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Comment No. 29 Date 10 March 1986 3.3.86

ETHICAL REVIEW COMMITTEE, ICDDR,B. DHAKA - 12

Principal Investigator DR. BRAD Kay Trainee Investigator (if any) None

Application No. 86-014 Supporting Agency (if Non-ICDDR,B) UNDP

Title of Study Assessment of Antitoxic Project status:

Immunity Conferred by the Oral Whole Cell (X) New Study

Killed Cholera Vaccine against Non-O1 Rabbit Model. ( ) 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: N/A

(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 an overview (all other requirements will be submitted with individual studies
   X Protocol (Required)
   X 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 Ctte. 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.

Bradford C. Kay
Principal Investigator

Trainee

MAR 31 1986

SECTION I - RESEARCH PROTOCOL

1. Title : Assessment of Antitoxic Immunity Conferred by the Oral Whole Cell B-subunit Cholera Vaccine Against Non-01 Vibrionaceae

2. Principal Investigator : Dr. Bradford A. Kay

Co-Investigators Dr. Ivan Ciznar
Dr. Jeff R. Harris
Dr. John D. Clemens

3. Starting Date : March 01, 1986

4. Completion Date : February 28, 1987

5. Total Direct Costs : $ 16,620.00

6. Scientific Program Head :

This protocol has been approved by the Disease Transmission Working Group.

Signature _____

Date 19 Feb 1986

7. Abstract Summary :

A number of extracellular factors have been associated with the virulence of the Vibrionaceae. These may include cholera toxin, heat stable toxin, heat labile toxin and a variety of other agents which include hemolysins, permeability factors and other ill-defined necrotizing or toxic agents.

The killed whole cell B-subunit oral cholera vaccine now being evaluated in Matlab represents an attempt to effectively immunize a susceptible population against the B subunit of cholera toxin as well as to a variety of somatic antigens of V. cholerae which may likewise be important in protective immunity. In view of reports that several members of the Vibrionaceae have variable abilities to produce cholera toxin or cholera-like toxin, we propose to examine the production of CT or CT-like toxins made by members of the Vibrionaceae other than serotype 01 V. cholerae. Bacterial strains to be tested will be those isolated from a defined population of diarrhoeal patients examined in association with the post-vaccination surveillance program being carried out in Matlab. Isolates will be biochemically and serologically identified as completely as possible and then tested for their ability to produce toxins which are cross reactive with CT. Results of this study will give information as to (a) the relative numbers of CT or CT-like toxin producing Vibrionaceae in the vaccinated population, (b) the efficacy of the oral vaccine to protect against these organisms, and (c) a clearer understanding of the scope of protection the oral vaccine imparts to immunized individuals.

8. Reviews :

a. Ethical Review Committee _____

b. Research Review Committee _____

c. Director _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective :

We propose to investigate the existence of toxins produced by non-01 Vibrionaceae isolates which may be identical or similar to Cholera toxin.

The aim is to determine if the Oral Cholera Vaccine has the capability of eliciting antibodies with cross reactivity to toxins produced by members of the non-01 Vibrionaceae.

2. Background :

Members of the Vibrionaceae exclusive of the 01 serotype of *V. cholerae* are, with mounting frequency, being implicated in a variety of human clinical disorders. *Aeromonas*, *Plesiomonas*, Non 01 serotype *V. cholerae* and at least nine additional *Vibrio* species (NCV's) are currently identified as human pathogens (1,2). These organisms have been associated with gastroenteritis, wound infections, septicemia and miscellaneous infections including meningitis, peritonitis, otitis, endocarditis and cystitis (3,4). This protocol deals specifically with the members of the Vibrionaceae exclusive of 01 serotype *V. cholerae*. These organisms will be referred to in this proposal as the "non 01 Vibrionaceae".

A number of extracellular factors have been associated with the virulence of the non 01 Vibrionaceae. Among these are the production of cholera (cholera-like?) toxin, heat-stable enterotoxin (positive result in the infant mouse assay used to

detect Escherichia coli heat-stable toxin), heat-labile toxin that induces steroidogenesis and increases cAMP in Y1 adrenal cells and a variety of hemolysins and permeability factors (1,2,5,6,7,8)

Since February, 1985, members of the Vibrionaceae have routinely been isolated from patients attending several treatment centers in the Matlab area as part of the surveillance for the current Oral Cholera Vaccine trial. Preliminary data show that approximately one third of patients attending these centers will yield cultures positive for a member of the Vibrionaceae, exclusive of serotype 01 V. cholerae. Of these non-01 Vibrionaceae positive patients, aproximately 70% will possess no other identifiable pathogen. Therefore, there is gathering evidence that non-01 Vibrionaceae may play a significant role in the etiology of diarrhoea in this population. These observations, coupled with the ever-increasing body of world literature implicating newly identified members of the Vibrionaceae in diarrhoeal disease, warrant their further examination.

