

CONSENT FORM

EFFECT OF CHARCOAL-G_{MI} ON CHOLERA

The Cholera Hospital is trying a new medicine to reduce the amount of diarrhoea in cholera. We would like you to participate in a study to find out whether this medicine that removes the cholera poison from your intestine will reduce your diarrhoea. You will receive either the new medicine or the same medicine without the part that binds the cholera poison. Except for this medicine you will be treated in the best and safest way possible. You will have tests done on your blood to insure that your treatment is adequate.

If you do not wish to be a part of this study you will still receive care for your cholera, as is required. You are also free to leave the study at any time should you wish to do so.

Investigator's signature

Date _____

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REVIEW BOARD ON THE USE OF HUMANS

CRL

Principal Investigator W.B. Greenough, III Trainee investigator (if any) _____

Application No 78-022 Supporting Agency (if Non-CRL) _____

Title of study Clinical Trial of Cholera Project status:
Ganglioside in cholera & E.coli enterotoxin Diarrhea
() New Study
() Continuation with change
() No change (do not fill out rest of ...)

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 possibly 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

- 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 to be submitted with individual studies).
- Protocol (Required)
- Abstract summary (Required)
- Statement given or read to subjects re 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 Board for review.

I agree to obtain approval of the Review Board on Use of Human Volunteers for any changes involving the rights and welfare of subjects before making such change.

W.B. Greenough, III
Principal Investigator

Trainee

SECTION 1 - RESEARCH PROTOCOL

- 1) Title: Clinical Trial of Charcoal GM₁ Ganglioside in Cholera and E. Coli enterotoxin Diarrhea.
- 2) Principal Investigators: W.B. Greenough, III and a Clinical Research Centre Physician
Jan Holmgren
Lars Svennerholm
- 3) Starting Date: September 1978
- 4) Completion Date: December 1978
- 5) Total Direct Cost: \$ 10,500

6) Abstract Summary: Mono Sialosylganglioside (GM₁) firmly binds cholera toxin neutralising its toxic properties. This ganglioside can be attached to finely divided charcoal thus forming a non absorbable matrix for the toxin receptors. Using this material and a control preparation of charcoal without ganglioside patients with cholera will be treated with material sufficient to bind all toxin produced by V.Cholerae in their intestinal tract. If toxin produced in the gut lumen is important in increasing the fluid loss we anticipate that patients receiving charcoal GM₁ will have a milder course than those receiving charcoal alone or simply ordinary fluid replacement. Should this prove to be the case charcoal GM₁ will be used prophylactically in a selected population which is at high risk to contract cholera. If the only toxin important to the pathogenesis of cholera is that produced by vibrios closely attached to the mucosal cells then the charcoal GM₁ approach will not have appreciable effect. This information in itself would be important to know. Should the charcoal GM₁ be effective in cholera similar studies would be done in enterotoxigenic E.Coli diarrhea.

7) Reviews:

- a) Research Involving Human Subjects: _____
- b) Research Committee: _____
- c) Director: _____
- d) BMRC: _____
- e) Controller/Administrator: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objectives:

- a) To evaluate the ability of charcoal - G_{MI} (C- G_{MI}) to bind V. cholerae and E. coli enterotoxins and to reduce duration of secretion and total stool output in clinical cases.
- b) If this study gives promising results, to evaluate the prophylactic value of C- G_{MI} against cholera in high risk population (family contacts of index cases).

2. Background: At present there has been no practical advantage taken in human cholera of the wealth of information about the binding of cholera toxin or its mode of action. One of the vulnerable points that can prevent the action of toxin on the intestine is at its binding site. In nature or experimentally toxin can be prevented from reaching its intestinal receptor by antitoxic antibodies, G_{MI} ganglioside, or by prior blocking of the receptor by the binding subunits of the toxins. The present protocol proposes an innovative approach to the practical application of knowledge of receptors to prevent or modify disease in humans.

There is now very strong evidence that the monosialosylganglioside G_{MI} is the membrane receptor to which cholera toxin must bind before it can induce intestinal secretion and diarrhoea (1-3). Thus, it has been shown that 1) G_{MI} specifically binds and inactivates cholera toxin in equimolar proportions. (4,5); 2) a direct correlation exists between the cellular G_{MI} content and the number of toxin receptors in intestinal cells of various species (6,8); 3) chemical modifications of cholera toxin affect binding to cells and to isolated G_{MI} to the same extent (9); 4) incorporation of exogenous G_{MI} into the membrane of target cells increases the number of binding receptors as well as the biologic responsiveness to toxin (3,6); and 5) binding of cholera toxin to cell membranes specifically prevents the G_{MI} from being oxidized by galactose oxidase (3).

