

IMMUNOLOGIC CONTROL OF DIARRHEAL DISEASE DUE
TO ENTEROTOXIGENIC ESCHERICHIA COLI:
REACTOGENICITY, IMMUNOGENICITY, AND EFFICACY
STUDIES OF PILI VACCINES

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Center for Vaccine Development, Division of
Infectious Diseases, University of Maryland
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BACKGROUND:

Recognizing that acute travelers' diarrhea is one of the major causes of loss of effectiveness of United States military personnel assigned to less-developed areas and recognizing that enterotoxigenic Escherichia coli (ETEC) are the most frequent etiologic agent of travelers' diarrhea,¹⁻⁵ we have undertaken a long-term program aimed toward development of effective immunizing agents to control ETEC diarrhea.

Infection-Derived Immunity

In 1978 in studies supported by the U.S. Army Medical Research and Development Command, we demonstrated by means of challenge studies in volunteers that an initial clinical diarrheal infection due to ETEC strains B7A (O148:H28, LT⁺/ST⁺, type 1 somatic pili) or H10407 (O78:H11, LT⁺/ST⁺, CFA/I, type 1 somatic pili) conferred significant protection against diarrhea upon re-challenge with the homologous strain.^{6,7} Although they did not develop diarrhea, the re-challenged "veterans" excreted as many ETEC as the ill control volunteers. Based on these observations, we hypothesized that the operative mechanism of immunity probably involved intestinal secretory IgA (SIgA) directed against the organelles of adhesion of ETEC (adhesion pili). We further hypothesized that SIgA prevented adherence of ETEC to small intestinal mucosa whence the pathogens were cleared by peristalsis to the colon, an intestinal site wherein they do not exert pathophysiologic changes.

Adhesion Pili in Human Enteropathogens

Because of the notable efficacy of purified adhesion pili vaccines in veterinary studies employing purified K88, K99 and 987-type pili as immunizing agents,⁸ we considered a role for purified pili vaccines in preventing ETEC diarrhea in man. One of the factors determining the feasibility for effective use of such vaccines in man relates to identification of the organelles of adhesion of human ETEC strains and characterization of their antigenic heterogeneity.

Colonization factor antigens I and II (CFA/I, CFA/II) are adhesion pili that have been identified in some human ETEC pathogens and are analogous in many ways to K88 and K99 pili of animal ETEC.⁸⁻¹⁰ A study was carried out to determine the frequency with which CFA/I and II pili were found in a series of 36 ETEC strains isolated from 36 cases of acute travelers' diarrhea and to relate pili phenotype to toxin phenotype. The strains were provided by Dr. R.B. Sack of Baltimore City Hospital. We found that CFA/I or II were encountered in 4 of 9 LT⁺/ST⁺ strains (44%) but in only 3 of 13 LT⁻/ST⁺ strains (23%) and in none of 14 LT⁺/ST⁻ strains.⁸ Volunteer challenge studies with ETEC strains lacking CFA/I and II established the fact that most of these strains were indeed pathogenic despite the absence of CFA; they caused unequivocal diarrhea, colonized the intestine and stimulated immune responses.¹¹ These observations inferred that there must exist additional adhesins other than CFA/I and II and that such antigens would have to be identified, characterized and purified for inclusion in a polyvalent pili vaccine in order to provide broad-spectrum protection.¹¹

Many of the ETEC pathogens lacking CFA/I and II were found to possess type 1 somatic pili.^{11,12} Such pili, however, are found as commonly in normal colonic flora E. coli and are not usually considered to be a virulence property in the same sense as CFA pili. Nevertheless, type 1 somatic pili do bind to receptors on epithelial cells and such binding can be prevented by specific anti-pili antibody.¹⁵

Type 1 Somatic Pili Vaccines

We decided to evaluate type 1 somatic pili as a potential immunizing agent in the hope that, if successful, inclusion of this antigen(s) might enhance and broaden the spectrum of protection against ETEC of a future polyvalent (CFA/I, CFA/II, etc.) pilus antigen vaccine.^{3,14} A collaboration was entered into with Dr. Charles C. Brinton, Jr., whose laboratory prepared purified type 1 somatic pili from ETEC strain H10407.

In the safety, immunogenicity and efficacy studies carried out in volunteers, the type 1 somatic pili vaccine was administered by the parenteral route. (Note - one of the two Human Volunteer Research Committees that reviews the clinical protocols restricted initial use of this vaccine to the parenteral route). The immunologic rationale for proceeding with this route of immunization was the recognition that parenteral immunization in man can stimulate appearance of local SIgA antibody in persons immunologically primed to the antigen.^{15,16} It is presumed that most persons are already primed to respond to type 1 somatic pili vaccine because of immunologic stimulation stemming from colonization of the colonic mucosa with type 1 piliated E. coli normal flora.

During contract years 1979 and 1980, we demonstrated that parenteral type 1 somatic pili vaccine was safe, stimulated brisk circulating anti-pili antibody responses and was significantly protective in the first of three efficacy studies utilizing the homologous organism (H10407) as the challenge strain (see Appendix A). In the two subsequent challenges with E. coli H10407 significant protection was not encountered. The initial group of vaccinees challenged had all received at least one maximal dose (1800 mcg) of vaccine which stimulated significant rises in antibody to O78 antigen as well as to pili. The subsequent two groups immunized and challenged received modified immunization schedules (900 mcg primary and 450 mcg booster doses) which resulted in anti-pili antibody responses approximately 75% as high as those found in the first group but in little or no rise in O antibody. Thus it was unclear to what extent vaccine-induced anti-O antibody may have influenced the significant protection observed in the initial group of vaccinees challenged. Work carried out during the past contract year was intended to clarify this situation.

WORK ACCOMPLISHED DURING 1981 CONTRACT YEAR

The major tasks undertaken in the 1981 contract year included:

- 1) Elucidation of the role of vaccine-induced anti-type 1 pili antibody in

mediating protection against challenge with selected ETEC strains.

2) Development of practical, reliable and accurate assays to measure anti-body to CFA/I, CFA/II, type 1 somatic pili and O antigens in human serum and intestinal fluids.

3) Analysis of the local intestinal as well as the serum antibody response to CFA/I, type 1 somatic pili, heat-labile enterotoxin (LT) and O antigen in volunteers following immunization and challenge with ETEC.

4) Immunogenicity studies in rabbits to assess intestinal IgA antibody response following enteral immunization with purified CFA/II pili.

5) Preliminary safety/immunogenicity studies in volunteers with a candidate attenuated E. coli vaccine strain to prevent ETEC diarrhea.

Role of Anti-Type 1 Pili Antibody in Mediating Protection Against ETEC

In the three efficacy challenge studies in which recipients of type 1 somatic pili vaccine prepared from Strain H10407 were challenged with E. coli H10407, conflicting results were obtained (Appendix A). It was possible that vaccine-induced O antibody may have been partly (or largely) responsible for the efficacy encountered in the first challenge study. Furthermore, Strain H10407 possesses CFA/I adhesion pili as well as type 1 somatic pili and thus is not the class of ETEC organism against which type 1 pili vaccine was envisioned to play a role. Rather, type 1 somatic pili vaccine was envisioned as protecting against ETEC strains which lack CFA/I and II but possess antigenically related type 1 somatic pili.

Two definitive clinical experiments were designed to rule out the effect of O78 antibody as a confounding variable and clarify the protective role (if any) of antibody to type 1 somatic pili. For these studies two challenge strains were carefully selected. Both challenge strains lack CFA/I and II pili and are of O:H serotypes, distinct from E. coli H10407 (O78:H11), the strains from which the vaccine pili were prepared. One of the challenge organisms, Strain B7A, (O148:H28) possesses type 1 somatic pili of an antigenic type only loosely related

to that of Strain H10407 (Table 1). In contrast, Strain A 338 C5 (O27:H7) possesses type 1 somatic pili of an antigenic variety apparently identical to the type 1 somatic pili of strain H10407. (Note - Early information from Dr. Printon's laboratory suggested a close serologic similarity between the type 1 somatic pili of strains B7A and H10407 but in subsequent testing this turned out not to be correct).

A group of eight individuals were immunized with 1800 mcg primary and 450 mcg booster doses of type 1 somatic pili vaccine given one month apart. One month following the booster inoculation the eight vaccinees and six controls were challenged with 10^{10} B7A organisms (Table 2). Diarrheal illness occurred in five of eight vaccinees and four of six controls. The lack of vaccine efficacy in this instance was expected, since neither the type 1 somatic pili nor the O antigen of the challenge strain are related to the type 1 pili or O antigen of the strain from which vaccine was prepared.

The second more definitive study involves challenge with Strain A 338 C5. Since this strain is not one which had previously been given to volunteers, a preliminary challenge study was required to verify its pathogenicity and identify an appropriate challenge inoculum. Accordingly, 5×10^8 A 338 organisms were fed with NaHCO_3 to five volunteers. Mild to moderate diarrhea occurred in all five and the clinical syndrome closely resembled that of naturally-occurring travelers' diarrhea (Table 3).

Following demonstration of the suitability of Strain A 338 C5 as a challenge organism, a group of 15 volunteers were immunized with 1800 mcg primary and 450 mcg booster parenteral doses of H10407 type 1 somatic pili vaccine. This group of vaccinees and a group of approximately 10 unimmunized control volunteers will participate in an efficacy challenge study in November, 1981 utilizing E. coli A 338 C5 as the challenge strain. Results of this challenge study should clarify if highly-specific antibody to type 1 somatic pili can prevent ETEC diarrheal infection.

Measurement of Circulating and Intestinal Antibody to ETEC Antigens

In order to compare the magnitude and kinetics of immunoglobulin class-specific immune responses of man to various ETEC antigens, it was necessary to develop practical, reliable assay systems. Enzyme-linked immunosorbent assay (ELISA) systems were successfully developed for measurement of serum IgG and intestinal IgA antibody to CFA/I, CFA/II, O antigen, LT and type 1 somatic pili.

The ELISA for CFA/I antibody will be described in some detail as an illustrative example:

1) Preparation of Antigen

A rough E. coli C strain was obtained from Dr. Werner Maas of New York University into which a CFA/I plasmid had been transferred. This rough strain was cultivated on CFA agar at 37°C to encourage expression of CFA/I pili and suppress type 1 somatic pili. The existence of CFA/I on the strain and absence of type 1 pili was verified by hemagglutination patterns and agglutination by anti-CFA/I antiserum. The bacterial cells were harvested and the pili sheared by agitation in an Omnimixer. Bacterial cells were initially separated from pili by high-speed centrifugation. Pili present in the supernate were purified by two ammonium sulfate precipitations. CFA/II pili were purified in the identical manner from E. coli strain M424 C1 (O6:H18).

