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Attachment 1.
(FACE SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 28/7/92

Principal Investigator M. John Albert Trainee Investigator (if any) Nil

Application No. 92-022 Supporting Agency (if Non-ICDDR,B) _____

Title of Study Production and characterization of monoclonal antibodies to the virulence-associated factors of enteropathogenic Escherichia coli for use as diagnostic reagents.

Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population: NA
   (a) Ill subjects Yes No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve: NA
   (a) Physical risks to the subjects Yes No
   (b) Social Risks Yes No
   (c) Psychological risks to subjects Yes No
   (d) Discomfort to subjects Yes No
   (e) Invasion of privacy Yes No
   (f) Disclosure of information damaging to subject or others Yes No
3. Does the study involve: NA
   (a) Use of records, (hospital, medical, death, birth or other) Yes No
   (b) Use of fetal tissue or abortus Yes No
   (c) Use of organs or body fluids Yes No
4. Are subjects clearly informed about: NA
   (a) Nature and purposes of study Yes No
   (b) Procedures to be followed including alternatives used Yes No
   (c) Physical risks Yes No
   (d) Sensitive questions Yes No
   (e) Benefits to be derived Yes No
   (f) Right to refuse to participate or to withdraw from study Yes No
   (g) Confidential handling of data Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No
5. Will signed consent form be required: NA
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) Yes No
6. Will precautions be taken to protect anonymity of subjects Yes No NA
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

M. John Albert
Principal Investigator

Trainee

A-031963

APPLICATION FOR PROJECT SUPPORT

1. PRINCIPAL INVESTIGATOR : M. John Albert, Ph.D.[a]

2. OTHER INVESTIGATORS : Firdausi Qadri, Ph.D.[a]
Tasnim Azim, Ph.D.[a]
James B. Kaper, Ph.D.[b]
Michael S. Donnenberg, M.D.[b]

[a]International Centre for Diarrhoeal Disease Research, Bangladesh, GPO Box 129, Dhaka 1000, Bangladesh

and

[b]Center for Vaccine Development, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, MD, USA

3. TITLE OF PROJECT : Production and characterization of monoclonal antibodies (MAbs) to the virulence-associated factors of enteropathogenic *Escherichia coli* (EPEC) for use as diagnostic reagents

4. STARTING DATE : When money becomes available

5. DATE OF COMPLETION : Two years from starting date

6. TOTAL BUDGET REQUESTED : US$ 62,125

7. FUNDING SOURCE : Not known

8. HEAD OF PROGRAMME : Prof. R. Bradley Sack
Associate Director
Laboratory Sciences Division

9. AIMS OF THE PROJECT

a) General aim

Production of MAbs to virulence-associated factors of EPEC for use as diagnostic reagents.

b) Specific aims

The two well-recognized virulence factors of EPEC are:

1) EPEC adherence factor (EAF) whose production is mediated by a large plasmid. EAF is responsible for adherence of EPEC in localized clusters to cultured mammalian cells *in vitro*. Recent unpublished data suggest that bundle-forming pili (BFP) composed of 19.5-kDa protein are most likely the EAF.

2) Product(s) responsible for attaching-effacing (AE) lesions in the intestinal mucosa of affected animals. A structural gene called EPEC attaching-effacing (*eae*) gene located in the bacterial chromosome has been identified as one of the genes responsible for this lesion; the product of *eae* gene is a 94-kDa outer membrane protein (OMP).

The objectives of the project are: (a) to produce and characterize mouse MAbs to the EAF and the *eae*-specified OMP, (b) use these MAbs for development of assays for the laboratory diagnosis of EPEC diarrhoea.

10. SIGNIFICANCE

EPEC are one of the major causes of diarrhoea in children in the developing countries. Traditionally, laboratories have relied on serogrouping with

commercial antisera for identifying EPEC strains. This has often led to misdiagnosis since H-typing is not normally attempted. Recent research has identified important virulence factors of EPEC. Production of MAbs to these factors and their wider availability offer great potential for accurate diagnosis of EPEC infection.