3. Rationale :

If antibodies to the Oral Cholera vaccine are cross reactive with toxins produced by various members of the Vibrionaceae, there may be evidence for in-vivo protection of vaccine recipients to a broader range of pathogens than just the 01 serotype of V. cholerae. Such a finding could be of great significance in light of the apparent magnitude of infection by these organisms in this vaccine population and worldwide.

B. SPECIFIC AIMS

In order to accomplish the objectives of this research protocol, the following specific aims must be accomplished :

1. The identification to species of all non-01 Vibrionaceae isolated from vaccine trial surveillance cultures.

2. The examination of a statistically significant number, of non-01 Vibrionaceae isolates for the production of toxins that cross react with CT as determined by the Y1 adrenal cell assay, GM1 ganglioside ELISA and cross immunoelectrophoresis.

C. METHODS OF-PROCEDURE

1. Identification of Isolates

Non-01 Vibrionaceae will be identified to species according to the methods described by Popoff and Veron (9), Colwell et al. (10) Farmer et al.(2) and Furniss et al.(11). Only well identified strains will be evaluated for cross reactivity of antigens to CT or complete vaccine.

2. Selection of Isolates

In order to increase the probability of examining only genuine etiologic strains of the non-01 Vibrionaceae, the following criteria will apply :

a. GM1 Ganglioside Assay :

All isolates of the species Vibrio exclusive of 01 serotype V. cholerae, will be tested by this method (approximately 250 isolates). In addition, Aeromonas isolates (irrespective

of species) will be selected according to the following criteria :

i. All Aeromonas from children under the age of 5 years (approximately 360 isolates).

ii. All Aeromonas from persons who received two or more doses of either oral cholera vaccine or placibo (approximately 200 isolates).

iii. All Aeromonas isoltes from a 20% random sample of surveillance patients not in either category i or ii above whose laboratory numbers end in either "0" or "5". (approximately 90 isolates).

Therefore, approximately 650 Aeromonas and 250 Vibrio isolates will be tested by the GM1 Elisa assay method.

b. Y1 Adrenal Cell Assay

All isolates which give positive results in the GM1 ELISA assay will be further tested by the Y1 Adrenal Cell assay. In addition, a random sample of up to 200 strains representing all species of the non-01 Vibrionaceae isolated will be tested by the Y1 method.

c. Cross Immunoelectrophoresis (XIE).

All positive isolates from the GM1 or Y1 assay methods will be examined by XIE. Antisera to CT, 01 V. cholerae and to the complete oral vaccine will be run against cellular and extracellular antigens of strains tested.

3. Propigation of isolates

All strains for antigen studies will be grown overnight in either syncase broth (5,12) or caseamino yeast extract broth (13).

Cross immunoelectrophoresis (XIE) Method :

A modification of the original method described by Kroll (14) will be carried out as follows :

Briefly, TRITON buffer (ionic strength 0.02, pH 8.2) will be incorporated into 1% agarose (BioRad). The first dimension separation will be carried out at 5 mAmp/per plate, for 45 minutes. The second dimension electrophoresis will be carried out at 1.5 mAmp/plate for 18 hours. The dimension of the plate will be 5x5 cm, the volume of antigen 20 ul and the volume of incorporated antiserum in the second dimensional gel 20 ul/cm^2. After electrophoresis, the gel will be washed, dried, stained with CBB (Pharmacia, Sweden) and destained.

Preparation of the whole cell extract:

Cells from the 18 h culture will be separated by centrifugation at 16,000 X G for 15 minutes. The pellet will be diluted in a minimal amount of distilled water and sonicated at 20 KHz, 75 watts using a microtip in six, 5-second intervals. After sonication, an equal volume of TRITON buffer will be added to the lysate and cells will be further extracted by mixing with glass beads on a rotary shaker for 20 minutes. After extraction, the lysate will be centrifuged at 25,000 G for 20 minutes, the supernatant will be dialyzed overnight and the remaining fraction lyophilized.

Preparation of rabbit antisera:

Rabbit antisera will be prepared against the following antigens : whole cell extracts of both Inaba and Ogawa serotypes of V. cholerae as well as the B-subunit. Whole cell mixture, whole cells only and purified cholera toxin. The immunization schedule is as follows. Immunization will start with the first I.V. dose equivalent to 10^6 cells, followed after one week by the second dose of 5.10^7 cells. Then, four doses (10^7, 5.10^8, 10^8, 5.10^9) will be injected at two-day intervals. After a one week interval, the last dose of 10^9 cells will be injected, and one week after the last dose, the sera will be collected.