Method

Purified CFA/I and CFA/II pili antigens were applied to wells of polystyrene microtiter plates. For each well with antigen a corresponding control well was designated without antigen which, except for the absence of antigen, was otherwise treated identically.¹⁷ Based on experiences with ELISA to detect antibody to other protein antigens (including cholera toxin¹⁷ and type 1 somatic pili)⁴, we anticipated that an antigen concentration circa 10 mcg/ml would be optimal. Accordingly some preliminary studies were carried out utilizing antigen concentrations of 1.0, 5.0 and 10 mcg/ml. Pre- and post-challenge sera from five volunteers who experienced induced diarrheal infection due to E. coli H10407

(which possesses CFA/I) were two-fold serially diluted (1:100 to 1:6400) and added to the appropriate wells. Following standard incubation and washing procedures, alkaline phosphatase-conjugated goat anti-human IgG was added, incubated and washed. Enzyme substrate (para-nitrophenyl phosphate) was added to generate color and the reaction was stopped after 30 minutes by the addition of 3 M NaOH. Optical density was measured at 400 nm wave length in a microspectrophotometer. Net optical density (net O.D.) refers to the resultant value when the O.D. of the control well is subtracted from the O.D. of the corresponding test well with antigen and is a quantitative expression of the level of antibody.¹⁷ These preliminary checkerboard studies demonstrated that an antigen concentration of 5 mcg/ml was optimal.

Selection of Single Dilution of Serum for Testing

Pre-challenge and peak post-challenge serum IgG-ELISA titers to CFA/I and II antigens of five volunteers who experienced H10407 diarrhea are shown in Table 4. Four of five volunteers manifested significant (four-fold or greater) rises in titer to CFA/I antigen but not to CFA/II. In subsequent studies it became apparent that sera need not be titered but could be assayed at a single dilution of 1:400, thereby greatly enhancing the practicality, economy and simplicity of the assay.

We next set out to statistically define the magnitude of rise in net O.D. of the post-challenge over the pre-challenge sera that would represent a significant rise. To accomplish this, paired pre- and post-challenge sera were tested from a negative control population comprising 27 persons who developed diarrhea following ingestion of bacterial pathogens that lack CFA/I including enteropathogenic *E. coli*, some ETEC and *Vibrio cholerae* (Figure 1). The mean change in net O.D. between the paired specimens of these persons was 0.01 ± 0.06 (range -0.17 to +0.14). Thus the mean plus three standard deviations gives a value of 0.19. We thus selected a rise in net O.D. of 0.20 or greater as significant. None of the 27 persons in the negative control group had rises by this criterion (Figure 1).

Pre- and multiple post-challenge (days +10, +21 and +28) sera were tested from 38 volunteers who developed diarrhea following ingestion of E. coli H10407. When tested against CFA/I at a single serum dilution of 1:400, 18 of 38 persons (47%) had rises in net O.D. of 0.20 or greater. None had significant rises against CFA/II antigen.

Magnitude and Kinetics of the IgG-ELISA CFA/I Antibody Response

The magnitude of the 18 significant rises in CFA/I antibody (expressed as net O.D. rises) ranged from 0.22 to 1.00. The kinetics of the IgG anti-CFA/I response are shown in Table 5 and 6. Of the 18 individuals who manifested significant rises in CFA/I antibody, in only five of 18 were the rises present by day +10, indicative of an anamnestic response in immunologically-primed subjects. In the remaining 13 the IgG-ELISA CFA/I antibody peaked on day +21 or +28, indicative of a primary immune response. It is of further interest to note that the magnitude of the anti-CFA/I antibody response (expressed as net O.D.) was significantly greater in the five primed persons than in the 13 un-primed responders on day +10 (0.90 vs. 0.11) and on day +28 (0.67 vs. 0.34).

No significant correlation was found between the height of the anti-CFA/I immune response and the inoculum size of H10407 organisms ingested or the severity of diarrhea (determined by total diarrheal stool volume) (Table 6).

Pre- and post-challenge sera were also tested from 10 individuals who had asymptomatic infection following ingestion of E. coli H10407. Only one (10%) had a significant rise in IgG ELISA CFA/I antibody. This person was immunologically-primed and showed a prominent anamnestic response which peaked by the tenth day post-challenge.

Measurement of Serum and Intestinal Fluid Antibody to ETEC Antigens

During the past contract year, we had an opportunity to apply the various assay systems to detect antibody to pertinent ETEC antigens. The specimens tested were sera and intestinal fluids from the initial group of volunteers vaccinated with type 1 somatic pili vaccine and from these vaccinees and unimmunized controls who

participated in the first challenge study; a description of the antibody assays and results are contained in Appendix A along with a discussion of the findings.

Briefly, pertinent points include the observations that:

- 1) Neither serum nor intestinal fluid levels of antibody to type 1 somatic pili rose in vaccinees or controls in response to challenge with ETEC H10407.

- 2) In contrast, most controls had significant rises in titer of serum and intestinal fluid antibody to O78 antigen, CFA/I and LT.

- 3) Immediately prior to challenge, intestinal fluids of the vaccinees appeared to have more IgA antibody to type 1 somatic pili than intestinal fluids of control volunteers suggesting that immunization with parenteral type 1 pili vaccine may have stimulated secretion of local antibody.

Preliminary Immunogenicity Studies in Animals with Purified CFA/II Pili

Based on their similarities to K88 and K99 pili of animal ETEC strains we have always presumed that purified CFA/I and CFA/II pili would comprise the initial components of a polyvalent pili vaccine to prevent human ETEC diarrhea. Our studies with type 1 somatic pili were undertaken in the hope of broadening the spectrum of protection of a pili vaccine and under the assumption that other groups would proceed with immunogenicity studies of purified CFA/I and CFA/II pili.

While there have been some reports of human studies with purified CFA/I, there have been no reports to date of studies with CFA/II. Accordingly, we decided to initiate an evaluation of the safety, immunogenicity, and efficacy of purified CFA/II vaccine administered orally. This portion of our work-scope involved intensive collaboration with the Department of Gastroenterology (Col. Edgar Boedeker and Capt. Christopher Cheney) and the Department of Bacterial Diseases (Dr. Samuel B. Formal) of WRAIR. CFA/II pili from an O6:H16 strain provided by the CVD were purified according to a method developed by Capt. Cheney and Col. Boedeker. Purified CFA/II pili were then used to immunize Thiry-Vella intestinal loops in rabbits surgically prepared in Dr. Formal's laboratory.⁸ Measurement

of IgA antibody in intestinal washes and IgG antibody to CFA/II in serum from the immunized rabbits were carried out at the CVD utilizing ELISA techniques perfected during the past contract year.

Following surgery to prepare Thiry-Vella loops, the rabbits were allowed one week to stabilize prior to initiating immunization. In the initial studies CFA/II pili were instilled into the loops of rabbits once weekly for three weeks. Separate rabbits received 0.1, 1.0 or 2.0 mg doses. The Thiry-Vella loops were irrigated daily with 20 ml of isotonic saline and the wash fluid collected and frozen for antibody assays. These intestinal washes were tested for IgA antibody to CFA/II. The magnitude and kinetics of the antibody responses are shown in Figure 2. The 0.1 mg dosage stimulated virtually no detectable intestinal antibody whereas the peak response occurred in the rabbits immunized with 2.0 mg doses. Because of the recognition that frequent and large antigenic stimuli are required to prime the intestinal SIgA immune system, we next immunized Thiry-Vella loops of rabbits with 2.0 mg doses of CFA/II, twice weekly for eight weeks. By this regimen it was found that antibody levels stayed elevated for at least two months (Figure 3).

Preliminary Evaluation in Man of a Candidate Attenuated Strain to Prevent ETEC Diarrhea

During this contract year we initiated another approach toward development of immunizing agents against ETEC. In this instance we began exploring the possibility of immunization with an attenuated E. coli strain that possesses adhesion pili but lacks enterotoxins. We were able to obtain an E. coli O6:H16 strain from Dr. Bernard Rowe of the Central Public Health Laboratory, Colindale, England that possesses CFA/II but does not produce LT or ST when assayed by sensitive assays including Y-1 adrenal cells,¹⁹ ELISA²⁰ and the infant mouse test.^{21,22} The strain, E1392/75 2A, was also tested for the presence of genes encoding E. coli LT and/or ST by the colony blot hybridization technique. An LT DNA probe was pre-

pared from the recombinant plasmid EWD 299 and two different ST probes (representing both porcine and human ST) were prepared according to the method of Mosley, et al.²⁵ The DNA probes were labelled with ^{32}P and hybridized to total DNA from colonies lysed on nitrocellulose filters. The resulting autoradiograph demonstrated that strain 1392/75 2A was completely devoid of genes encoding E. coli LT or ST. These phenotypic and genotypic results are summarized in Table 7.

In August, 1981 strain 1392/75 2A was fed to eight volunteers in doses of 10^9 or 10^{10} organisms with NaHCO_3 (Table 8). At each dosage one of four volunteers manifested extremely mild diarrhea. One individual had two small loose stools totaling only 304 ml. during a 48 hr. period, while the other had four very small loose stools comprising only 245 ml in total. None of the eight volunteers experienced malaise, nausea or significant abdominal cramps. From 3 of the 8 volunteers we were able to document colonization of the proximal jejunum by the vaccine candidate strain.

The very mild diarrhea experienced by 2 of 8 volunteers who ingested this non-toxicogenic colonizing strain is reminiscent of the findings of Smith²⁴ who worked with non-toxicogenic K88-positive E. coli strains in neonatal piglets. They noted that strains possessing both enterotoxin and K88 caused severe diarrhea; variants lacking ent⁺ and K88 plasmids produced no diarrhea; and variants having K88 but no toxin stimulated definite but mild diarrhea. The mild diarrhea associated with K88-positive, toxin-negative strains was attributed to the effect of heavy colonization of the proximal small intestine.

In a further analogy, we have found that 20% of 50 recipients of a highly adhesive non-toxicogenic Vibrio cholerae attenuated vaccine strain (Texas Star)²⁵ manifested mild diarrhea that was clearly not related to enterotoxin production.²⁶ Volunteers were shown to be avidly colonized in the proximal jejunal by Texas Star. The proximal small intestine is the critical anatomic site of host/parasite interaction in both ETEC diarrhea and cholera and is therefore the pivotal target site for immunization. Based on experiences with both E1392/75 2A and Texas Star

it would appear that the price that will have to be paid in exchange for adhesion of attenuated vaccine strains to proximal jejunum will be appearance of a few small volume loose stools in 20% of vaccine recipients.

Consequent to these initial encouraging observations with strain E1392/75 2A we shall proceed to insert into the strain a stable vector plasmid encoding genes for high copy production of the immunogenic, non-secretogenic B subunit of LT. It is also intended that volunteer efficacy challenge studies will be carried out to assess the protective efficacy of E1392/75 2A with and without B subunit.

