Library (2)

Attachment 1.

Date September 18, 1989

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Ms. Setarunnahar Saha

Trainee Investigator (if any) N/A

Application No. 89-008

Supporting Agency (if Non-ICDDR,B) N/A

Title of Study "Studies on the New cholera toxin"

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
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
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
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
   (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 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.

(PTO)

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

Setarunnahar Saha DEC 04 1989

89-008
25/9/89

APPLICATION FOR PROJECT GRANT

1. INVESTIGATOR : Ms. Setarunnahar Saha
2. TITLE OF THE PROJECT : Studies on the "New cholera toxin"
3. STARTING DATE : As soon as fund is available
4. COMPLETION : Two years from starting date
5. TOTAL BUDGET REQUESTED : US$ 32,700
6. FUNDING SOURCE :
7. PROGRAMME COORDINATOR : Dr. S. Tzipori S. Tzipori
Associate Director
Laboratory Sciences Division

8. AIMS OF PROJECT :

a) General aim:

Purification of the new cholera toxin and its physicochemical and immunobiological studies.

b) Specific aims:

1) The NCT from CT gene-positive (CT^+) and CT gene-negative (CT^-) strains of *Vibrio cholerae* 01 (5698 and X-392 respectively) will be purified to quantitate the yield of the toxin by these strains.

2) Antiserum against pure NCT will be prepared for the immunobiological study of the NCT.

3) Rabbit/mouse will be immunized by the NCT to see the protective antitoxic immunity against NCT induced diarrhoea.

A-033780

4) Monoclonal antibodies will be prepared using pure NCT.

5) The purified NCT will be characterized in respect to its physicochemical immunobiological and molecular properties (molecular weight, subunit structure, etc.) to elucidate its relationship with virulence factors of *V. cholerae*, such as Shiga-like toxins, haemolysin and protease.

6) Attempt will be made to develop an *in vitro* method for the detection of the NCT (such as Biken, coagglutination, etc.).

7) Application of *in vitro* test to determine the frequency of occurrence of the NCT in *V. cholerae* strains collected at ICDDR,B.

c) Significance:

The above mentioned detail study on this poorly characterized NCT will help to determine any immunobiological relationship of this toxin with other virulence factors of *V. cholerae*. It will also be helpful to understand the disease process of cholera and the role of NCT (if any) in the pathogenesis of the disease. Further, the study shall be of paramount significance not only for our understanding of the pathogenesis and epidemiology of the disease but also

in developing candidate live oral vaccine strains against cholera.

9. ETHICAL IMPLICATIONS : Not applicable

10. BACKGROUND :

A noble approach to protect against cholera is to develop or isolate cholera toxin gene-negative (CT^-) strains of *V. cholerae* O1 and to use them as live oral candidate vaccines. The approach effectively eliminates the possibility of reversion of full atoxic strains to full toxigenicity. Testing of such strains starting from Texas Star-SR (Honda and Finkelstein, 1979) to CVD103 (Levine *et al.*, 1988) has indicated the efficacy in volunteers, but unfortunately diarrhoea was apparent in several subjects. Further, isolation of the CT^- strains from patients with severe diarrhoea has also been reported (Morris *et al.*, 1988; Roger *et al.*, 1988; Honda *et al.*, 1988). Since the central dogma of cholera work has been that production of cholera toxin (CT) is the prerequisite for diarrhoea, these observations (diarrhoea by CT^- strains) have led to a substantial reevaluation of the pathogenesis of the disease. The possibility that other toxic factors such as haemolysin, protease and Shiga-like toxins may contribute to diarrhoea has also been proposed (Editorial, 1986).

The preliminary study of Sanyal *et al.* (1983) in ICDDR,B indicated the elaboration of a new cholera toxin (NCT) by

