Date

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator DR. F.C. Patra

Application No. 84-032

Title of Study Studies on the secretory effects of cholera toxins on rat jejunum and ileum.

Trainee Investigator (if any) x

Supporting Agency (if Non-ICDDR,B) x

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:
   (a) Ill subjects — Yes No
   (b) Non-ill subjects — Yes No
   (c) Minors or persons under guardianship — Yes No
   (NA)
2. 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
   (NA)
3. 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
   (NA)
4. 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
   (NA)
   (f) Right to refuse to participate or to withdraw from study — Yes No
   (g) Confidential handling of data — Yes No
   (NA)
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes No NA
5. Will signed consent form be required:
   (a) From subjects — Yes No NA
   (b) From parent or guardian (if subjects are minors) — Yes No NA
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.

F.C. Patra — 19 JUL 1984

Principal Investigator — Trainee

84-032
18/7/84

SECTION I - RESEARCH PROTOCOL

1. TITLE : Studies on the secretory effects of cholera toxins on rat jejunum and ileum

2. PRINCIPAL INVESTIGATOR : Dr. F.C. Patra

   CO-INVESTIGATORS : Dr. K.A. Al-Mahmud
   Dr. A.S.M. Hamidur Rahman
   Mr. K. Alam
   Mr. Akbar Ali

   CONSULTANTS : Prof. S.C. Sanyal
   Dr. W.B. Greenough III

3. STARTING DATE : July 15, 1984

4. COMPLETION DATE : July 15, 1985

5. TOTAL DIRECT COST : US $17,271

6. SCIENTIFIC PROGRAM HEAD :

   This protocol has been approved by the PTWG Working Group

   Signature of the Scientific Program Head

   Date 16-7-84

7. ABSTRACT SUMMARY

Non-toxigenic V. cholerae O1 strains have been reported from diverse environmental sources, from human intestinal and extra-intestinal infections. But these strains have not been recognized as a cause of acute diarrhoea. Culture filtrates of all these strains have been shown to cause significant accumulation of fluid in adult rabbit ileal loops, and it has been suggested that these non-toxigenic V. cholerae O1 strains elaborate a toxin not yet known, which is different from the known cholera toxin in antigenic nature, receptor site, mode of action and genetic homology. The proposed research plan intends to study the effect of the culture filtrate of non-toxigenic V. cholerae O1 strains on the small intestine of rat using an in vivo perfusion technique.

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8. REVIEWS

a. Research involving human subjects _____

b. Research Review Committee _____

c. Director _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective

To study the effects of a new cholera toxin on the small intestine of rat using an *in vivo* perfusion technique.

2. Background

Till very recently it was known that strains of *Vibrio cholerae* O1 produce only one enterotoxin (CT) of importance governed by one genome (1.2). Isolation of non-toxigenic *V. cholerae* O1 strains have been reported from diverse invironmental sources in different countries (3). Such strains have also been isolated from human intestinal and extra-intestinal infections. Many of these strains failed to demonstrate any homology with CT gene (4,5). These strains, therefore, have not been recognized as a cause of acute diarrhoea, even though for almost a decade, numerous cases of acute diarrhoea had been attributed to inexplainable contamination of the varous types of marine envorinments. The first scientific report appeared in 1980 (6) concerning a mini outbreak where the disease was traced to cooked crabs from local marshes.

It was shown by Sanyal *et al* (2) that live cells of all these strains caused significant accumulation of fluid in ligated adult rabbit ileal loops and diarrhoea in infant rabbits. They also obtained similar results using sterile culture filtrates (7) and have concluded that these *V. cholerae* O1 strains produce a toxin not previously recognised and that the new toxin is different from the known CT in antigenic nature, receptor site, mode of action and genetic homology (7).

Aziz *et al* in a pioneering study (8) have successfully used rats as a cholera model by injecting culture filtrates of Vibrio cholera into rat ileal loops. Sandhu *et al* (9), while studying the inhibitory action of Loperamide on cholera-toxin-induced small intestinal secretion have used rat jejunum in an *in Vivo* perfusion technique. In their studies 75 µg of CT was used as a challange dose. Klipstein and Engert (10) have also used rat jejunum in *in vivo* perfusion studies to demonstrate secretory activities of semipurified

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2. Background (contd.)

preparations of the heat-labile (LT) and heat-stable (ST) enterotoxins of Escherichia coli, Klebsiella pneumoniae and Enterobactoer cloacoe. Swabb et al (11) in their studies testing the efficacy of berberine as an antisecretory agent have used rat ileum as a model to induce CT induced secretion. They have modified their study design by constructing 2 loops in the same rat ileum so that one loop was used as the test and the other as a control following challange with 75 µg of purified cholera toxin. By this modification they could avoid the inter-animal variability of the response to CT.