Antiserum against CT and complete vaccine will be prepared by I.V. injection of three doses of CT (100 ug, 100 ug, 150 ug) given at one week intervals. The sera will be collected one week after the last dose.

GM1 Ganglioside Assay

Polyvinyl microtiter "U" plates (Cooke Engineering) will be used for ELISA procedures. GM1 ganglioside (Supelco, Inc.) will be used in a concentration of 1 ug/ml, diluted in phosphate buffered saline. Guinea pig anti-CT(1:2,500) and goat anti-Guinea pig antisera (1:1000) (Antibodies Incorporated, Davis, Calif.) will be diluted for use in phosphate buffered saline (PBS) tween with 1% fetal calf serum. Goat anti-guinea pig globulin will be conjugated with alkaline phosphatase (Sigma type VII). p-Nitrophenyl phosphate (Sigma 104 phosphate substrate) will be used as the substrate for the enzyme reaction.

Isolates of suspected toxin-producing strains will be grown at 30°C using syncase broth. Cultures will be aerated (50 rpm, roller drum) during the incubation period.

The ELISA assay will be performed following the procedure of Sack et al. (13), a summary of which is given the Table.

The blocking antibody assay will be performed by incubating CT antiserum with an equal volume of purified CT in uncoated wells of a microtiter plate. The plate will be gently agitated for 1 hour. One hundred ul of this mixture will be added to step 3 (see table) and the assay performed as outlined. The concentration of toxin used in the blocking assay will be four times the dose giving an OD of 1.00

Y1 Adrenal Cell Assay Procedures

The procedures for the Y1 adrenal cell assay were described by Sack and Sack (15) are a modification of the original method of Donta et al. (16). Briefly Y1 cells will be grown to confluency in 96 well miniculture plates (Cook Engineering). Cell free bacterial supernatants will be grown and prepared in Caseamino Yeast Extract broth as previously described. Supernatants (0.05 ml) will be applied to monolayer and allowed to remain for 10 minutes. After removal of the supernatant, cells will be washed and fresh tissue culture medium applied. Cells will then be incubated in 5% CO_2 for 6 hours and observed for morphological changes. After the 6 hour reading, cells will be further incubated overnight and then observed for morphological changes indicating toxin activity.

In cases where toxic activity is demonstrated, duplicate culture supernatants will be treated with anti-cholera toxin antiserum and retested. Neutralization of toxic effects with specific antisera will confirm the toxic nature of the supernatant.

D. SIGNIFICANCE

The Oral Cholera Vaccine trial now in progress offers an unusual opportunity to study the role of non-01 Vibrionaceae in diarrhoeal illness. The post vaccine surveillance system monitors all cases of diarrhea for which patients seek medical attention in Matlab or any of the several field substations. Specimens are rapidly transported to the Matlab microbiology laboratory where they are examined in great detail. At least three different media are used for isolation of the Vibrionaceae alone. The primary focus of these efforts are to identify and confirm any cases of V. cholerae among vaccine or placebo recipients. However, the broader effect is to offer a unique opportunity to assess the importance of members of the Vibrionaceae in this population. According to this proposal, Vibrionaceae isolates will be identified to genus and species, their capacity to produce CT or CT-like toxins will be assessed, the antigens shared between species will be examined and the antigens shared between them and components of the complete killed whole cell B subunit Oral Vaccine will be determined. These results should allow a more complete understanding of the significance of these organisms in the study population as well as a deeper understanding of their overall pathogenic potential. In addition, the examination of the antigenic profiles of these species may suggest improved methods for their identification as well as

describe their pathogenic interactions. Finally, the identification of common antigens between these bacteria and the antigens of the oral cholera vaccine will assist in determining if this vaccine might protect against non-01 Vibrionaceae infections.

E. FACILITIES REQUIRED

All facilities required are currently operational at ICDDR,B. No addional facilities will be necessary.

F. COLLABORATIVE ARRANGEMENTS

Collaborative immunological studies are being planned with faculty from Dhaka University, these studies will be especially related to the cross immunoelectrophoresis. For this collaboration a faculty member would work at the ICDDR,B during sabbatical leave from the University.

