ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date April 19th 19

Principal Investigator Dr. IVAN CIZNAR Trainee Investigator (if any) 28

Application No. 84-016 Supporting Agency (if Non-ICDDR,B)

Title of Study POTENTIAL CONTRIBUTION OF ENDOTOXIN TO ENTEROTOXIC/TOXIC ACTIVITIES OF V.CHOLERAE AND E COLI

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).

Source of Population:
(a) Ill subjects — Yes (No)
(b) Non-ill subjects — Yes (No)
(c) Minors or persons under guardianship — Yes (No)

Does the study involve:
(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)

Does the study involve:
(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)

Are subjects clearly informed about:
(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:
(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
7. Check documents being submitted herewith to Committee:

___ Umbrella proposal - initially submit a overview (all other requirements will be submitted with individual studies).
✓ Protocol (Required)
✓ Abstract Summary (Required)
NA 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)
NA Informed consent form for subjects
NA Informed consent form for parent or guardian
NA 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.

laboratory stock cultures will be used. The study does not involve human subject.

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

Ivan Ciznar
Principal Investigator

Trainee

SECTION I - RESEARCH PROTOCOL

1. Title : Potential contribution of endotoxin to enterotoxic/toxic activities of V. cholerae and E. coli.

2. Principal Investigator : Dr. Ivan Ciznar

   Co-Investigators : Drs. T. Butler, M.I. Huq and K.A. Al-Mahmud

3. Starting Date : May 1, 1984

4. Completion Date : April 30, 1985.

5. Total Direct Cost : US$ 34,080.00

6. Scientific Program Head : Dr. Thomas C. Butler

   This protocol has been approved by the Host Defense WG

   Signature of the Programme Head : Thomas Butler

   Date : 11 April 1984

7. Abstract Summary:

In recent years more attention has been focused to the "free endotoxin" and its role in protection against and exacerbation of gram negative bacterial infections. Since this endotoxin is released into the surroundings in various quantities depending on the strain and environment it seem to be interesting to analyse whether any difference in this respect exist among enteropathogens and whether the endotoxin could contribute to effects observed in some experimental models used for detection of enterotoxins.

Endotoxin spontaneously released into the media by:

a. toxigenic strains of V. cholerae

b. CT gene negative V. cholerae

c. E. coli (EPEC)

d. E. coli (ETEC)

will be determined. The quantity of endotoxin released in various growth phases by above mentioned pathogens will be correlated with other virulence markers. Rabbits tolerant to endotoxin will be used tc estimate a possible effect of endotoxin in the skin test.

8. Reviews:

a. Research involving Human Subjects : _____

b. Research Review Committee: _____

c. Director : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective:

a. To characterize quantitatively and qualitatively endotoxin spontaneously released from live cultures of CT gene deficient *V. cholerae* 01 from toxinogenic *V. cholerae* 01 strains as well as from *E. coli*/EPEC and ETEC strains/.

b. To examine whether releasing of endotoxin by live cells has any relation to enterotoxin production by the strains.

c. To examine whether the released endotoxin has any impact in the results of diagnostic tests particularly on the rabbit skin test.

2. Background:

Endotoxin lipopolysaccharide (LPS) from gram-negative bacteria can cause multitude of biological effects and initiate a chain secondary reactions in a host. It also functions as a barrier for release of periplasmic enzymes to the surroundings (Irvin et al, 1975) as well as for penetration of small molecules into the cell (Nakae and Nikaido, 1975) Antibiotic resistance and resistance to lysozyme, complement and antibody is also related to the structure of LPS (Schlecht and Westphal, 1970), Sanderson et al, 1974, Rowley, 1976). Variety of these functions has always led to question to what extent could endotoxin participate in reactions revealed whether in experimental model or in clinical picture of a patient infected by enteropathogens. This questions has become more attractive due to findings on releasing of endotoxin into the surroundings by live cells without any physical or chemical attack (Pike, Chandler, 1974

Novotny et al, 1975; Johnson et al, 1975; Irving et al, 1975; Holmgren et al, 1971). Despite the many data on chemistry and immunochemistry of lipopolysaccharide (Westphal et al, 1983) as well as pathophysiologic reactions elicited in experimental animals by endotoxin there is still lack of correlation or causative association between clinical symptoms and experimental responses to endotoxin. In most experiments endotoxin isolated by the hot phenol-water extraction has been used (Westphal, Jann., 1965). Though useful for biochemical and immunochemical studies it might be less appropriate for biological studies due to the fact that the extraction procedure by phenol does not represent conditions in-vivo for releasing of endotoxin. Thus the product of phenol-water extraction might be biologically different from the spontaneously released substance. Some literature data have shown that the difference exists in Shigella sonnei (Romanowska et al, 1970).