these CT^- *V. cholerae* O1 strains. Recent immunobiological studies with the NCT revealed-the presence of this NCT in CT gene-positive (CT^+) *V. cholerae* O1 strains including the hypertoxigenic strain 569B (Sanyal *et al.*, 1987; Saha and Sanyal, 1988, 1989a) and the antigenic identity among the NCT produced by CT^+ and CT^- strains (Saha and Sanyal, 1988, 1989b). This NCT was found to differ from known CT in antigenic nature, receptor site, mode of action and genetic homology (Sanyal *et al.*, 1984; Setarunnahar Saha, 1989, Ph.D. Thesis submitted in the Institute of Medical Sciences, BHU, India). Further, rabbit ileal loop reacting unit per ml of crude NCT (26.66 unit/ml) preparation indicates the high yield of the NCT in syncase medium by CT^- *V. cholerae* O1 strains. In physico-chemical characterisation the NCT was found to be heat-labile, resistant to trypsin and sensitive partially to pronase and completely to papain as judged by their biological activity in rabbit ileal loops. The molecular weight of the NCT was found to be 61,000 which was judged only by SDS-PAGE. All these studies focused the NCT as a challenge to the prevailing concept in pathogenesis, epidemiology and development of vaccine against the disease.

Research plan

The CT^+ (569B) and CT^- (X-392) strains of *V. cholerae* O1 will be used from the ICDDR,B culture stock. The NCT will be prepared in Syncase medium (Finkelstein *et al.*, 1966). The sterile filtrate will be concentrated by sequential

passage through 100,000 and 10,000 molecular weight cut-off ultrafiltration membranes and then by 80% ammonium sulphate precipitation. The centrifuged precipitate will be dialysed against 0.02 M phosphate buffered saline (PBS) of pH 7.2 at 4°C. The non-dialysable material will be passed through ion exchange chromatography columns and will be eluted with gradient salt concentration in buffer. The fractions will be tested for enterotoxic activity in rabbit ileal loop model of De and Chatterjee (1953). The purity and homogeneity of the toxin fraction will be tested by polyacrylamide gel electrophoresis (PAGE) (Davis, 1964). The molecular weight of the purified NCT will be determined using sephadex column chromatography following the method of Andrews (1964). SDS-PAGE will be used to explore the presence of any subunit structure and to determine the molecular weights of the subunits, if any, using the method of Laemmli (1970). The yield of the NCT by both CT^+ and CT^- strains of *V. cholerae* 01 will be quantitated by assaying the pure toxin in rabbit ileal loops (Saha and Sanyal, 1989).

Adult rabbit/mouse will be used to study the protective antitoxic immunity in either ligated small bowel loops or intact small intestine. Peroral immunization will be done to see the role of antibody against the NCT induced diarrhoea following the method of Lange and Holmgren (1978).

Antiserum against the purified NCT will be prepared by immunizing adult rabbits (Saha and Sanyal, 1988).

Inbred 8-14 weeks old BALB/C mice of either sex will be immunized with pure NCT following the method of Svennerholm *et al.* (1986) and monoclonal antibodies will be prepared using the technique developed by De St. Groth and Scheidegger (1980). The hyperimmune antiserum and monoclonal antibodies of the NCT will be used for the subsequent immunobiological studies.

For physicochemical characterization stability of the toxin to heat, pH, proteolytic enzymes (trypsin, pronase, papain) and different chemicals [sodium dodecyl sulphate (SDS), urea, mercaptoethanol, dithiothereitol, etc.] will be tested. Immunobiological relationship of the toxin with Shiga-like toxin will be tested by gel-diffusion test using the pure NCT against anti-Shiga toxin and by *in vivo* neutralization test. Any haemolytic and proteolytic activity will be tested by tube dilution (using human, sheep, rabbit erythrocytes) and milk agar plate methods, respectively.

The frequency of occurrence of the NCT in *V. cholerae* strains of diverse origin, collected at ICDDR,B over last 15 years, will be determined by *in vitro* tests.

REFERENCES

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4. De St. Groth, S.F. and Scheidegger, D. (1980) Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods, 35:1-21.

5. Editorial. (1986) Oral cholera vaccines. Lancet, ii:722-723.

6. Finkelstein, R.A., Atthasampunna, P., Chulasamaya, M. and Charunmethee, P. (1966) Pathogenesis of experimental cholera: biologic activities of purified procholeragen A. J. Immunol., 96:440-449.

7. Honda, S., Shimoirisa, K., Adachi, A., Saitok, Asano, N., Taniguchi, T., Honda, T. and Miwatani, T. (1988) Clinical isolates of *Vibrio cholerae* 01 not producing cholera toxin. Lancet, ii:1486.

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9. Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

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15. Sanyal, S.C., Neogy, P.K.B., Alam, K., Huq, M.I. and Al-Mahmud, K.A. (1989) A new enterotoxin produced by *Vibrio cholerae* 01. J. Diar. Dis. Res., 2:3-12.