The proposed research plan intends to study the effects of this new toxin on the jejunum and ileum of the rat intestine by an in vivo perfusion technique. Since the toxin has not been purified, at present the culture filtrate of the CT gene negative strains of V. cholerae 01 will be used.

3. Rationale

The proposed study will provide us with further information regarding the action of this newly recognised cholera toxin on the jejunal and ileal parts of the rat intestine i.e. whether this toxin increases the secretion of electrolytes and water in the intestine similar to CT. Since the model to be used is an in vivo perfusion technique using the whole of rat jejunum and ileum, the study will be akin to a physiological state.

B. Specific plan

- to set up an in vivo perfusion model using the whole of rat jejunum and ileum combined and also using only the rat ileum alone.

- to obtain baseline net flux (lumen→serosa) values of an electrolyte solution, similar in electrolyte composition to that of rat plasma, by using the marker perfusion technique.

- to study the secretory effect of known CT on this model, which will act as positive control.

- to study the secretory effects (if any) of the newly identified cholera toxin in this model.

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C. METHODS AND PROCEDURE

Source of the new cholera toxin produced by CT gene negative V. cholerae O1 strains:

Culture filtrates prepared in Richardson's medium as described by Sanyal et al (7) will be prepared and preserved at - 20°C Before use in perfusion studies, the toxin preparation will be tested for positive reaction in adult rabbit ileal loops and the batch which will cause maximum fluid accumulation will be used in the perfusion studies.

Composition of the perfusion solution:

The solution will contain Sodium-140. Potassium-5 and Chloride-145, all in mmol/L. It will also contain glucose 5 mmol/L. In addition, it will contain polythylene glycol (PEG), mol wt 4000 (Sigma) 5.0 g/L as a nonabsorbable marker.

Perfusion technique:

General :

Male adult rats will be used as subjects. Rats will be kept in special cages during fasting period to prevent corpophagia. After fasting (water day-1, and 5% dextrose day-2, allowed ad lib) for a period of 48 hours the rats will be anaesthetised by intraperitoneal injection of Nembutal 40 mg/kg. On laparatomy, a canula (proximal canula) will be introduced into the jejunum about 1 to 2 cms distal to the dudeno-jejunal flexure and tied securely. Similarly, the distal canula will be introduced about 2 to 4 cm proximal to the ileo-caecal junction. The canulated segment will be washed gently using the perfusion solution. Perfusion experiment will be started by infusing perfusion solution through the proximal canula at the rate of 0.4 ml/min at a temp. of about 37°C . The rate of infusion will be maintained by using a peristaltic pump. After allowing 45-min to achieve a steady state, the perfusate will be collected from the distal canula over ice. Details of the perfusion technique have been described elsewhere (12).

Towards a later stage of the study the perfusion technique adopted by Swabb et al (11) will used. Two ileal loops of about 18 to 20 cms in length and separated by 10 to 12 cms of ileum will be constructed. Perfusion studies will be conducted by attaching proximal and distal canulae in the loops, In this technique one loop would be randomly used for the test and the other loop will serve as control.

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Study sequence:

a) For standardisation and to obtain base line values:

Perfusion technique will be similar as mentioned above (General). After 45 minutes of equillibration, perfusate will be collected for 4, 15 min-perfusion-period. Ten successful experiments will be conducted. And also for base line values another ten successful experiments will be conducted. In these experiments the study segment will be incubated with 5 ml of perfusion solution for 2 hrs following which after 45 minutes of equillibration period perfusate will be collected for 3, 15 min-perfusion period.

b) for testing positive control :

Perfusion technique will be similar as mentioned above (General). After introducing the proximal and distal canulae, the perfused segments will be washed using the perfusion solution as described above (General).