It has been recognized that endotoxin possesses an activity to associate specifically and non-specifically with biological membranes (Kabir et al, 1970) and this can trigger an sequence of events at the level of cell, organ or the macroorganism (Morrison, 1983). It can be speculated that free endotoxin released at the attachment and/or adhesion of pathogen to a receptor on a membraneous structures of the host could amplify or inhibit some of the pathogen activities. Besides this the blebs of outer membrane carrying endotoxin may deliver other toxic products of the pathogen to distant sites (Sparling, 1983). Furthermore, some data indicate that bacterial proteins enhance the host's susceptibility to LPS toxicity raising a possibility that small amounts of LPS, acting synergestically with other bacterial components may contribute significantly to septicaemic effects (Rietschel et al, 1982). Another aspect of this problem seems to reside in interpretation of results from presently used laboratory methods for

detection of enterotoxins. Endotoxin present in culture filtrates could interfere due to its membrane associating activity with other substances giving reactions leading to non-correct interpretation. In order to understand better these relations it is important to have more data on spontaneous release of endotoxin from enteric bacteria with different pathogenic mechansism. Such results could also help to improve discriminating capability of methods particularly the skin test which have been used for detection of enterotoxic/toxic activities of enteropathogens.

3. Rationale:

Pathogenic mechanisms of many enteroinfections are still poorly understood Though many factors contributing to a complex pathogenic picture of enteroinfections have been defined, there are still some which need to be clarify. Endotoxin has been known for years as a toxic component than can elicit spectrum of pathophysiologic and pharmacologic effects but its role during the infections process caused by non-toxinogenic and other toxic factors producing enteropathogens is not clear. The study intends to explore the contributions of "free endotoxin" to some activities presented in-vitro by toxinogenic and non-toxinogenic strains of V. cholerae and E. coli. These data can contribute to better understanding of pathogenic mechanisms in enteroinfections as well as may improve toxicity testing procedures.

B. SPECIFIC AIMS:

1. To determine the amount of endotoxin released in-vitro by toxinogenic and nontoxinogenic V. cholerae and E. coli during the various phases of the growth curve.

2. To test a possible contribution of endotoxin to reactions observed in experimental models used for detection on enterotoxicity.

C. METHODS OF PROCEDURE

1. Strains selection: One strains of the each species will be selected.

   a. V. cholerae 01, toxinogenic

   b. E. coli/ETEC/LT (+)

   c. E. coli/EPEC/

   To these isolates one strain of V. cholerae 01 that failed to demonstrate any homology with CT or LT genes will be added. Non-pathogenic strain of E. coli from reference strains at ICDDR,B will be also included.

2. Prepration of culture filtrates: For V. cholerae Richardson's medium (1969), for E. coli, Evans et al (1973) medium will be used. Incubation of the cultures will be done on a shaking water bath at 37°C. In preliminary experiments growth curves of all the tested strains will be examined by counting of C.F.U. after appropriate dilution. Simultaneously O.D. of the culture will be measured. Then samples will be taken from the cultures in the second and third part of log phase as well as from the first part of the stationary phase for estimation of endotoxin and enterotoxins. Cultures will be centrifuged at 4°C for 30 min at 20,000 g and supernates filtered through milipore membranes and stored at -20°C in aliquots for further experimental work.

3. Biological and immunochemical tests:

   a. Adult rabbit ileal loop test for CT by Kasai and Burrows (1966), and the skin permeability test for CT, LT by Craig (1965) will be used.

e. Suckling mice assay for ST by Dean et al (1972).

c. Limulas lysate test (LAL) for endotoxin by Levin and Bang (1968). Modificatien of Difco-Pyrotest will be used.

d. Double diffusion get test for endotoxin by Ouchterlony (1964) with 10 times concentrated culture filtrates, specific antiserum and LPS extracted by phenol water procedure (Westphal, Jann 1965) will be used.

e. In order to separate a bulk of endotoxin a culture filtrate of each strain will be precipitated with amonium sulphate, the precipitate will be dialyzed and separated on a column of Sephadex G-100. Fractions over 100,000 between 90,000 and 50,000 of molecular weight will be cut off and retested for biological activity in various dilutions. In samples obtained by this procedure a ratio of endotoxin/enterotoxin or enterotoxin like substances will be different of the original culture filtrate and will enable to compare the activities presented by the different components in the skin test.

f. Samples with higher concentration of endotoxin from each strains will be tested for the skin permeability on rabbits rendered tolerant to endotoxin to see any change and/or effect of endotoxin. Contrary to the tolerance the Schwarzmann local reaction will be used to enhance the effect of endotoxin if present in the tested samples.