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Table 1

COMPARISON OF THE SEROLOGIC SIMILARITY OF TYPE 1 SOMATIC PILI OF
THREE ENTEROTOXIGENIC ESCHERICHIA COLI STRAINS IN QUANTITATIVE
BACTERIAL AGGLUTINATION TESTS

<u>Antigen</u>	<u>Reciprocal Agglutination Titer</u> <u>Against H10407 Type 1 Pili Rabbit Antiserum</u>
<u>E. coli</u> H10407 with type 1 somatic pili [*]	640
<u>E. coli</u> H10407 without type 1 pili [†]	0
<u>E. coli</u> B7A with type 1 somatic pili [*]	40
<u>E. coli</u> B7A without type 1 pili	0
<u>E. coli</u> A338 C5 with type 1 somatic pili [*]	640
<u>E. coli</u> A338 C5 without type 1 pili	0

^{*} type 1 piliation promoted by stagnant growth in Mueller-Hinton broth for 48 hours

[†] type 1 piliation suppressed by growth on solid CFA agar for 24 hours

Table 2

RESPONSE OF TYPE 1 SOMATIC PILI VACCINE RECIPIENTS AND UNIMMUNIZED CONTROLS
FOLLOWING INGESTION OF 10^{10} ORGANISMS OF ENTEROTOXIGENIC E. COLI STRAIN B7A⁺

	<u>Mean Incubation (hrs.)</u>	<u>Diarrhea Attack Rate</u>	<u>Mean Diarrheal Stool Volume per Ill Volunteer</u>	<u>Mean No. Loose Stools per Ill Volunteer</u>	<u>Positive Stool Cultures</u>
Vaccinees	20	5/8 *	0.9 liters (0.3-2.3) **	14.2 (3-43)	8/8
Controls	31	4/6	0.6 (0.3-1.0)	4.3 (3-6)	6/6

* No. ill/No. challenged

** (range)

⁺ type 1 somatic pili of strain B7A are not serologically closely related to the vaccine pili

Table 3

CLINICAL AND BACTERIOLOGIC RESPONSE OF VOLUNTEERS FOLLOWING INGESTION OF ENTEROTOXIGENIC *ESCHERICHIA COLI*
STRAIN A338 C5 (O27:H7, LT-/ST+)

<u>Inoculum</u>	<u>Mean Incubation (hrs.)</u>	<u>Diarrhea Attack Rate</u>	<u>Mean Stool Volume per ill Volunteer</u>	<u>Mean No. loose Stools per ill Volunteer</u>	<u>Positive Jejunal Fluid Cultures</u>	<u>Positive Stool Cultures</u>
5×10^8 with NaHCO_3	17.3	5/5 *	0.94 liters (0.37 + 1.78) †	6.6	3/5	5/5

* No. ill/No. challenged

† (range)

Table 4

PRE- AND PEAK POST-CHALLENGE TITERS OF IgG ELISA CFA/I AND CFA/II ANTIBODY
IN FIVE VOLUNTEERS WHO DEVELOPED DIARRHEA FOLLOWING INGESTION OF
E. COLI H10407 (O78:H11, LT+/ST+, CFA/I)

		<u>CFA/I</u>	<u>CFA/II</u>
4002-1	Pre-	400*	200
	Peak	1600	200
4002-5	Pre-	200	200
	Peak	800	400
4002-4	Pre-	400	<200
	Peak	1600	<200
4002-10	Pre-	200	200
	Peak	3200	400
4002-11	Pre-	200	<200
	Peak	400	200

* reciprocal titer

Table 5

MAGNITUDE AND KINETICS OF THE IgG ELISA ANTI-CFA/I ANTIBODY RESPONSE IN 18 INDIVIDUALS WHO MANIFESTED SIGNIFICANT ANTIBODY RISES FOLLOWING EXPERIMENTAL DIARRHEA DUE TO E. COLI H10407 (O78:H11, LT+/ST+, CFA/I)

	Day Post-Challenge			
	0	+10	+21	+28
18 persons who showed significant rises in CFA/I antibody	0.14*	0.33	0.42	0.43
5 persons who had anamnestic anti-CFA/I responses (indicative of immunologically-primed status)	0.16	0.90	0.82	0.67
13 persons who exhibited primary anti-CFA/I antibody responses	0.13	0.11	0.33	0.34

* mean net O.D. of sera tested at 1:400 dilution

Table 6

MAGNITUDE OF RISE IN NET O.D. BETWEEN PRE- AND PEAK POST-CHALLENGE
SERUM SPECIMENS IN 18 PERSONS WHO MANIFESTED SIGNIFICANT INCREASES
IN IgG ELISA CFA/I ANTIBODY FOLLOWING DIARRHEA DUE TO E. COLI H10407

<u>Volunteer</u>	<u>Rise in Net O.D.</u>	<u>Total Diarrheal Stool Volume</u>	<u>Antibody Rise Present by Day +10</u> *
4002-11	0.22	1.77	-
4002-16	0.23	1.70	-
4002-5	0.23	0.25	-
4002-13	0.24	1.66	-
2014-8	0.25	2.98	-
2014-5	0.26	3.11	-
2014-11	0.26	3.76	-
4002-12	0.27	1.38	-
4006-16	0.29	2.34	-
4002-4	0.30	9.86	-
4006-4	0.33	2.78	-
4002-1	0.34	3.82	-
2011-12	0.38	0.52	-
4002-15	0.39	0.50	+
2014-3	0.64	0.63	+
4006-2	0.79	0.45	+
4002-14	0.95	6.40	+
2009-2	1.00	6.78	+

* anamnestic response indicative of prior immunologic priming

Table 7

MICROBIOLOGIC AND GENETIC CHARACTERISTICS OF ESCHERICHIA COLI
STRAIN EI392/75 2A (06:H16) CANDIDATE VACCINE STRAIN

Mannose-Resistant Hemagglutination:		Agglutination with CFA/II Antiserum	Tests for LT		Tests for ST		Genetic Probes	
Bovine RBCs	Human RBCs		Y1 Adrenal	ELISA	Infant Mouse	LT	Porcine ST	Human ST
-	-	+	-	-	-	-	-	-

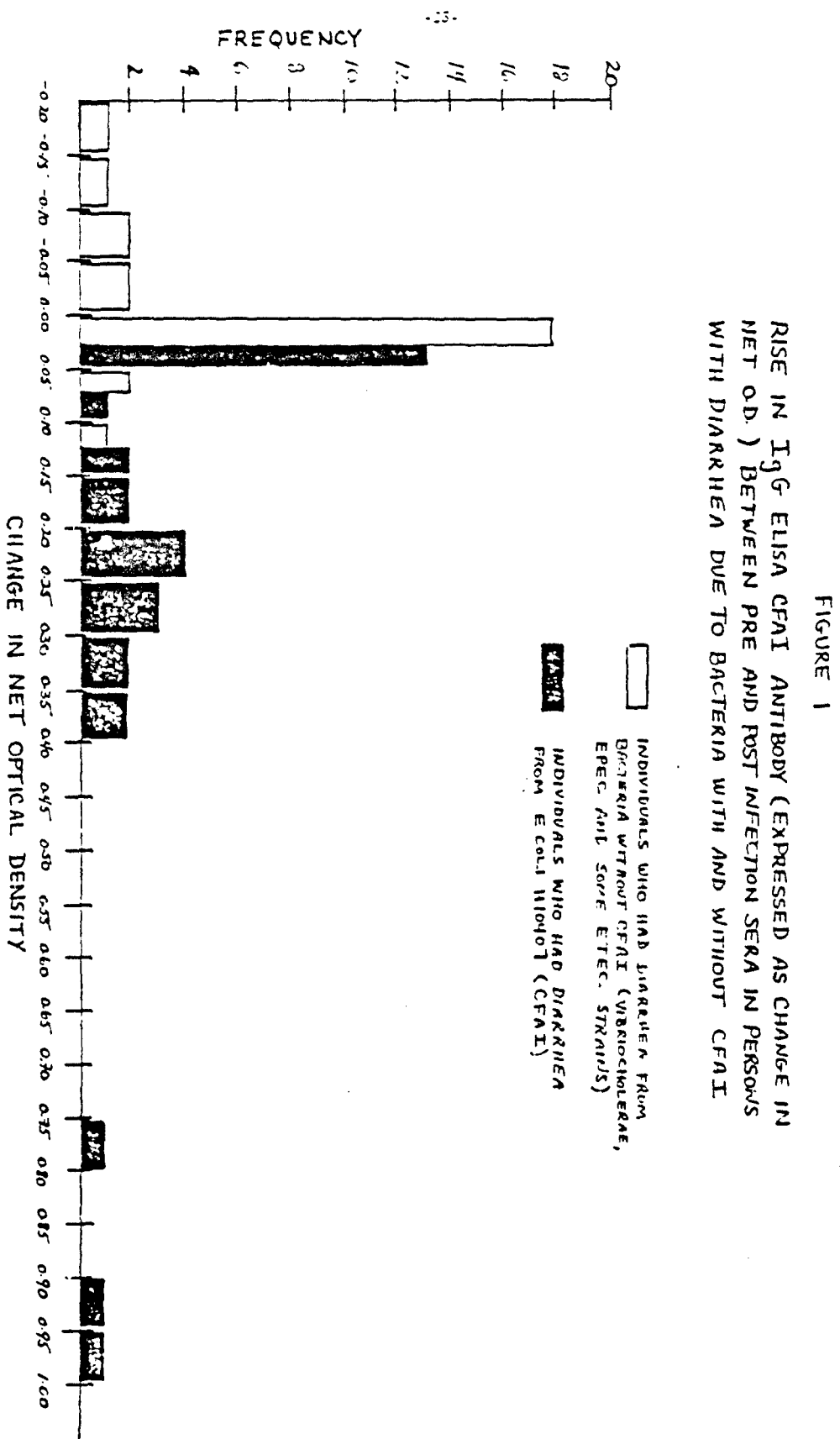
Table 8

CLINICAL AND BACTERIOLOGIC RESPONSE OF VOLUNTEERS FOLLOWING INGESTION OF
NON-TOXIGENIC CFA/II-POSITIVE ESCHERICHIA COLI VACCINE CANDIDATE STRAIN E 1392/75 2A

<u>Inoculum</u>	<u>Incubation (hrs.)</u>	<u>Diarrhea Attack Rate</u>	<u>Stool Volume of III Volunteer</u>	<u>No. loose Stools of III Volunteer</u>	<u>Positive Jejunal Fluid Cultures</u>	<u>Positive Stool Cultures</u>
10^9 (with NaHCO_3)	47	1/4*	0.30 liter	2	1/4	4/4
10^{10} (with NaHCO_3)	33.5	1/4	0.24	4	2/4	4/4
Totals		2/8			3/8	8/8

* No. ill/No. challenged

RISE IN IgG ELISA CFAT ANTIBODY (EXPRESSED AS CHANGE IN NET OD.) BETWEEN PRE AND POST INFECTION SERA IN PERSONS WITH DIARRHEA DUE TO BACTERIA WITH AND WITHOUT CFAT



