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IMMUNOLOGIC CONTROL BY ORAL VACCINES OF DIARRHEAL DISEASE DUE TO
ENTEROTOXIGENIC ESCHERICHIA COLI AND SHIGELLA

ANNUAL / FINAL REPORT

20030128378

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June 30, 1988

Supported by:
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21701-5012

Contract No. DAMD 17-83-C-3074
DAMD 17-78-C-8011

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designated by other authorized documents.

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AD-A203 376

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE			4. PERFORMING ORGANIZATION REPORT NUMBER(S)		
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION Center for Vaccine Development Department of Medicine		6b. OFFICE SYMBOL (If applicable)		7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) University of Maryland Baltimore, MD 21701-5012		7b. ADDRESS (City, State, and ZIP Code)			
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (If applicable)		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-83-C-3074 DAMD17-78-C-8011	
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012		10. SOURCE OF FUNDING NUMBERS			
		PROGRAM ELEMENT NO. 64758A		PROJECT NO. 3S4- 64758D849	
		TASK NO. AA		WORK UNIT ACCESSION NO. 071	
11. TITLE (Include Security Classification) (U) Immunologic Control by Oral Vaccines of Diarrheal Disease Due to Enterotoxigenic <u>Escherichia coli</u> and <u>Shigella</u>					
12. PERSONAL AUTHOR(S) Myron M. Levine					
13a. TYPE OF REPORT Annual/Final		13b. TIME COVERED FROM 12/1/77 TO 8/31/87		14. DATE OF REPORT (Year, Month, Day) 1988 June 30	
15. PAGE COUNT 50					
16. SUPPLEMENTARY NOTATION Annual Report covers period January 1, 1986 - August 31, 1987.					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	RA 1, Enterotoxigenic <u>E. coli</u> ; diarrheal disease; CFA/I, CFA/II; Immunization; <u>Salmonella</u> ; <u>Shigella</u> ; oral vaccine; Genetically engineered vaccine; enteropathogenic <u>E. coli</u> .		
06	13				
06	03				
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Travelers' diarrhea in several different clinical forms represents an important source of morbidity and loss of efficiency among United States Military personnel deployed in less-developed areas of the world. The single most common recognized etiologic agent of travelers' diarrhea is enterotoxigenic <u>Escherichia coli</u> , while the major cause of the dysenteric form of travelers' diarrhea (i.e. accompanied by diarrheal stools with blood and mucus) is <u>Shigella</u> . Research carried out under this contract was aimed at developing safe and effective immunizing agents to prevent these diarrheal infections of military importance. Candidate oral vaccines against ETEC that were evaluated included purified CS1 and CS3 colonization factor fimbriae and a prototype attenuated strain that expresses CS1 and CS3 fimbriae but does not elaborate LT or ST toxins. The live oral vaccine gave the best secretory IgA antifimbrial antibody response. Studies with <u>Shigella</u> included carrying out a dose-response with pathogenic <u>S. sonnei</u> to establish a model to assess the efficacy of candidate <u>Shigella</u> vaccines. Strain 5076-1C,					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian			22b. TELEPHONE (Include Area Code) 301-663-7325		22c. OFFICE SYMBOL SGRD-RMI-S

Block 19 continued:

a modification of attenuated Salmonella typhi strain Ty21a into which is introduced the 120 Md plasmid of S. sonnei, was found to be safe and immunogenic as a live oral Shigella vaccine. Some lots of vaccine provided significant protection against experimental challenge, while others did not. Vaccine candidate 7931-1-2-9, consisting of an E. coli K-12 containing chromosomal genes for expression of the type and group antigens of S. flexneri 2a and the 140 Md flexneri invasiveness plasmid was evaluated for safety, immunogenicity and efficacy. This vaccine caused some adverse reactions at a dose of 10^7 organisms. Recipients of lower, non-reactogenic doses were not protected against experimental challenge with pathogenic S. flexneri 2a.

FOREFWORD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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BACKGROUND

Diarrheal diseases represent one of the principal causes of morbidity and loss of efficiency among military personnel deployed in less-developed areas of the world (1-8). Measures must be developed to diminish the incidence of and loss of productivity due to travelers' diarrhea in military populations. The most common recognized agent of travelers' diarrhea in U.S. adults visiting or working in less-developed countries is enterotoxigenic Escherichia coli (ETEC) (9-14). Shigella, the major causative agent of bacillary dysentery, is often the second or third most frequently isolated agent in studies of the etiology of travelers' diarrhea (9,13). Although the incidence of diarrheal illness due to Shigella is much lower than due to ETEC, the severity of the average case is much greater, so that a soldier is lost from his duties for a longer period of time. Furthermore, Shigella infections can be transmitted by direct contact involving very low infective inocula (as few as 10 Shigella organisms can cause clinical illness (15). ETEC infections, in contrast, require much larger inocula (circa 10^6 organisms) and are typically transmitted by contaminated food and water vehicles (16). Thus, both ETEC and Shigella represent infectious agents of considerable military importance.