11. ETHICAL IMPLICATIONS

No experiments with humans and collection of human samples are involved. However, standard procedures will be used for immunization of mice and production of ascites fluid.

12. BACKGROUND

Diarrhoeal disorders are a major cause of morbidity and mortality in children in developing countries. It is estimated that in developing countries, enteric infections collectively cause >1 billion episodes of disease and up to 5 million deaths annually; diarrhoeagenic *E. coli* collectively contributes to >6 million cases and up to 0.4 million deaths worldwide (1). While diarrhoea due to EPEC in industrialized countries is limited to occasional outbreaks in nurseries and daycare centres (2,3) it occurs at a high incidence in children in developing countries (4-6).

EPEC were traditionally identified by serotyping. The World Health Organization's definition of EPEC includes the following serogroups: 026, 055, 086, 0111, 0114, 0119, 0125, 0126, 0127, 0128, 0142 and 0158 (7) although there is evidence for expansion of this list (8).

Although EPEC were the first category of diarrhoeagenic *E. coli* to be identified in the early part of this century (9), the pathogenic mechanism(s) by which they cause diarrhoea remained undefined until recently. It is now recognized that the majority of EPEC strains when inoculated into cultured mammalian cell lines, such as HeLa and HEp-2, adhere in a pattern called localized adherence (LA). The LA phenotype is characterized by clusters of bacteria adhering to localized regions of tissue culture cells. LA is associated with a high-molecular-weight plasmid (~60 mDa) which is present in most EPEC strains. This plasmid is called the EPEC adherence factor (EAF) plasmid, the product of which presumably confers LA (10). EPEC strains cured of this plasmid lose the ability to exhibit the LA phenotype, and the introduction of this plasmid into a non-pathogenic laboratory strain of *E. coli* confers the ability of LA to the host strain. A 1-kb DNA fragment called the EAF probe cloned from a region essential for the expression of LA of one such plasmid is highly sensitive and specific for identifying EPEC that exhibit LA in tissue culture cells *in vitro* (11).

The identity of LA factor remained elusive so far. Several OMPs have been proposed as possible candidates and these include a 94-kDa OMP (12), a 29- to 32-kDa OMP (13) and 45- and 82-kDa OMPs (14). However, Giron *et al.* (15) found that when EPEC possessing EAF plasmid were grown on solid medium containing blood or adherent to HEp-2 cell monolayer, express rope-like bundles of filaments termed bundle-forming pili (BFP) that create a network of fibres which bind individual organisms together. When cured of the EAF plasmid, the strains neither expressed BFP nor grew as adherent colonies. Furthermore, an antiserum to BFP reduced the capacity of EPEC to infect cultured epithelial cell monolayer (15).

BFP are composed of a repeating subunit with an apparent molecular weight of 19.5-kDa. The NH_2-terminal amino acid sequence of this polypeptide obtained from an EPEC strain B171 (serotype O111:NM) revealed homology with the toxin-coregulated pili (Tcp) of *Vibrio cholerae*. This may point to the similarity of attachment functions for these two structures in these bacteria. The evidence that BFP are the most likely candidates for EAF came from direct cloning and sequencing studies. Donnenberg *et al.* (31) have previously generated 22 non-invasive mutants of an EPEC strain E2348/69 (serotype O127:H6) by Tn*phoA* mutagenesis, 7 of which were found to have insertions within a 500 bp region of the EAF plasmid. These mutants are deficient in LA. Using a DNA probe derived from the fusion junction of one of these mutants, the insertions were mapped to a region previously found to be involved in LA. DNA sequencing of cloned fusion junctions has revealed a 579 bp open reading frame. The predicted amino acid sequence of the protein encoded by this reading frame is similar to that of BFP from the strain B171, and the predicted molecular weight of the protein, which is 18, 716 Da is comparable to that estimated for BFP from the EPEC strain B171. In addition, the open reading frame is closely related at the DNA sequence level to the TcpA gene encoding the toxin-coregulated pilus of *V. cholerae* (Donnenberg *et al.*, submitted for publication).

