# IMMUNOLOGIC CONTROL OF DIARRHEAL DISEASE DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI

ANNUAL REPORT

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JANUARY 1984

(covering contract period 1/1/83 to 12/31/83)

Supported by:

U.S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3074

Center for Vaccine Development

Department of Medicine

University of Maryland School of Medicine

Baltimore, Maryland 21201





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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

| REPORT DOCUMENTATION PAGE | | READ INSTRUCTIONS BEFORE COMPLETING FORM |
|---|---|---|
| 1. REPORT NUMBER | 2. GOVT ACCESSION NO. AD-A155812 | 3. RECIPIENT'S CATALOG NUMBER |
| 4. TITLE (and Subtitle) Immunologic Control of Diarrheal Disease Due to Enteotoxigenic Escherichia coli | | 5. TYPE OF REPORT & PERIOD COVERED Annual Report Period 1/1/83-12/31/83 |
| | | 6. PERFORMING ORG. REPORT NUMBER |
| 7. AUTHOR(s) Myron M. Levine, M.D., D.T.P.H., Robert E. Black, M.D., M.P.H., Mary Lou Clements, M.D., M.P.H., James B. Kaper, Ph.D. | | 8. CONTRACT OR GRANT NUMBER(s) DAMD17-83-C-3074 |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS Center for Vaccine Development Department of Medicine University of Maryland School of Medicine Baltimore, Maryland 21201 | | 10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 63750A.3M463750D808-AA-027 |
| 11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command, Fort Detrick, Fredrick, Maryland 21701-5012 | | 12. REPORT DATE January 1984 |
| | | 13. NUMBER OF PAGES 51 |
| 14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) | | 15. SECURITY CLASS. (of this report) unclassified |
| | | 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE |

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Enterotoxigenic Escherichia coli; Diarrheal Disease; CFA/I, CFA/II ;Immunization Salmonella shigella; Oral Vaccine;Genetically Enginerred Vaccine

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A long-term program has been undertaken to develop effective immunizing agents to control enterotoxigenic Escherichia coli (ETEC) diarrheal disease. Acute travelers' diarrhea is a major cause of loss of effectiveness in United States Military personnel assigned in less-developed areas and that ETEC are the most frequent etiologic agent of acute travelers' diarrhea. Two separate approaches are being followed to develop vaccines against ETEC. One involves purification of colonization factor antigen fimbriae (pili) and their use as oral vaccines. The second approach involves a genetically-engineered (cont.)

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20. ABSTRACT:

non-enterotoxigenic strain to be used as an oral vaccine.

Another major pathogen responsible for travelers' diarrhea and dysentery in military personnel is Shigella. A close collaboration with the Department of Bacterial Diseases of the Walter Reed Army Institute of Research involves clinical studies to assess the safety, immunogenicity and efficacy of candidate oral Shigella vaccines developed by Dr. S.B. Formal and co-workers in the Department of Bacterial Diseases at WRAIR.

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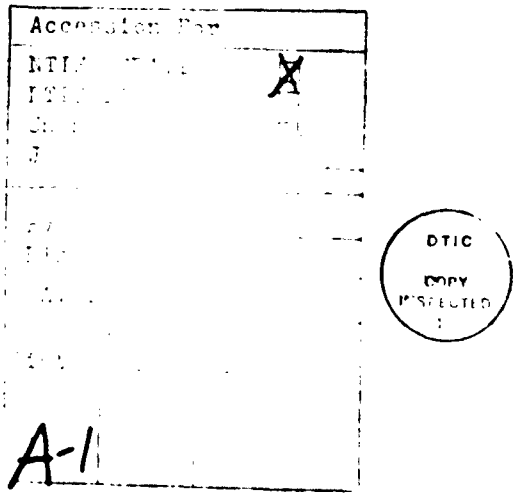
## FOREWORD

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) 78-23, Revised 1978).



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BACKGROUND:

A long-term program has been undertaken to develop effective immunizing agents to control enterotoxigenic Escherichia coli (ETEC) diarrheal disease. This program stems from the recognition that acute travelers' diarrhea is a major cause of loss of effectiveness in United States Military personnel assigned in less-developed areas and that ETEC are the most frequent etiologic agent of acute travelers' diarrhea (1-12).

Two separate approaches are being followed to develop vaccines against ETEC. One involves purification of colonization factor antigen fimbriae (pili) and their use as oral vaccines to stimulate antibodies that will prevent attachment of ETEC to mucosa of the proximal small intestine, the anatomic site that ETEC must colonize in order to cause diarrhea. This work involves collaboration with the Department of Gastroenterology, Walter Reed Army Institute of Research (WRAIR). The second approach to prevention of ETEC being followed at the CVD involves a genetically-engineered non-enterotoxigenic strain to be used as an oral vaccine. Each approach has potential advantages as well as drawbacks.

Another major pathogen responsible for travelers' diarrhea and dysentery in military personnel is Shigella. While a less frequent cause of illness than ETEC, the severity of the clinical syndrome due to Shigella makes its prevention an important objective. Therefore, we have been involved in a close collaboration with the Department of Bacterial Diseases of the Walter Reed Army Institute of Research. This collaboration involves clinical studies to assess the safety, immunogenicity and efficacy of candidate oral Shigella vaccines developed by Dr. S.B. Formal and co-workers in the Department of Bacterial Diseases at WRAIR.

WORK ACCOMPLISHED DURING 1983 CONTRACT YEAR

The major achievements during the 1983 contract year so far include:

1.) Investigation of the antigenic composition of purified colonization factor antigen II (CFA/II) vaccine in view of recent reports of antigenic heterogeneity within the entity referred to as CFA/II.

2.) Purification to homogeneity of the CS1 and CS3 antigens of CFA/II.

3.) Studies of the safety and immunogenicity of purified CFA/II fimbriae oral vaccine in man.

4.) Selection of a CFA/II-positive ETEC challenge strain for efficacy studies of CFA/II vaccine in volunteers.

5.) A challenge study in volunteers to assess the efficacy of purified CFA/II oral vaccine.

6.) Study of the immune response in man to CS1 and CS3 antigens following immunization with CFA/II vaccine or following experimental challenge with pathogenic ETEC bearing CFA/II.

7.) Modification of a plasmid containing cloned genes encoding the production of the B subunit of human LT and insertion of the plasmid into a non-enterotoxinogenic E. coli strain bearing CFA/II.

8.) Studies in volunteers to assess the safety, immunogenicity and efficacy of an oral Salmonella typhi/Shigella sonnei bivalent vaccine.

Definitions

Heretofore in the scientific literature the terms pili and fimbriae have been used rather interchangeably to refer to the non-flagellar, hair-like appendages found on the surface of many bacteria. Recently, several groups of investigators have suggested restricting the term pili to refer to sex pili, the structures involved in bacterial conjugal transfer of DNA. This would

leave the term fimbriae to refer to the remaining surface organelles including type 1 somatic fimbriae and various mannose-resistant hemagglutinins and epithelial cell adhesins.

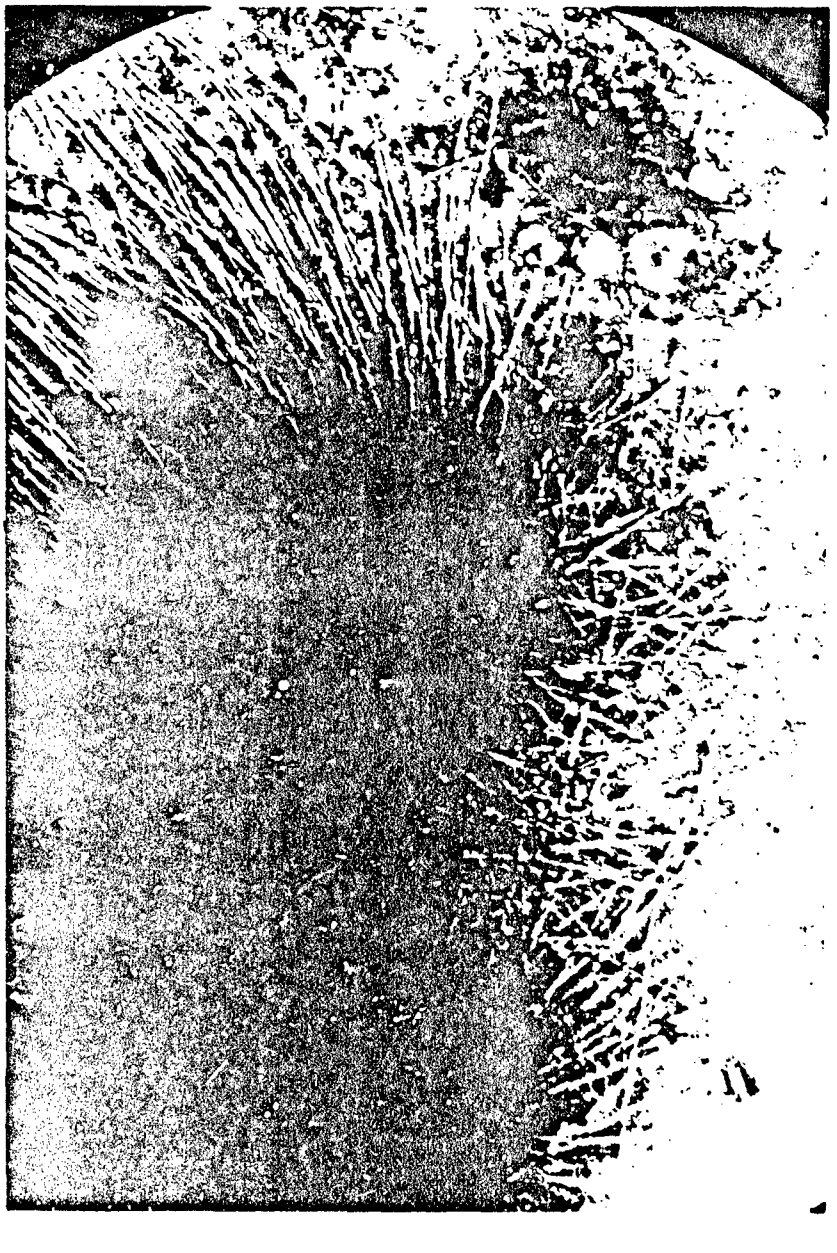
It should be noted that most "classic" fimbriae are long, rigid structures 4-8 nm in width that are easily visible with negative staining techniques in electron-photomicrographs (Fig. 1). Some examples are somatic type 1, 987P, and CFA/I fimbriae. However, some fimbriae are extremely thin (2-3 nm in width), wiry rather than rigid, and difficult to visualize in electron photomicrographs. Examples of the thin type (which are sometimes called fibrils) include K88 and F41 fimbriae (13).