Collaboration for taxonomic identification of isolates will be conducted through the following individuals and institutions :

Dr. Rita Colwell
University of Maryland
College Park, MD, USA

Dr. Patricia Desmarchelier
Commonwealth Institute of Health
University of Sydney, Australia

Dr. Paul Blake
Centers for Disease Control
Atlanta, Georgia, USA

Table 1 - GM1 ganglioside ELISA for determination of CT

Step	Diluent[a]	Concn	Vol per well (ul)	Incubation period	Incubation period
1. Precoat plate with GM1 ganglioside	PBS	1 ug ml	100	Overnight	Room temp
2. Wash 3 x with PBS-Tween			200	3 min	Room temp
3. Add test sample	Undiluted or diluted PBS-Tween with 1% FCS		100	Overnight	Room temp
4. Wash 3 x with PBS-Tween			200	3 min	Room temp
5. Add guinea pig anti-CT	PBS-Tween 1% FCS	1:2,500[b]	100	1h	37 °C
6. Wash 3 x with PBS-Tween			200	3 min	Room temp
7. Add enzyme-labeled goat anti-guinea pig globulin	PBS-Tween with 1% FCS	1:1,000[b]	100	1h	37 °C
8. Wash 3 x with PBS-Tween			200	3 min	Room temp
9. Add substrate	10% DEA buffer	1 mg/ml	100	45 min	37 °C
10. Stop reaction with NaOH		3M	25		
11. Read OD at 405 nm					

[a]PBS, Phosphate-buffered saline; FCS, fetal calf serum; DEA, diethanolamine (13)

[b]Variable with titer of antiserum

REFERENCES

1. von Graevenitz, A. 1985. Aeromons and Plesiomons. IN: Lennette, E., A. Ballows, W. Hauster and J. Shadomy, Eds. Manual of Clinical Microbiology, 4th Ed. Am.Soc.Microbiol., Wash. DC.

2. Farmer III, J., F. Hikman-Brenner and M. Kelly, 1985. Vibrio. IN: Lennette, E., A. Ballows, W. Hauster and J. Shadomy, Eds. Manual of Clinical Microbiology, 4th Ed. Am.Soc.Microbiol. Wash. DC.

3. Blake, P., R. Weaver and D. Hollis, 1980. Diseaes of Humans (Other than Cholera) caused by Vibrios. Ann. Rev.Microbiol. 34: 341-367.

4. Tison, D., and M. Kelly, 1984. Vibrio Species of Medical Importance. Diag.Microbiol. Infect.Dis. 2:263-276.

5. Yamamoto, K., M. Al-Omani, T. Honda, Y. Takeda, and T. Miwatani, 1984. Non-01 Vibrio cholerae Hemolysin : Purification, Partial Characterization and Immunological Relatedness to El Tor Hemolysin. Infect.Immun. 45:192-196.

6. Finkelstein, R., 1984. Cholera. In: Bacterial Vaccines. Academic Press, Inc. New York.

7. Burke, V., J. Robinson, R. Berry and M. Gracey. 1981. Detection of Enterotoxins of Aeromonas hydrophila by a Suckling-Mouse Test. J.Med.Microbiol. 14:401-408.

8. Pitarangsi, C., P. Escheverria, R. Whitmire, C. Tirapat, S. Formal, G. Dammin and M. TingtaLapong. 1982. Enteropathogenicity of Aeromonas hydrophila and Plesiomonas shigelloides : Prevalence among Individuals with the without Diarrhea in Thailand. Infect.Immun. 35:666-673.

9. Popoff, M. and M. Veron, 1976. A taxonomic study of the Aeromons hydrophila - Aeromons punctata Group. J.Gen.Microbiol. 94:11-22.

10. Colwell, R. Ed. 1984. Vibrios in the Environment. John Wiley and Sona, New York.

11. Furniss, A., J. Lee and T. Donovan, 1978. The Vibrios. Maidstone Public Health Laboratory, London.

12. Finkelstein, R., K. Fujita and J. LoSpalluto 1971. Procholeragenoid:an aggregated intermediate in the formation of colleragenoid. J.Immunol. 107:1043-1051.

13. Sack,D., S. Huda, P.Neogi, R. Daniel, W. Spira, 1980. Microtiter Ganglioside Enzyme-linked Immunosorbent Assay for Vibrio and Escherichia coli Heat Labile Enterotoxins and Antitoxin. J.Clin.Microbiol. 11:35-40,

14. Kroll, J. 1973. Crossed-line Immunoelectrophoresis. IN:Axelsen, H., J. Kroll, B. Wecke, eds. A Manual of Quantitative Immunoelectrophoresis. Blackwell Scientific Publications, Oxford.

15. Sack, D. and R. Sack, 1975. Test for enterotoxigenic Escherichia coli using Y1 adrenal cells in miniculture. Infect.Immun. 11: 334-336.