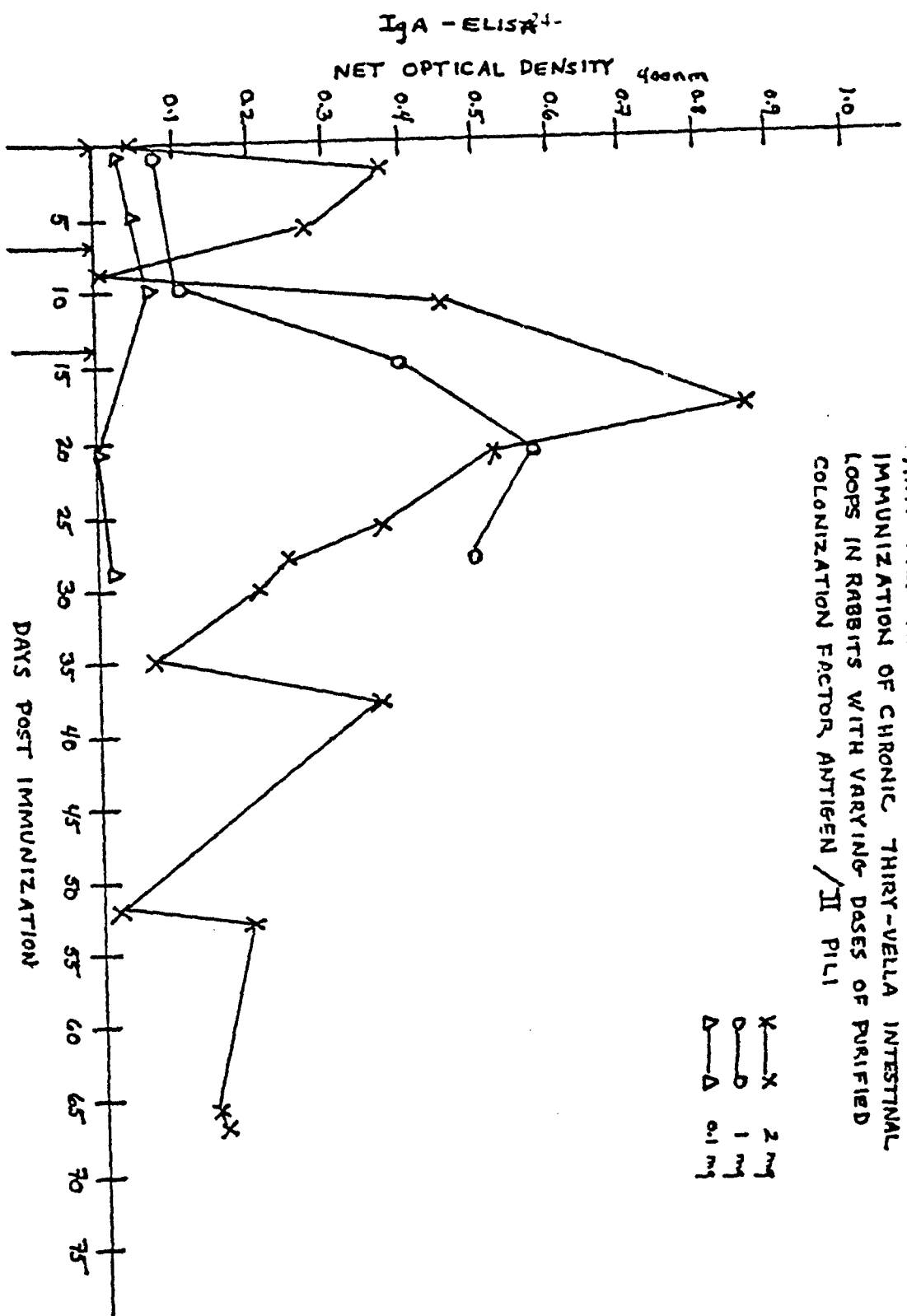
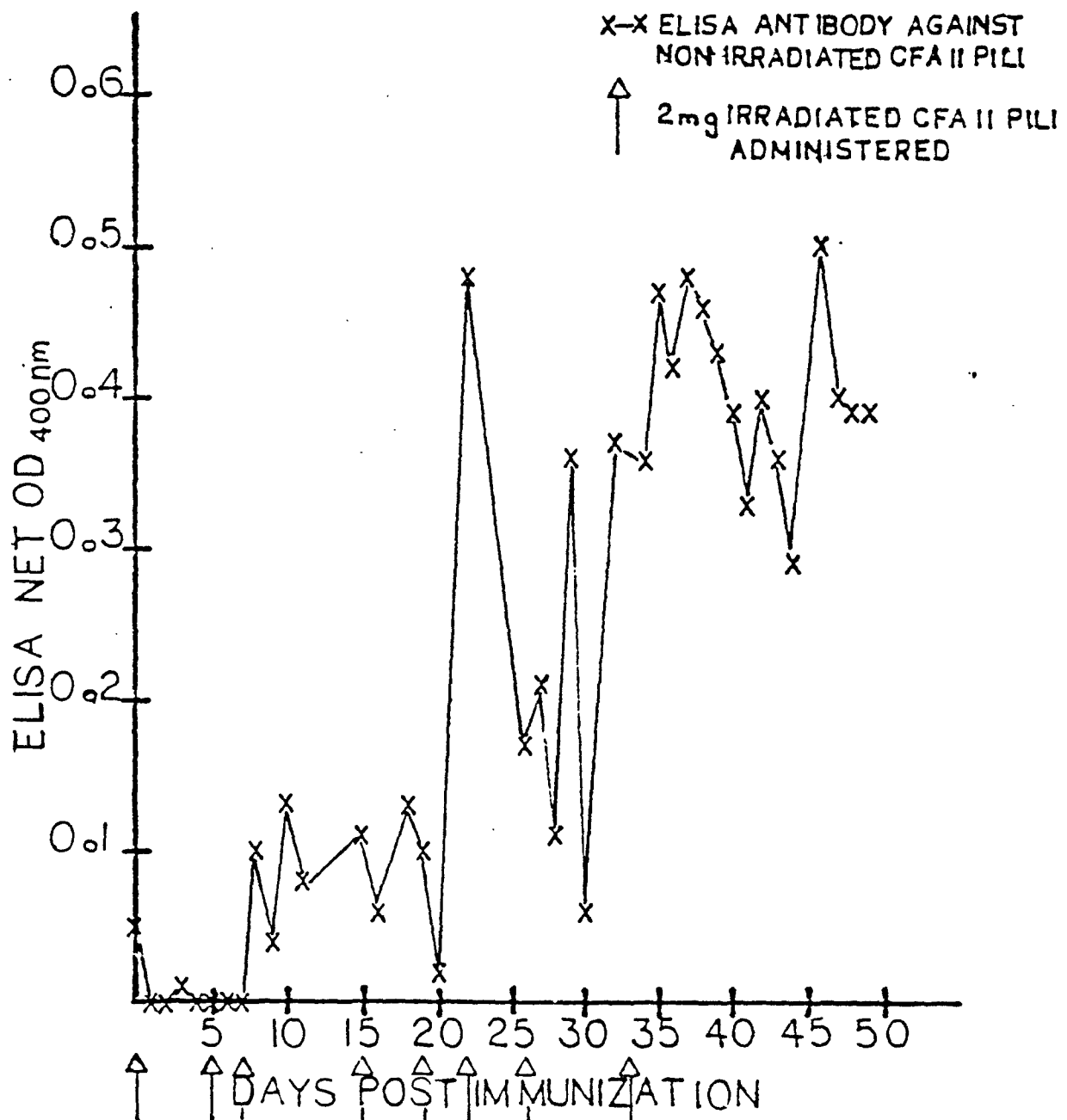


FIGURE 2

ANTI-PILI ANTIBODY IN GUT WASHES FOLLOWING
IMMUNIZATION OF CHRONIC THIRY-VELLA INTESTINAL
LOOPS IN RABBITS WITH VARYING DOSES OF PURIFIED
COLONIZATION FACTOR ANTIGEN/II PILI

FIGURE 3

IgA ANTI-PILI ANTIBODY MEASURED BY
ELISA IN INTESTINAL WASHES (DILUTED 1:20)
FROM A CHRONIC THIRY-VELLA INTESTINAL LOOP
OF A RABBIT IMMUNIZED WITH EIGHT 2.0mg DOSES
OF PURIFIED CFA / II PILI



CONTRACT-RELATED PUBLICATIONS DURING
THE PAST CONTRACT YEAR

- 1) Levine, M.M., Black, R.E., Brinton, C.C., Jr., Clements, M.L., Fusco, P., Hughes, T.P., O'Donnell, S., Robins-Browne, R., Wood, S., Young, C.R. Reactogenicity, Immunogenicity and Efficacy Studies of Escherichia coli Type 1 Somatic Pili Parenteral Vaccine in Man Scand. J. Infect. Dis. in press.
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- 7) Levine, M.M., Young, C.R., Hughes, T.P., O'Donnell, S., Black, R.E., Clements, M.L., Robins-Browne, R., Lim-Y-L. Duration of Serum Antitoxin Response following Vibrio cholerae Infection in North Americans: Relevance for Sero-epidemiology. Am. J. Epidemiol. in press.
- 8) Candy, D.C.A., Leung, T.S.M., Mak, R.H.K., Harries, J.T., Marshall, W.C., Phillips, A.D., Robins-Browne, R., Chadwick, M.V., Levine, M.M. A Fimbrial Antigen Mediating Adhesion and Hemagglutination of Escherichia coli from Children. Submitted for publication.
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REACTOGENICITY, IMMUNOGENICITY AND
EFFICACY STUDIES OF ESCHERICHIA COLI TYPE 1
SOMATIC PILI PARENTERAL VACCINE
IN MAN

Running Head: E. coli Pili Vaccine

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ABSTRACT

Purified type 1 somatic pili from enterotoxigenic Escherichia coli (ETEC) strain H10407 (O78:H11) was evaluated as a parenteral immunizing agent in the hope that this antigen might enhance a contemplated polyvalent pilus vaccine. Intramuscular inoculation with 45, 90, 900 or 1800 mcg of pili vaccine was tolerated without incident in 82 volunteers. Six of 15 persons who received a 28 day booster of 1800 mcg developed local reactions while none of 52 persons receiving 180 or 450 mcg boosters evinced such reactions. Pili vaccine did not significantly alter intestinal transit time, absorptive capacity or the prevalence of colonic E. coli bearing type 1 somatic pili of the H10407 antigenic variety.

All vaccinees developed significant rises in circulating IgG antibody to type 1 somatic pili, the magnitude of the response being directly proportioned to the vaccine dose. None of the vaccinees had significant rises to CFA I or II pili nor to heat-labile enterotoxin. However, many had rises in O antibody, particularly among those inoculated with 1800 mcg.