Please note that whereas last year we used the term CFA/II pili, henceforth we will refer to CFA/II fimbriae.

Antigenic Heterogeneity within CFA/II

The fimbrial antigen CFA/I was first described in an ETEC strain of O78:H11 serotype (14). Antisera to this fimbrial antigen was originally prepared by absorption technique (14). Rabbits were immunized with O78:H11 bearing CFA/I (strain H10407). Immune sera were then repeatedly absorbed with an O78:H11 strain (H10407P) lacking CFA/I until the antiserum failed to agglutinate the latter but still agglutinated the former bacteria. Even using these early sera prepared by absorption it was clear that CFA/I fimbriae found on diverse serotypes were antigenically homogeneous. As purified CFA/I and antisera against purified CFA/I became available, these initial impressions of antigenic homogeneity were confirmed.

Later Evans et al (15) described a second fimbrial surface antigen associated with ETEC of O serogroups distinct from CFA/I and termed it CFA/II. The prototype strain of Evans et al, PB176, was serotype O6:H16. As other investigators began to study ETEC for the prevalence of CFA/II, it became apparent that a degree of antigenic heterogeneity existed. The first



Electron photomicrograph of E. coli bearing CFA/I fimbriae

FIGURE 1

definitive study of antigenic heterogeneity within the entity CFA/II was published in 1982 by Cravioto et al (16). These workers prepared heat-saline extracts of the surface antigens of a series of ETEC strain of several serotypes and biotypes bearing "CFA/II". They also prepared anti-CFA/II antisera by the absorption method utilizing CFA/II-positive E. coli of several serotypes and biotypes as the vaccines in rabbits and CFA/II-negative variants for the absorptions.

Using the above-mentioned E. coli surface antigen extracts and antisera in immunodiffusion in agar studies, Cravioto et al (16) made the following observations:

1.) virtually all CFA/II-positive E. coli possess a common surface antigen which they called component 3 (some strains have only component 3);

2.) in addition to component 3, depending on serotype and biotype, most CFA/II-positive strains possess one of two other surface antigens referred to as components 1 and 2. Components 1 and 2 were noted to migrate more slowly in the immunodiffusion gels than component 3. Cravioto et al noted that when examined under the electronmicroscope, they found components 1 and 2 to be fimbriae, whereas component 3 was considered to be a nonfimbrial surface antigen.

In late 1982, C.J. Smyth (17) published a paper that further investigated heterogeneity in CFA/II. Smyth also described three antigens which he referred to as coli surface antigens 1 (CS1), 2 (CS2) and 3 (CS3). These turned out to be identical to components 1, 2 and 3 of Cravioto et al (16). Smyth partially purified CS1, CS2 and CS3. By treating the partially-purified antigens with sodium dodecyl sulfate (SDS) and subjecting the preparations to electrophoresis in polyacrylamide gel (PAGE), he was able to assign approximate molecular weights to the protein subunits that make-up the

fimbrial polymers. CS3 was approximately 14.8 kDal, CS2 15.3 kDal and CS1 16.3 kDal in size. Smyth reported that CS1 and CS2 were strong hemagglutinins, while CS3 was not a hemagglutinin.

Characterization of our CFA/II Purified Fimbriae Vaccine in Light of New Information

The CVD-WRAIR CFA/II vaccine was prepared from E. coli M424-C1 an O6:H16 strain of biotype A (18). The vaccine revealed a single major protein subunit (circa 15 kD) band when subjected to SDS-PAGE. (A very faint, thin second band was barely visible at a MW slightly higher than the major band. Little attention was ever paid to this minor band and its very existence was questioned). When examined under the electron microscope, only one homogeneous type of fimbriae was clearly visible.

Since O6:H16 biotype A strains, according to both Smyth (17) and Cravioto et al (16), produce both CS1 and CS3 antigens, we considered it imperative to re-characterize both our purified CFA/II vaccine as well as the strain from which it was prepared, in light of the new information. Accordingly strain M424-C1 and a sample of our purified CFA/II vaccine were sent to Cyril Smyth at Trinity College, Dublin for analysis. Smyth reported to us that approximately 95% of the protein content of our vaccine was CS3 antigen and corresponded to the major subunit band visible on the SDS-PAGE. Smyth further noted that he does not consider CS3 to be fimbrial in morphology. He reported that the vaccine also contained CS1 antigen which corresponds to a second faint protein subunit band of slightly higher MW visible on SDS-PAGE; this was the very faint band that we had previously disregarded as an artifact due to overloading of the gel.

Smyth also reported to us that the fimbriae visible in electron photomicrographs of the CFA/II vaccine were CS1 fimbriae (representing less than 5% of the total protein in the vaccine) and not CS3 antigen which

comprises 95% of the vaccine protein! Smyth was unable to provide a morphological description of CS3. He was unsure of whether or not it was fimbrial in nature but at that point was agreeing with the Colindale group that it was a non-fimbrial surface antigen.

The above preliminary information came as a bit of a scientific shock to us and raised a series of fundamental questions about the vaccine and about CFA/II that had to be answered in order for us to proceed rationally in further vaccine development. These questions are posed below.

1.) What is the morphologic nature of CS3?

2.) Are both CS1 and CS3 hemagglutinins?

3.) Are both CS1 and CS3 expressed in vivo in the small intestine of man?

4.) Do both CS1 and CS3 function as adhesins attaching E. coli to receptors on enterocytes?

5.) Do both CS1 and CS3 stimulate immunologic responses in man in the course of natural infection with a strain that produces both antigens?

6.) Is an ETEC strain which in vitro produces only CS3 pathogenic for man? Will man mount an anti-CS3 immunologic response to clinical infection with such a strain?

7.) If persons are immunized with purified CFA/II oral vaccine containing both CS3 and CS1 antigens in a ratio of 20:1, will both antigens elicit a serologic response?

It is obvious that characterization of CS1 and CS3 antigens, documentation of their morphology and antigenic relatedness, and measurement of immune responses to these antigens could not proceed without the availability of purified CS1 and CS3 preparations. Since these antigens in pure form had not heretofore been described, the CVD undertook an intensive effort to successfully purify CS1 and CS3 antigens in the shortest possible

time. Minute amounts of anti-CS1 and anti-CS3 prepared by absorption techniques, were provided by C. Smyth and represented an invaluable and precious reagent in these efforts.

Purification of CS1 Antigens

Unfortunately, for the purification of CS1, no naturally-occurring strain has been reported that elaborates only CS1. Therefore, we attempted to purify CS1 from M424-C1 (O6:H16), the strain from which the CFA/II vaccine is made. This strain produces both CS1 and CS3.

The general approach to purification of CS1 that we took involved the use of ultracentrifugation in an isopycnic cesium cloride gradient, a method adapted by Mr. G. Marley and previously reported to be successful for the purification to homogeneity of CFA/I and type 1 fimbriae.

A suspension of E. coli M424-C1 was streaked onto baking pans containing CFA agar. Following overnight incubation at 37°C, the bacterial cells were harvested, suspended in 20 ml phosphate buffered saline (pH 7.2), and sheared in a Sorvall Omnimixer. The sheared homogenate was clarified by repeated centrifugation (8000 x g). Outer membrane vesicles were removed by centrifugation at 18,000 rpm (SS-34 rotor). The supernatant (containing fimbriae) was removed and centrifuged at much higher speed, 39,000 rpm (SW-41 rotor). The pellet, which now contains fimbriae, was resuspended in 2ml PBS.

The suspension containing fimbriae was applied to an isopycnic cesium chloride gradient of density 1.29 g/cm$^3$. Following ultracentrifugation (SW 41 rotor, 36,000 rpm overnight), distinct hydrophobic bands were noted close to the meniscus. These bands were collected by puncturing the side of the tube with a 21 gauge needle and aspirating with a syringe.

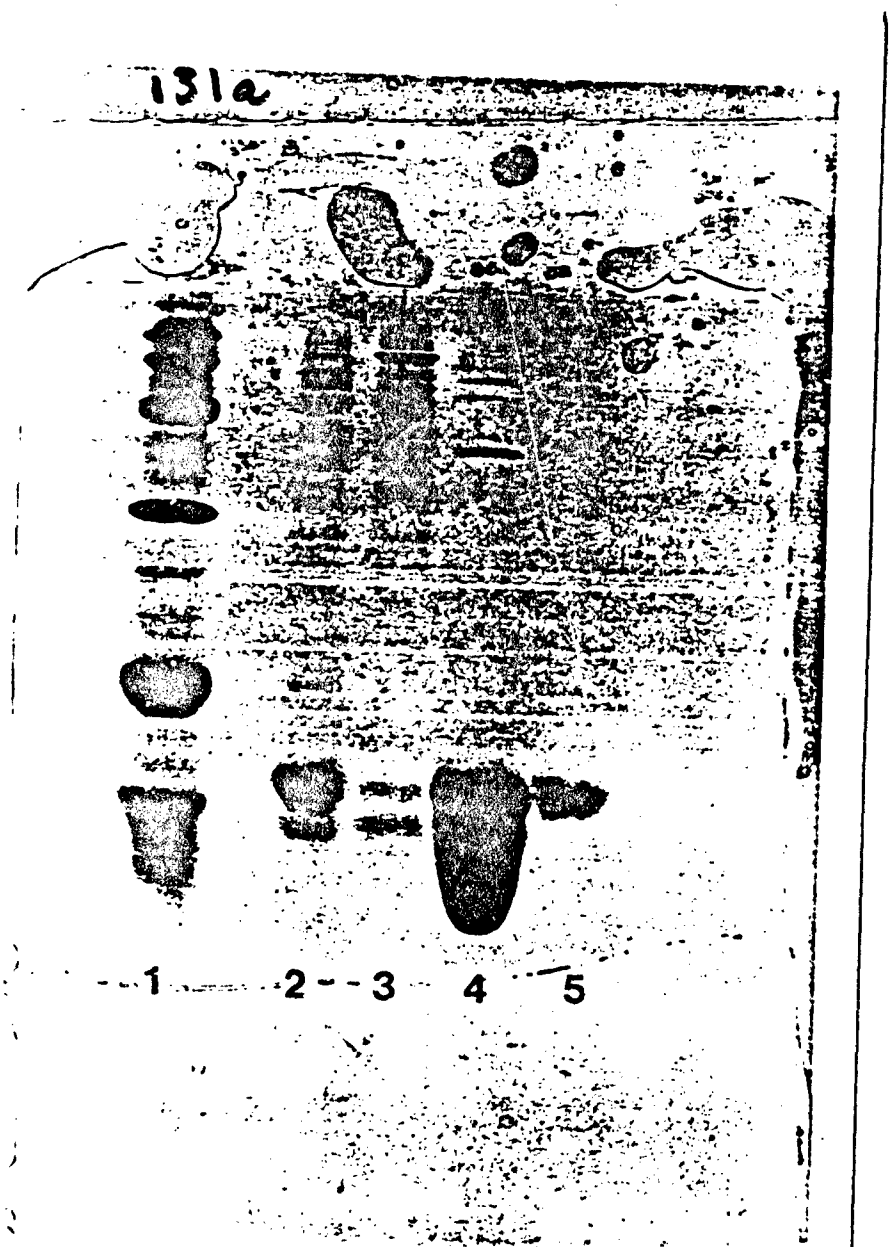
The presence of CS1 and CS3 in the bands was monitored by Ouchterlony immunodiffusion in gel studies using Smyth's antisera and characterization of subunits by SDS-PAGE, and immunoblotting (Western blotting) technique using