A second phenotype that is identified as an attribute of virulence of EPEC is the ability to produce characteristic histopathology known as attaching and effacing (AE) lesions in the intestines of affected infants (16), experimental animals (17) and on tissue culture cells (18). The AE lesion is characterized by the close adherence of bacteria to the enterocyte, effacement of microvilli, disruption of cytoskeleton and "cupping" of the enterocyte membrane around bacteria. High concentrations of filamentous actin are

present in the host cell beneath the attached bacteria, a characteristic which was utilized to develop the fluorescein-actin-staining (FAS) assay for the detection of the AE lesions on tissue culture cells. The FAS assay which is utilized as a rapid screening test for the AE lesions makes use of fluorescein isothiocyanate-labelled phalloidin to detect polymerized actin (19).

Using Tn*phoA* mutagenesis, Jerse *et al.* (20) identified a chromosomal gene called *eae* (for *E. coli* attaching and effacing) that is necessary for AE activity. It has a 2817 bp open reading frame capable of encoding a 102-kDa protein. A DNA probe derived from this gene hybridized to 100% of EPEC serogroups that showed the AE activity on tissue culture cells as well as other *E. coli*, such as enterohaemorrhagic *E. coli* (EHEC) and RDEC-1 (an EPEC of weanling rabbits). The predicted amino acid sequence derived from the nucleotide sequence of *eae* shared significant homology to that of the invasion of *Yersinia pseudotuberculosis*, suggesting that perhaps the two proteins could serve similar functions. A laboratory strain of *E. coli* K-12 when transformed with *eae* gene failed to produce FAS activity on tissue culture cells (20), and a Tn*phoA* mutant of EPEC strains E2348/69 in which *eae* gene was inactivated produced a "shadow" phenotype in FAS assay indicating some degree of actin condensation. This suggested that *eae* locus is necessary, but not sufficient for AE activity and that other loci are involved in the production of the AE lesions.

Antiserum to a 128-kDa Eae-*phoA* protein expressed by a *eae*:Tn*phoA* mutant strain JPN15.96 (derived from the strain E2348/69) recognized a 94-kDa membrane protein as the product of *eae* gene from the wild-type parent strain and a collection of wild-type EPEC strains. Moreover, sera obtained from volunteers recovering after infection with E2348/69 recognized a 94-kDa OMP

from E2348/69, as well as from its EAF plasmid-cured derivative JPN15.1. This suggested that the 94-kDa protein initially mistaken as the product of EAF plasmid is indeed the product of the *eae* gene (21). Although EHEC strains and RDEC-1 hybridized with the *eae* probe derived from the EPEC strain E2348/69 antiserum to the 128-kDa Eae-*phoA* fusion protein of the EPEC derivative, JPN15.96 failed to recognize any protein from these strains. This may suggest that the product of *eae* genes in EPEC, EHEC and RDEC-1 strains are antigenically heterogeneous although they may serve similar functions in the production of the AE lesions (21).

Data obtained so far suggest that the plasmid- and chromosomal-mediated factors act synergistically to produce disease, and a three-stage infection process has been envisaged. The first stage involves initial adherence of the bacterium to the enterocyte which is mediated by the EAF plasmid. This is followed by the intimate attachment and effacement of the enterocyte surface with the formation of pedestal-like or cup-like structures (AE lesions). The third stage of the infection is characterized by the entry of bacteria into the enterocyte (22).

LA phenotype can occur independent of chromosomal genes, and likewise, AE phenotype can occur without the EAF plasmid (10,18). However, *in vivo* and *in vitro* data suggest that the EAF plasmid is required for the increased infections of EPEC strains (22). The postulated mechanism by which this is achieved is by increasing the expression of genes responsible for the AE activity or by increasing initial adherence, thus producing contact so that the AE activity can occur more efficiently. It has been shown that the EAF plasmid in the human EPEC strain E2348/69 and the plasmid encoding AF/R1 pili in the rabbit RDEC-1 strain can be substituted by genes encoding other

adherence factors to provide initial adherence to the cells to facilitate actin condensation (22,23).