Three challenge studies were carried out with E. coli H10407 to assess vaccine efficacy. In the initial study the vaccinees were either protected against diarrhea (2 of 6 vaccinees versus 7/7 of controls) or had milder disease than the controls. In two subsequent challenges with H10407 significant protection was not seen. It was not clear whether protection exhibited by the vaccinee group in the first challenge was due to O antibody, pili antibody, or both acting synergistically.

To clarify this, a group of the immunized volunteers were challenged with ETEC strain B7A which is a different serotype (O143:H23) lacks CFA/I or II pili, but possesses type 1 somatic pili antigenically distantly related to those of H10407. Attack rates and severity of illness were similar in both vaccinee and control groups.

A-3.

Appendix A

While most volunteers challenged with E. coli H10407 developed significant rises in circulating antibody to CFA/I, LT and O antigen, none had rises to type 1 somatic pili. It is unclear if this is due to immune tolerance to this antigen when encountered enterally or whether these pili are not present in vivo in ETEC initiating diarrhea in the proximal small intestine.

In summary, parenterally inoculated type 1 somatic pili were safe and highly immunogenic in man but did not consistently induce protection. Further studies are planned to clarify the role of antibody to type 1 somatic pili in mediating protection.

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) are an important cause of traveler's diarrhea (27), infant diarrhea in less-developed countries (4) and colibacillosis of piglets and calves (18,28). Various types of pili (hair-like organelles on the bacterial cell surface) have been found to serve as virulence factors for ETEC of man and animals. Vaccines consisting of K88, K99 and 987-type pili have been given safely to pregnant cows and gilts. Calves and piglets suckled on immunized mothers are significantly protected against lethal diarrhea when challenged with ETEC bearing the homologous pilus antigen (1,29,32,37).

Volunteer studies have demonstrated that an initial episode of diarrhea due to either ETEC 87A (22) or H10407 (24) conferred significant protection against illness upon re-challenge with the homologous strain. Despite being clinically protected volunteers undergoing re-challenge excreted as many ETEC per gm of stool as ill controls. Non-bactericidal secretory IgA (SIgA) antibody directed against the organelles of adhesion of ETEC could explain these observations.

If the colonization factors and organelles of adhesion of human ETEC could be identified, characterized antigenically, and purified they could be evaluated as immunizing agents. Evans et al have described two antigenically distinct pilus-like (fimbrial) surface antigens found in many human ETEC strains (11-14). Referred to as colonization factor antigens I and II (CFA/I, II), these pili are analogous to K88 (18) and K99 (23) of animal ETEC strains: genes for their production are plasmid-encoded; they do not appear when bacteria are cultured at 18°C; they confer on bacteria the property of mannose-resistant hemagglutination. CFA/I and II tend to be associated with strains of a limited number of O serogroups that produce both heat labile (LT) and heat-stable (ST) enterotoxins (26,35). Based on their analogy to K88 and K99, it is hoped that CFA/I and II pili will successfully immunize against ETEC strains bearing these antigens.

Many ETEC strains colonize the intestine, cause diarrheal illness, and stimulate immune responses but lack CFA/I and II (16,23,26,35). The antigens responsible for adhesion of such strains have not yet been identified. Many of these ETEC pathogens lacking CFA/I and II possess type 1 somatic pili (5,26). These pili are associated with mannose-sensitive hemagglutination of guinea pig erythrocytes (34) and genes encoding their production are chromosomal (44). Although type 1 somatic pili are found with equal frequency among normal E. coli flora and their role, if any, in pathogenesis of ETEC is not presently known, they have been shown to bind E. coli to receptors on intestinal epithelial cells in vitro (17,33,39). Furthermore, binding can be inhibited by monospecific anti-pilus antibody (17).

We decided to assess type 1 pili as a potential immunizing agent in the hope that inclusion of this antigen might enhance and broaden the spectrum of protection against ETEC by a future polyvalent pilus antigen vaccine.

METHODS AND MATERIAL

Vaccine

Pili were purified from E. coli H10407 in the laboratory of Charles C. Brinton, Jr. by a modification of the pilus crystal solubilization/recrystallization method previously described (6). Strain H10407 (078:H11) elaborates both heat-labile (LT) and heat-stable (ST) enterotoxins and possesses CFA/I as well as type 1 somatic pili. To prepare the vaccine, the strain was cloned according to colonial type and grown under conditions in which only its type 1 pili are expressed (5). Purified type 1 somatic pili were stored in 50 ml vials in a concentration of 1800 mcg of purified pili protein per ml with merthiolate as preservative. The vaccine was tested by C.C. Brinton, Jr. for general safety, sterility and product safety according to standard procedures recommended by the Bureau of Biologics of the Food and Drug Administration, U.S.A.

Volunteers

Volunteers were college students and other healthy adults (mean age 24 years). Reactogenicity and vaccine efficacy challenge studies were carried out in the 22 bed Isolation Ward of the Center for Vaccine Development. The protocol was reviewed by the University of Maryland Hospital Human Volunteer Research Committee and the Clinical Review Sub-Panel of the National Institute of Allergy and Infectious Diseases. The studies were explained to volunteers in detail and signed, witnessed consent was obtained. Documentation of the informed nature of consent and of the pre-inoculation health status of the volunteers was carried out as previously described (20-22).

Immunization

Initial Dose Response Studies

Sequential groups of 3-6 volunteers (21 in total) were parenterally inoculated in the triceps muscle with 45, 90, 900 or 1800 mcg doses of purified pili vaccine. Twenty-eight days thereafter 15 of the 21 volunteers received a booster IM inoculation with 1800 mcg of pili vaccine. Volunteers were given an inoculation with the same volume of physiological saline in the other arm in order to assess local reactions due to the act of IM inoculation. Neither the volunteer nor the examining physician was told which arm received vaccine and which received saline.

Modified Immunization Schedules

A second group of 21 volunteers received primary immunization as outpatients with 900 mcg of purified pili followed 28 days later by a booster inoculation of 180 mcg (10 persons) or 450 mcg (11 persons).

A third group of 11 outpatient volunteers was immunized with an 1800 mcg primary dose of pili vaccine followed 28 days thereafter by booster inoculation of 450 mcg. Twenty-nine other individuals who received only a single dose, a different dosage schedule or were lost to serologic follow-up are included only in reactogenicity data.

Efficacy Challenge Studies

Approximately four to six weeks following inoculation with the booster dose

of pili vaccine consenting vaccinees and comparable numbers of control volunteers ingested virulent ETEC in challenge studies to assess vaccine efficacy.

Inocula and Challenge

Challenge organisms including E. coli H10407 and E. coli B7A (O148:H28, LT/ST) were thawed from storage in skim milk at -70°C and streaked onto casamino acid yeast extract (CAYE) agar. After 12 hr incubation at 37°C , 30 piliated colonies were used to heavily inoculate each of six CAYE agar plates for incubation at 37°C . Twelve hours later the CAYE agar cultures were harvested with saline (0.85%) and dilutions made in saline. Inocula of H10407 and B7A were given to volunteers with NaHCO_3 as previously described (22,24). Inoculum size was quantitated pre and post challenge by replicate pour-plate technique.

Clinical Observations

Initial Dose/Response Study

The initial group of 21 vaccinees was kept under close observation on the Isolation Ward for two days post-inoculation with the pili vaccine. Oral temperatures were taken every six hours and the injection sites were examined for erythema, heat, induration and tenderness.

Subsequent Immunization Schedules

All subsequent volunteers immunized as out-patients were examined once daily 24 and 48 hours post-inoculation.

Challenge:

Volunteers participating in challenge studies of vaccine efficacy were examined daily starting three days prior to ingestion of the virulent organisms. Oral temperatures were taken every six hours and repeated within five minutes if they were 37.7°C or above. All stools and vomitus were collected in plastic cholera seats, examined by a nurse or physician and the volumes measured. Stools were graded on a five point scale (21): grades 1 (fully formed) and 2 (soft) were considered normal; grade 3 denoted thick liquid stool, grade 4 designated opaque-watery stool,

and grade 5 represented rice-water stools. Diarrhea was defined as any one of the following: 1) three or more loose (grade 3-5) stools of any volume within 48 hrs.; 2) at least two loose stools within 48 hours surpassing 200 ml. in volume; 3) a single loose stool if ≥ 300 ml in volume. Prior to discharge all volunteers received a five day course of oral neomycin (500 mg six hourly) to eradicate fecal carriage of the virulent ETEC strain (21,24).

Tests of Gastrointestinal Function

Prior to primary immunization, prior to booster inoculation and before and after challenge with virulent ETEC, intestinal absorption and transit time were measured in most of the volunteers who participated in the initial dose response and efficacy studies.

Intestinal transit time was determined by Carmine red dye technique (10). A 500 mg oral dose of dye was ingested by the volunteers and the stools examined for evidence of the dye.

Intestinal absorption was assessed by D-xylose absorption (7,30) and excretion tests (40). After an overnight fast, volunteers micturated, provided a baseline blood specimen and ingested 5.0 gm of D-xylose in 250 ml of distilled water. An additional 250 ml of water was then consumed. Blood for measurement of D-xylose was collected after one hour (7,30). For D-xylose excretion tests urine was collected for five hours after ingestion of the monosaccharide (40). D-xylose was quantitated by the method of Roe and Rice (36).

Sera and Intestinal Fluids

Serum specimens were collected before and 10, 21 and 28 days after primary and booster immunization. Sera were also obtained from participants in the efficacy challenge studies before and 10, 21 and 28 days following ingestion of ETEC.

Volunteers ingested polyvinyl chloride intestinal tubes before and eight days after challenge with ETEC to collect 100 ml of jejunal fluid for measurement of

local antibody. Jejunal fluids were centrifuged to remove particulate matter, the SIgA content was quantitated by radial immunodiffusion (25) and the fluids were lyophilized. Fluids were reconstituted to a standard SIgA level of 20 mg % prior to testing for antibody.

Antibody Assays

Measurement of circulating IgG anti-type 1 somatic pili antibody was carried out in the Pittsburgh laboratory. The remaining serologic assays including measurement of circulating antibodies to CFA/I, CFA/II, heat-labile enterotoxin (LT) and H10407 O antigen and intestinal secretory IgA antibodies to type 1 somatic pili, O antigen, CFA/I and CFA/II were performed in the Baltimore laboratory.

Antibody to Type 1 Somatic Pili

Type 1 pili antibody in serum was measured by an enzyme-linked immunosorbent assay (ELISA) (C.C. Brinton, manuscript in preparation).

Pili antibody levels were standardized using a high titer standard serum from a volunteer who was immunized with two parenteral inoculations of purified H10407 type 1 somatic pili vaccine. Serial dilutions of the standard serum were tested in every series of assays and a curve was constructed. Antibody in the unknown sera was quantitated from the standard curve relative to an arbitrary level of 56,000 assigned to the standard serum.