CS1 and CS3 antisera (19,20). Several successive isopycnic gradients were required to completely separate CS1 from CS3. CS1 and CS3 banded at buoyant densities close to one another. However, with repeated gradients it was possible to isolate pure CS1 bands from bands at other buoyant densities that contained CS3 and outer membrane proteins. The yield of CS1 was only 150 mcg per pan of harvested bacterial cells. Nevertheless, the material obtained, albeit in low yield, was shown to be pure CS1: 1) on SDS-PAGE only a single protein subunit band was visualized (Fig. 2, lane 5), 2) the purified material formed precipitin lines with anti-CS1 but not with anti-CS3 in Ouchterlony immunodiffusion tests, 3) when tested with CS1 and CS3 antisera by the sensitive Western blot technique, reaction occurred only with CS1 antiserum (Fig. 3, see legend). Analysis of this material by electron microscopy revealed fimbriae of a uniform type (Figure 4). This purified CS1 was utilized as antigen to measure anti-CS1 in sera and intestinal fluids of vaccinated and challenged volunteers.

With the arrival of a senior biochemist in July (Dr. R. Patnaik), we began devising modifications to greatly increase the yield and step up the rate of production of purified CS1. This will provide purified material for use an antigen in further serological studies as well as antigen for immunization of rabbits to obtain pure anti-CS1.

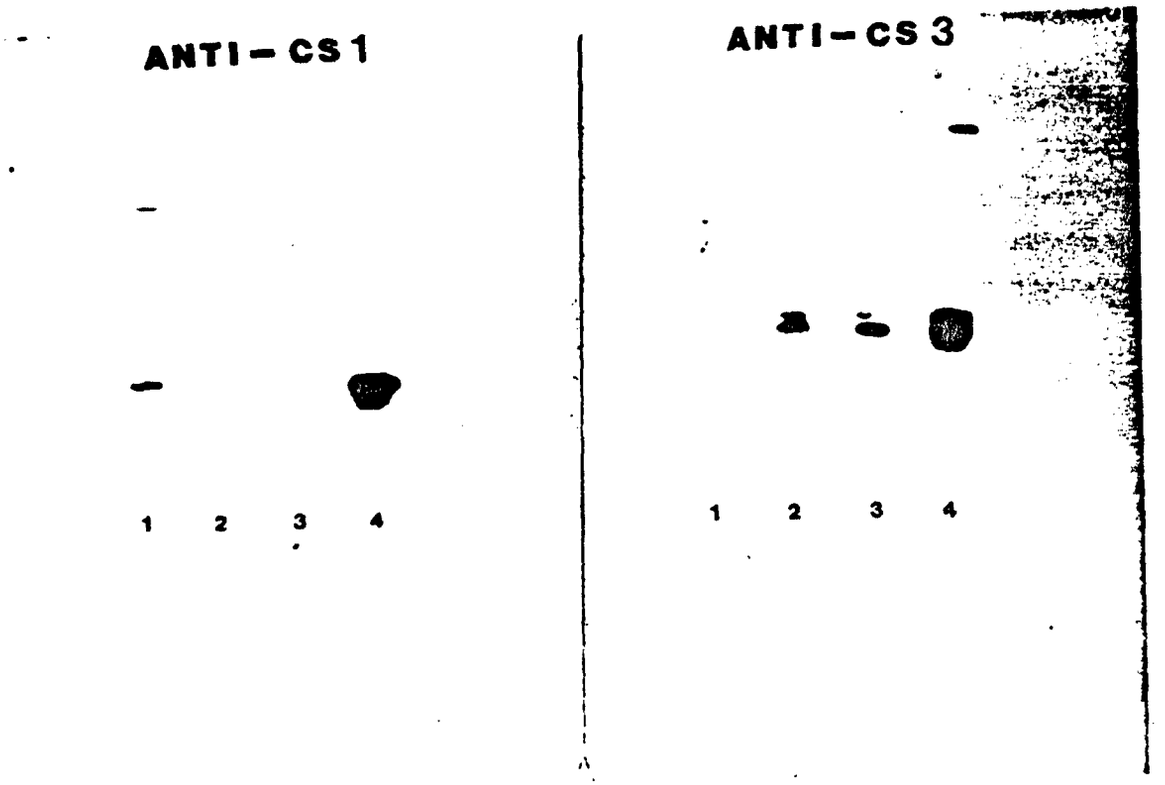
Purification of CS3 Antigen

For purification of CS3 antigen we selected an ETEC strain, E9034A (O8:H9), that in vitro elaborates only CS3 antigen. Ms. Polly Ristaino of the CVD Electron Microscopy Laboratory examined bacterial cells of strain E9034A by negative staining technique. On approximately 5% of agar-grown cells she was able to visualize extremely fine (1-3 nm), wiry, fibrillar type fimbriae. Pans of CFA agar were heavily inoculated with E. coli E9034A and incubated overnight. Bacterial cells were harvested and sheared by standard technique,



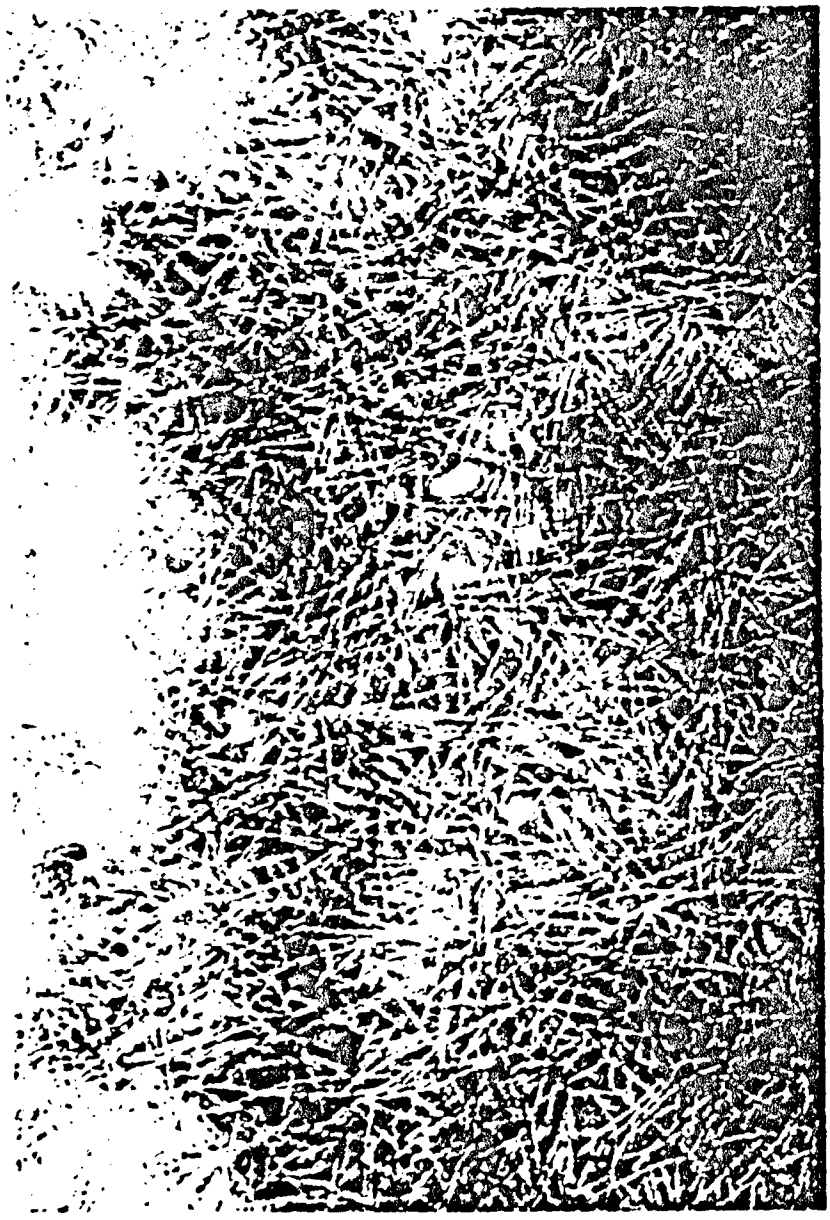

Sequential purification of CS-1 antigen run on 20% SDS-PAGE.
Lane 1: Molecular weight markers (92.5 kDal, 66.2 kDal, 45.0 kDal, 31.0 kDal, 21.5 kDal, 14.4 kDal)
lanes 2-5: Results of successive isopycnic CsCl gradients; Lane 5 represents purified CS-1 antigen.