ENTEROTOXIGENIC E. COLI

The Center for Vaccine Development of the University of Maryland School of Medicine has been involved in a long-term program to develop immunizing agents to prevent ETEC diarrhea. This program has involved studies of the pathogenesis of ETEC diarrhea, of the human immune response and the evaluation of

certain prototype vaccine candidates. Studies carried out under Research Contract DAMD17-78-C-8011 paved the way for studies performed under Research Contract DAMD17-83-C-3074. Under Research Contract DAMD17-78-C-8011 a volunteer model of experimental enterotoxigenic E. coli diarrhea was established (17). This allowed studies to assess the importance of specific virulence properties and the first studies to assess whether immunity occurs following clinical infection with ETEC. In the mid-1970s, for example, there was great skepticism expressed by microbiologists, epidemiologists and infectious disease consultants as to whether ETEC strains that express only the heat-stable (ST) but not the heat-labile (LT) enterotoxin were pathogenic for humans. Levine et al (18) fed to volunteers an ETEC strain, 214-4, that elaborates only ST. This strain, which had been originally isolated from a traveler with diarrhea, caused clear-cut diarrhea in volunteers, demonstrating that ST-only ETEC strains can indeed be pathogenic. Also in the mid-1970s, fimbrial colonization factors were identified among human ETEC pathogens. The first such fimbrial antigen, colonization factor antigen I (CFA/I) was described by Evans et al (19,20). Later Evans et al (21) described CFA/II. These investigators implied that all human ETEC strains must possess these fimbrial colonization factors, in order to be pathogenic. Levine et al (22) examined a series of ETEC challenge strains known to cause diarrhea in volunteers and showed that several of the strains did not express CFA/I or II yet were pathogenic. Levine et al hypothesized that other antigenically distinct colonization factors must exist among human ETEC pathogens

besides CFA/I and II; this was subsequently proven to be correct (23-25).

Under Research Contract DAMD17-78-C-8011, Levine et al showed that clinical infection with an ETEC strain that elaborates both LT and ST, strain B7A (O148:H28), provided significant protection against re-challenge nine weeks later with the same organism (17). Diarrhea occurred in only 1 of 8 re-challenged volunteers but in 7 of 12 controls ($p=0.05$). This homologous re-challenge study was the first demonstration of infection-derived immunity to ETEC and represented a hallmark observation to help direct the development of vaccines against ETEC. It was notable that although the re-challenged volunteers were clinically protected they shed the ETEC challenge strain with the same frequency as control volunteers. This suggested that the mechanism of protection against ETEC did not involve gut bactericidal mechanisms. Levine et al hypothesized that intestinal secretory IgA (SIgA) directed against relevant ETEC antigens was the mediator of protection. The two most likely antigens hypothesized as being involved were LT and fimbrial colonization factors. A subsequent challenge study was designed to determine whether infection-derived anti-LT by itself could protect. Volunteers who convalesced from diarrhea due to LT/ST ETEC strain B7A (O148:H28) were re-challenged several weeks later with a strain of a different O:H serotype, E2528C1 (O25:NM), that elaborates only LT. Despite the fact that the volunteers had manifested a serological response to LT after illness with B7A, they were not protected against challenge with E2528C1. The results of this clinical research study suggested

that anti-LT by itself may not be protective. As a consequence, subsequent research focused on studies of anti-colonization factor immunity. Anti-colonization immunity was envisioned to involve SIgA antibodies directed against surface antigens of the bacteria concerned with colonization of the proximal small intestine, the critical anatomic site of the host/bacteria interaction that leads to diarrhea. This would prevent ETEC from attaching to receptors on enterocytes of the proximal intestine. The bacteria would then be swept into the large intestine, still in a viable state, where they would proliferate unimpeded in the lumen. This hypothesis could explain the results of the volunteer studies described in Reference 17.

Virtually all ETEC strains express type 1 somatic fimbriae (22,26), as do the vast majority of normal intestinal flora E. coli. In this sense there is no evidence that type 1 somatic fimbriae are a virulence property of ETEC. Type 1 somatic fimbriae are characterized by their ability to hemagglutinate guinea pig erythrocytes, a property that is inhibited by D-mannose. It is likely that the function of type 1 fimbriae is to anchor E. coli to mucus in the large intestine, allowing colonization of that anatomic site by the E. coli. It was suggested by Brinton et al (27) that stimulation of an immune response to type 1 somatic fimbriae might nevertheless provide protection against colonization of the proximal small intestine by ETEC. Brinton et al purified type 1 somatic fimbriae of strain H10407 to be used as a parenteral vaccine. This vaccine was shown to be well-tolerated and to elicit both serum IgG and intestinal IgA responses against type 1 somatic fimbriae (28,29). In one of three challenge studies volunteers

were protected against challenge with pathogenic strain H10407; it was deemed likely, however, that anti-O78 antibody stimulated by contaminating LPS in the fimbrial vaccine probably mediated the protection seen in that one study. When immunized volunteers were challenged with an ETEC strain of a distinct O:H serotype that expressed type 1 somatic fimbriae antigenically closely related to those of H10407, no protection was seen. The conclusion drawn from these studies was that a vaccine based on stimulating an immune response to type 1 somatic fimbriae is not of great value in protecting against ETEC.

ETEC is an important pathogen causing diarrhea among neonatal and infant herd animals, including calves and piglets. The causative strains are of different O:H serotypes from those that cause human disease. The fimbrial colonization factors present in the animal strains are also antigenically distinct; they include K88, K99, F41 and 987-pili. Vaccines consisting of purified K88, K99 or 987 fimbriae were used by veterinary investigators to immunize pregnant sows and cows (30-33). Newborn piglets and calves suckled on immunized mothers were significantly protected against challenge with otherwise lethal doses of ETEC strains expressing the homologous fimbriae. Immunity was not cross-protective. These impressive results in veterinary studies stimulated studies of vaccines in man intended to stimulate anti-colonization factor immunity.