There are interesting leads on the mechanism of diarrhoea. Raised intracellular calcium concentrations in cells infected with EPEC (25) and increased phosphorylation of myosin light chain, a cytoskeletal protein known to affect actin organization, have been found at the sites of the AE lesions in infected cells (26). From these findings, it is postulated that extensive phosphorylation of myosin light-chain, activated by high concentration of calcium at the sites of EPEC contact, irreversibly destroys microvillus function with the accumulation of actinomycin within the AE lesions. Such brush border damage could cause diarrhoea by reduction of absorptive capacity of the intestinal mucosa. It is also possible that phosphorylation of cellular proteins involved in the ion transport across the membrane could result in secretory diarrhoea (26).

EPEC can be diagnosed in the laboratory by (a) serotyping (7), (b) LA phenotype in tissue culture assay or EAF probe assay (11), and (c) AE activity using FAS assay or DNA probe assay (19,20). However, none of these assays is easily amenable to laboratories in the developing countries where EPEC diarrhoea is most prevalent. In an attempt to develop a simpler assay for use in developing countries, we developed a polyclonal antibody-based ELISA for the detection of LAF+EPEC (24). This was accomplished by using an antiserum to the LAF+EPEC strain E2348/69 after absorption with its isogenic LA- derivative MAR-20. The ELISA was found to be 100% sensitive and specific in detecting LA+EPEC serogroups. It was thought that the antiserum contained antibodies to a product(s) associated with EAF plasmid, but this was not proven.

Nevertheless, this suggested that an immunodiagnostic test can be developed based on the virulence-associated factor(s) of EPEC. Polyclonal antibody-based ELISA has the limitation of difficulty in preparing highly specific reagent each time and its limited supply. In this regard, we plan to develop MAbs to the product of EAF plasmid (19.5-kDa protein) and that of *eae* gene (94-kDa protein) and explore their use as possible immunodiagnostic reagents. It is expected that production of useful MAbs will help in the wider distribution and use of these reagents for diagnosis of EPEC diarrhoea.

13. EXPERIMENTAL DESIGN

Purification of immunizing antigens

BFP: BFP, which is the product of the EAF plasmid will be purified from the EPEC strain B171 (serotype O111:NM) by previously described methods (15). Briefly, bacteria grown on trypticase soy agar supplemented with 5% defibrinated sheep blood will be suspended in monoethanolamine buffer (MEB). The BFP will be sheared from bacterial bodies in an Omnimixer, precipitated by the addition of ammonium sulfate and resuspended in MEB. The purity of the preparation will be checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a gradient gel.

94-kDa protein: The *eae* gene-specified 94-kDa protein will be purified from the OMP of EPEC strain E2348/69 as described previously (21). Briefly, bacteria grown in Luria broth to early stationary phase will be broken by French press, membrane proteins will be extracted with Triton X-100 and separated by SDS-PAGE. The identity of the protein will be confirmed by a previously prepared 128-kDa Eae-*phc*A protein (20,21).

MAb production: Standard methods will be followed for the production of MAbs (27).

Immunization of mice: Female BALB/c mice will be used for the production of hybridoma secreting antibodies. A total of three doses, each dose containing 100 µg of protein will be given per mouse. The first dose will be mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously. The booster doses will be given at 4-week intervals without any adjuvant (intraperitoneally, followed by intravenously) (28).

Production of hybridomas: Approximately 10^8 cells from pooled spleens of two immunized mice will be fused with 5×10^7 myeloma cell sp2/0 (29) by using polyethylene glycol 4000. After fusion, cells will be grown in hypoxanthine-aminopterin-thymidine selection medium in 96-well microtitre plates.

Screening and selection of hybridomas: Supernatants harvested from hybridomas will be tested in ELISA with respective purified antigens and EPEC expressing *eae*-encoded protein and BFP. Hybridomas of interest will be cloned twice by limiting dilution.