FIGURE 2




Western Blot analysis of purified CS-1 and CS-3 antigens with monospecific antisera.
Lane 1: CS-1 preparation
Lanes 2 & 3: CS-3 preparations
Lane 4: Vaccine prepared from ETEC strain M424/C1

FIGURE 3



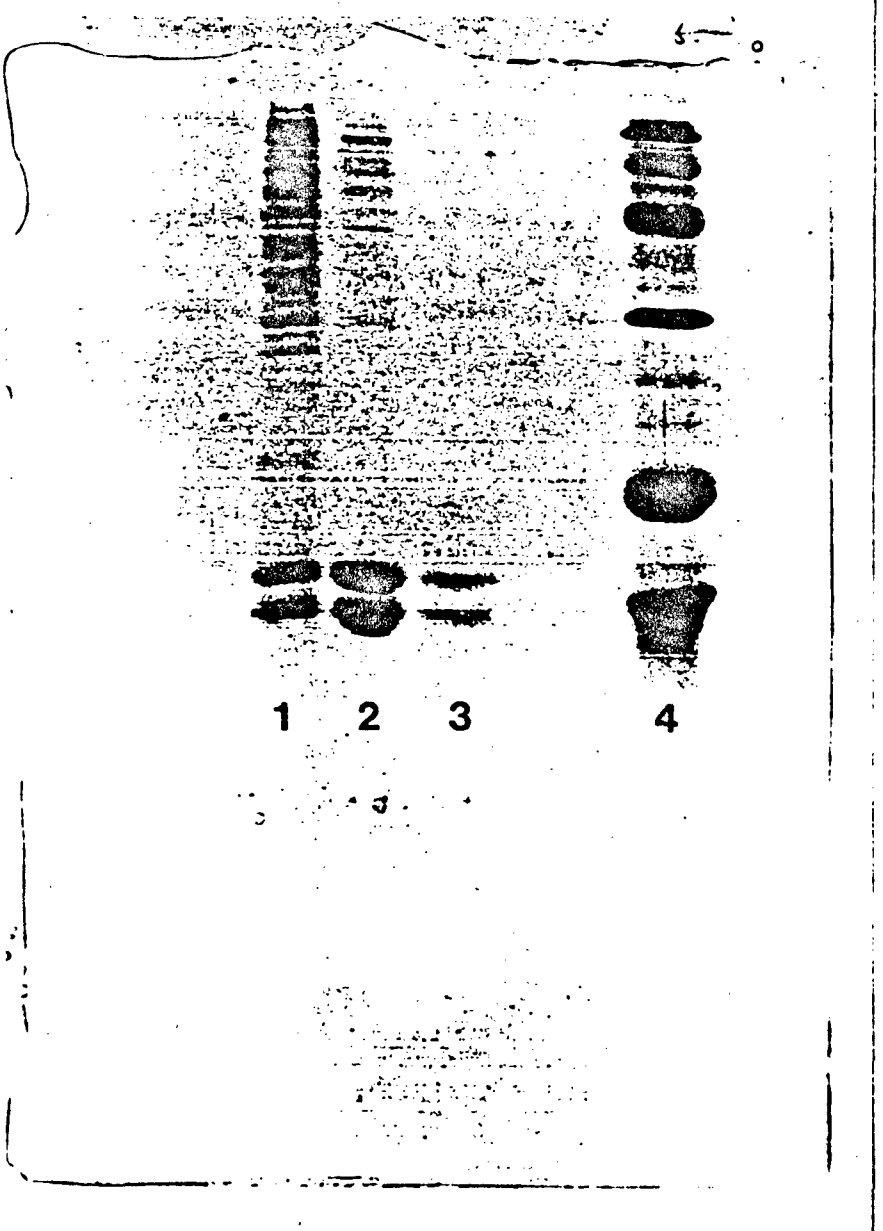
P-2
CS1 from M424C1 Wnlags - Topband U
1% DTA
30,100x

FIGURE 4 - Electron photomicrograph of purified CS1 antigen. Homogeneous fimbriae are visible.

and centrifuged 12,000 rpm x 30 min. The supernatant was filtered through a sterile 0.8 mu Millipore and the filtrate was subjected to 20% and 40% sequential ammonium sulfate precipitations. The 40% precipitate was saved and dialysed against 0.1 M sodium phosphate (pH 7.0) to a final volume of 11 ml. This material was chromatographed on a Sepharose CL-6B (Pharmacia) column (2.5 x 20 cm) which had been equilibrated in 100 mM sodium phosphate with 6 M guanidine hydrochloride. (The 6 M guanidine denatures fimbriae polymers into their individual subunits.) Thirteen 3 ml fractions from this column were collected, dialysed against phosphate buffer and analyzed by SDS-PAGE. (The dialysis removes the guanidine, thereby creating conditions in which the subunits reaggregate to form fimbriae.)

Middle fractions from the Sepharose CL-6B column, which revealed the most intensely staining low molecular weight bands on the SDS-PAGE, were pooled (Fig. 5, lane 2). These fractions were contaminated with high MW protein (Fig. 5, lane 2). Guanidine HCl (6 M) was added to the pooled material to denature fimbriae into subunits, whereupon the material was applied to a Sephacryl S-200 (Pharmacia) column which had been equilibrated with 100 mM sodium phosphate and guanidine HCl. Twenty 5 ml. fractions were collected, dialysed against 100 mM sodium phosphate buffer (pH 8.0) and analyzed by SDS-PAGE (Fig. 5, lane 3). Two fractions eluted from the Sephacryl S-200 column revealed only two small polypeptide bands upon Coomassie Blue staining with MW of approximately 14.5 kDal and 15.5 kDal (Fig. 5, lane 3). Contamination with high MW material was no longer evident.

Although the high MW contaminants were removed by Sephacryl S-200 chromatography, we were left with two distinct subunits, very similar in MW (Fig. 5, lane 3). By Ouchterlony immunodiffusion studies we showed that this material formed precipitin lines with CS3 but not CS1 antibody. However, it was not clear if only one or both of the two subunits were CS3. In order to




Purification of CS-3 antigen run on 20% SDS-PAGE.

Lane 1: 40% ammonium sulfate precipitate from E9034 homogenate
Lane 2: Pooled fractions from Sepharose CL-6B colum.
Lane 3: Pooled fractions from Sephacryl S-200 column.
Lane 4: Molecular weight markers (92.5 kDal, 66.2 kDal, 45.0 kDal, 31.0 kDal, 21.5 kDal, 14.4 kDal)

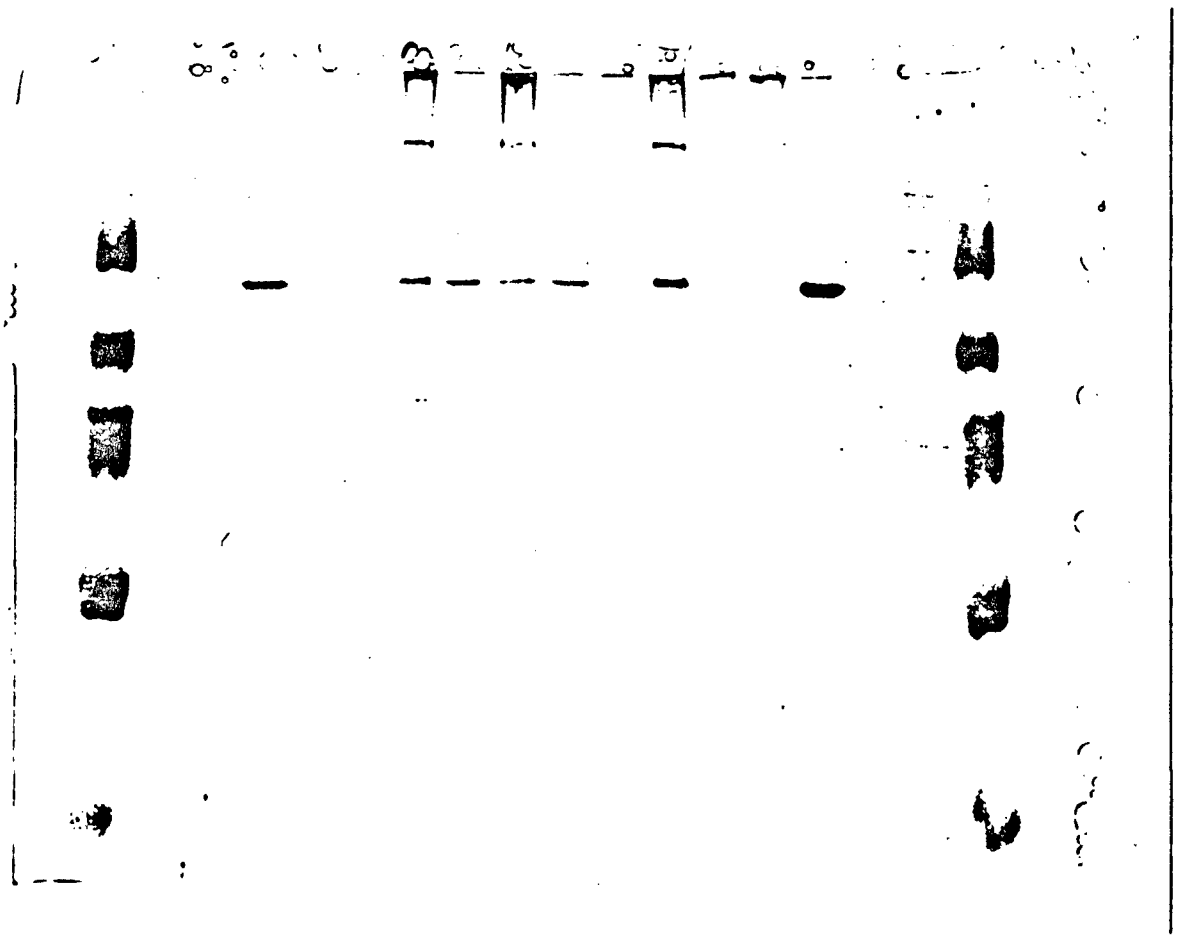
FIGURE 5

answer this question we carried out Western blot analysis (immunoblotting) by the technique of Towbin et al (19) and Burnett (20) with minor modifications. Western blot results revealed that both low molecular weight bands reacted specifically with CS3 antiserum (Fig. 3). Neither band reacted with CS1 antiserum (Fig. 3).

A non-denaturing PAGE (i.e. without SDS) showed that the Sephacryl S-200 column fractions that had been dialysed against 0.1 M sodium phosphate (pH 8.0) were reaggregated into large polymers (Fig. 6). This purified CS3 antigen was examined under the electron microscope using negative staining by Ms. Polly Ristaino. She was unable to visualize typical fimbriae; nor did she see the very thin, wiry, fibrillar type of fimbriae that she had visualized on the surface of strain E9034A, from which the CS3 had been purified. Recognizing that some fibrillar forms of fimbriae (e.g. F41 fimbriae) are notoriously difficult to visualize by electron microscopy, we sent strain E9034A and the purified CS3 antigen to Dr. Sam To, Pitman Moore Co., New Jersey. He is highly recognized as an electron microscopist who is particularly skilled at visualization of bacterial surface organelles. Sam To confirmed Ms. Ristaino's observation of thin, wiry fimbriae on E9034A and also was able to visualize and record these fibrillar type fimbriae in the purified CS3 preparation. He is currently preparing high quality electron photomicrographs for us.