In order for ETEC to cause disease in man they must be able to colonize the small intestine, overcoming the usually potent peristaltic defense mechanism (34). Fimbrial colonization factors

which bind the ETEC to receptors on enterocytes of the small intestine protect the bacteria from the effects of peristalsis. Under Research Contract DAMD17-83-C-3074 we sought to identify the fimbrial colonization factors associated human ETEC, to study the serum and intestinal SIgA immune response to these fimbrial antigens and to evaluate candidate vaccines intended to elicit anti-fimbrial immunity.

Fimbrial Colonization Factors among ETEC Strains in Relation to Toxin Phenotype

Levine et al (26,35) examined a series of ETEC strains from patients with travelers' diarrhea isolated in various parts of the world for the presence of the fimbrial colonization factors recognized at that time including CFA/I and CFA/II. CFA/I or CFA/II was identified in 7 of 10 LT/ST strains but in only 2 of 26 LT-alone or ST-alone strains.

Identification of New Fimbrial Colonization Factors of ETEC

It was obvious that there must exist additional fimbrial colonization factors other than CFA I and CFA II because these antigens were never associated with ETEC strains of certain common O serogroups isolated from diverse geographic sources, including O27, O115, O148, O159 and O167 (34). Investigators at the Central Public Health Laboratory, Colindale, England reported the existence of antigenically distinct fimbrial antigens in ETEC strains of serogroups O25, O27, O115, O148 and O167 (23). This new antigen was originally referred to as PCF E8775. Subsequently it was shown that E8775 represents a family of three distinct antigens that were

referred to as CS4, CS5 and CS6 (36,37). CS6 is common to virtually all the E8775 ETEC strains but often in conjunction with CS4 or CS5. CS4 and CS5 are rigid fimbriae circa 6-7 nm in diameter. The morphology of CS6 has not yet been described.

The last major ETEC serogroup not to be associated with a known fimbrial antigen was O159, particularly of the common serotype O159:H4. Investigators at the Center for Vaccine Development identified an antigenically distinct fimbrial colonization factor antigen in O159:H4 strains, which they referred to as PCF O159:H4 (25).

Antigenic Heterogeneity in Strains Expressing CFA/II

When Evans et al (19,20) reported the existence of a plasmid-mediated fimbrial colonization factor (CFA/I) in LT/ST strains of serogroups O78, O25 and O63, other investigators confirmed this observation and exchanges of prototype strains and anti-CFA/I antisera among different laboratories throughout the world demonstrated that there was no antigenic heterogeneity within CFA/I of diverse sources. Evans et al had originally identified CFA/I on the basis of the property whereby strains expressing this factor hemagglutinate human type A and bovine erythrocytes despite the presence of D-mannose (which prevents the hemagglutinating properties of type 1 somatic fimbriae).

Evans et al (21) subsequently described another fimbrial colonization factor, which they referred to as CFA II. CFA II was reported to confer on ETEC strains the property of mannose-resistant hemagglutination of bovine erythrocytes and was

found to be associated with ETEC strains of serogroups O6 and O8. However, when other investigators attempted to corroborate the findings of Evans et al and when reference strains were exchanged, much confusion occurred as discrepancies were found to exist. A major step towards resolution of this confusing situation was made when Cravioto et al (38) reported that antigenic heterogeneity existed within CFA/II. These investigators discovered that so-called CFA/II was not a single antigen but was rather a family of three distinct antigens; some ETEC strains expressed only one of the antigens, while others concomitantly expressed two of the antigens. Cravioto et al referred to these three distinct antigens as components 1, 2 and 3. Independently, Smyth (39) also discovered that CFA/II comprised a family of three separate antigens, which he referred to as Coli Surface antigens 1, 2 and 3 (CS1, CS2 and CS3). Smyth reported that virtually all strains bearing CFA/II express CS3; in addition many also express CS1 or CS2 in conjunction. No strains were found which express CS1 and CS2 simultaneously. Furthermore, Smyth showed that which CS antigens are expressed is a function of the serotype and biotype of the ETEC strain. Smyth (39) showed that CS1 and CS2 antigens were fimbrial in nature and consisted of rigid structures 6-7 nm in diameter. In contrast, the morphology of CS3, the antigen common to virtually all "CFA/II" strains was not ascertained. Similarly, investigators at the Central Public Health Laboratory, Colindale, England described CS3 as being non-fimbrial and amorphous (40,41).

Under contract DAMD17-83-C-3074, we were investigating anti-colonization factor immunity, particularly among CFA/II

strains. Thus, it became imperative to study more intensively the characteristics of CS3, the common antigen of the CFA/II family. Levine et al (42) purified CS3 and CS1 to homogeneity and prepared antisera against these antigens. The specificity of the antisera was demonstrated in immunoblotting studies utilizing purified CS1 and CS3. Levine et al (42) confirmed that CS1 consisted of rigid fimbriae with a diameter of 6-7 nm. However, for the first time the morphology of CS3 was also described and was found to consist of flexible, wiry, fibrillar type of fimbriae with a diameter of 2-3 nm. Other fibrillar type fimbriae include K88 and F41 found in animal ETEC isolates. By means of gold-immunolabelling electron microscopy, Levine et al (42) confirmed that the fibrillar fimbrial structures that they visualized were indeed CS3.