16. Donta, S., H. Moon and S. Whipp, 1974. Detection of heat labile E. coli enterotoxin with the use of adrenal cells in tissue culture. Science. 183: 334-336.

ABSTRACT SUMMARY

The purpose of this study is to assess the potential cross reactivity between antigens of the whole cell B-subunit oral cholera vaccine and extracellular antigens produced by non 01 serotype V. cholerae and non cholera members of the Vibrionaceae. Cross reactivity of antigens produced by these organisms will be evaluated by the GM1 ganglioside assay to Cholera Toxin (CT), the Y1 adrenal cell assay and Cross Immunoelectrophoresis (XIE) to antiserum to CT, whole cells of 01 V. cholerae and the complete oral vaccine.

No human subjects will be used for this protocol. There is no risk to any patient population and informed consent is not required. Bacterial isolates will be identified by laboratory number only. No contact with patients by interview or other means will be necessary for completion of this protocol.

The direct benefits of this study to patients contemporaneous with this protocol are negligable as the work will not be completed during the active stages of infection. However, a more thorough understanding of the protection or lack of protection of the Oral Cholera vaccine currently being tested against members of the Vibrionaceae has far reaching and potentially significant results to this or any other population at risk due to enteric infections by the Vibrionaceae.

SECTION III - BUDGET

ICDDRB BUDGET PROPOSAL--1986

Program Name: DTWG
Project/Protocol/Branch: VIBRIO ANTIGENS
Principle Investigator: B.A.KAY
Budget Code:
Protocol No:

SUMMARY BUDGET

3100 Local Salary	6300
3200 Intl Salary	11484
3300 Consultants	0
3500 Travel Local	0
3600 Travel International	0
3700 Supplies	4420
3800 Other costs	500
4800 Inter Departmental	5400
Total Direct Operating	28104
Capital Expenditure	0
TOTAL DIRECT COST	28104
DIRECT COST EXCLUDING INTERNATIONAL SALARY	16620

PERSONNEL REQUIREMENT (Local)

	No/Pos	Man mon	Amount
A Staff	0	0	0
B Recrt	0	6	0
C Al frm		33	6300
Sub	0	39	6300
D Sep	0	0	0
E Al to		0	0
Sub	0	0	0
TOTAL	0	39	6300

MANPOWER ALLOCATED FROM OTHER AREA-LOCAL

Job	Level	Bdg Cd	No/pos	Man mo	$/mo	Amount
RES OFFICER	GS5	060401	1	12	220	2640
LAB TECHNICIAN	GS3	060401	1	12	140	1680
RES OFFICER	GS5	030100	1	6	220	1320
SEC II	GS5	060401	1	3	220	660
TOTAL				33		6300

PERSONNEL - INTERNATIONAL

	No pos	Man mo	Amount
Direct		0	0
Recrt		0	0
Al frm		3	11484
Sub		3	11484
Sep		0	0
Al to		0	0
Sub		0	0
TOTAL		3	11484

MANPOWER ALLOCATED FROM OTHER AREA--INTERNATIONAL

Person	Bdg no	Man mo	$/mo	Amount
B. KAY	010100	3	3828	11484
				0
TOTAL		3		11484

SUPPLIES AND MATERIALS

A/C	ITEMS	AMOUNT
3701	Drugs	
3702	Glassware	200
3703	Hosp supply	
3704	Stationary	200
3705	Chem, media	500
3706	Uniform	
3707	Fuel	
3708	Lab supply	1500
3709	Housekeep	
3710	Janitorial	
3711	Tool&spares	
3712	Non stock	1000
	SUBTOTAL	3400
3713	FREIGHT	1020
	TOTAL	4420

OTHER COSTS

A/C	ITEMS	Amount
3800	Maintenance	
3900	Rent,comm,util	
4100	Bank charges	
4200	Legal	
4300	Print, pub	500
4400	Entertainment	
4500	Service charges	
4600	Staff development	
	TOTAL	500

INTERDEPARTMENTAL SERVICES

A/C	ITEMS	Amount
4801	Computer	0
4802	Trans, Dhaka	100
4803	Trans,Matlab	
4804	Water Transport	
4805	Trans,Teknaf	
4806	Xerox	100
4807	Pathology	
4808	Microbiology	4500
4809	Biochemistry	
4810	X-ray	
4811	I.V.	200
4812	Media	500
4813	Patient hosp.	
4814	Animal	0
4815	Med Illustration	
4817	Telex	
4818	Outpatient Care	
4830	Trans sub	
	TOTAL	5400