Safety and Immunogenicity of Purified CFA/II (CS1, CS3) Oral Vaccine in Man

Ten healthy adult volunteers received eight 1.7 mg doses of oral CFA/II vaccine (two doses weekly for four weeks). Vaccine was given with 2.0 gm $NaHCO_3$ and 90 minutes after 300 mg cimetidine to counteract possible adverse effects of gastric acid. No vaccinee experienced nausea, vomiting, fever, diarrhea, notable abdominal cramps or other adverse reactions.



Non-denaturing gel (15% acrylamide) of reaggregated samples from isopycnic CsCl gradient of crude M424/C1.
Outer Lanes: Molecular weight markers (92.5 kDal, 66.2 kDal, 45.0 kDal, 31.0 kDal, 21.5 kDal, 14.4 kDal)

FIGURE 6

Sera were collected before and 10, 21 and 28 days after the onset of vaccination. Jejunal fluids were collected prior to and on the 14th and 29th days following initiation of vaccination. A summary of the serologic results is shown in Table 1. Only two of 10 vaccinees had significant rises in serum antibody tested against the CFA/II (CS1, CS3) vaccine as antigen by IgG-ELISA methodology. These sera were then tested against partially-purified and purified CS1 and CS3 antigens. Both vaccinees had rises against partially-purified CS antigen (which contained high MW outer membrane protein contaminants), and one had a significant rise against purified CS3. Neither of these two volunteers manifested significant rises in serum antibody against purified CS1.

The same two vaccinees who exhibited significant rises in serum IgG-ELISA antibody to the vaccine as antigen, also demonstrated significant rises in SIgA antibody in jejunal fluid when tested against vaccine antigen by IgA-ELISA. These vaccinees did not manifest significant rises when their jejunal fluids were tested against purified CS1 and CS3 antigens.

It is not yet clear why in these preliminary serologic assays the sera and jejunal fluids of the few vaccinees who manifested rises against vaccine as antigen usually did not react with purified CS1 and CS3 antigens. It is possible that the small amount of high MW outer membrane protein that remains in the CFA/II vaccine is reacting with antibody in the ELISA. It is also possible that the conditions of purification of CS1 and CS3 antigens, as described in the previous sections, altered the proteins, rendering them less able to combine with antibody. Lastly, it is possible that the CFA/II oral vaccine, was in fact poorly immunogenic in man and stimulated minimal, barely detectable, levels of antibodies. These antibodies may have been of low avidity and unable to combine with the highly manipulated, purified CS1 and

TABLE 1

SUMMARY OF SERUM AND INTESTINAL FLUID ANTIBODY RESPONSE IN VOLUNTEERS IMMUNIZED WITH ORAL CFA/II (CS1, CS3) VACCINE*

| | | FIMBRIAL ANTIGENS: | | | |
|---|---|---|---|---|---|
| | CFA/II VACCINE (CS1, CS3) | PARTIALLY-PURIFIED CS3 | PURIFIED CS3 | PARTIALLY-PURIFIED CS1 | PURIFIED CS1 |
| SIGNIFICANT RISES IN SERUM IgG ANTIBODY** | 2/10† | 2/10 | 1/10 | 0/10 | 0/10 |
| SIGNIFICANT RISES IN INTESTINAL SIgA ANTIBODY** | 2/10 | 0/10 | 0/10 | 0/10 | NT†† |

* 1.7 MG TWICE WEEKLY FOR 4 WEEKS.

† NO. WITH SIGNIFICANT SEROCONVERSIONS/NO. VACCINEES TESTED.

** MEASURED BY ELISA.

†† NT, NOT TESTED.

CS3. The greater frequency of seroconversions to partially purified and purified CS3 rather than CS1 antigens could easily be explained by the fact that the vaccine contained 20 times more CS3 than CS1.

As we will note in subsequent sections, the highly purified CS1 and CS3 antigens were in fact quite useful in detecting antibody responses in persons who participated in experimental challenge studies with ETEC possessing CS1 and CS3. Therefore, the purified antigens cannot be faulted. Since we have repeatedly detected brisk and prominent SIgA anti-CFA/II responses in intestinal washings of rabbits mucosally immunized by Col. Boedeker and his group (21,22), the ability of the vaccine as an immunogen (at least if applied directly to the mucosa) has been established.

It cannot be ruled out that the CFA/II vaccine was adversely affected by gastric contents (despite our precautions) or by intestinal proteases. To at least rule out the possible adverse effect of gastric acidity, we propose to administer vaccine next time directly into the duodenum of vaccinees via intestinal tube.

SELECTION OF CFA/II-POSITIVE ETEC CHALLENGE STRAIN FOR VACCINE EFFICACY STUDIES

It was necessary to identify an ETEC strain to utilize in challenge studies to assess vaccine efficacy. We required a strain that possesses the same antigenic components of CFA/II as contained in our vaccine (CS1 and CS3 antigens) but of a serotype distinct from O6:H16, the strain from which the vaccine was prepared. Two potential challenge strains were kindly provided by Bernard Rowe, Central Public Health Laboratory, Colindale. These include strain E24377A (O139:H28), which produces LT and ST and elaborates both CS1 and CS3, and strain E9034A (O8:H9), which produces LT and ST but in vitro elaborates only CS3. There were several justifications for our interest in the latter strain even though it did not appear (at least in vitro) to produce

CS1. First, our vaccine is composed of 95% CS3 and only 5% CS1 antigen. Second, we felt it important to determine if a strain that lacks CS1 and produces only CS3 following in vitro culture would be pathogenic for man. Third, we wished to determine if we could indirectly elicit evidence of in vivo production of CS1 by this strain by means of measuring antibody to CS1. Lastly, O8:H9 is a very common CFA/II serotype, second only to O6:H16.

Based on considerable experience with multiple other ETEC challenge strains for which $5x10^8$ organisms is the optimal challenge dose (giving rise to diarrhea in 50-70% of volunteers), we administered $5x10^8$ organisms (with $NaHCO_3$) of strain E24377A to five volunteers, while five others received the same dose of strain E9034A. As seen in Table 2, four of five who received the former, and two of five who ingested the latter developed typical travelers'diarrhea syndrome.

Preliminary Challenge Study to Assess the Efficacy of Oral CFA/II Vaccine

Approximately one month after completion of vaccination, eight vaccinees agreed to participate in a challenge study along with nine unimmunized control volunteers. All were given $5x10^8$ organisms of strain E24377. Six of nine controls developed diarrhea (67%) versus three of eight vaccinees (38%) (vaccine efficacy 43%). (Table 3) The severity of illness was comparable in the two groups.

In view of the poor antibody response that followed initial studies of this oral vaccine in these volunteers, the failure to observe more potent protection is not surprising. We shall now have to determine the factors responsible for the surprisingly meager antibody response following oral administration of this vaccine to volunteers, in contrast with the striking mucosal antibody response that was observed when intestinal loops of rabbits were directly vaccinated.

Table 2

CLINICAL, BACTERIOLOGIC AND IMMUNOLOGIC RESPONSE OF VOLUNTEERS

FOLLOWING INGESTION OF ENTEROTOXIGENIC E. COLI STRAINS

E24377A (CS1, CS3) AND E9034A (CS3)

| Challenge Strain | Diarrhea | Mean Diarrheal Stool Volume for Ill Volunteer | Mean No. Loose Stools per Ill Volunteer | Positive Coprocultures | Significant Rise in Serum IgG Antibody to: Vaccine (CS1,CS3) | Purified CS3 | Purified CS1 |
|---|---|---|---|---|---|---|---|
| E24377A* | 4/5† | 1081 ml (498-2135) | 10.5 (5-27) | 5/5 | 2/5 | 1/5 | 1/5 |
| E9034A* | 2/5 | 1443 (775-2092) | 7 (5-9) | 5/5 | 1/5 | 1/5 | 1/5 |

* $5x10^8$ organisms

† No. positive/No. volunteers challenged

TABLE 3

CLINICAL RESPONSE FOLLOWING CHALLENGE OF IMMUNIZED (8 ORAL DOSES OF CFA/II PILI VACCINE) VOLUNTEERS AND CONTROLS WITH ENTEROTOXIGENIC *E. COLI* STRAIN E24377A (O139:H28, CS1, CS3).

| | Diarrheal Attack Rate | Mean Diarrheal Stool Volume per Ill Volunteer | Mean No. Loose Stools per Ill Volunte |
|---|---|---|---|
| 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).

Immune Response to CS1 and CS3 Antigens in Volunteers Following Challenge with CFA/II-Positive ETEC

The challenge strain selection study and the vaccine efficacy challenge study provided sera and intestinal fluids from a total of 19 unimmunized volunteers, 14 of whom were challenged with strain E24377A, which bears both CS1 and CS3, and five of whom received strain E9034A, which elaborates only CS3 in vitro. These specimens were tested for antibodies to CFA/II vaccine and purified CS1 and CS3 antigens. Results of the 14 volunteers who ingested strain E24377A are shown in Table 4. Intestinal fluids collected prior to and eight days after challenge are being tested for SIgA antibody. Preliminary results show that three of nine persons tested so far had significant SIgA-ELISA rises to CFA/II vaccine antigen.

These data demonstrate that following ingestion of an ETEC strain bearing CS1 and CS3, at least one-half of persons manifest significant rises in serum antibody to purified CS1 and CS3 antigens. These data provide evidence that both antigens are expressed in vivo and imply that both may be involved in pathogenesis of diarrhea.

One of five volunteers who ingested strain E9034A manifested a significant rise to CFA/II vaccine used as antigen in an ELISA . This individual also had a very prominent antibody rise to purified CS3 and a less notable, but still significant, rise to CS1. E9034A does not elaborate CS1 in vitro; however, the appearance of a rise in antibodies to purified CS1 antigen suggest that it may be produced in vivo. Although this strain does not produce CS1 in vitro, it possesses the genes for CS1 (H. Smith, personal communication).