Animal Studies with Purified CS1/CS3 Combination Vaccine

Studies were carried out in rabbits to determine whether enteral immunization with purified CS1/CS3 fimbriae vaccine would inadvertently elicit immunological tolerance to fimbriated organisms inoculated parenterally. This was a safety concern expressed by one of the Human Volunteer Committees that reviewed the proposed clinical protocols (29). The basis for the concern was the observation that in some species certain antigens administered orally induce tolerance to those antigens when they are presented parenterally (43-45). Since some vaccinees might at some later time in life have to mount immune responses to E. coli in the course of bacteremia secondary to urinary tract infection, cholecystitis, etc., it was considered prudent to carry out animal studies. As

shown in Levine et al (29), rabbits immunized enterally into chronic Thiry-Vella intestinal loops, mounted brisk SIgA antifimbrial antibody responses; nevertheless, these rabbits responded following parenteral inoculation with fimbriated E. coli bearing the homologous fimbriae.

Rabbits were given multiple oral doses of purified CS1/CS3 fimbriae vaccine, with NaHCO_3 and cimetidine; a total of 1.4 mg was given to each rabbit. Approximately one month after completion of immunization, the rabbits were challenged with pathogenic ETEC of strain E24377A by the RITARD technique (reversible intestinal tie acute rabbit diarrhea) model. There was no difference in attack rate between the immunized and the control rabbits (46).

Clinical Studies with Purified CS1/CS3 Fimbriae

A group of volunteers received multiple oral doses of purified CS1/CS3 fimbriae vaccine prepared from strain M424-C1 (O6:H16, biotype A) (77,78). In order to protect the protein vaccine from the possible deleterious effects of gastric acid, volunteers received cimetidine several hours before ingesting vaccine, in order to diminish gastric acid production, while vaccine was administered concomitantly with NaHCO_3 to neutralize gastric acid. Only 2 of 10 vaccinees manifested a significant rise in either serum IgG or intestinal SIgA antibody to CS1 or CS3 fimbriae (Table 1). These volunteers participated in a challenge study to assess vaccine efficacy. Not surprisingly, there was no evidence of vaccine efficacy elicited by the fimbrial vaccine (Table 2). These studies are summarized in Levine et al (46).

It was not clear why the CS1/CS3 purified fimbrial vaccine elicited such a poor response in humans when it had stimulated prominent SIgA anti-fimbrial antibody responses when applied to the mucosa of chronic Thiry-Vella loops of rabbits (29). Investigators in the Department of Gastroenterology at the Walter Reed Army Institute of Research showed that the antigenicity of purified CS1/CS3 vaccine was adversely affected by gastric contents even at pH 7.0 (48). Presumably this is due to the effects of proteolytic enzymes that are having demonstrable, albeit diminished, activity even at neutral pH. To confirm the hypothesis that gastric contents adversely affect the purified CS1/CS3 fimbriae, we immunized a group of volunteers with purified CS1/CS3 vaccine by direct intraduodenal inoculation via intestinal tube and collected intestinal fluid on several occasions post-inoculation to measure antibody. Under these circumstances, significant rises in SIgA anti-fimbrial antibody were detected in 4 of 5 vaccinees, versus only 2 of 10 who received oral vaccine ($p=0.046$, Fisher's Exact test, 1 tail) (Table 3). It became apparent from these studies that if purified fimbriae are to be used as an oral vaccine they will have to be delivered by means of enteric-coated capsules or some other mechanism that completely protects the vaccine protein from exposure to gastric contents.

Clinical Studies with a Prototype Live Oral Vaccine against ETEC

E1392-75-2A is an O6:H16 biotype A isolate that expresses CS1 and CS3 fimbrial antigens but does not elaborate LT or ST and fails to hybridize with LT and ST gene probes. This spontaneous

laboratory mutant, derived from an LT/ST strain, was evaluated in volunteers for safety, immunogenicity and efficacy as a live oral vaccine. The characteristics of strain E1392-75-2A are summarized in Levine et al (29). In initial clinical studies it was found that a single dose of E1392-75-2A given with buffer stimulated brisk serum and intestinal SIgA responses in the majority of vaccinees, including all who received doses $\geq 10^{10}$ organisms. The vaccine was generally well-tolerated. However, reminiscent of what has been encountered in recipients of some live oral cholera vaccines (49,50), approximately 10% of the volunteers who ingested E1392-75-2A developed some loose stools (29).

In order to explore the magnitude and kinetics of the immune response elicited by E1392-75-2A and whether anti-fimbrial immunity is protective, a group of volunteers were given a single oral 5×10^{10} organism dose of the prototype vaccine strain. A strong serum and intestinal SIgA antibody response to CS1 and CS3 fimbriae was recorded (Table 4). Moreover, the geometric mean titer of gut SIgA anti-fimbrial antibody (416) was approximately 10-fold higher than that noted in volunteers immunized with three 5 mg doses of purified CS1/CS3 vaccine given intraduodenally via intestinal tube. These data show that live vaccines interact with the host intestinal immune system in a manner both qualitatively and quantitatively different from inactivated vaccines.