To detect intestinal IgA antibody to type 1 pili in jejunal fluids an IgA-ELISA was employed. Alternate wells of polystyrene microtiter plates were coated with 100 μ l of purified type 1 pili (10 mcg/ml). For each antigen-coated test well there was included a background control well without antigen. Two-fold dilutions of jejunal fluids were inoculated into test and control wells, incubated and washed. An affinity chromatography-purified goat anti-human IgA to which alkaline phosphatase was conjugated was added to the wells, incubated, washed and substrate added (45). After 30 minutes the reaction was stopped and the optical

density (O.D.) measured with an ELISA colorimeter (9). The term net O.D., the resultant value when the O.D. of the antigen-free control well is subtracted from that of its corresponding test well, is an expression of antibody level (45).

Antibody to CFA Pili

Sera and jejunal fluids were tested for IgG and IgA class antibody, respectively, to CFA/I and II. One hundred μ l of purified CFA/I or II (5 μ g/ml for serum, 10 μ g/ml for intestinal fluids) pili was adsorbed to every other well of polystyrene microtiter plates. For each antigen-coated test well there was a corresponding antigen-free background control well which was identically treated. Jejunal fluid (diluted two-fold serially) or serum at a single dilution (1:400) was added to both test and control wells. Incubation of the wells, washes, addition of alkaline phosphatase-conjugated IgG or IgA, development of color, cessation of reaction and measurement of O.D. followed a standard ELISA method previously described (45). A rise in net O.D. of 0.20 or greater of the post-immunization (or post-challenge) serum over the pre-specimen was considered significant. The titer of antibody in an intestinal fluid was considered to be the highest dilution that gave a net O.D. of 0.20 or above: four-fold or greater rises between pre- and post-challenge specimens were considered significant.

Antibody to Heat-Labile Enterotoxin:

Antibody to E. coli LT was measured by the Y-1 adrenal cell neutralization assay as previously described (22).

Antibody to O Antigens:

Circulating antibody to the somatic O antigens of E. coli H10407 and B7A (O148:H28) was assayed by microtiter passive hemagglutination technique as previously described (21). Lipopolysaccharide (O) antigen was prepared by the method of Morrison and Levy (31) from a clone of E. coli H10407 lacking both type 1 somatic and CFA/I pili. O antigen was prepared by the method of Young et al

(46) from a piliated clone of E. coli B7A (which possesses type 1 somatic pili antigenically not closely related to those of E. coli H10407). Antigen was adsorbed to glutaraldehyde-treated (3) sheep erythrocytes and added to double dilutions of heat inactivated (56°C, 30 min), adsorbed (with sheep erythrocytes) test samples (19,22). Reactions were read after incubation for 2 hrs at 37°C and 16 hours at 4°C (19,22). High titer lapine antisera from rabbits immunized with H10407 and B7A served as positive controls.

For measurement of intestinal IgA O antibody serial two-fold (1:8-1:64) dilutions of pre- and eight day post-challenge jejunal fluids were assayed by microtiter ELISA utilizing H10407 heat alkaline extracted lipopolysaccharide as antigen (10 mcg/ml) (46).

Stool Cultures

During Immunization:

Stool specimens from 15 vaccinees were collected before and 28 days after primary immunization and plated on Levine's eosin-methylene-blue (EMB) agar. Fifteen clones per specimen were inoculated into separate tubes of Mueller-Hinton broth (5 ml) and incubated resting for 48 hrs. at 37°C (23). Sub-cultures were made into Mueller-Hinton broth and similarly cultured. The culture tubes were centrifuged, the supernatants discarded and the bacterial pellets tested for agglutination with antiserum to purified H10407 type 1 somatic pili; the proportion of cultures reacting with specific antibody was recorded.

During Challenge:

Stool specimens or rectal swabs were collected daily throughout the challenge study (including at least two pre-challenge specimens) and inoculated onto Levine's EMB agar. Ten colonies with a typical E. coli metallic sheen were sub-cultured onto slants of trypticase soy agar in screwtop tubes. After 18 hrs. incubation at 37°C these clones were tested for agglutination with rabbit antisera to E. coli

H10407 (or B7A), H10407 type 1 somatic pili and CVA/I pili (during H10407 challenge only). The isolates were also tested for LT production by Y-1 adrenal cell assay as previously described (12,38).

RESULTS:

Initial Dose Response Study

Clinical: _

No erythema, induration, heat, tenderness, fever or malaise occurred in any of the 21 volunteers who received primary immunization with varying doses of purified pili vaccine (Table I). Among the fifteen persons who received an 1800 mcg booster inoculation no systemic adverse reactions were noted but six vaccinees developed objective local adverse reactions including induration, heat or erythema (Table I). The local reactions of these six persons were without respect to the size of their primary inoculations, i.e. 45 (2), 900 (2), or 1800 (2) mcg doses of vaccine. These reactions were evident 24 hrs. post-inoculation but, with one exception, were gone by 48 hrs; they were described as mild to moderate by the volunteers. In no instance did nausea, vomiting, diarrhea or fever occur.

Gastrointestinal Function: _

Approximately two-thirds of the vaccinees had measurements of intestinal transit time and D-xylose absorption before and 28 days following primary immunization. In no instance were abnormal values noted. The mean intestinal transit times pre-immunization (mean 29 hrs., range 12-72 hrs.) were similar to those of post-immunization (mean 20 hrs., range 2-72 hrs.) in the 14 persons tested.

Sixteen recipients of pili vaccine (Table II) had D-xylose absorption tests performed prior to and 28 days after primary immunization; the levels before (mean 12.6 ± 4.6 mg/dl) and 28 days after vaccination (mean 14.7 ± 4.1 mg/dl) were similar ($p > 0.1$, paired Student's t test).

Six vaccinees who received 1800 mcg booster doses of pili vaccine went on to participate in the first challenge study. These individuals provided an opportunity to analyze D-xylose absorption at four points in time (Table II): pre-

immunization (Day 0), pre-booster (day +28), pre-challenge (day +57) and post-challenge (day +64). There were no significant differences in the mean one hour blood xylose levels between day 0 (14.8 ± 2.7 mg/dl), day +28 (14.4 ± 4.0 mg/dl), and day +57 (15.7 ± 2.8 mg/dl) (Table II).

Colonic E. coli

Stool specimens from 15 vaccinees were cultured prior to and 28 days after primary immunization. The prevalence of normal E. coli flora that possess type 1 somatic pili of the H10407 antigenic variety were recorded. In 12 of 15 vaccinees prior to immunization, 40-100% of normal E. coli colonic flora was agglutinated by antiserum to H10407 type 1 somatic pili (Table III). In ten persons the prevalence of piliated E. coli remained unchanged. In two persons, E. coli possessing type 1 somatic pili of the H10407 antigenic variety disappeared 28 days post-immunization, while piliated E. coli appeared on day 28 in three persons in whom they were absent pre-immunization. These data demonstrate that vaccine did not alter the prevalence of normal E. coli colonic flora bearing type 1 somatic pili of H10407 antigenic variety.

Antibody to Type 1 Somatic Pili

All 21 immunized persons in the initial dose/response study developed four-fold or greater rises in antibody to type 1 somatic pili following primary immunization, including the seven volunteers who received low doses of 45 or 90 mcg of purified pili vaccine.

In 14 individuals who received a booster, the kinetics of the immune response in relation to antigenic load and the effect of booster immunization are seen in Figure 1. Although antibody rises were evident by day +10, levels peaked on day 28 after the first dose of vaccine. Antibody levels attained following primary immunization were clearly related to antigenic load. The GMT (122) on day 28 of vaccinees who received 900 or 1800 mcg. was significantly higher than the GMT (21)

of those who received 45 or 90 mcgs. By 28 days after booster inoculation of the low dose group with an 1800 mcg immunization (56 days after primary dose), the GMT rose four-fold to a titer of 82. In the group who received 900 or 1800 mcg primary doses, the 1800 mcg booster inoculation did not stimulate a further rise in GMT between day 28 (164) and day 56 (159) (Figure 1).

Antibody to CFA Pili

None of the vaccinated individuals developed significant rises in circulating IgG antibody to CFA/I or II either after primary or booster immunization.

Antibody to Heat-Labile Enterotoxin

No significant rises in LT antitoxin occurred following primary or booster immunization with pili vaccine.

Antibody to Somatic O Antigen:

Significant rises in antibody to O antigen of E. coli H10407 were found in 2 of 7 volunteers inoculated with 45 or 90 mcg primary doses of vaccine, in 1 of 4 who received 900 mcg, and in all 10 who received 1800 mcg (Table IV). In all but one instance antibody peaked on day 10 and titers were significantly higher in recipients of the 1800 mcg dose. In contrast, none of the 21 vaccinees developed four-fold or greater rises to the heterologous O antigen (O148) of E. coli B7A.

Initial Vaccine Efficacy Study

Clinical

One month after booster inoculation with 1800 mcg of pili vaccine, six vaccinees in the initial group immunized agreed to participate in a challenge study along with seven unimmunized control volunteers. Following ingestion of 5×10^8 virulent E. coli H10407 bacteria, all seven controls developed diarrheal illness (efficacy study #1, Table V). Three controls passed copious rice-water stools resulting in cholera-like total diarrheal stool volumes of 3.8, 7.5 and 9.9 liters; two controls required intravenous fluids for several hours to maintain hydration.

Two of six vaccinees developed diarrheal illness ($p=0.04$, two-tailed Fisher's Exact Test) one being cholera-like in severity (6.4 liter stool volume). While ill controls experienced malaise (7 of 7) and vomiting (6 of 7), none of the vaccinees, ill or well, had these complaints. However, the incubation time, total stool volume, number of loose stools, and duration of illness manifested by the two vaccinees was similar to that seen in the controls.

Excretion of E. coli H10407:

Despite clinical protection all vaccinees, as well as controls, excreted virulent E. coli H10407. Within 48 hrs. post-challenge all volunteers were shedding E. coli H10407 as the predominant aerobic coliform. Of 1200 E. coli clones picked from stool cultures during the first five days post-challenge, 1183 (98.6%) were strongly agglutinated by lapine H10407 antiserum. Of 602 clones agglutinated with specific antisera for H10407 type 1 somatic and CFA/I pili all had both types.

D-xylose Absorption Tests

The one hour blood xylose levels fell significantly ($p<0.05$, Table II) in the seven control volunteers post-challenge in comparison with pre-challenge levels. The mean one hour blood D-xylose level was also significantly lower in the group of six vaccinees in comparison with pre-challenge and pre-immunization levels ($p<0.01$, Table II). While four of six vaccinees had significant drops in D-xylose absorption following challenge, the two vaccinees with diarrhea had the most prominent falls in blood xylose levels between pre- and post-challenge specimens; 16.7 mg/dl fell to 2.1 and 13.6 to 2.8 mg/dl.

D-xylose excretion tests were performed on five hour urine collected from vaccinees and controls pre and post-challenge. All 13 volunteers had normal values (>1.2 gm/5 hr. urine volume) pre-challenge. Six of seven ill controls, both ill vaccinees and one of four well vaccinees had abnormal test results post-challenge.