SIGNIFICANCE

The role of endotoxin in pathogenesis of enteroinfections as well as in reactions presented by enteropathogens in experimental models used for enterotoxicity testing is not clear. This study intends to explore the releasing of endotoxin from toxigenic and non-toxigenic V. cholerae and E. coli. Moreover, the study plans to analyse the contribution of released endotoxin to biological reactions in experimental models. If these points are elucidated this could improve quality of the diagnostic procedures

used for detection of enterotoxins and could have implications towards better understanding of the nature of the pathogenic mechanisms in enteroinfections.

E. FACILITIES REQUIRED

1. ICDDR,B Microbiology Laboratory and Animal House facilities will be shared.

2. Laboratory for separation and analysis of biologically active macromolecules will be needed. The instruments needed include;

Laboratory colums, fraction collector, UV recorder, UV/VIS spectrophotometer peristaltic pump, horizontal and vertical electrophoresis, preparative ultracentrifuge and accessories. Part of these equipments have already been in possession of ICDDR,B. Additional equipments will be transfered to ICDDR,B by Pharmacia Fince Chemical, Sweden for purpose of the course on Biochemical techniques in separation of macromolecules. The course will be held at ICDDR,B in September this year.

This laboratory should be established at ICDDR,B not only for the purpose of this protocol but also for other protocols presently in preparation and further protocols oriented to study of molecular aspects of pathogenicity, pathogenesis and host defence. Moreover such facilities offer unique opportunity for training and education of international, national and ICDDR,B students in research methodology oriented to better knowledge in the above mentioned field.

F. COLLABORATIVE ARRANGEMENTS

Contact will be maintained with Prof. Dr. E. Rietschel, Forschuginstitute Borstel, Institut fur Experimentlle Biologie and Medicine, West Germany

concerning the possible analysis of action of free and bound LPS on macrophages.

Contact will also be maintained with Dr. G. Bartkova, Research Institute of Preventive Medicine, Bratislava, Czechoslovakia, regarding an immunochemical analysis of LPS.

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SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICE

Name	Position	% or No. of Day	Annual Salary	Project Requirement Taka	Project Requirement Dollar
Dr Ivan Ciznar	Prin. Investigator	30%			12,600
Dr T. Butler	Co-Investigator	5%			3,500
Dr I. Huq	Co-Investigator	10%			6,800
Dr K.A. Al-Mahmud	Co-Investigator	15%		18,500	
Technician (Micro)		50%		12,000	
Technician (Animal)		40%		12,000	
Research Officer (to be named)		50%		35,000	
				77,500	22,900

2. SUPPLIES AND MATERIALS

	Unit Cost	Amount Required	Project Requirement Taka	Project Requirement Dollar
New Zealand adult albino rabbits	140.00	150	21,000	
Suckling mice 50 tests	16.00	250	4,000	
Media				500
Chemicals				600
LAL test				600

3. EQUIPMENT

Separation unit of Pharmacia Fine Chemicals 5,000

4. PATIENT HOSPITALIZATION

None

5. OUTPATIENT CARE

None

6. TRANSPORT (ICDDR,B)

None

		Taka	Dollar
7.	TRAVEL & TRANSPORT OF PERSONS		
	None		
8.	TRANSPORT OF THINGS		100
9.	RENT, COMMUNICATION		
	None		
10.	PRINTING & REPRODUCTION	7,000	
11.	OTHER SERVICES		
	None		
		32,000	6,700

B. BUDGET SUMMARY

		Taka	US Dollars
1.	Personnel Services	77,500	22,900
2.	Supplies and Materials	25,000	1,700
3.	Equipment		5,000
4.	Patient hospitalization		
5.	Outpatent Care		
6.	Transport (ICDDR,B)		
7.	Travel & Transportation		100
8.	Transport of Things		
9.	Rent, Communication and Utilities		
10.	Printing and Reproduction	7,000	
11.	Other Contractual Services		
		109,500	29,700

US$4,380

Total US$ 34,080

Salaries US$ 25,000

Operation Cost US$ 9,080

Conversion rate: 1 US$ = Tk.25.00