Challanging with Cholera Toxin (CT):

Partially pure cholera toxin obtained from S.H. Richardson will be used. Cholera Toxin will be diss-lved in 5 ml of perfusion solution, which will be injected into the perfused segment by the proximal canula, after which both the proximal and distal canula will be closed. Two hours incubation time will be allowed for the toxin to act. Following this, the perfused segment will be washed and after 45 minutes of equillibration period, perfusate will be collected for 4, 15 minute-perfusion-period.

Dose of Cholera Toxin:

Initially, 100 µg of CT will be used. If after 3 successful experiments, no secretion of water and electrolyte occurs as determined by analysis of the perfusate (see below), then 200 µg of CT will be used. Likewise, 300, 400, 500 µg of CT will be used for the challange dose, if necessary.

If after challange with 500 µg of cholera toxin, no secretory response is elicited, then preparation of the rats for the experiment will be done as described by Aziz et al (8), and 500 µg of CT will be used for the challange.

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Dose of Cholera Toxin: (contd)

If secretory response of the mucosal cells by using any of the doses of CT, as mentioned above, becomes excessive as determined by the magnitude of water and electrolyte secretion, a lower dose of the toxin will be used.

c) for testing new cholera toxin:

The culture filtrates of the CT gene negative V. cholerae O1 strains will be used, in the increasing doses of 1 ml, 2ml, 3ml, 4 ml, 5 ml if no secretory response is observed in the lower dose. The culture filtrate will be diluted with appropriate amount of perfusion solution to give a final volume of 5 ml.

The details of the perfusion technique will be similar as for testing the positive control.

Every week the potency of the culture filtrate will be tested in the rabbit loop.

Ten successful experiments will be conducted.

d) for testing deactivated new cholera toxin:

The new cholera toxin preparation will be heated at 100°C for 15 minutes. Ten successful experiments will be conducted by using this preparation.

e) for testing negative control:

The culture medium used to grow the bacteria will be used as negative control. Five ml of the filtrate of the medium (the medium alone being incubated for 18 hours) will be used for the challange. After the exposure time of 2 hours and proper washing and allowing 45 minutes of equillibration, perfusate will be collected for 4, 15 minutes perfusion period.

Ten successful experiments will be conducted.

Perfusion studies in the ileum:

The study sequence will be repeated in the ileal segments. Details of the operative technique have been described elsewhere (11). Out of the 2 study segment one will serve as control and the other will be used as test segment at random. The study sequence b, c, d, e will be repeated. Ten successful experiments will be conducted in each series.

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Calculations:

Net transport of water and electrolyte can be calculated from the change in PEG concentration and the electrolyte concentration by the following formula (13).

The subscript I and F refer to initial and final concentration. IR refers to the infusion rate (in ml/min). In these studies, absorption is the measured disappearance of a substance from the intestinal lumen during the time interval of perfusion. Entry is the measured appearance (secretion) of a substance during a time interval in luminal fluid. Calculations are performed as follows :

1) PEG ratio (PEG R) = (PEG I)/(PEG F)

2) H_2O absorption % = 100 (1 - PEG R)

3) H_2 absorption, ml/15min = $\frac{H_2O, \% \text{ (IR) } (15)}{100}$

4) Na^+, $(\text{or } K^+)$ (or Cl) absorption mol/15min.
$((Na^+_I - (Na^+_F \times \text{PEG R})) (15) \times (1 \text{ R})$

Statistical calculations will be done by using student's t test.

Analysis:

PEG will be measured by turbidimetric method. Na, K will be measured by using flame photometer and chloride by a chloridometer. Osmolality will be measured by freezing poing depression using an Osmette automatic osmometer (Precision System Inc.).

Facilities required:

The existing facilities at the animal lab, biochemistry lab and microbiology lab will be utilised.

REFERENCES

1. Holmes, R.K., Bramucci, M.G., Twiddy, E.M. : Genetics of Toxinogenesis in Vibrio Cholerae and Escherichia Coli contrib Microbiol Immunol 1979; 6: 165-77.

2. Sanyal, S.C., Alam, K., Neogi, P.K.B., Huq, M.I., and Al-Mahmud, K.A. : A new cholera toxin (letter). Lancet 1983; 1: 1337.

3. World Health Organisation. Scientific Workingroup. Cholera and other Associated Diarrhoeas. Bull WHO 1980; 58: 353-74.