Antibody to Type 1 Somatic Pili

As shown in Table VI, neither vaccinees nor controls had four-fold (or even two-fold) rises in levels of circulating type 1 somatic pilus antibody. There were too few volunteers to relate protection to level of pili antibody.

Prior to and eight days after challenge, jejunal fluids were collected from five vaccinees and six controls. Prior to and eight days after challenge none manifested significant rises in IgA antibody to type 1 pili. It is notable, however, that the mean net O.D. of intestinal fluids of vaccinees pre-challenge was considerably higher at all dilutions than that of the controls (Table VII), suggesting that the former group had increased levels of local jejunal antibody to type 1 pili.

Antibody to CFA Pili:

Post-challenge, 4 of 6 vaccinees and 6 of 7 controls had significant increases in circulating IgG antibody to CFA I pili (Table VI). Two of five vaccinees and 1 of 6 controls, from whom intestinal fluids were available for testing, manifested significant rises in titer of intestinal IgA antibody to CFA/I pili. No significant rises were found in serum or intestinal fluid of any volunteers to CFA/II pilus antigen.

Antibody to LT:

Following challenge with virulent E. coli H10407, six of seven controls and four of six vaccinees developed significant rises in circulating LT antitoxin. In both groups, antitoxin peaked on day 21 (Table VI).

Antibody to O Antigen:

All 13 volunteers, both vaccinees and controls, developed significant rises in serum antibody to E. coli H10407 O antigen (Table IV). O antibody showed a distinctive peak in both groups on the tenth day post-challenge. Among the vaccinees the baseline pre-challenge titers were low; the highest reciprocal titer

was 32. While none of the vaccinees or controls tested had measurable intestinal IgA O antibody pre-challenge, all individuals tested manifested significant rises in response to challenge.

Modified Immunization Schedules

Studies were undertaken to identify an immunization schedule that would be immunogenic without causing local reactions when a booster dose was given. Volunteers who received primary inoculation with 900 mcg pili vaccine and boosters of either 180 or 450 mcg showed, no adverse reactions. Mean antibody levels one month following the booster were comparable in the two groups (Table VIII) and were only 25% below the GMT found in eight persons in the initial dose/response study who received 1800 mcg boosters following either 900 or 1800 mcg primary inoculations. With this immunization schedule only 9 of 21 (43%) individuals developed significant rises in O antibody and the titers were much lower than in the initial group immunized.

Subsequent Challenge Studies with E. coli H10407

A group of volunteers immunized with the 900 mcg primary/450 mcg booster schedule were challenged with 5×10^8 E. coli H10407 organisms. The strain and inoculum size were the same as used in the initial challenge study to ensure that change in vaccine dose represented the only variable between the two clinical experiments. The difference in attack rates between vaccine (3/6) and control (7/8) groups was not significant (Study #2, Table V).

Since the inoculum size employed in the previous challenge, 5×10^8 , was high and probably represents an inoculum much larger than would occur in nature, we challenged a second group of volunteers immunized by the 900/450 mcg schedule with 10^7 H10407 organisms. While the attack rate with this smaller inoculum was lower in the control group (3/11) it remained high in the vaccinees (3/4) (Study #3, Table V).

Challenge with E. coli B7A

Two studies were designed to clarify the relative role of O and type 1 somatic pili antibodies in mediating protection. In the first study a group of vaccinees immunized with 1800 mcg primary and 450 mcg booster doses was challenged with 10^{10} E. coli B7A. Strain B7A (O148:H23) is a different serotype than H10407, does not possess CFA/I or II and elaborates type 1 somatic pili but of an antigenic variety only distantly related to the type 1-pili of H10407. No evidence of vaccine efficacy was seen. Five of eight vaccinees and 4 of 6 controls developed diarrhea of comparable severity (Study #4, Table V).

A final challenge study is planned involving an ETEC strain of a serotype distinct from H10407, lacking CFA/I or II but possessing type 1 somatic pili closely related to those of strain H10407.

DISCUSSION

These studies represent the first evaluation in man of the safety, immunogenicity and efficacy of purified type 1 somatic pili vaccines. They were undertaken to determine whether type 1 somatic pilus antigens might have a role in a multivalent pilus antigen vaccine for ETEC diarrhea.

Single IM doses of 45, 90, 900 and 1800 mcg caused no adverse reactions. Although 6 of 15 individuals who received booster inoculations of 1800 mcg had objective local adverse reactions, none were encountered among 52 persons who received booster doses of 180 or 450 mcg. The local reactions following the 1800 mcg dose booster were apparently due to antigen-antibody interaction since they were not seen after primary immunization with the same dose of vaccine. It is not clear whether the reactions were mediated by anti-pili antibody or antibody to the small amount of H10407 endotoxin (O antigen) also present in the vaccine preparation.

Although the vaccine stimulated high levels of circulating antibody it did not adversely affect gastrointestinal function: intestinal transit time,

D-xylose absorption tests (Table II) and the prevalence of normal E. coli colonic flora possessing type 1 somatic pili of H10407 antigenic variety (Table III) were not significantly altered.

Purified pili vaccine did not stimulate rises in antibody to CFA/I, CFA/II pili or LT antitoxin. However, all persons who received 1800 mcg as primary immunization (and 27% of those receiving lower dose primary immunizations) developed significant rises in antibody to H10407 O antigen. The titers peaked on day 10 and fell rapidly thereafter, typifying the predominantly IgM class antibody response to O antigen (8). We are confident that the antibody measured by HA was indeed to H10407 O antigen and not to type 1 pili that may have contaminated the O antigen preparation. To preclude such confusion H10407 O antigen was deliberately prepared from a clone devoid of both type 1 somatic and CFA/I pili. Furthermore, no rises were recorded when sera were tested against an O antigen prepared from E. coli B7A which has an O antigen serologically distinct from H10407 (O148 vs. O78). The appearance of antibodies to H10407 O antigen following immunization with "high" doses of pili vaccine probably represents contamination of the vaccine preparation with minute quantities of O antigen. Studies by Greisman et al. (15) have shown that man will develop significant rises in circulating O antibody in response to minuscule parenteral doses of E. coli O lipopolysaccharide (0.001-0.01 mcg/kg) if the immunizing preparation also contains protein.

By the time of challenge with virulent ETEC H10407, the short-lived circulating IgM anti-O titers had dropped and approximated pre-immunization titers. In contrast, long-lived IgG class anti-pili antibody remained at high levels. Pre-challenge level of intestinal IgA antibody to type 1 pili (expressed as mean net O.D.) was higher in vaccinees than controls ($p=0.10$, Student's t test) (Table VII). On the assumption that most individuals are immunologically primed to

type 1 somatic pili consequent to antigenic stimulation by E. coli flora in the colon, it is conceivable that two parenteral doses of pilus vaccine may have stimulated increased production of local intestinal anti-pilus antibody (42,43).

Although the number of volunteers involved in the initial challenge study was small, the difference in attack rates between the control (7/7) and immunized (2/6) groups was significant ($p=0.04$). Protection in the vaccinees occurred even though a large inoculum was administered. Furthermore, illness in the two vaccinees appeared qualitatively milder since vomiting, and malaise did not occur -- such symptoms were the rule in the ill controls. All challenged vaccinees, including those without illness, excreted virulent ETEC H10407. This is not surprising since we similarly observed in homologous rechallenge studies of E. coli H10407 (24) and 87A (22) that fecal excretion of virulent ETEC was not diminished in spite of clinical protection. Consequent to the homologous re-challenge studies we hypothesized that protection was mediated by a mechanism that was not bactericidal and probably involved antibody at the mucosal surface that interfered with adhesion of bacteria to critical mucosal sites (22).

The protective mucosal antibody due to parenteral immunization may have been either serum-derived or produced locally. There exist other examples wherein parenteral vaccines exert notable effects in the intestine. Parenteral killed whole cell cholera vaccines, for example, provide protection, albeit short-lived, against Vibrio cholerae, a pathogen which (like ETEC) does not invade intestinal mucosa. While protection due to such cholera vaccines has been correlated with the levels of serum vibriocidal antibody stimulated, it has also been shown that parenteral immunization boosts levels of specific local IgA antibody to cholera antigens in immunologically-primed persons (42,43).

Whatever the derivation of the antibody responsible for protection in the

initial challenge study, it was not clear whether such antibody was directed against pili, O antigen or both. In two subsequent challenges with E. coli H10407 significant protection was not seen. The modified immunization schedule employed to immunize these groups of volunteers (900 mcg primary/450 mcg booster) resulted in a slightly lower GMT of anti-pili antibody but markedly lower anti-O antibody levels.

In the first of two definitive experiments to rule out the role of O antibody as a confounding variable, a group of vaccinees immunized with 1800 mcg primary and 450 mcg booster doses were challenged with ETEC B7A which lacks CFA/I or II and possesses type 1 somatic pili only distantly related to H10407. H10407 type 1 pili vaccine did not protect against B7A challenge. In a final study in this series we will challenge vaccinees and controls with an ETEC strain of a different serotype than H10407 that lacks CFA/I and II but possesses type 1 somatic pili antigenically closely related to those of H10407. Results of this challenge study should definitively elucidate the protective role, if any, of antibody directed against type 1 somatic pili.

One of the sidelights of these studies was the opportunity to intensively examine the immunologic response to E. coli H10407 antigens, particularly during the initial challenge study. Following oral challenge, most volunteers showed prominent circulating immune responses to LT, CFA/I pili and O antigen. However, neither vaccinees nor controls manifested significant rises in titer of circulating or local jejunal antibody to type 1 somatic pili. The reasons for the lack of immune response to this antigen following oral challenge are unclear. It is possible that constant antigenic stimulation of colonic mucosa by type 1 pilated normal E. coli flora, beginning early in life, leads to immune tolerance to this antigen in adults when it is presented enterally (2). Parenteral immunization apparently can overcome such tolerance if it exists, as the cir-

culating and preliminary local antibody data from the initial group of vaccinees suggest. Alternatively, one can argue that E. coli H10407 may not express type 1 somatic pili on its surface in vivo as it interacts with proximal small intestinal mucosa in the course of initiating diarrheal infection. Studies are currently underway to assess this critical point.

Type 1 somatic pili have been shown to be safe and highly antigenic when given as a parenteral vaccine. However, results of the challenge studies carried out so far are too inconclusive to allow argument for or against inclusion of such antigens in a vaccine against ETEC.