TABLE 4

SIGNIFICANT RISES IN SERUM ANTIBODY TO CFA/II VACCINE AND PURIFIED CS1 AND CS3 ANTIGENS MEASURED BY IgG-ELISA IN CONTROL VOLUNTEERS CHALLENGED WITH CFA/II-POSITIVE ENTEROTOXIGENIC E. COLI

| | | Test Antigens | | |
|---|---|---|---|---|
| Volunteer No. | Diarrhea | CFA/II Vaccine | Purified CS3 | Purified CS1 |
| EC 7002-1 | + | - | - | - |
| EC 7002-5 | + | + | + | + |
| EC 7002-6 | - | + | - | - |
| EC 7002-8 | - | + | + | + |
| EC 7002-10 | + | - | + | - |
| EC 7002-11 | - | - | - | - |
| EC 7002-13 | + | + | + | + |
| EC 7002-15 | + | + | + | + |
| EC 7002-17 | + | + | + | + |
| EC 2027-3 | + | + | - | + |
| EC 2027-5 | + | + | + | + |
| EC 2027-1 | - | - | - | - |
| EC 2027-2 | + | - | - | - |
| EC 2027-4 | - | - | - | - |
| Totals | 9/14 | 8/14 | 7/14 | 7/14 |

Comparison in Rabbits of the Immunogenicity of CFA/II Vaccine Sterilized by Two Different Methods

CFA/II fimbriae vaccine was prepared under the supervision of Dr. Sanford Berman at the Forest Glen facility of WRAIR and the vaccine was sterilized by one of two ways, with gamma radiation or formaldehyde. The two vaccine preparations sterilized in two different ways were used by Col. Boedeker and colleagues to immunize Thiery-Vella intestinal loops of rabbits. Intestinal washings from these loops were sent to the CVD where they were tested by Charles Young for SIgA anti-CFA/II. As shown in Figs. 7 and 8, vaccine sterilized in both ways was immunogenic and stimulated prominent and prominent and equivalent antibody levels. Fig. 9 shows the impressively rapid anamnestic response in the loop of a rabbit stimulated with formalin-sterilized vaccine several months after completion of primary vaccination. As seen in Fig. 10, rabbits immunized with vaccine sterilized by either method developed SIgA antibodies to both CS1 and CS3 as detected by IgA-ELISA using purified CS1 and CS3 as antigen.

Vaccination of Rabbits with CFA/II Vaccine and Challenge by RITARD

Evans et al (23) and de la Cabada et al (24) reported that rabbits orally immunized with purified CFA/I fimbriae were protected from diarrhea when challenged with ETEC in the reversible intestinal tie adult rabbit diarrhea (RITARD) model (25).

We undertook a comparable study with CFA/II vaccine. Thirteen young adult rabbits were given eight 0.8 mg doses of CFA/II oral vaccine (two doses weekly for four weeks). The vaccine was administered with antacid to neutralize gastric acidity. The 13 vaccinated rabbits were challenged one month after completion of vaccination along with 13 control rabbits. In the RITARD method of challenge, an abdominal incision is made in anaesthetized rabbits, the caecum is ligated and a reversible slip knot ligature is put

FIGURE 7

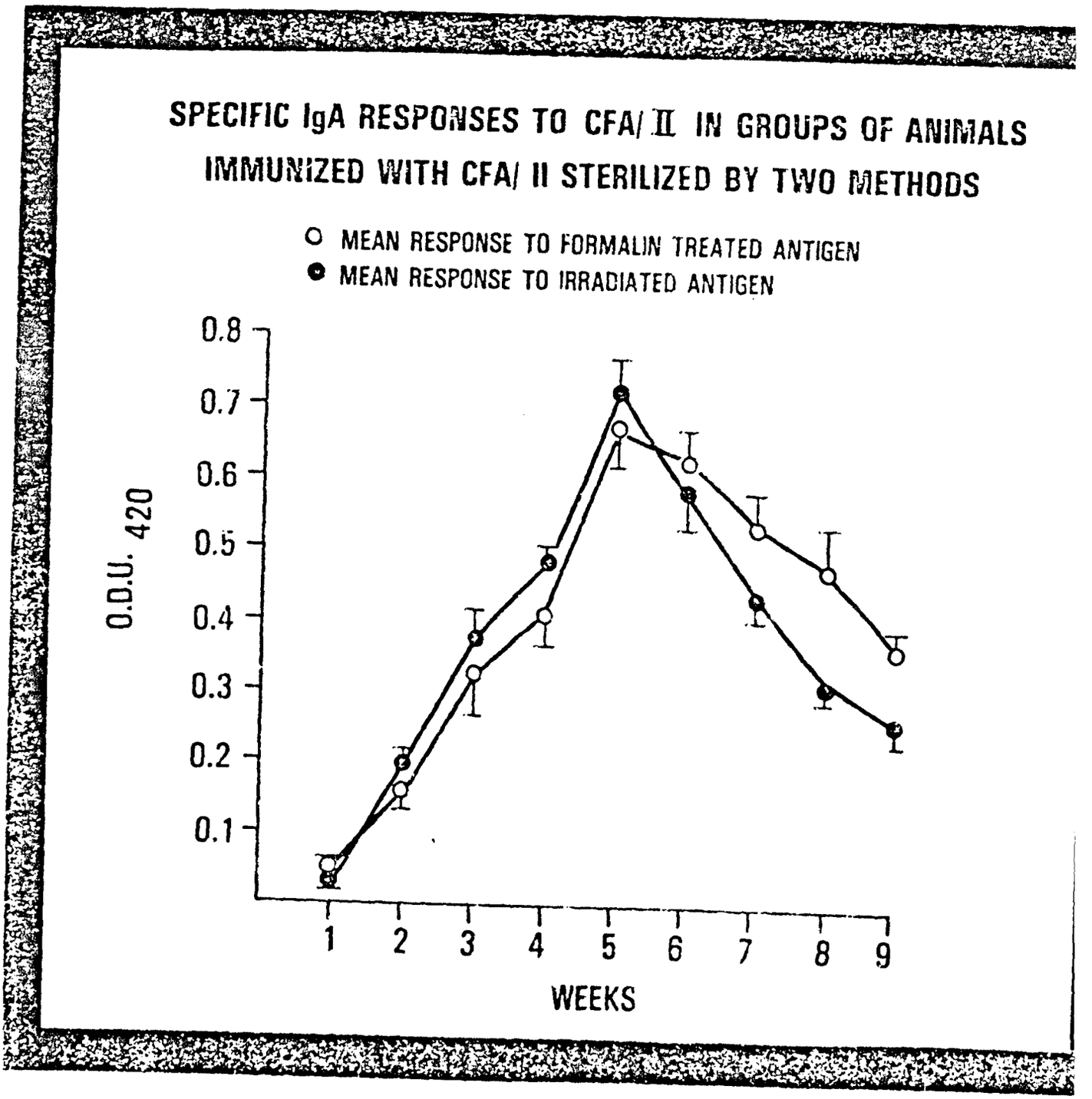

SPECIFIC IgA RESPONSES TO CFA/ II IN GROUPS OF ANIMALS IMMUNIZED WITH CFA/ II STERILIZED BY TWO METHODS
MEAN RESPONSE TO FORMALIN TREATED ANTIGEN
MEAN RESPONSE TO IRRADIATED ANTIGEN
O.D.U. 420
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
WEEKS