It has been shown that experimental cholera, induced by either live cholera vibrios or preformed cholera enterotoxin in ligated intestinal loops rabbits, can be completely prevented by the simultaneous presence in the perorally administered G_{MI} ganglioside could be practically useful by (a) prophylactically preventing development of cholera and (b) reducing the duration and volume of diarrhoea in cholera cases by blocking any further delivery of toxin to the intestinal cells. On the other hand it is not possible to remove, either by G_{MI} nor by other means, the toxin which has already bound to the intestine. However, as mentioned above, gangliosides can become incorporated in the plasma membrane of mammalian cells (10,6,3). In the case of G_{MI} this results in generation of additional receptors for cholera toxin, which increases the susceptibility of the intestine to experimental cholera. To avoid the paradoxical situation that under certain conditions the administered G_{MI} may render the intestine more rather than less sensitive to cholera toxin it is important to prevent this membrane incorporation of the ganglioside. The safest means to achieve this is by coupling the ganglioside via its ceramide portion to a solid matrix.

In recent years it has become apparent that acute diarrhoeal diseases other than cholera are often also produced by enterotoxigenic bacteria. Thus, certain strains of E. coli produce one or both of two different enterotoxins, one being labile (LT) and the other stable (ST) on heating. Such bacteria are a common cause of diarrhoea in many countries and are probably responsible for the majority of diarrhoeal illness among international travellers. E. coli LT is structurally related to cholera toxin as shown by immunological methods. It also binds to ganglioside G_{MI} , and in experimental animals G_{MI} can prevent LT from causing any intestinal fluid output (11, 13). This gives hope that matrix-coupled G_{MI} might be prophylactically effective against E. coli LT-induced diarrhoea in man also.

B. SPECIFIC AIMS:

1. To determine whether charcoal bound G_{MI} ganglioside will decrease the severity of human cholera.

3. Rationale:

It is not known whether the toxin elaborated by V.cholerae swimming freely in the gut lumen is important or trivial in the pathogenesis of cholera. With GM₁ ganglioside bound to charcoal all toxin within the lumen of the intestine can be neutralized. If this free toxin is an important mediator of fluid loss it is expected that the specific toxin absorbant (charcoal GM₁ ganglioside) will decrease the rate and duration of diarrhea.

This knowledge thus has both practical as well as theoretical implications.

B. SPECIFIC AIMS:

1. To determine whether charcoal bound GM₁ ganglioside will decrease the severity of human cholera.

2. To see whether cholera can be prevented by charcoal-bound G_{MI} ganglioside.

C. METHODS OF PROCEDURE:

Preparation of charcoal-coupled G_{MI}-ganglioside

Calf brains are homogenised with phosphate buffer and the brain lipid is extracted with chloroform-methanol 1/2 by volume. The lipid extract is freed from the brain residue by filtration through fuller earth. To the lipid extract is added water to give a final ratio of chloroform-methanol water 1/3/1.4, by volume. After mixing two solvent phases will occur. The gangliosides, partitioned in the upper methanol-water phase, are isolated from the extract by the passage through a strong cation exchange resin in hydrogen form and then attached to a column with Spherosil-DEAE-dextran in acetate form.

The gangliosides are eluted from the Spherosil column with 0.5 M potassium acetate in ethanol. After dilution the extract with water the gangliosides are absorbed to charcoal W3SL under continuous agitation - the amount of charcoal is determined from the desired concentration of ganglioside coupled to the charcoal.

Higher gangliosides are converted to G_{MI}-ganglioside by suspending the charcoal with absorbed gangliosides in 0.2 M formic acid and heating. The charcoal-G_{MI} is collected by centrifugation, rinsed with potassium hydrogen carbonate and dried at room temperature on a heated drum.

Control assay on the charcoal-coupled G_{MI}-ganglioside

A portion of the gangliosides attached to the charcoal can be extracted by boiling with chloroform-methanol water 60/30/34.5, by volume. Approx. 50% of the gangliosides will be released from the charcoal by extraction, but the released lipids are a representative sample of the gangliosides and derivatives absorbed to the charcoal. Their percentage composition is determined by thin-layer chromatography and scanning of the developed fractions. This test is a control on the hydrolysis of higher gangliosides to G_{MI}-ganglioside, and that no measurable amounts of other substances than ganglioside G_{MI} and its derivatives are coupled to the charcoal.