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17. Setarunnahar Saha and Sanyal, S.C. (1988) Cholera toxin gene-positive *Vibrio cholerae* 01 Ogawa and Inaba strains produce the new cholera toxin. FEMS Microbiol., Lett., 50:113-116.

18. Setarunnahar Saha and Sanyal, S.C. (1989a) Neutralization of cholera toxin by antiserum against crude enterotoxin of cholera toxin gene-positive *Vibrio cholerae* 01 in rabbit ileal loop model. Indian J. Med. Res., 89:119-122.

19. Setarunnahar Saha and Sanyal, S.C. (1986b) Immunobiological relationship among new cholera toxins produced by CT gene-negative strains of *Vibrio cholerae* 01. J. Med. Microbiol., 28:33-37.

20. Svennerholm, A.M., Wikstrom, M., Lindblad, M. and Holmgren, J. (1986) Monoclonal antibodies to *Escherichia coli* heat-labile enterotoxins: neutralising activity and differentiation of human and porcine LTs and cholera toxin. Med. Biol., 64:23-30.

11. PUBLICATIONS OF INVESTIGATOR (last 5 years)

Dr. Setarunnahar Saha

1) Sanyal, S.C., Setarunnahar Saha, Saha, S.K. and Ahsan, C.R. (1987) Immunologic and genetic relationship between enterotoxins, pp.669-678. In: Progress in Venom and Toxin research. P. Gopalakrishnakone and C.K. Tan (eds.). National University of Singapore, Singapore.

2) Sanyal, S.C., Saha, S.K., Shukla, B.N., Saha, S., Sing, N.P. and Agarwal, R.K. (1987) Human, Poultry and Water isolates of *Campylobacter jujuni* produce CT-like toxin in Charles-Foster strains of rats, pp.44-52. In: Proceedings of First Asian Congress on Anaerobic bacteria in Health and Disease. A. Mehta and N. Kochar (eds.), Bombay, India.

3) Setarunnahar Saha and Sanyal, S.C. (1988) Cholera toxin gene-positive *Vibrio cholerae* 01 Ogawa and Inaba strains produce the new cholera toxin. FEMS Microbiol. Lett., 50:113-113.

4) Setarunnahar Saha, Chowdhury, A.A. and Farida Hug (1988) Aetiological agents of Meningitis in Bangladeshi children. Indian J. Med. Microbiol., 691):81-85.

5) Setarunnahar Saha and Sanyal, S.C. (1989) Immunobiological relationship among new cholera toxin produced by CT gene-negative strains of *Vibrio cholerae* 01. J. Med. Microbiol., 28:33-37.