FIGURE 8

# SPECIFIC MUCOSAL IgA RESPONSES TO NATIVE CFA/II IN ANIMALS IMMUNIZED WITH CFA/II STERILIZED BY TWO METHODS

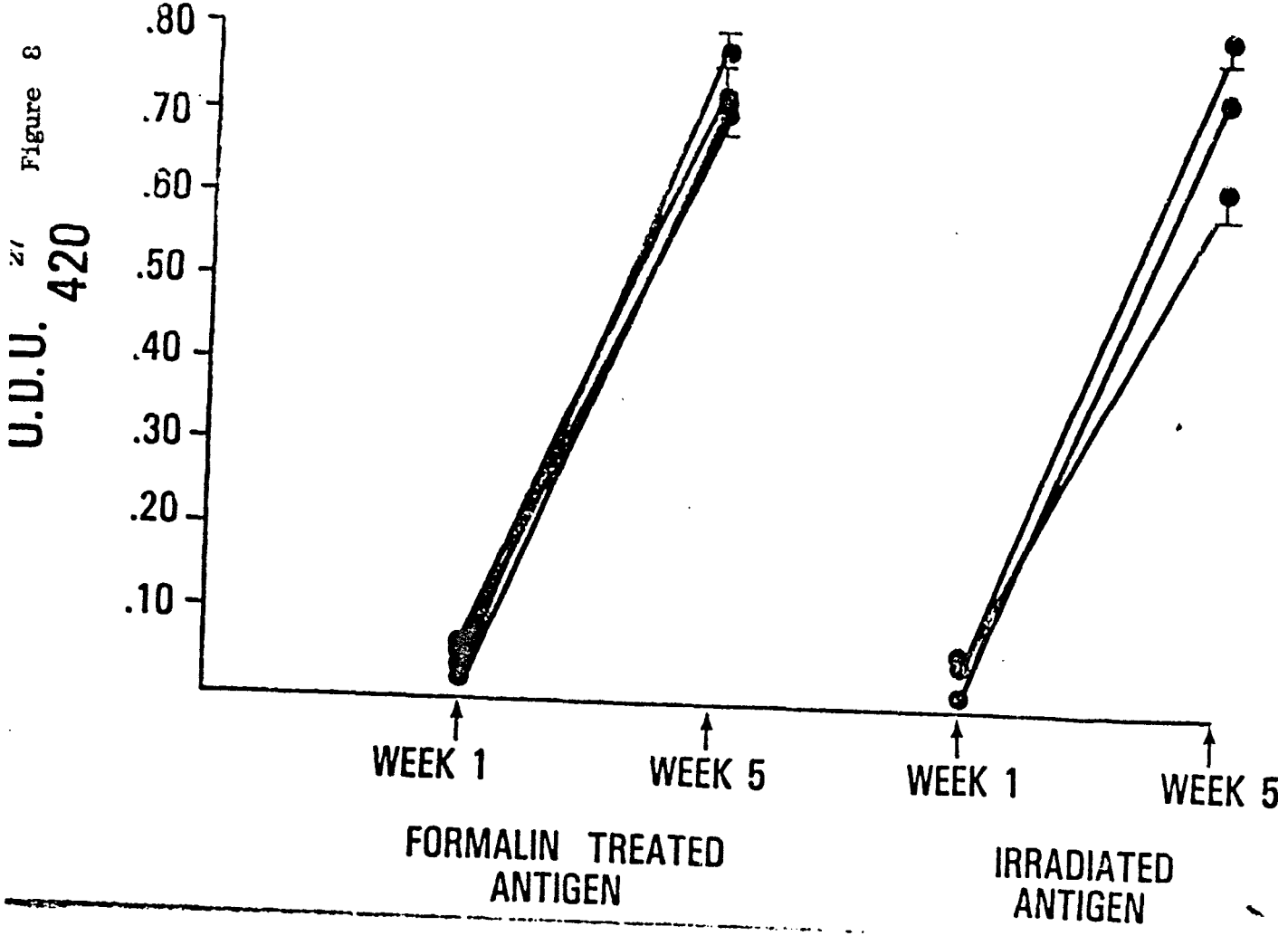



Figure 8

FIGURE 9

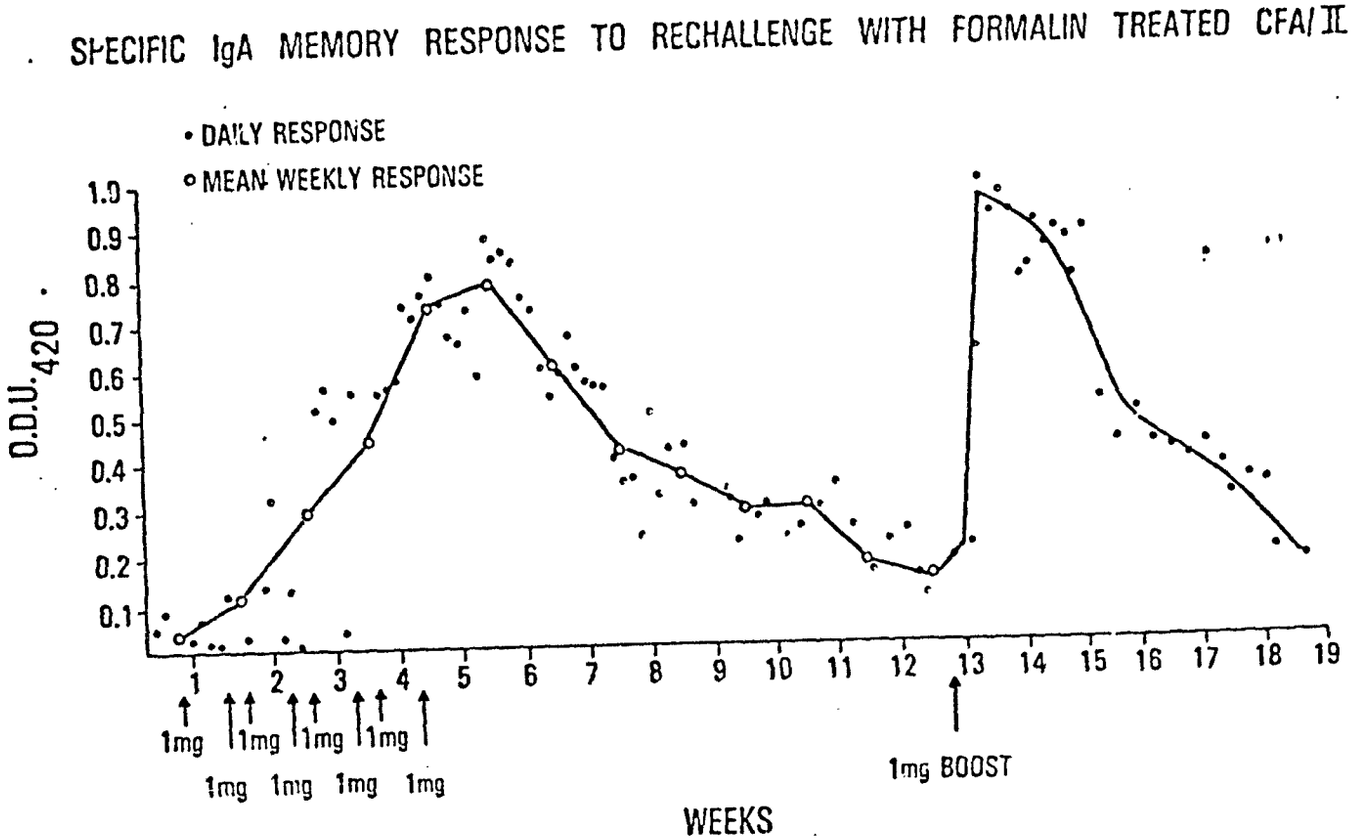
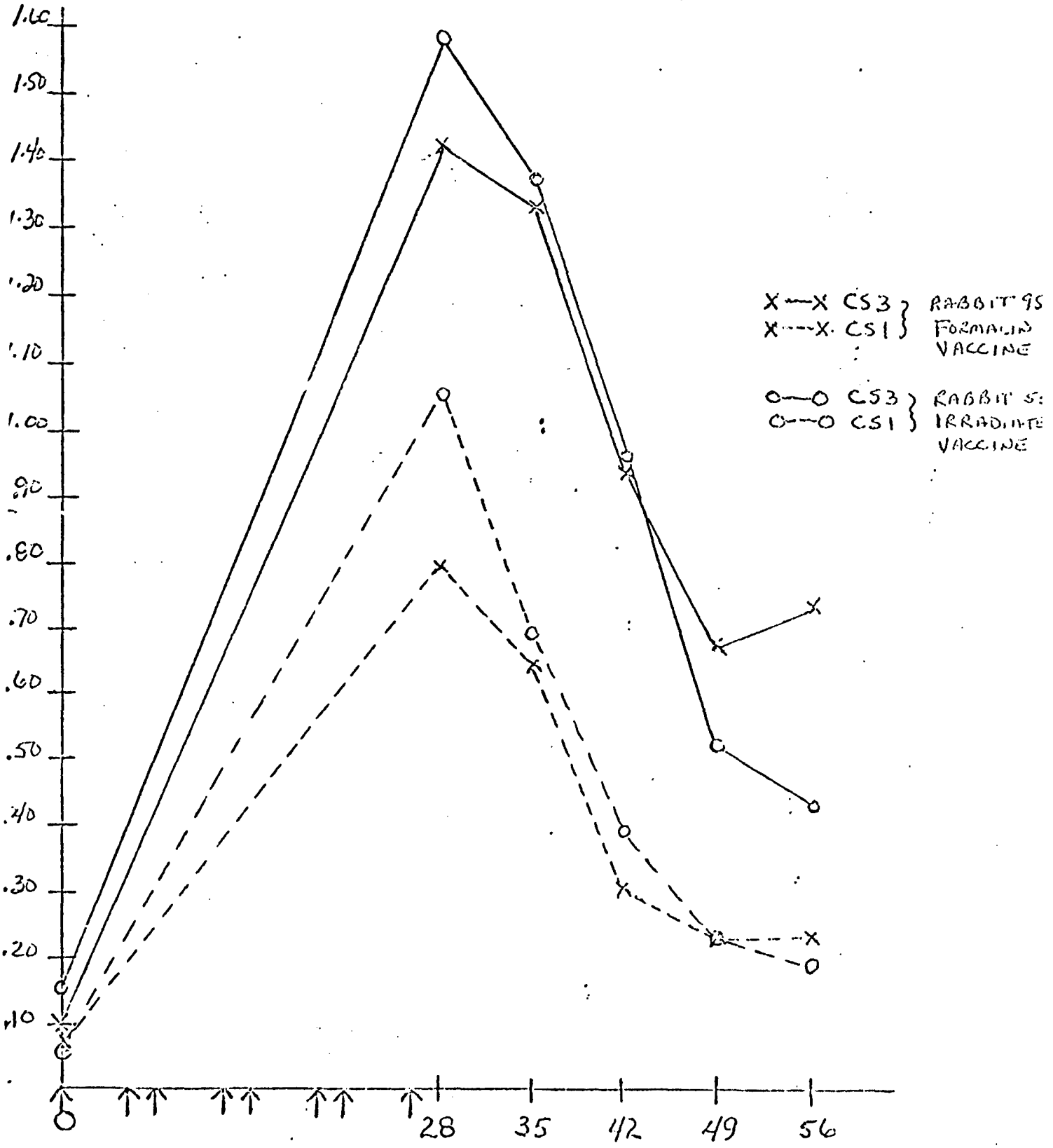

SPECIFIC IgA MEMORY RESPONSE TO RECHALLENGE WITH FORMALIN TREATED CFA/II
• DAILY RESPONSE
o MEAN WEEKLY RESPONSE
O.D.U.420
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
1mg
1mg 1mg 1mg 1mg
1mg 1mg 1mg 1mg
1mg BOOST
WEEKS

FIGURE 10

IgA RESPONSE IN INTESTINAL FLUIDS FROM CHRONIC THIERY-VELLA LOOPS IN RABBITS IMMUNIZED WITH EIGHT 1 MG DOSES OF CFA/II PILI



around the distal ileum, and the string is exteriorized. The challenge inoculum (in this experiment $10^{10}$ E. coli E24377A) is inoculated directly into the proximal jejunum or distal duodenum. The animal is closed and after 2 hrs. the reversible ileal tie is removed. Of the 13 vaccinated and control rabbits challenged in this manner, nine in each group developed diarrhea (Table 5). The lack of efficacy of the CFA/II vaccine in orally vaccinated rabbits parallels the poor vaccine efficacy in the initial vaccination challenge study in man (where vaccine efficacy was only 42%). It is not known if oral vaccination of rabbits elicits an intestinal secretory antibody response as does vaccination of isolated rabbit Thiery-Vella loops. This is currently being investigated. If it turns out that oral vaccination of rabbits with CFA/II fimbriae elicits a poor antibody response, whereas direct vaccination of rabbit intestinal Thiery-Vella loops give excellent responses, we will have learned that the normal gastrointestinal tract in some way adversely effects the vaccine. This may be due to gastric acidity, intestinal proteases, intestinal motility or inability of the fimbriae to successfully pass through the mucus layer to reach the epithelial surface. Each of these factors can be investigated and means devised to deliver the fimbriae to the mucosal surface in an improved manner.