A group of volunteers who received a single oral dose (5×10^{10} organisms) of E1392-75-2A were challenged one month later with 10^8 organisms of pathogenic strain E24377A; the latter strain elaborates LT and ST and CS1 and CS3 but is of a different

serotype (O139:H28). Thus if E1392-75-2A were to show protection against challenge with E24377A it would be on the basis of anti-fimbrial immunity. As shown in Table 5, only 3 of 12 vaccinees developed diarrhea versus 6 of 6 controls ($p < 0.005$, 75% vaccine efficacy). Moreover, as shown in Table 6, bacteriological studies demonstrated that the mechanism of protection was by preventing E24377A organisms from colonizing the proximal small intestine. The proportion of individuals excreting the challenge organism and the mean level of excretion was the same in vaccinees and controls. However, only 1 of 12 vaccinees tested had positive duodenal cultures versus 5 of 6 controls ($p < 0.004$). Thus in the vaccinees anti-colonization immunity was evident and was correlated with SIgA antibody to CS1 and CS3 fimbriae (Table 4). As a consequence of these studies, we have undertaken to develop strains of E. coli that express fimbrial colonization factors and B subunit of LT but do not elaborate biologically active LT or ST. It is hoped that such strains will colonize the intestine and stimulate potent immune responses without causing adverse reactions.

Studies of the LT Antitoxin Response

A somewhat surprising revelation made in the early 1980s was that there were antigenic differences between the LT elaborated by porcine ETEC strains and that of human ETEC strains (51-53). Indeed, antigenically, the difference was about as great as that between cholera toxin and either LT. Consequent to these reported antigenic differences in human LT (LTh) and porcine LT (LTp), we undertook to assess the immune response to LTh, LTp and cholera toxin in persons infected with ETEC or with cholera. These studies

resulted in methods for the specific serodiagnosis of ETEC and cholera infection and provided helpful aid for seroepidemiological studies (54). These studies are summarized in Levine et al (54).

ENTEROPATHOGENIC E. COLI

Enteropathogenic E. coli (EPEC) represent another major category of diarrheagenic E. coli (34,55). EPEC belong to certain classical O:H serotypes, do not elaborate LT or ST and do not manifest Shigella-like enteroinvasiveness. Studies of the pathogenesis of EPEC diarrhea have greatly enhanced our knowledge of novel mechanisms by which E. coli cause diarrhea.

It was discovered that most EPEC of classical serotypes adhere to HEp-2 cells in tissue culture by the formation of microcolonies, giving rise to a pattern of adherence referred to as localized adherence (LA) (56-59). This was shown to be a property mediated by a plasmid circa 60 Md in size, the so-called EPEC adherence factor (EAF) plasmid (57-59). Transfer of the plasmid to E. coli HB 101 was accompanied by transfer of the LA property, while loss of the EAF plasmid from an EPEC strain was followed by loss of the ability to manifest LA (57). A 1 kb fragment of the EAF plasmid has been shown to function as a sensitive and specific DNA probe to identify EPEC (60,61).

Studies in volunteers have shown that the 60 Md EAF plasmid is necessary for expression of the full pathogenicity of EPEC strains (62). The plasmid has been shown to encode the production of a 94 Kd protein against which humans mount a serological response (62). This outer membrane protein is a candidate antigen for future vaccines against EPEC.

SHIGELLA

Inactivated Shigella organisms utilized as parenteral whole cell vaccines have failed to protect monkeys or man in experimental challenge studies or humans in controlled field trials (63-65), despite stimulating high titers of circulating antibody. In contrast, some attenuated strains of Shigella used as live oral vaccines have been safe and protective in volunteer challenges and in controlled field trials (66-70). In particular, the streptomycin-dependent (SmD) Shigella strains of Mel et al (67-69) and the T₃₂ S. flexneri 2a strain of Istrati et al (71) have shown considerable promise as safe and protective vaccines (67-72). Nevertheless, these attenuated strains have a number of drawbacks that encourage research to prepare improved attenuated strains. Both the SmD and the T₃₂ strains represent mutants with undefined genetic lesions responsible for the attenuation; as a consequence, it is theoretically possible for these strains to undergo genetic reversion to a virulent state. Indeed, streptomycin-independent revertants of the SmD S. sonnei vaccine strain occurred with some lots (73), although there was no evidence that these particular revertants were capable of causing disease. The SmD vaccines required four doses to provide primary immunization and annual boosters to maintain protection (69). An ideal live oral Shigella vaccine would require fewer doses. The SmD vaccines caused vomiting in a small percentage of children after the first dose of vaccine. Ideally, a Shigella vaccine should not be associated with any adverse reactions. A prototype vaccine created by Dr. S.B. Formal and coworkers at the Walter Reed

Army Institute of Research in the mid 1970s consisted of an E. coli K-12 expressing the group and type specific antigens of S. flexneri 2a. This vaccine was well-tolerated but colonized the intestine poorly, elicited weak immune responses and did not provide significant protection in experimental challenge studies in volunteers (74).