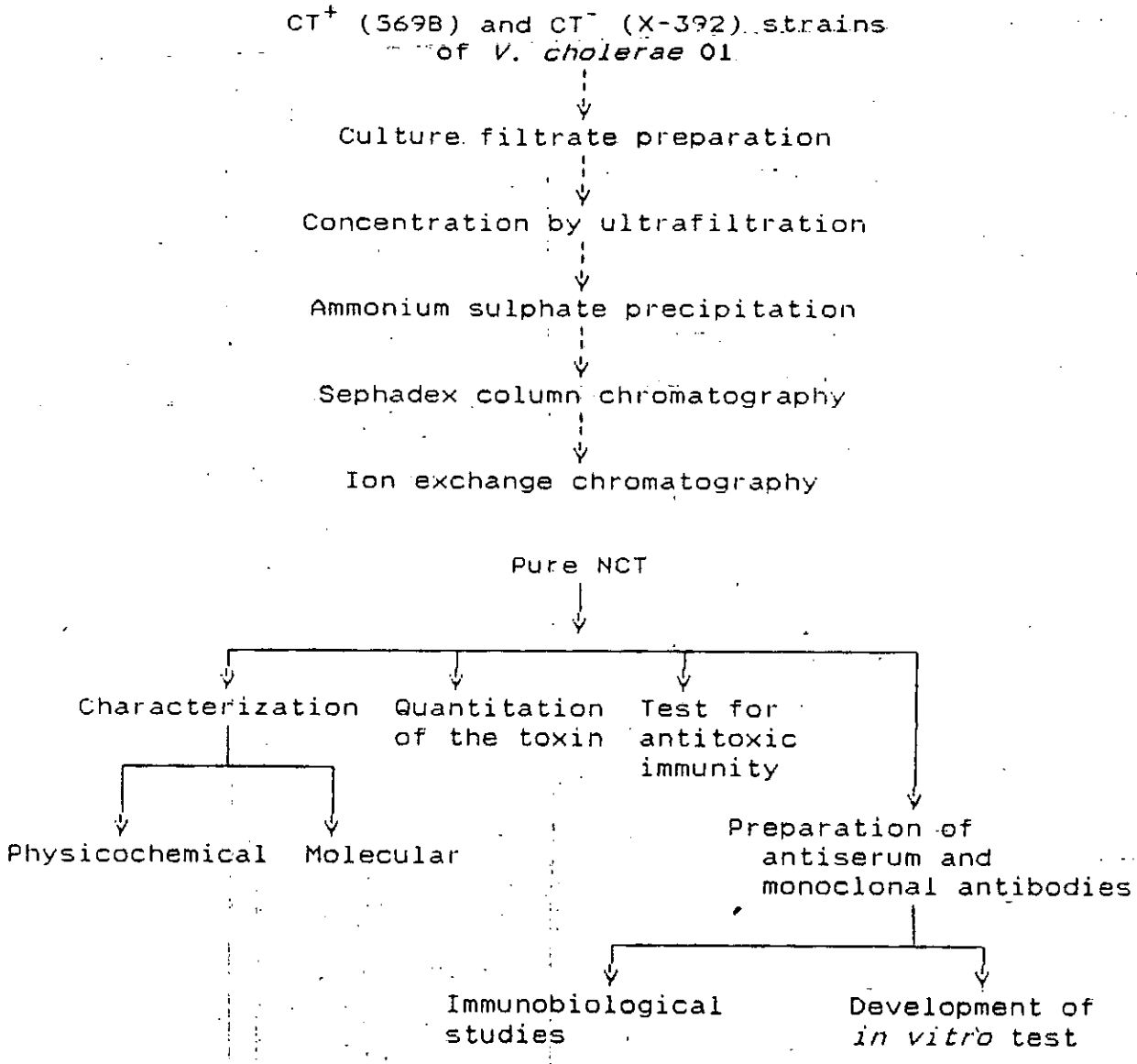
6) Setarunnahar Shaha and Sanyal, S.C. (1989) Antiserum against the crude enterotoxin of cholera toxin gene-positive *Vibrio cholerae* 01 neutralises the new cholera toxin in rabbit ileal loop model. Indian J. Med. Res., 89:119-122.

7) Sanyal, S.C. and Setarunnahar Saha. (1988) The new cholera toxin: A challenge to the prevailing concepts in pathogenesis epidemiology and development of a vaccine against the disease. BIOTEK InDIA '88 (Biotechnology in Health Care and Medicine), pp.1-12.

8) Satarunnahar Saha and Sanyal, S.C. (1988) Immunobiological relationship of new cholera toxin produced by cholera toxin gene positive/negative *V. cholerae* 01 (Abstract). XIIth National Congress of Indian Association of Medical Microbiologist, Trivandrum, India.

12. FLOW CHART (sequence of tasks within time frame)

12.1. Purification of the NCT:



12.2 First year

1. Pruficiation, characterization and quantitation of the NCT. Test of the NCT for any haemolytic and protease activities.

2. Antiserum preparation against pure NCT.

3. Attempt to develop an *in vitro* test to identify the NCT.

Second year

1. Preparation of monoclonal antibodies

2. Immunobiological studies to see the relationship of NCT with SLTs.

3. Studies to see the role of antitoxic immunity of NCT to protect the NCT induced diarrhoea.

4. Application of the *in vitro* test to determine frequency of occurrence of the NCT in *V. cholerae* strains.

13. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATOR:

14. BUDGET

		1st year	2nd year
a)	Personel	7,500.00	8,000.00
b)	Operating costs	7,000.00	8,000.00
c)	Capital equipments	0.00	0.00
d)	Travel	0.00	0.00
e)	Computing, other	1,000.00	1,200.00
TOTAL:		15,500.00	17,200.00

GRAND TOTAL: US$ 32,700.00

A. Instruments to be used:

1. Incubator with shaker
2. Centrifuge (high speed, cold)
3. Ultrafiltration system
4. Column chromatography system
5. PAGE system
6. Inverted phase contrastt microscope, etc.