The activity of the charcoal-G_{MI} preparations are controlled by toxin neutralization assays. The neutralization of an exact amount of cholera toxin is determined for each batch by comparison with standard series of charcoal-G_{MI} ganglioside with defined concentrations. (For details, see under the next section).

The release of gangliosides from the charcoal has been tested with a large number of solvents and chemicals. These assays have been performed with labelled G_{MI} - ganglioside and the concentration of ganglioside has varied between 10 to 0.1 umole G_{MI}/g charcoal. Less than 5% G_{MI} ganglioside was removed by water with a large number of different electrolytes, amino acids, sugars and proteins in varying concentrations, further with ethanol or ethanol water mixtures at 37°C, methanol, propanol and acetone. Less than 5% G_{MI} ganglioside was eluted from the charcoal by human gastric and duodenal juices and human duodenal mucosa. It has not been possible to determine whether gall bladder bile or various bile salts in concentration of 5mM or higher will release more than 5% of G_{MI} ganglioside since the bile salts will remove fine charcoal particles attached to the standard sized charcoal particles and these fine particles are trapped in the bile salt micelles and cannot be removed for an assay of the G_{MI} released from the charcoal.

Ability of C-G_{MI} to bind cholera toxin

The ability of C-G_{MI} preparations to fix cholera toxin was evaluated in vitro experiments. Lyophilized crude toxin (NIH, preparation 001 after dialysis against distilled water for 48 h) was dissolved in a glycine-NaCl buffer, pH 7.5 to a concentration of 30 mg/ml., being equivalent to 37.5 ug/ml pure toxin. To 50-200 ul of this toxin was added an equal volume of C-G_{MI} (or for control purposes unlabelled charcoal) suspended in the same buffer to various densities. The mixture was incubated on a roller drum at 23°C for 1 h, the C-G_{MI} or unlabelled charcoal sedimented by centrifugation and the supernatant analysed for remaining unbound toxin by means of single radial immunodiffusion according to Mancini using a toxin-specific antiserum. The results have shown that whereas the unlabelled charcoal under these conditions bind negligible amounts of toxin, the G_{MI} preparations bind toxin in amounts directly proportional to the coupled G_{MI} over a wide range until sterical hindrance begins to reduce the ratio (10 umol G_{MI}/g charcoal). At 5 Umol G_{MI}/g charcoal 1 g of C-G_{MI} fixes approximately 5 mg cholera toxin, i.e. about 1 ug toxin/umole bound G_{MI}. In vivo protection against

experimental cholera has been tested in rabbits, in which 20-25 small bowel loops were ligated. Into the loops were injected 1 ml of either live cholera vibrios (569B) or crude cholera toxin in graded doses mixed shortly prior to injection with 0.1 ml of C-G_{MI}, unlabelled charcoal or buffer only. Fluid accumulation was registered 18 h after charcoal tested in 1-5 mg/loop gave only little (2-fold) increased resistance to toxin challenge and no protein at all against live vibrio challenge, C-G_{MI} provided very significant protection against both types of experimental cholera.

Thus it can be seen in the table at e.g. 5 μ mol G_{MI} per g charcoal the in vivo protection obtained against toxin challenge closely matched the previously mentioned in vitro binding capacity of the C-G_{MI} preparation, i.e. about 5 mg toxin was bound and thereby inactivated per g C-G_{MI}. Using 5 mg of this C-G_{MI} preparation per loop the protective factor was about 40-fold against toxin and live vibrios. The better protection against toxin is probably explained both by the fact that there was an incubation of toxin with C-G_{MI} prior to the injection into the loops and by a presumed mucus penetration and epithelial adherence of a fraction of the vibrios, which should make their released toxin inaccessible to C-G_{MI}. However, the data suggest that the later fraction, at least as studied in rabbit ligated loops, was relatively small and show that continuous binding of luminal toxin is of prime importance for protection against live vibrio challenge in this model.