Genetically-Engineered Attenuated E. coli Live Oral Vaccine

Another approach toward prevention of ETEC diarrhea in man involves the use of attenuated strains of E. coli bearing several critical antigens. In theory such a strain would colonize the small intestine of man, following ingestion of a single dose, and would stimulate a potent immune response to the critical antigens without causing significant adverse reactions. The advent of recombinant DNA technology makes possible the construction of such

RESPONSE OF RABBITS IMMUNIZED WITH PURIFIED CFA II VACCINE AND UNIMMUNIZED CONTROLS TO BACTERIAL CHALLENGE (RITARD) WITH *E. COLI* STRAIN E24377/A 0139:H28 (LT+/ST+; CFA II+, CS1+, CS3+)

| | Rabbit | Wt. (Kg) | Dose | Diarrhea Day +1 | +2 | +3 | +4 | +5 | Diarrheal Illness |
|---|---|---|---|---|---|---|---|---|---|
| Controls: | | | | | | | | | |
| | 176 | 2.6 | $10^{10}$ | - | - | - | - | - | - |
| | 177 | 1.9 | $10^{10}$ | - | - | - | - | - | - |
| | 178 | 1.77 | $10^{10}$ | - | - | - | - | - | - |
| | 179 | 1.63 | $10^{10}$ | - | - | - | - | - | - |
| | 180 | 2.1 | $10^{10}$ | - | - | + | - | - | + |
| | 187 | 2.33 | $10^{10}$ | + | - | - | + | - | + |
| | 186 | 1.86 | $10^{10}$ | - | + | - | - | - | + |
| | 190 | 1.94 | $10^{10}$ | - | - | - | - | - | - |
| | 188 | 2.73 | $10^{10}$ | - | - | + | - | - | + |
| | 189 | 2.44 | $10^{10}$ | - | + | + | + | + | + |
| | 197 | 2.10 | $10^{10}$ | - | + | - | - | - | + |
| | 196 | 1.76 | $10^{10}$ | - | + | - | - | - | + |
| | 198 | 1.87 | $10^{10}$ | - | + | - | - | - | + |
| | | | | | | | | | 8/13 (61.5% |
| Vaccinees: | | | | | | | | | |
| | 184/955 | 2.36 | $10^{10}$ | - | - | - | - | - | - |
| | 183/959 | 3.23 | $10^{10}$ | - | - | - | - | - | - |
| | 182/958 | 3.34 | $10^{10}$ | + | + | + | - | - | + |
| | 181/957 | 2.72 | $10^{10}$ | + | + | + | + | - | + |
| | 192/962 | 3.60 | $10^{10}$ | - | - | - | - | - | - |
| | 191/968 | 3.36 | $10^{10}$ | - | - | + | + | + | + |
| | 193/960 | 3.67 | $10^{10}$ | - | - | + | - | - | + |
| | 194/961 | 3.61 | $10^{10}$ | - | + | - | - | - | + |
| | 195/967 | 3.63 | $10^{10}$ | - | - | - | - | - | - |
| | 200/979 | 3.04 | $10^{10}$ | + | + | - | - | - | + |
| | 201/980 | 2.85 | $10^{10}$ | - | - | - | - | - | - |
| | 203/982 | 2.70 | $10^{10}$ | - | + | - | - | - | + |
| | 202/981 | 3.18 | $10^{10}$ | - | + | - | - | - | + |
| | | | | | | | | | 8/13 (61.5% |

strains by genetic engineering. Ideally, a single smooth E. coli vaccine strain would be genetically-engineered to produce large quantities of CFA/I, CFA/II, other CFA antigens, B subunit of human LT, and perhaps, an ST toxoid.

The spontaneous occurrence in the laboratory of an LT-/ST-CFA/II-positive variant of a previously enterotoxigenic O6:H16 LT+/ST+ strain offered us the opportunity to investigate the behaviour of an attenuated E. coli vaccine prototype in man. The properties of this strain, E. coli 1392/75-2A, which was provided by Dr. Bernard Rowe of the Central Public Health Laboratory, Colindale, U.K., are summarized in Table 6. As previously reported (26), this prototype vaccine strain was given to adult volunteers in doses of $10^9$, $10^{10}$, or $6x10^{10}$ organisms with $NaHCO_3$. All recipients excreted the strain, most had serologic responses, and the strain was recovered from cultures of jejunal fluid of 14 of 19 vaccinees (Table 7). Two vaccinees, however, developed mild diarrhea, presumably as a consequence of colonization of the proximal small intestine.

Strain E1392/75-2A has the potential to serve as a recipient for insertion of high copy plasmid vectors containing recombinant DNA encoding CFA/I, LT B subunit and other relevant antigens. Thus this contract year we inserted into strain E1392/75-2A a plasmid containing cloned genes encoding the production of the B subunit of human LT. The plasmid was derived from pWD615 provided by Dr. Walter Dallas. Dr. Dallas cloned the genes for LT into pBR322. The resultant plasmid was cut with the restriction endonuclease Xba I, the only site of which is in the A subunit gene. This left "sticky" ends four base pairs in length. These "sticky" ends were blunted by adding the complementary bases which were filled in with DNA polymerase. The two blunt ends of the plasmid were ligated using a ligase. The resultant plasmid,

Table 6

MICROBIOLOGIC AND GENETIC CHARACTERISTICS OF *ESCHERICHIA COLI*

STRAIN E1392/75-2A (O6:H16)* CANDIDATE VACCINE STRAIN

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

*Spontaneously-derived laboratory derivative of a previously enterotoxigenic (LT+/ST+) strain; kindly provided by Dr. Bernard Rowe, Central Public Health Laboratory, Colindale, England.

+DNA hybridizations with cloned LT and ST genes to detect homologous sequences.

Table 7

CLINICAL, BACTERIOLOGIC AND IMMUNOLOGIC RESPONSE OF VOLUNTEERS FOLLOWING INGESTION OF NON-ENTEROTOXIGENIC CFA/II-POSITIVE *ESCHERICHIA COLI* VACCINE CANDIDATE E1392/75 2A

| Vaccine Dose (viable organisms) | Diarrhea | Positive Cultures | | Significant Serum Antibody Rises to: | | |
|---|---|---|---|---|---|---|
| | | Stool | Jejunal Fluid | O6 | CFA/II | LT |
| $10^9$ | 0/4[a] | 4/4 | 1/4 | 2/4 | 0/4 | 0/4 |
| $10^{10}$ | 1/4[b] | 4/4 | 2/4 | 3/4 | 2/4 | 0/4 |
| $6 \times 10^{10}$ | 1/11 | 11/11 | 11/11 | 7/11 | 5/11 | 0/11 |
| Totals | 2/19 | 19/19 | 14/19 | 12/19 | 7/19 | 0/19 |

[a]No. positive/No. volunteers vaccinated.

[b]This individual passed 4 loose stools totalling 243 ml in volume.

[c]This individual passed 9 loose stools over three days totalling 1197 ml in volume.

pWD615, contains a net addition (insertion) of four base pairs in the middle of the A subunit gene. This shifts the reading frame and completely disrupts the correct transcription and translation of A subunit.

Dr. James Kaper of the CVD added a gene encoding mercury resistance to pWE615 and deleted 200 base pairs from the gene encoding tetracycline resistance. The resultant plasmid is designated, pJBK95. This plasmid was introduced into E. coli E1392/75-2A; the resultant strain, E. coli CVD 201, elaborates the B subunit of human LT and is resistant to mercury but sensitive to tetracycline. This strain will be evaluated in volunteers for safety, immunogenicity and efficacy. It is also currently being used to orally immunize rabbits which will be subsequently challenged with ETEC by RITARD method.

Attenuated Salmonella typhi/Shigella sonnei Bivalent Vaccine

An ideal Shigella vaccine does not presently exist. Nevertheless, previous experiences have demonstrated that some attenuated strains used as oral vaccines can successfully immunize against shigellosis (27). The most extensively studied oral attenuated Shigella vaccines were streptomycin-dependent strains. However, they suffered multiple drawbacks: they required multiple doses to stimulate primary immunity, the genetic lesion responsible for attenuation was unknown, reversion to streptomycin-independence (although not to pathogenicity) occurred with some strains.

Formal and co-workers at WRAIR have developed a new oral Shigella vaccine candidate that is highly promising (28). This vaccine, strain 5076-1C, was prepared by conjugating into an established attenuated Salmonella typhi vaccine strain (Ty21a) the genes that encode production of the O antigen specific polysaccharide of Shigella sonnei (28). Ty21a has been extensively tested in adult volunteers and in children. Field trials in Egypt and Chile have documented its complete safety and efficacy. Ty21a, which penetrates

intestinal mucosa, is completely devoid of one enzyme (UDP-galactose-4-epimerase) and has diminished activity of two others (galactokinase and galatose-1-phosphate uridyl transferase) that are involved in the incorporation of exogenous galactose into the lipopolysaccharide O antigen of S. typhi. When grown in the presence of galactose, smooth lipopolysaccharide O antigen is produced. However, because of the lack or decreased activity of the above enzymes, intermediate products of galactose metabolism accumulate, leading to bacterial cell lysis. This accounts for both the immunogenicity and the safety of Ty21a. Modified S. typhi strain 5076-1C possesses the same characteristics as its Ty21a parent but, in addition to S. typhi O antigen, it possesses on its surface S. sonnei O antigen. Immunity to Shigella is serotype-specific and thus is believed to be dependent on Shigella O antigens as critical immunogens (27).

S. typhi vaccine strain 5076-1C bearing S. sonnei antigen was given to 14 volunteers at the CVD who ingested three doses (day 1,3,7), each containing $1.5 \times 10^9$ viable vaccine organisms. Minimal excretion of vaccine organism was documented in stool cultures of 6 of 14 vaccinees; isolated organisms were agglutinated by both S. typhi and S. sonnei O antisera. Sera and intestinal fluids will be examined for levels of antibody to both S. typhi and S. sonnei O antigens.

A challenge study to assess vaccine efficacy was carried out in which 10 vaccinees and eight controls ingested $6 \times 10^2$ S. sonnei organism (78% were Form I colonies) of pathogenic strain 53G. Definite clinical illness occurred in six of eight controls but in only two of ten vaccinees (73% vaccine efficacy) ($p=0.054$) (Table 8). Based on these extremely encouraging results a second vaccination and challenge study with this vaccine is scheduled.

Table 8

CHALLENGE* STUDY TO ASSESS THE EFFICACY
OF SALMONELLA TYPHI/SHIGELLA SONNEI HYBRID
VACCINE STRAIN 5076-1C

| Group | Clinical Illness | Diarrhea | Mean Stool Volume per Ill Volunteer | Mean No. Loose Stools Per Ill Volunteer | Blood and Mucus in Stool | Fever | Positive Copro-cultures |
|---|---|---|---|---|---|---|---|
| Controls | 6/8† | 5/8 | 2245 ml (300-7054) | 11 (4-25) | 4/8 | 5/8 | 7/8 |
| p=0.054 | | | | | | | |
| Vaccinees** | 2/10 | 2/10 | 598 (205-992) | 9.5 (2-17) | 2/10 | 2/10 | 6/10 |

* Volunteers challenged with $6x10^2$ pathogenic S. sonnei strain 53G.

** Vaccinees received three $10^9$ organism doses with $NaHCO_3$ given within one week.

† No. positive/No. volunteers challenged.

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