B. Chemicals and media

Ammonium sulphate, casamino acid, salts, SDS, urea, acrylamide, bisacrylamide, agarose, DEAE sephadex A50, CM-cellulose, sephadex G-200, standard molecular weight markers, polyethylene glycole, Freund's complete and incomplete adjuvant, foetal calf serum, cell lines for monoclonal antibody production, myeloma cells, etc.

C. Glassware

Conical flasks, beaker, cylinder, petridish, slides, etc.

D. Rabbits, mice.

SPECIFIC COMMENTS

This project may hopefully contribute to increase the knowledge on cholera pathogenesis and might help to explain the phenomenon by which CT– *V. cholerae* strains may cause diarrhea in up to 50 % of challenged volunteers.

Unfortunately the application lacks a summary of the data hitherto achieved in the characterization of NCT - therefore it is difficult to evluate the originality of this project. The approach to purify the toxin is sound, but will probably only result in partaily purified toxin . Thus, it is important to exclude contamination with LPS that might induce high titers of anti-LPS antibodies in immune sera - it will be necessary to eliminate such antibodies by absorption before evaluating the protective effect of polyclonal anti-NCT sera! Furthermore, there is no indication of the quantities of NCT expected in culture filtrates, making it impossible to evaluate the feasibility to purify sufficient amounts of toxin for immunizations and characterization of immunological relationship with other toxins, and for the selection of monoclonal antibodies etc. This information is also important in evaluating the potentials of developing suitable in vitro tests for detection of NCT.

Therefore I suggest that the PI expands the background information somewhat to enable a better evaluation of the feasibility of the project!

From: Ms. Setarunnahar Saha

Clarification of the question raised by the reviewer:

1. The proposal is modified with expanded background as suggested by the reviewer.

   The preliminary characterisation of the NCT revealed that the toxin is a heat-labile, trypsin resistant protein which is sensitive completely to papain and partially to pronase as judged only by its biological activity in rabbit ileal loop.

   The effect of the proteolytic enzymes (trypsin, papain, pronase) on this toxin is further included in this protocol. This overlapping can be justified by the following point:

   In this study the effect of these enzymes will be determined at the molecular level by PAGE and by Sephadex gel filtration to demonstrate the activity of the enzymes if any, on any site of the toxin molecule which might not be the functional part for biological activity.

2. The molecular weight of the NCT was found to be 61,000 in SDS-PAGE.

   In this protocol, the molecular weight of the toxin will be determined by gel filtration as mentioned in the text. to see the exact molecular weight of the whole biologically active toxin moiety and to confirm whether any part of the molecule is missed in SDS-PAGE (13%).

3. The possibility of LPS contamination in the purified NCT is very less as these molecules are supposed to be eliminated at the time of Sephadex gel filtration. However, keeping the suggestion of the reviewer in mind, the purified toxin will be tested for the presence of LPS and any contaminating LPS will be removed by ultracentrifugation.

   Further, anti-LPS antibody in the antiserum (if any) will be removed by affinity chromatography (Svennerholm, A.M., 1974, Ph.D. thesis submitted in Goteborg University, Sweden).

4. The yield of the NCT from CT$^-$ strains of *V. cholerae* O1 were observed to be quite high. The ammonium sulphate precipitated culture filtrate (100 times concentrated) preparation from CT$^-$ strain X-392 was found to contain 26.66 rabbit ileal loop reacting unit per ml where one unit contains 24 µg of protein (Saha and Sanyal, 1989b;

Getarunnahar Saha. 1989. Ph.D. thesis submitted to the Institute of Medical Sciences, BHU, India).

On the basis of this observation, we will hopefully be able to purify sufficient amount of the NCT for the proposed work.

P2:CLARIF

GÖTEBORGS UNIVERSITET
Institutionen för medicinsk mikrobiologi
UNIVERSITY OF GÖTEBORG *Department of Medical Microbiology*

September 1, 1989

Dr Saul Tzipori
Associate Director
Laboratory Sciences Div
Int Centre for Diarrhoeal Disease
Research
GPO Box 128 Dhaka 1000
Bangladesh

Dear Dr Tzipori,

Re: Revised project proposal "Studies on the new cholera toxin"

In my opinion Dr Setarunnahar has answered the questions raised by me in my review of her protocol adequately. In relation to her specific clarifications I have the following comments:

1.,2. I think it is justified to repeat the proteolytic enzyme studies on the basis pointed out. I also find it important to do further characterization of the molecular weight of NCT in SDS-PAGE and in immunoblot analyses using specific antisera.