Because of the above-mentioned shortcomings of the earlier live oral Shigella vaccines, Formal et al at WRAIR produced two new prototype Shigella vaccines. The first prototype consisted of attenuated Salmonella typhi strain Ty21a into which was introduced the 120 Md invasiveness plasmid of S. sonnei. This hybrid strain, 5076-1C, expresses the O antigens of both S. sonnei and S. typhi (75). The second prototype oral Shigella vaccine prepared at WRAIR in the mid 1980s consists of E. coli K-12 into which was introduced the chromosomal genes encoding the group and type specific O antigens as well as the invasiveness plasmid S. flexneri 2a (76). Results of clinical studies evaluating the safety, intestinal colonizing potential, immunogenicity and efficacy of these vaccine candidates are presented below.

Strain 5076-1C, S. typhi-S. sonnei Bivalent Oral Vaccine

Strain 5076-1C was grown on solid agar and lyophilized at the Forest Glen Vaccine Production Facility of WRAIR. Three separate clinical studies were carried out with lots # 2 and # 5, involving vaccination of volunteers with three 2×10^9 organism doses given on an every other day schedule. Approximately one month after completion of vaccination the immunized volunteers and a similar number of controls were challenged with 5×10^2 pathogenic S. sonnei (76). Results of these three vaccination/challenge studies

are presented in detail in Black et al (76). The vaccination was very well-tolerated and provided significant protection against diarrhea and dysentery. Based on the safety and efficacy of 5076-1C vaccine found with lots # 2 and # 5, a large lot of 5076-1C vaccine was prepared at Forest Glen with a sufficient number of doses to carry out field trials of efficacy in Israel, Chile and Thailand. However, this lot of vaccine, lot # 8, failed to confer significant protection in volunteers against experimental challenge. Furthermore, two separate lots of 5076-1C vaccine lyophilized at the Swiss Serum and Vaccine Institute after growth in liquid broth failed to provide significant protection of volunteers in experimental challenge studies of vaccine efficacy.

E. coli/S. flexneri 2a Vaccine Strain 7931-1-2-9

Strain 7931-1-2-9 designates the E. coli K-12 that expresses S. flexneri 2a O antigen and possesses the flexneri invasiveness plasmid; this strain invades HeLa cells. In step-wise fashion involving three cohorts of volunteers, strain 7931-2-9 was fed to a total of 17 individuals in doses of 5×10^6 (2 volunteers), 5×10^7 (2 volunteers) or 10^9 (13 volunteers). As summarized in Table 7, four vaccinees manifested adverse reactions including two who had fever, one who had mild diarrhea and a fourth who experienced a single small dysenteric stool with blood and mucus. The serological responses of the vaccinees are also summarized in Table 7. As with previous live oral Shigella vaccines, rises in serum O antibody were seen in approximately only one-fourth of vaccinees. Notably, the only individual who manifested rises in serum O antibody to both O antigen and to the

invasiveness-associated outer membrane proteins was the individual who experienced diarrhea during vaccination; this individual also had a rise in intestinal SIgA antibody to O antigen.

Since all the adverse reaction had occurred in recipients of the 10^9 organism dose of vaccine and lower doses of vaccine appeared to be well-tolerated (albeit involving small numbers of vaccinees), the next group of volunteers were given three 5×10^6 organism doses of vaccine with buffer. No adverse reactions were recorded among these 13 vaccinees (Table 8). Accordingly, a challenge study was planned to assess vaccine efficacy. As a preliminary, a dose response was carried out with pathogenic S. flexneri 2a to determine the appropriate dose for the challenge model. Eight volunteers ingested 10^2 and eight received 10^3 pathogenic S. flexneri 2a organisms of strain 2457T. Results, summarized in Table 9, show that 4 of 8 individuals who ingested 10^2 organisms manifested clinical illness; in three individuals this involved diarrhea, fever and dysentery. At a dose of 10^3 organisms of pathogenic strain 2457T, the attack rate for clinical illness was 5 of 8. A dose of 10^2 was selected for the challenge study to assess vaccine efficacy.

Eight volunteers who had received three 5×10^6 organism doses of 7931-2-9 vaccine were challenged (approximately one month after completion of vaccination) with 10^2 pathogenic S. flexneri 2a, along with 8 unimmunized control volunteers. Also challenged at the same time were a volunteer who had ingested a single 5×10^7 organism dose of vaccine and another who had received a single 10^9 organism dose. Results of this challenge study,

summarized in Table 10, were disappointing. There was no evidence of vaccine efficacy. Indeed, 4 of 10 vaccinees developed clinical illness, of whom all four had dysentery and three had fever. The results of these studies show that if the modified E. coli K-12 approach to preparing Shigella vaccine candidates is to be further pursued, the next candidates must be less reactogenic and more immunogenic. There is some expectation that with appropriate genetic modifications this may be achievable.

TABLE 1

Summary of Serum and Intestinal Fluid Antibody Response In
Volunteers Immunized with Oral CFA/II (CS1, CS3) Vaccine*

	CS1 & CS3 <u>Fimbrial Antigens</u>
Significant Rises in Serum IgG ELISA Antibody	2/10 [†]
Singificant Rises in Intestinal SIgA ELISA Antibody	2/10

*1.7 mg twice weekly for 4 weeks. Volunteers took
cimetidine 3 hours before ingesting vaccine with NaHCO₃.

[†]No. with significant seroconversion/No. vaccinees tested.