4. Kaper, J.B., Levine, M.M. :Cloned Cholera Enterotoxin Gens in the Study and Prevention of Cholera. Lancet 1981; 2: 1162-63.

5. Kaper, J.B., Mosley, S.L., Falklow, S. : Molecular Characterisation of Environmental and Non-toxigenic Strains of Vibrio Cholerae. Infect Immun 1981; 32: 66L-67.

6. Cholera in 1981. Weekly Epidemiological Rec. 1982; 57: 131-32.

7. Sanyal, S.C., Alam, K., Neogi, P.K.B., Huq, M.I., and Al-Mahmud, K.A. : A new Enterotoxin produced by Vibrio Cholerae O1. J Dir Dis Res 1984; 2: 3-12.

8. Aziz, K.M.S., Mohsin, A.K.M., Hare, W.K.? Phillips, R.A. : Using the Rat as a Cholera 'Model'. Nature 1968; 220: 814-15.

9. Sandhu Bhupinder, Tripp, J.H., Candy, D.C.A., Harries, J.T. : Loperamide Inhibits Cholera-Toxin-Induced Small-intestinal Secretion. Lancet 1979; 2: 689-9¼.

10. Klipstein F.A. and Engert R.F. : Reversal of jejunal water secretion by glucose in rats exposed to coliform enterotoxins. Gastroenterology 1978; 75: 255-262.

11. Swabb E.A., Tac Yuan-heng and Jordan Leila: Reversal of cholera toxin-induced secretion in rat ileum by luminal berberine. Am J Physiol 1981, 4 G 248 - G 252.

12. Patra, F.C., Mahalanabis, D., and Jalan, K.N. Stimulation of Sodium and Water Absorption by Sucross in the Rat Small Intestine. Acta Paediatric Scand. 1982; 71: 103-107.

13. Lavinson, R.A., and Schedl, H.P. Absorption of Sodium, Chloride, Water and Simple Sugars in Rat Small Intestine. Am J Physiol 1966: 939-42.

SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICE

Name	Position	% or No. of Day	Annual Salary	Project Requirement Taka	Project Requirement Dollar
Dr. F.C. Patra	Prin. Investigator	40%			8,000
Dr. K.A. Al-Mahmud	Co-Investigator	10%		20,400	
Dr. A.S.M.H. Rahman	-do-	20%		20,600	
Mr. K. Alam	-do-	20%		20,600	
Mr. Akbar Ali	-do-	5%		7,800	
Dr. S.C. Sanyal	Guest Scientist				
Dr. W.B. Greenough	Consultant				
Technician (Animal)		10%		5,500	
				74,900	8,000

2. SUPPLIES AND MATERIALS

	Unit Cost	Number Required	Project Requirement Taka	Project Requirement Dollar
New Zealand White Rabbit:				
Adult	250	25	6,250	
Rat: Adult	20	200	4,000	
Media				100
Laboratory Tests				3,000
Anaesthetic Agents			2,000	
			12,250	3,100

3. EQUIPMENT

Peristalitic Pump with Accessories	1		1,225
Surgical Instruments		1,000	
Glassware		6,000	
Water bath with Thermostatic Control	1		500
P.V.C. Tubing			100
Syringe, Needle, etc.			100
		7,000	1,925

4. PATIENT HOSPITALIZATION	None
5. OUTPATIENT CARE	None
6. TRANSPORT (ICDDR?B)	None
7. TRAVEL & TRANSPORT OF PERSONS	Nil
8. TRANSPORT OF THINGS	Nil
9. RENT, COMMUNICATION	Nil
10. PRINTING & REPRODUCTION	7,000
11. MISCELLANEOUS	5,000

B. BUDGET SUMMARY

	TAKA	DOLLAR
1. PERSONNEL SERVICES	74,900	8,000
2. SUPPLIES & MATERIALS	12,900	3,100
3. EQUIPMENTS	7,000	1,925
4. PATIENT HOSPITALIZATION	-	-
5. OUTPATIENT CARE	-	-
6. TRANSPORT (ICDDR,B)	-	-
7. TRAVEL & TRANSPORTATION	-	-
8. TRANSPORT OF THINGS	-	-
9. RENT, COMMUNICATION AND UTILITIES	-	-
10. PRINTING AND REPRODUCTION	7,000	-
11. MISCELLANEOUS	5,000	-
	1,06,150	13,025

contd. next