Specificity of hybridomas: Ascites tumours will be induced in pristine-primed BALB/c mice with the hybridomas. The specificity of hybridomas of interest (ascites fluid) will be checked against respective purified antigens in ELISA and immunoblot, and also in ELISA against a number of genetic constructs and wild-type *E. coli* as shown below in the table:

Organism	Phenotype	Description
EPEC	EAF$^+$ *Eae*$^+$	Wild type strains isolated from clinical cases
ETEC	EAF$^-$ *Eae*$^-$	-do-
EHEC	EAF$^-$ *Eae*$^+$	-do-
EIEC	EAF$^-$ *Eae*$^-$	-do-
EAggEC	EAF$^-$ *Eae*$^-$	-do-
E. coli K-12	EAF$^-$ *Eae*$^-$	Laboratory strain of *E. coli*
JPN15	EAF$^-$ *Eae*$^+$	Wild-type EPEC E2348/69 cured of EAF plasmid
JPN15 (p^{MAR7})	EAF$^+$ *Eae*$^+$	Cured wild-type EPEC with the reintroduced EAF plasmid
JPN15.96	EAF$^-$ *Eae*$^-$	JPN15 with Tn*phoA* insertion in *eae*
JPN15.96 (p^{MAR7})	EAF$^+$ *Eae*$^-$	JPN15.96 with EAF plasmid
JPN15 (p^{ILI4})	AFA$^+$ *Eae*$^+$	JPN15 with plasmid encoding afimbrial adhesin
JPN15.96 (p^{ILI4})	AFA$^+$ *Eae*$^-$	JPN15.96 with plasmid encoding afimbrial adhesin
JPN15 (p^{SH2})	Pil$^+$ *Eae*$^+$	JPN15 with plasmid encoding type I pili
JPN15.96 (p^{SH2})	Pil$^+$ *Eae*$^-$	JPN15.96 with plasmid encoding type I pili

[Partly adapted from ref. 22]

EPEC = enteropathogenic *E. coli*
ETEC = enterotoxigenic *E. coli*
EHEC = enterohaemorrhagic *E. coli*
EIEC = enteroinvasive *E. coli*
EAggEC = enteroaggregative *E. coli*

Class and subclass of MAbs: The class and subclass of MAbs of interest will be determined by ELISA. The wells of microtitre plates will be coated with serum-free culture supernatant of antibody-producing hybridoma as antigen. The wells will be reacted with rabbit anti-mouse immunoglobulins (IgG_1, IgG_{2a},

IgG_{2b}, IgG_3, IgM and IgA), followed by enzyme-linked) goat anti-rabbit conjugates and development with appropriate substrate.

Development of immunodiagnostic tests using MAbs

ELISA: ELISA for the detection of the *eae*-specified protein and the EAF plasmid-encoded BFP-bearing *E. coli* will be developed according to the format described elsewhere (24). *E. coli* grown in trypticase soy agar supplemented with 5% sheep blood agar will be suspended in carbonate-bicarbonate coating buffer to give 10^9 organisms per ml and used to coat wells of microtitre plates. The wells will then be reacted with appropriate MAbs followed by detection of MAbs with enzyme-linked anti-mouse immunoglobulins and substrate. Appropriate cut-off point optical density (OD) for positivity will be established in comparison with OD values obtained with negative controls. The ELISA will be validated with well-characterized EPEC strains.

Agglutination test: If successful in developing specific MAbs of the IgG class, these MAbs will be used to sensitize Cowan I strain of *Staphylococcus aureus* bearing protein A or latex particles by standard methods (30). The sensitized *S. aureus* or latex particles will be used in slide agglutination tests to detect EPEC bearing the relevant antigens. If successful in developing MAbs of IgM class, these will be utilized in direct agglutination of bacteria.