TABLE 2

Clinical Response Following Challenge of Immunized (8 Oral Doses of CFA/II Pili Vaccine) Volunteers and Controls with Enterotoxigenic E. coli Strain E23477A (0139:H28, CS1,CS3)

	<u>Diarrheal Attack Rate</u>	<u>Mean Diarrheal Stool Volume Per Ill Volunteer</u>	<u>Mean No. Loose Stools Per Ill Volunteer</u>
Controls	6/9*	576 ml (442-782)**	5 (3-7)
Vaccinees	3/8	847 (510-1290)	5.3 (2.7)

*No. ill/No. volunteers challenged.

** (Range).

TABLE 3

Immunogenicity of Purified CFA/II (CS1, CS3) Fimbriae
Administered Orally or Enterally

<u>Method of Vaccination</u>	<u>Significant Rises (>4-Fold) in SIgA Intestinal Fluid Antibody to CFA/II (CS1,CS3)</u>
----------------------------------	---

Oral*	2/10**
-------	--------

p=0.046++

Enteral†	4/5**
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*1.7 mg doses twice weekly for four weeks
(total 13.6 mg); vaccine given after
cimetidine and NaHCO₃ treatment.

†5 mg doses on day 1, 14, and 28.

**No. seroconverters/No. vaccinated.

++Fisher's Exact Test, single tail.

TABLE 4

Immunogenicity of Live Oral Non-Enterotoxigenic E. coli Vaccine
Bearing CFA/II Fimbriae E1392/75-2A

	Significant Rises <u>Antigen</u>	Geometric Mean Titer		
		<u>Pre-</u> <u>Vaccination</u>	<u>Post</u> <u>Vaccination</u>	<u>Pre-</u> <u>Challenge</u>
CFA/II (CS1, CS3)	10/10	5	416	315
06	10/10	6	91	165
0139	0/10	2	2	2
LT	0/10	2	2	2

TABLE 5

Efficacy of a Single Dose of Live Oral E. Coli Vaccine E1392-75-2A
(06:H16,CS1,CS3) in Protecting Against Diarrhea Following Challenge with
5X10⁹ E. coli E24377A (0139:H28, LT⁺/ST⁺, CS1,CS3)

<u>Group</u>	<u>Diarrheal Attack Rate</u>	<u>Severity of Diarrhea</u> <u>Per Ill Volunteer:</u>	
		<u>Mean No.</u> <u>Stools</u>	<u>Mean</u> <u>Stool Volume</u>
Controls	6/6*	8.8 (2-18) ⁺	1147 ml (315-1855)
Vaccinees	3/12	3.7 (2.5)	713 (229-1110)

p<.005

*No. ill/No. challenged.

⁺(Range)

TABLE 6

Bacteriologic Findings in E1392-75-2A Vaccinees and
Controls Following Challenge with Enterotoxigenic E. coli
Strain E24377A (0139:H28, LT⁺/ST⁺, CS1, CS3)

<u>Group</u>	<u>Duodenal Cultures</u>	<u>Stool</u>
Controls	5/6* (7X10 ³) ⁺	6/6 (8X10 ⁸)
		p<.004
Vaccinees	1/2 (10 ¹)	12/12 (1X10 ⁸)

*No. Positive/No. volunteers challenged.

⁺(Mean no. E. coli 24377A per gm stool or
duodenal fluid.)

TABLE 7

SHIGELLA CVD 2002 - CLINICAL, MICROBIOLOGIC AND IMMUNOLOGIC RESPONSE
TO VACCINATION WITH E. COLI/S. FLEXNERI 2A VACCINE STRAIN 7931-1-2-9

STUDY AND VOLUNTEER NUMBER	DOSE	CLINICAL RESPONSE TO VACCINATION			VACCINE STRAIN EXCRETION IN STOOL DURATION (DAYS)	IMMUNOLOGY - 4-FOLD R _I BY ELISA			
		DIARRHEA	DYSENTERY	FEVER		SERUM LPS(IgA)	SERUM LPS(IgG)	PCP(IgG)	JEJUNAL FLUID LPS(sIgA)
2002 -17	6 (5 X 10 ⁶) X 3	-	-	-	9	-	-	- *	NT
-18	6 (5 X 10 ⁶) X 3	-	-	-	7	-	-	- *	-
-8	7 (5 X 10 ⁷) X 3	-	-	-	12	-	-	- *	+
-12	7 (5 X 10 ⁷) X 3	-	-	-	7	-	-	-	+
-2	9 10	-	-	4b 101	4	+	-	+	-
-3	9 10	-	-	-	5	-	-	-	-
-7	9 10	c 435 ML	-	-	3	-	-	- *	-
-9	9 10	-	-	-	4	-	-	+	-
-10	9 10	d 80 ML	+	-	4	-	-	- *	-
-11	9 10	-	-	-	4	-	-	+	-
-13	9 10	-	-	-	4	+	-	-	+
-14	9 10	-	-	-	4	+	-	+	-
-15	9 10	-	-	-	4	-	-	-	NT
-16	9 10	-	-	e 100	3	-	+	-	+
-19	9 10	-	-	-	4	-	-	+	-
-20	9 10	-	-	-	4	-	-	-	-
-21	9 10	-	-	-	-	-	-	-	-
TOTAL ADVERSE REACTIONS:			4/17			3/17	1/17	5/17	4/15

b 27 HOURS AFTER VACCINATION, ALSO HAD MALAISE AND HEADACHE

c 83 HOURS AFTER VACCINATION

d 1 SMALL DYSENTERIC STOOL 43 HOURS AFTER VACCINATION

e 27 HOURS AFTER VACCINATION, ALSO HAD GROSSLY BLOODY GRADE 2 STOOL,
MALAISE, HEADACHE, ABDOMINAL CRAMPS

* HAD POLYPEPTIDE RESPONSES BY WESTERN BLOT IN PRE AND POST IMMUNIZATION SERA

+ PCP = PLASMID CODED POLYPEPTIDE

NT = NOT TESTED

TABLE 8

SHIGELLA CVD 2003 - CLINICAL, MICROBIOLOGIC AND IMMUNOLOGIC RESPONSE
TO VACCINATION WITH E. COLI/S. FLEXNERI 2A VACCINE STRAIN 7931-1-2-9