Subjects

Adult male patients presenting to the Treatment Centre of CRL, Dacca who are moderately to severely dehydrated and dark field positive for V. cholerae will be admitted to the Clinical Research Centre. Hydration will be accomplished according to the degree of dehydration then maintenance provided to match stool losses. This will be done by the intravenous route. Patients will receive charcoal, charcoal-G_{MI} ganglioside or charcoal alone according to a table of random numbers. Venous blood will be drawn for specific gravity and electrolytes at admission, four hours, 24 hours and discharge. Intake and output will be measured every eight hours. All other

care will be according to the clinical indications. No antibiotics will be used. The dose of charcoal would be calculated as follows:

Total gut volume 2 liters C-G_{MI}/mg/ml live vibrio challenge give 20 fold protective margin. Thus one gram per liter gut volume would be estimated to give a large margin of protection. The C-G_{MI} will be given over every hour in 1 gram dose to insure a constant protective level during the period of rapid transit time. When the stool volume decreases below 50 ml/kg/24 hours the dose will be decreased to 1 gram every 4 hours. It is estimated that if there is a large difference between groups a total of 15 patients would be needed in each category or a total of 45 individuals. If a definite effect is seen on the course of cholera then the study would be extended to E. coli diarrhoea selecting patients of a similar degree of illness who are dark field negative. If no cholera patients are available at the time of the study then the E. coli patients may be taken first. The design of any prophylactic use of the charcoal-G_{MI} ganglioside will await analysis of the clinical studies. The approach would be select an index case with cholera; then go to the household and administer the charcoal-G_{MI} ganglioside suspension to close contacts observing the secondary routes.

In the prophylactic studies the charcoal matrix with or without ganglioside would be given every 4-6 hours to insure that a substantial amount of binding material would always be present in the gut.

Risks to Subjects

Charcoal is a remedy that is in standard use in medical practice to diminish gas in the intestine. G_{MI} ganglioside is a normal component of our foods. There would seem to be no risk involved in this study to human subjects. No measurements will be made that are not a part of good medical care. Since Tetracycline will not be given however, there will be prolongation of diarrhoea in some instances. The patients will be informed of this as well as the nature of the medicine to be used.

D. SIGNIFICANCE:

There are two major points of significance to this study.

1. At a theoretical level the question "does toxin generated in the gut lumen produce a significant component of the illness?" Can be answered unequivocally.
2. Can a toxin binding maneuver alter the course of cholera? Since the ingredients are cheap charcoal and animal brain such an approach might reduce diarrhoea sufficiently to allow more patients to be treated by oral electrolyte replacement, reducing costly or unavailable intravenous fluid requirements.

E. FACILITIES REQUIRED:

1. Office space for the principle Investigator and guest investigator.
2. Laboratory space for toxin testing may be desirable although most of this work will be done in Sweden.
3. Hospital Resources. Patients will remain in the hospital or average of 4 days. Estimating 50 patients in each study there will be 200 patient days for the cholera and 200 for the E. coli study. The staff of the study ward will be needed for the measurements to be made and care of the patients.
4. Then space will be needed if a preventive study is carried out.
5. A field worker will be needed if a preventive study is done.

F. COLLABORATIVE ARRANGEMENTS:

This study will be done in collaboration with the Department of Bacteriology, University of Goteborg, Sweden. Dr. Jan Holmgren will be in Dacca as as a guest investigator during the time of this study.

BIBLIOGRAPHY

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3. Moss, J., Fishman, S.H., Manganeillo, V.C., Vaughan, M., and Brady, R.O., "Functional incorporation of ganglioside into intact cells: induction of cholera for responsiveness. Proc. Nat. Acad Sci: 73: 1034-1037 (1976).
4. Van Heyningen, W.E., Carpenter, C.C.J., Pierce, N.F., and Greenough III, "Deactivation of cholera toxin by ganglioside". J. Infect. Dis. 124: 415-416 (1971).
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6. Holmgren, J., Lonroth, I., Mansson, J.E., and Svennerholm, L., "Interaction of cholera toxin and membrane G_{MI} ganglioside of small intestine". Proc. Nat. Acad. Sc: 72: 2520-2524 (1975).
7. Gascayne, N., and Van heyningen, W.E., "Binding of cholera toxin by various tissues". Infect. and Immun. 12: 3547-3557 (1973).
8. Gill, M., "The mechanism of action of cholera toxin". Advances in cyclic Nucleotide Res. 8: (1977).
9. Holmgren, J., and Lonroth, I., "Cholera toxin and the adenylates cyclase-activating signal". J. Infect. Dis. 133: 664-674 (1976).
10. Geatre Casas, P., "Gangliosides and membrane receptors for cholera toxin". Biochem. 12: 3558-3566.