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15. TASKS OF INVESTIGATORS

Dr. M. J. Albert :	Principal investigator and initiator of the project. He will oversee the project and write reports.
Dr. F. Qadri and Dr. T. Azim	They will supervise the Research Assistant and carry out key parts of experiments
Dr. J. Kaper and Dr. M. Donnenberg	They will prepare and provide purified antigens and provide advice on the proper execution of the project

16. BUDGET

First year

a)	Personnel			
	Research Assistant			US$ 7,000
b)	Operating costs			
	Animals, tissue culture medium, enzyme-conjugate; CO_2 gas cylinder, liquid nitrogen, glassware, etc.	...		4,000
c)	Capital equipment			
	Biohazard cabinet	...	US$ 8,000	
	Water-bath	...	3,000	
	Refrigerator	...	1,000	
				12,000
d)	Travel			
	Airfare	...	12,000	
	Living expenses	...	2,000	
				14,000
e)	Other costs (communication, publication, etc)			1,000
				US$ 38,000

Second year

Salary + Reagents	... US$ 7,700 + 4,000		11,700
Total (first + second years)			US$ 49,700
Overhead (25%)	...		12,425
GRAND TOTAL			US$ 62,125

Justification of budget

a) Personnel

We will require the service of a full-time Research Assistant in Dhaka. The Research Assistant we have in mind is Mr. Ansaruzzaman, who is at present employed at NOA level in an ongoing project, which will be completed by the end of 1992.

b) Operating cost

These are costs of disposables which are essential for carrying out the study.

c) Capital equipment

Currently we do not have biohazard cabinet, water-bath and refrigerator for exclusive use in the MAb laboratory. This has given rise to contamination problems. We plan to overcome these problem by purchasing these equipments for exclusive use.

d) Travel and living expenses

We seek two return economy fares from Baltimore to Dhaka to enable Drs. Kaper and Donnenberg to visit Dhaka, and also one return economy fare from Dhaka to Baltimore for Dr. Albert to visit Baltimore. Living expenses relate to projected visit of three days to one week duration.

e) Other costs

These are necessary expenses for successful completion of the project.

MJA:mh/J8:MABS2.PRT

Title: Production and characterization of monoclonal antibodies (MAbs) to the virulence-associated factors of enteropathogenic Escherichia coli (EPEC) for use as diagnostic reagents.

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	✓		
Adequacy of Project Design	✓		
Suitability of Methodology	✓		
Feasibility within time period		✓	
Appropriateness of budget	✓		
Potential value of field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☑

b) with qualification ☐

- on technical grounds ☐
- on level of financial support ☐

I do not support the application ☐

Name of Referee: DR. S. KNUTTON

Signature: Stuart Knutton Date: 2-7-92

Position: Lecturer

Institution: Institute of Child Health,
University of Birmingham,
Francis Road,
Birmingham B16 8ET.
England.

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Title: Production and characterisation of monoclonal antibodies (Mabs) to the virulence-associated factors of enterpathogenic *Escherichia coli* (EPEC) for us as diagnostic reagents

PI: Dr John M Albert

Reviewer: Dr Stuart Knutton

The proposal 'Production and characterisation of monoclonal antibodies (Mabs) to the virulence-associated factors of enteropathogenic *Escherichia coli* (EPEC) for use as diagnostic reagents' by Alberts et al. is essentially a straightforward project using well developed methods and is designed to develop a Mab based dignostic ELISA test for EPEC. I am in agreement with the proposers that O:H serotyping, assays based on tissue culture cell adhesion and DNA probe tests are inappropriate for most routine diagnostic laboratories especially in developing countries whereas a Mab based ELISA test would be more feasible. Previous work has shown the feasibility of developing a polyclonal antibody based diagnostic ELISA test for EPEC but the proposes rightly appreciate the limitations of reproducibility inherent in this approach.

The originality and timeliness of the present proposal stems from important recent progress in defining 2 specific virulence associated surface antigens of EPEC (the plasmid encoded bundling pilus antigen and the chromosomally encoded 94kDa outer membrane protein product of the *eae* gene) which could form the basis for a Mab based diagnostic test.

Available evidence indicates that Mabs recognising epitopes of the 94kDa protein should be highly specific for 'attaching effacing' *E. coli* and, because it is now apparent that there are differences in the eae genes between EPEC and enterohemorrhagic *E. coli*, it should be possible to produce a Mab which recognises a specific epitope found only on the EPEC protein.