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TABLE I

CLINICAL RESPONSE OF VOLUNTEERS TO PARENTERAL
IMMUNIZATION WITH VARYING DOSES OF PURIFIED H10407
TYPE 1 SOMATIC PILI VACCINE

<u>Dose</u>	<u>Following Initial Vaccine Dose</u>			<u>Following 1800 mcg. Booster Dose</u>		
	<u>Fever</u>	<u>Malaise</u>	<u>Local Reactions</u>	<u>Fever</u>	<u>Malaise</u>	<u>Local Reactions</u>
45 mcgs.	0/3*	0/3	0/3	0/3	0/3	2/3
90 mcgs.	0/4	0/4	0/4	0/3	0/3	0/3
900 mcgs.	0/4	0/4	0/4	0/3	0/3	2/3
1800 mcgs.	0/10	0/10	0/10	0/6	0/6	2/6

*No. with reactions/No. immunized

TABLE II

Appendix A

ONE HOUR D-XYLOSE BLOOD ABSORPTION
TESTS IN RELATION TO IMMUNIZATION WITH
TYPE 1 SOMATIC PILI VACCINE AND CHALLENGE
WITH ENTEROTOXIGENIC ESCHERICHIA COLI

<u>Group</u>	<u>Day*:</u>	<u>0</u>	<u>+28</u>	<u>+57</u>	<u>+65</u>
30 Normal Adults		14.2±5.2†	-	-	
16 Vaccinees		12.6±4.5	14.7±4.1	-	
6 Vaccinees**		14.8±2.7	14.4±4.0	15.7±2.8	7.7±4.7††
7 Controls		-	-	16.9±7.7	9.3±4.2†*

*Day 0 = pre-immunization

Day 28 = one month after primary immunization

Day 57 = one month after booster immunization and three days pre-challenge with ETEC

Day 65 = five days post-challenge with ETEC.

†mean ± standard deviation mg/dl.

**six vaccinees who participated in vaccine efficacy challenge study.

††Day 65 value significantly lower than day 0 and day +57 (p<0.01, Student's paired t test).

†*Day 65 value significantly lower than day pre-challenge (p<0.05, Student's paired t test).

TABLE III

PREVALENCE OF NORMAL COLONIC E. COLI FLORA
THAT POSSESS SOMATIC PILI OF H10407
ANTIGENIC VARIETY

<u>Volunteer</u>	<u>Day 0</u>	<u>Day +28</u>
4001-1A	0*	100
4001-2A	0	100
4001-3A	0	80
4001-6B	93	87
4001-7B	67	100
4001-8B	93	100
4001-10C	100	100
4001-11C	100	100
4001-12C	100	100
4001-13D	40	73
4001-14D	100	0
4001-15D	73	0
4001-16D	40	60
4001-17D	100	100
4001-18D	87	100

*% of 15 colonies tested that were agglutinated 5+ or 4+
by antibody to type 1 somatic pili of E. coli H10407

TABLE IV

ANTI-O ANTIBODY RESPONSE FOLLOWING PRIMARY IMMUNIZATION
WITH VARYING DOSES OF E. COLI H10407 PURIFIED
SOMATIC TYPE 2 PILI VACCINE.

<u>Vaccine Group</u>	<u>Antibody Rises*/No. Immunized</u>	Reciprocal Geometric Mean Titer†			
		<u>Day:0</u>	<u>+10</u>	<u>+21</u>	<u>+28</u>
45 or 90 mcg.	2/7	8.8	23.8	17.7	13.1
900	1/4	4.8	19	13.5	11.3
1800	10/10	2.6	276	111	64

*Four-fold or >

†Measured by passive hemagglutination

TABLE V

RESPONSE OF VACCINEES AND CONTROLS FOLLOWING INGESTION OF
ENTEROTOXIGENIC *ESCHERICHIA COLI* IN FOUR CHALLENGE
STUDIES OF PILI VACCINE EFFICACY

Efficacy Study	Challenge Strain (Inoculum)	Group	Mean Incubation (hrs.)	Diarrhea Rate	Mean Stool Volume per Ill Volunteer (liters)	Mean No. Loose Stools per Ill Volunteer	Positive Stool Culture
#1	H10407 (5x10 ⁸)	Vaccinees	37	2/6*	3.9** (1.4-6.4)	16 (15-19)	6/6
		Controls	35	7/7 p=0.04	4.0 (1.4-9.9)	18 (7-29)	7/7
#2	H10407 (5x10 ⁸)	Vaccinees	31	3/6	4.3 (0.9-10.6)	15.3 (5-31)	6/6
		Controls	37	7/8	3.0 (0.5-8.6)	12.3 (3-22)	8/8
#3	H10407 (10 ⁷)	Vaccinees	75	3/4	0.7 (0.3-0.8)	4.7 (1-11)	4/4
		Controls	44	3/11	1.2 (0.4-1.5)	15.3 (4-26)	11/11
#4	B76 (10 ¹⁰)	Vaccinees		5/8	0.9 (0.3-2.3)	14.2 (3-43)	8/8
		Controls		4/6	0.5 (0.3-1.0)	4.3 (3-6)	6/6

*No. positive/No. challenged

**Mean (range)

TABLE VI

Appendix A

CIRCULATING ANTIBODY TO TYPE 1 SOMATIC PILI,
LT, CFA/I, CFA/II AND O ANTIGEN IN VACCINEES AND
CONTROLS FOLLOWING CHALLENGE WITH 5×10^8 E. COLI H10407

Vaccinees	Clinical Illness	Antibody Assayed:				
		Type 1 Somatic Pili	O	LT	CFA/I	CFA/II
4002-2	+	-	++	+	+	-
4002-5	-	-	+	-	+	-
4002-10	-	-	+	+	+	-
4002-12	-	-	+	-	-	-
4002-14	+	-	+	+	+	-
4002-18	-	-	+	+	-	-
Total	2/6	0/6	6/6	4/6	4/6	0/6
<u>Controls</u>						
4002-1	+	-	+	+	+	-
4002-3	+	-	+	+	-	-
4002-4	+	-	+	-	+	-
4002-9	+	-	+	+	+	-
4002-11	+	-	+	+	+	-
4002-13	+	-	+	+	+	-
4002-16	+	-	+	+	+	-
Total	7/7	0/7	7/7	6/7	6/7	0/7

* + = significant rise.

SIGA ELISA ANTIBODY TO TYPE 1 SOMATIC PILI IN JEJUNAL FLUID OF
VACCINEES AND IN CONTROLS PRIOR TO CHALLENGE
WITH 5×10^8 E. COLI H10407

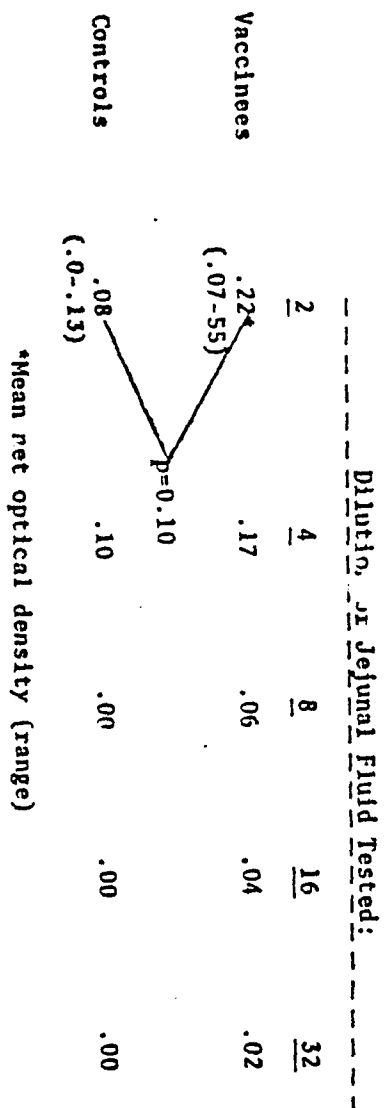


TABLE VIII

Appendix A

GEOMETRIC MEAN TITERS AFTER PRIMARY AND
 BOOSTER IMMUNIZATION OF VOLUNTEERS WITH
E. COLI TYPE 1 SOMATIC PILI VACCINE
 ACCORDING TO SEVERAL IMMUNIZATION SCHEDULES

<u>Primary (Day 0)</u>	<u>Booster (Day +28)</u>	<u>N</u>	Circulating ELISA Pili Antibody Reciprocal Titer $\times 10^{-3}$			
			<u>Day:</u>	<u>0</u>	<u>+28</u>	<u>+56</u>
900 mcg	180 mcg	10		3	94	111
900	450	11		3	88	123
900 (3) *						
or						
1800 (5)	1800	8		3	165	159

*These 8 volunteers were immunized in the initial dose response studies.

CIRCULATING ANTIBODY RESPONSE FOLLOWING
PRIMARY AND BOOSTER PARENTERAL IMMUNIZATION
WITH ESCHERICHIA COLI H10407 PURIFIED TYPE I
SOMATIC PILI VACCINE

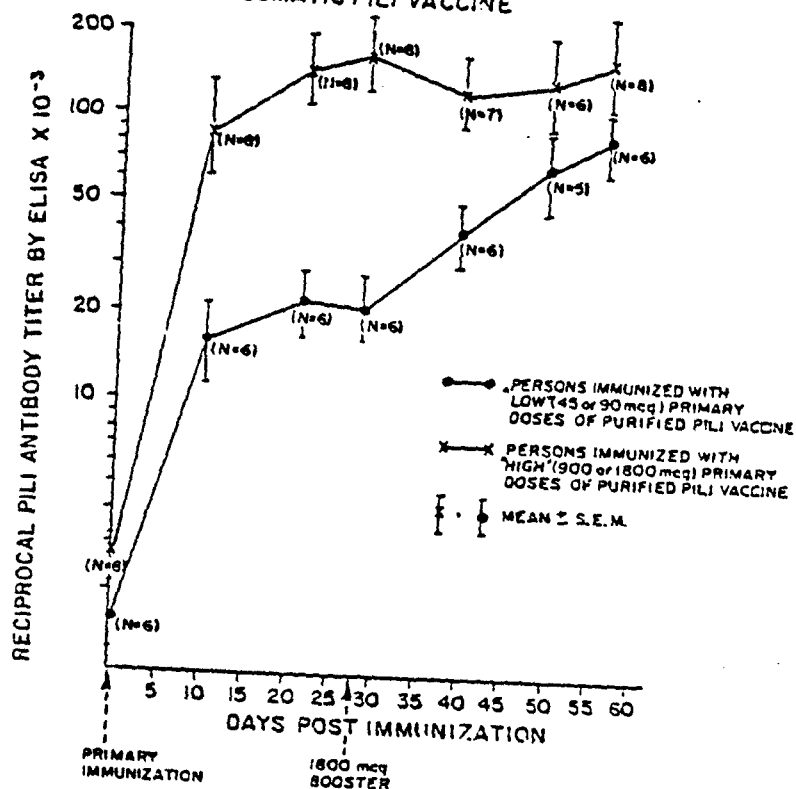


Figure 1

M.M. Levine et al

Reactogenicity, Immunogenicity
and Efficacy Studies of Escher. hibi
coli Type 1 Somatic Pili Parenteral
Vaccine in Man.

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