11. Pierce, N.F., "Differential inhibitory effects of cholera toxoids and ganglioside with enterotoxin of V. cholerae and E. coli". J. Exp. Med. 137: 1009-1023 (1973).
12. Zenner, T.V. and Metzger, J.F., "Comparison of the action of E. coli enterotoxin on the thynocyte adenylate cyclase-cyclic adenosine monophosphate system to that of cholera toxin and prostaglandin E. Infect and Immun. 10: 503-509 (1974).
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SECTION III - BUDGET
A. DETAILED BUDGET

PERSONNEL SERVICES

<u>Name</u>	<u>Position</u>	<u>% of Effort or number of days</u>	<u>Annual Salary</u>	<u>Taka</u>	<u>Dollars</u>
Dr. W.B. Greenough III	Investigator	10	50,780	-	5078
Dr. Jan Holmgren	Investigator	10	No cost to CRL		
Dr. I. Lonnoth	Investigator	10	No cost to CRL		
	Res. Physician	15	33,060	4,960	-
				4,960	5,078

SUPPLIES AND MATERIALS

<u>Items</u>	<u>Unit Cost</u>	<u>Amount Required</u>
Charcoal and charcoal G _{MI} ganglioside to be supplied by Dr. J. Holmgren		
No cost to CRL		
For toxin titren in Y. adrenal cells 100 tests	Tk. 7.50/test	750.00

EQUIPMENT

None

PATIENT HOSPITALIZATION

400 patient days Tk. 150.00 60,000

OUTPATIENT CARE

None

CRL TRANSPORT:

Mileage - Dacca: None

(Supplemental budget will be prepared if field study sector is to be implemented .

TRAVEL AND TRANSPORTATION OF PERSONS:

Local Travel

None

International Travel

½ roundtrip ticket Sweden to Dacca for

Dr. Holmgren

\$ 740

TRANSPORTATION OF THINGS:

No CRL Cost

Import of supplies from Sweden

RENT, COMMUNICATIONS & UTILITIES

None

PRINTING AND REPRODUCTION:

Publication cost reprints &/or page cost in journal

\$ 300

OTHER CONTRACTUAL SERVICES:

CONSTRUCTION, RENOVATION & ALTERATIONS

None

B. BUDGET SUMMARY

(Sept. '78 thru Dec. '78)

<u>Category</u>	<u>Taka</u>	<u>Dollar</u>
1. Personnel	4,960	5,078
2. Supplies	750	-
3. Equipment	-	-
4. Hospitalization	60,000	-
5. Outpatients	-	-
6. CRL Transport	-	-
7. Travel Persons	-	740
8. Transportation Things	-	-
9. Rent/Communication	-	-
10. Printing/Reproduction	-	300
11. Contractual Service	-	-
12. Construction	-	-
	<hr/>	<hr/>
Total	65,710	6,118

Total \$ 10,500

Conversion Rate \$ 1.00 = Tk. 15.00

Table

Exp.	Toxin challenge		Live vibrio challenge	
	ED ₅₀ ^a (μ g pure toxin)	PF ^b	ED ₅₀ ($\times 10^{-6}$)	PF
A.				
Charcoal				
1 mg/loop	0.46		3.0	
C-GM1 25 nmol/mg				
1 mg/loop	10.5	23	30	10
B.				
Charcoal				
2.5 mg/loop	0.45		NT	
C-GM1 10 nmol/mg				
2.5 mg/loop	16.5	37	NT	
C.				
Charcoal				
5 mg/loop	0.60		4.0	
C-GM1 5 nmol/mg				
5 mg/loop	25.0	42	75	19

^aToxin dose required for half-maximal fluid accumulation; determined with crude toxin and value translated into purified toxin based upon single radial immunodiffusion comparisons.

^bProtection factor estimated as the ratio between ED₅₀ for C-GM1 and ED₅₀ for unlabelled charcoal.

Abstract Summary

A clinical trial will be done with 40 patients having watery diarrhea darkfield positive for V.cholerae to determine whether Charcoal-bound GM₁ monosialosyl ganglioside will decrease the amount and/or duration of diarrhea.

This action is based on the known avid binding of cholera toxin by this ganglioside. Patients will be admitted to the Clinical Research Unit in Dacca. History and physical examination will be done and intravenous treatment given as indicated by degree of dehydration and stool losses.

Careful observation of intake and output will be done and blood drawn to determine the progress of treatment. Patients will receive either charcoal or charcoal with ganglioside attached according to a table of random numbers. The investigators will not know which preparation is being given to which patients.

1. Subjects will be uncomplicated adult males.
2. Informed consent will be obtained but since no information of a sensitive nature is to be taken no special precaution will be taken to protect privacy other than that normally observed for CRL medical records.
3. There will be no known risks to the subjects and potential benefits in this study. Charcoal is a standard medicine for flatulence and ganglioside a component of a normal diet which includes meat.
4. The patients will receive more intensive care and observation than is usual. This would be expected to be of potential benefit.