STUDY AND VOLUNTEER NUMBER	DOSE	** CLINICAL RESPONSE TO VACCINATION			VACCINE STRAIN EXCRETION IN STOOL DURATION (DAYS)	IMMUNOLOGY - 4-FOLD RISES BY ELISA			
		DIARRHEA	DYSENTERY	FEVER		SERUM LPS(IgA)	SERUM LPS(IgG)	PCP(IgG)	FLUID LPS(sIgA)
2003	-1	7 10	-	-	-	1	-	-	-
	-2	7 10	-	-	-	7	-	-	-
	-4	7 10	-	-	-	8	-	-	+
	-5	7 10	-	-	-	7	-	-	-
	-6	7 10	-	-	-	7	-	-	-
	-7	7 10	-	-	-	10	-	-	NT
	-8	7 10	-	-	-	5	-	-	NT
	-9	7 10	-	-	-	6	-	-	-*
	-10	7 10	-	-	-	11	-	+	++*
	-11	7 10	-	-	-	9	-	-	-*
	-12	7 10	-	-	-	11	-	-	-
	-13	7 10	-	-	-	7	-	-	-
	-14	7 10	-	-	-	8	-	-	-* NT
TOTAL ADVERSE REACTIONS: 1/13							1/13	2/13	1/10
TOTAL (2002 AND 2003): 5/30						3/30	2/30	7/30	5/25

* HAD POLYPEPTIDE RESPONSES BY WESTERN BLOT IN PRE AND POST IMMUNIZATION SERA

+ PCP = PLASMID CODED POLYPEPTIDE

6

** ACTUAL INOCULUM 5 X 10 CFU

NT = NOT TESTED

Table 9

SHIGELLA CVD 2001DOSE RESPONSE CHALLENGE STUDYSHIGELLA FLEXNERI 2a 2457T

<u>Volunteer</u>	<u>Mean no. of loose stools</u>	<u>Diarrheal stool volume</u>	<u>Dysentery</u>	<u>Peak fever</u>	<u>Coprocultures</u>
<u>Group A - 10²:</u>					
2001-1	-	-	-	-	-
-2	38	2069	+	103 ²	+
-3	-	-	-	-	+
-4	-	-	+	-	+
-5	-	-	-	-	-
-6	15	1178	+	103 ²	+
-8	30	1525	+	102 ⁸	+
-9	-	-	-	-	-
<u>Group B - 10³:</u>					
-10	-	-	-	-	+
-11	12	518	+	100 ⁷	+
-12	5	424	-	-	+
-13	-	-	-	-	+
-14	34	1354	+	102 ¹	+
-15	13	884	+	101 ³	+
-17	12	3028	+	104	+
-18	-	-	-	-	-

Table 10

SHIGELLA CVD 2004
CLINICAL AND MICROBIOLOGICAL RESPONSE TO CHALLENGE WITH
S. FLEXNERI 2A STRAIN 2457T (300 CFU) IN RECIPIENTS OF
E. COLI/S. FLEXNERI VACCINE 7931-1-2-9 VERSUS CONTROLS

VACCINEES	DIARRHEA	TOTAL NUMBER OF DIARRHEA STOOLS	DYSENTERY	FEVER	SHIGELLA STOOL EXCRETION (DAYS)
2004 -2	575	6	+	103 ⁸	3
-3	1885	15	+	104 ⁴	2
-5	227	7	+	-	5
-6	-	-	-	-	9
-8	-	-	-	-	-
-9	-	-	-	-	-
-12	548	6	+	100 ⁻²	>3
-14	-	-	-	-	-
-17	-	-	-	-	-
-18	-	-	-	-	-
CONTROLS					
2004 -1	383	4	+	101 ⁴	8
-4	-	-	-	-	-
-7	278	3	+	102 ⁴	4
-11	-	-	-	-	-
-13	73	2	+	100 ⁸	9
-15	-	-	-	-	5
-16	-	-	+	-	3
-19	676	6	+	100 ¹	6

* SINGLE GRADE 2 STOOL

NOTE: VACCINEE #6 RECEIVED SINGLE DOSE OF 10 CFU OF VACCINE
 IN SHIGELLA CVD 2002 STUDY (VOLUNTEER #2002 -2)

VACCINEE #8 RECEIVED 3 DOSES OF 5 X 10 CFU OF VACCINE
 IN SHIGELLA CVD 2002 STUDY (VOLUNTEER #2002 -8)

REMAINING VACCINEES RECEIVED 3 DOSES OF 5 X 10 CFU OF
 VACCINE IN SHIGELLA CVD 2003 STUDY

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