrolyte secretion detectable in vivo within 30 min in piglet jejunum; this secretion persists for several hours. These important results suggest a direct role for PKC in intestinal secretion.

A series of papers focused on STs. Dr. T. Hirayama confirmed previous reports that cholera and STs stimulate phosphorylation of brush-border cell membranes of jejunum. Dr. Y. Shimonishi and colleagues have determined the amino acid sequences of three different STs isolated from ETEC and *Yersinia enterocolitica* and have elucidated the structure-activity relation of synthesized analogues of these STs. Shorter analogues of *Yersinia* ST have almost the same activity. The substitution of Glu-Leu in *E. coli* STs results in a loss of toxic activity, a finding suggesting that the strong toxicity of *E. coli* STs is due to either one of these two amino acid residues.

A monoclonal-antibody ELISA identifying STa-producing ETEC was found by Dr. M. R. Thompson to agree 100% with RIA and the suckling mouse assay. Dr. M. B. Cohen assessed ST binding and ST-induced secretion on rat intestinal brush-border membranes and found that both processes are spontaneously reversible. Dr. Cohen suggested that various means of removing ST from its enterocyte receptor might be of therapeutic benefit. Finally, T. Honda discovered that 60% of *V. cholerae* non-O1 strains tested produce a new type of ST enterotoxin, as determined by amino acid sequence analysis. Dr. Honda also discovered that some non-O1 *V. cholerae* strains produce a hemolysin immunologically related to the ST produced by *Vibrio parahaemolyticus*. He hypothesized that this hemolysin may be responsible for the bloody diarrhea in some cases of infection with non-O1 *V. cholerae*.

Immunology and Vaccine Development

Iron-regulated outer-membrane proteins (IROMPS) that recognize and internalize the iron sideraphore are necessary for replication of *V. cholerae*. Dr. R. A. Finkelstein reported that monoclonal antibodies to IROMPS were bactericidal for *V. cholerae* and bacteristatic for several gram-negative enteropathogenic bacilli. Dr. Finkelstein is searching for cross-reactive antibodies to IROMPS in human milk in an attempt to explain how human milk, obtained from mothers never exposed to *V. cholerae*, inhibits that organism.

Dr. J. D. Clements has engineered model, live oral vaccines for *Salmonella* and the *V. cholerae*–ETEC diarrheas. He transformed attenuated *Salmonella typhi* strain Ty21a and an auxotropic *Salmonella enteriditis* strain, each with a recombinant plasmid coding for the nontoxic B subunit of *E. coli* LT. The derivative strains were avirulent, genetically stable,

and antigenic for the gut mucosa of experimental animals. Dr. Clements is studying the potential contribution of other virulence antigens, such as ST toxoid and cholera somatic antigens, in the formulation of an optimum hybrid vaccine.

Dr. M. M. Levine summarized his group's attempts to develop less diarrheagenic, attenuated cholera vaccines. A series of genetically engineered strains of *V. cholerae* were tested in volunteers. So far, observations suggest that retaining the ability to colonize the proximal small intestine, in the hope of being able to immunize with a single dose of vaccine, leaves the strains inherently reactogenic. The goal is to strike a critical balance between satisfactory colonization and immunogenicity and tolerable reactogenicity. Dr. Levine has also studied ways to stimulate intestinal secretory IgA antibody to fimbriae (pili) adhesions in animals and humans. He used purified fimbriae or an attenuated non-ETEC strain as an oral vaccine. The results in volunteers point to the superiority of live oral vaccines, rather than purified fimbriae, in stimulating such protective antibody to fimbriae.

The superior antigenicity of live, rather than killed, *V. cholerae* fed to mice was confirmed by Dr. Y. Zinnaka. Living antigen induced delayed cutaneous hypersensitivity and serum antibody, whereas killed antigen induced serum antibody only. Forced feeding compared with ad libitum feeding suppressed serum antibody production, probably, according to the authors, by enhancing splenic suppressor-cell activity.

In the final paper on vaccines, Dr. J. Clemens summarized the progress of an ongoing field trial of two killed, oral cholera vaccines in Bangladesh. One vaccine contains B subunit and killed whole cells of *V. cholerae*, and the other vaccine contains killed *V. cholerae* cells alone. In early-phase studies on 1,257 volunteers, the two vaccinees were totally free of side effects; they induced a significant rise in vibriocidal antibodies or in antibodies to cholera toxin in 85% of vaccines after two doses. However, antibody titers were much lower than those after natural cholera or attenuated oral vaccines. The ability of the two vaccines to prevent cholera will be determined and reported in 1986.

Pathophysiology

Dr. S. C. Whipp found that in contrast to *E. coli* LT and STa, the pig-specific, mouse-negative *E. coli* toxin STb causes morphological damage to the pig jejunum and has functional correlates in reduced sucrose activity and interference with active transport of alanine. Dr. S. H. Richardson provided an update of his studies on the genetic and cellular control of the response of inbred mice to cholera toxin. Finally, A. Ghoda presented preliminary data suggesting that the house musk shrew, *Suncus murinus*, may provide a new laboratory animal model for *V. cholerae* and *Shigella flexneri*.

Summary

Cholera outbreaks with high death rates continue in countries lacking rehydration therapy. Strains of *E. coli* without identified *E. coli* virulence markers but adhering to HEp-2 cells likely represent a new class of enteropathogens causing travelers' diarrhea. Erythromycin is effective treatment for *C. jejuni* dysentery if the antibiotic is started early in the course of illness. Monoclonal antibodies have helped identify new virulence antigens on the outer membrane of *V. cholerae*. Several antigenically distinct Shiga and ST toxins have been discovered. Most EPEC strains contain a unique 94-kilodalton plasmid likely coding for a surface adhesion factor responsible for virulence. Activation of PKC in jejunal epithelial cells is associated with intestinal secretion. Genetically engineered, attenuated hybrid vaccines against *S. typhi*, *V. cholerae*, and ETEC have been successful in animal models and, in some cases, in volunteers. The goal of oral, attenuated enteric vaccines will be to strike a critical balance between satisfactory colonization and immunogenicity and tolerable reactogenicity. Meanwhile, two killed oral vaccines against *V. cholerae* are being field tested in Bangladesh.

Dr. R. Bradley Sack, Chairman of the United States Cholera Panel, concluded that research on bacterial diarrhea is moving rapidly. Investigators must, however, (*1*) continue to investigate outbreaks as a way of finding new enteropathogens; (*2*) elucidate mechanisms producing bacterial toxin and antigen; (*3*) discover new bacterial virulence antigens for immunogens; and (*4*) improve therapy, vaccines, and rapid diagnostic tests.

The complete proceedings of the conference will be published by KTK Publishers (Tokyo). The next Joint Conference on Cholera and Cholera-Like Infections has been scheduled for July 1986 in Toyama City, Japan.