3. I am afraid that contaminating LPS in the NCT preparations will not be possible to eliminate through simple Sephadex gel filtration. Maybe one possibility to eliminate LPS would be through ultra- centrifugation although I think this approach has to be evaluated carefully. In the evaliation of the protective effect of anti-NCT antibodies it will be of great importance to test antibody preparations from which anti-LPS antibodies have been eliminated e.g. by absorption of sera on LPS affinity column as outlined or by using monoclonal antibodies against NCT.

4. The rabbit loop activity of NCT seems to be sufficient to allow purification of sufficient amounts of NCT for the work outlined in the protocol. However, the weight of one rabbit ileal loop reacting unit needs to be recalculated based on purified NCT.

In summary, I think that Dr Setarunnahar has answered the questions raised by me adequately and that there is good promise that this project may lead to new interesting information concerning the properties of NCTs. I suggest that her proposal is supported.

With kindest regards,

Yours sincerely

Ann-Mari Svennerholm
Professor

GÖTEBORGS UNIVERSITET
Institutionen för medicinsk mikrobiologi
UNIVERSITY OF GÖTEBORG *Department of Medical Microbiology*

FACSMILE COVER SHEET

From: Dept Medical Microbiology

Name: Jan Holmgren

Telefax No: 46 31 63 66 77

To: ICDDR,B

Name: Dr Saul Tzipori

Telefax No: 880 2 41 18 46

NUMBER OF PAGES 1 EXCLUDING COVER PAGE

MESSAGE:

Dear Dr Tzipori,

Attached you will find my evaluation of Dr S Saha's project grant application. As you can see from my comments I find this project to be good and worth supporting with some minor qualifications.

I look forward to seeing you in Copenhagen August 2.

With best regards.

Sincerely,

Jan Holmgren

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Detailed comments

This project represents an interesting and logical continuation of Dr Saha's previous work in this area together with professor S. Sanyal. The objective and specific aims of the project are highly worthwhile and the results can no doubt give important information. My only qualification would be that I am somewhat unclear to which degree these studies will differ from/add to the work already carried out in Dr Saha's thesis project and/or by Dr Sanyal. I just received a short résumé by Dr Sanyal of the work done on the new cholera (NCT) toxin in his laboratory (together with Dr Saha) in which he states: "NCT differs from the known CT in antigenic nature, receptor site, mode of action and genetic homology. It is a non-dialysable, heat-labile, trypsin-resistant, antigenic protein that can be separated as a single homogenous moiety in PAGE..... The molecular weight of NCT was determined to be 61,000 in SDS-PAGE.... and .... to be resistant to trypsin and sensitive partially to pronase and completely to papain". This description seems to overlap considerably with the general aim and the specific aim 5 of the application and this should be clarified with the investigator. Otherwise I find this to be a good and interesting project proposal well worth supporting.

Clarification of the question raised by reviewer

1. The purification of the New Cholera Toxin (NCT) aimed in this work will be from both cholera toxin positive (CT^+) and cholera toxin negative (CT^-) strains of *Vibrio cholerae* OI which will facilitate the quantitation of the NCT produced by the CT^+ strain. Further, the purification will be an obligatory part to fulfil other specific aims. The method of purification will be modified slightly on the light of the previous experience to increase the yield of toxin which was very low in earlier occasion (Ph. D. thesis submitted by Setarunnahar Sa[illegible] in the Institute of Medical Sciences, BHU, India).

   Immunobiological studies will include other specific aims (2-6) mentioned in the proposal.

2(a). Molecular weight of the NCT will be determined by gel filtration to see the exact molecular weight of the whole biologically active toxin moiety and to confirm whether any part of the molecule is missed in SDS-PAGE.

2(b). Part of the physico-chemical characterisation of the NCT, proposed in the protocol (specific aim - 5) will overlap the previous work. But, again these will be the confirmatory studies to characterise the toxin. However, in this study the effect of the enzymes on the NCT will be determined by PAGE, at the molecular level, which was done only on the basis of biological activity in the earlier study.