Evidence for the bundling pilus antigen being EAF is still not conclusive although the unpublished data presented in this application does provide further compelling evidence that it is. Even so, I am less convinced about the specificity of an ELISA assay for EPEC based on the bundling pilus antigen because, as the authors point out, there is evidence that not all EPEC strains produce EAF. It is possible of course that such EAF probe negative strains produce a related pilus antigen with epitopes in common with EAF. However, given these reservations I still believe it is probably worth evaluating tests based on both of these important EPEC virulence antigens although any priority should be given to producing Mabs to the 94 kDa protein.

As mentioned above, the experimental part of the project appears quite straightforward. My reading of the application indicates that a Mab laboratory already exists at ICDDR. I therefore can't forsee any problem with the feasibility of the project since there should be no problem in producing the required amounts of the purified antigens in Baltimore.

The budget proposals appear quite realistic to me for this type of study. If money was a major limiting factor one could make the case that it should only be necessary for either Dr Kaper or Donnenberg to travel to Dhaka although I can see, given that they would both be involved in the project, why they would both want to make a visit to ICDDR.

In summary, I believe this is a timely and important study which should not only lead to the development of new diagnostic tests for EPEC suitable for use in developing countries but also produce a collection of Mabs which would be valuable reagents for further studies of EPEC pathogenicity. I recommend approval.

Title: Production and characterization of monoclonal antibodies (MAbs) to the virulence associated factors of enteropathogenic Escherichia coli (EPEC) for use as diagnostic reagents.

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	X		
Adequacy of Project Design			X
Suitability of Methodology	X		
Feasibility within time period	X		
Appropriateness of budget	X		
Potential value of field of knowledge	X		

CONCLUSIONS

I support the application:

a) without qualification [ ]

b) with qualification [X]

- on technical grounds [ ]

- on level of financial support [ ]

I do not support the application [ ]

Name of Referee:

Signature: Peter Echeverria Date: June 21 1992

Position:

Institution:

AFRIMS Bangkok Thailand

The description ~~of the~~ problem in identifying EPEC and the pathogenesis of EPEC ~~is~~ first rate. However the lack of clinical study design must be addressed - Raise your $50

My main concern with this proposal is there is no mention of what children will be cultured and how the isolation rate of EPEC will be put in perspective. In fact, EPEC are a variable cause of endemic vs epidemic infantile diarrhea. While the CVD reports very high incidence rates in South America, it is difficult to know if they are reporting on endemic or epidemic disease. While it may be true that eae+ EAF+ E. coli are an important cause of diarrhea, it is very important that this be put into perspective. What will be the age of children, their nutritional status, and their other infections.

I think this proposal should be sent to Drs. Orskov or someone up who can perform serotyping. Drs. Orskov do not share this strictly mechanistic approach. For example what about DA EPEC O44 that are EAF- and eae-. At the least they need to compare O:H serotyping. This will increase the cost and number of collaborators but efforts to spend a greater proportion of the proposal of getting E. coli serotyped (the old method) would improve this proposal. The Japanese NIH are, in my opinion, much faster than Orskov or Rowe these days. Lior in Canada might be willing to offer serotyping capabilities.

I do not begin to understand ICDDB, B budgeting. I recalculated the 4 of $ 49,700. I don't have any problem with funds for personnel, capital equipment, or travel. To these, to add on 25% as overhead-and not itemize the cost of performing clinical bacteriology, testing for enteric viruses, intestinal parasites, or the cost of epidemiologist is not valid. As I understand ICDDR, B, they perform complete diagnostic studies on 4% of the cases of diarrhea. Do they plan to restrict their studies to 4% of infants ? What's an infant ?

Are they planning a case-control study which I think will double the cost of the study by 50% are not necessarily lead to any useful information since pathogens are frequent in "controls" unless these controls are selected very carefully. If they walk into an epidemic of EPEC O119:H6 they may find a very unrepresentative situation. Anyway, it's a start which should encourage similar studies in other populations. I wonder if this is an attempt to get laboratory support for another study they are not telling us about in this proposal.