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ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. M. Bennish

Application No. 85-004(P)

Title of Study "DEVELOPMENT OF AN ELISA TO MEASURE SHIGELLA TOXIN IN CLINICAL SPECIMENS (A PILOT STUDY)".

Trainee Investigator (if any)

Supporting Agency (if Non-ICDDR,B)

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.

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

Michael Bennish 27-1-85.

Principal Investigator

Trainee

SECTION 1 - RESEARCH PROTOCOL

(PILOT PROTOCOL)

1. TITLE: DEVELOPMENT OF AN ELISA TO MEASURE SHIGELLA TOXIN IN CLINICAL SPECIMENS

2. PRINCIPAL INVESTIGATOR: DR. MICHAEL BENNISH

CO-INVESTIGATORS: DR. MD. SHAHADAT HOSSAIN
DR. RUSSELL HARDY
DR. G. KEUSCH

3. STARTING DATE: 1 MARCH 1985

4. COMPLETION DATE: 1 SEPTEMBER, 1985

5. TOTAL INCREMENTAL COST: US $1,700

6. SCIENTIFIC PROGRAMME: THIS PROTOCOL HAS BEEN APPROVED BY THE DISEASE TRANSMISSION WORKING GROUP

SIGNATURE OF PROGRAMME HEAD: [signature]

DATE: 22/1/85

ABSTRACT

It has long been known that Shigella dysenteriae type I produces a protein toxin, and it has recently been shown that this toxin is produced by other Shigella species, as well as by other enteric enteropathogens, such as vibrios. Although the toxin is known to produce a number of biological effects in animal and cell models (fluid accumulation in the rabbit illeal loop, paresis and eventual death when injected intra-peritoneally in susceptible animals, cell death in HeLa cell monolayers), it's role in the pathogenesis of human disease remains undetermined. Recent advances in the chemical purification of the toxin have allowed for production of a mouse monoclonal antibody to the toxin. Using this monoclonal antibody an ELISA has been developed that has been shown to work well when used to measure purified or partially purified toxin from broth cultures of shigella. The next step which we plan to undertake in this study is to use this ELISA to measure toxin in clinical specimens, and to refine the assay procedure so that it can be done completed in a single day. To do this we will first, using purified toxin, vary the incubation periods of the various steps of the assay in an attempt to shorten the procedure. If we succeed in this we will then obtain stool from patients with a variety of types of diarrhea, and attempt to identify correlations with type of infection and clinical

manifestations. We will also identify patients with complications of shigellosis that are thought to be associated with a circulating toxin (seizures, hemolytic-uremic syndrome) and see if toxin can be identified in the appropriate body fluid (spinal fluid, serum, urine), or see if these patients have particularly high levels of toxin in thier stools.

OBJECTIVES

The objectives of this study are firstly to further develop the ELISA test for shigella toxin so that it is reproducible and conveniant for assaying toxin in clincal specimens from patients. Our second objective is to measure toxin clinical specimens from patients and see if there is any correlation between disease manifestations and toxin levels.

BACKGROUND

In the eighty or so years since it was discovered that Shigella dysenteriae type I produced a toxin that caused paresis when injected into the peritoneum of mice, we have learned a good deal about it's chemical structure and varied biologic effects in animal models of shigella infection or in in-vitro systems. However we still know next to nothing about what role it plays in the pathogenesis of human disease, and defining what role if any that it might have could shed some light on many of the currently poorly understood manifestations of shigella infections in humans.

The biologic effects of shigella toxin are known to include the following:

1. Classic "neurotoxic activity" which includes paresis, paralysis, and death when susceptible animals are given a parenteral injection of toxin.(1)
2. Cytotoxic activity, as manifested by the death of various continuous cell line monolayers when toxin is injected into the media in which they are growing, (2) or enteritis when injected into ligated rabbit illeal loops. (3)
3. Enterotoxic activity, as manifested by fluid accumulation when toxin preperations are injected into the ligated rabbit illeal loop. (4)

Shigellosis in humans presents with a variety of intestinal and extra-intestinal manifestations. (5) Although shigellosis is classically thought of as a dysenteric illness, we know that in certain ages and in certain settings, it more often can cause a watery diarrhea, which may or may not be associated with subsequent dysentery (4). Among the more severe complications are the hemolytic-uremic syndrome, which can often be fatal, and neurologic abnormalities, particularly seizures. How analogous any of the animal or in-vitro systems are for what we observe in humans is unclear. It might be speculated that the watery diarrhea phase of shigella could be produced by an enterotoxin, but we currently do not even know if the watery diarrhea is due to a secretory process or to alteration in absorbtion, or whether ii is due to small bowel abnormalities, or is, like the dysentery phase of the illness, a large bowel disease. Similarly, although the toxin produced by shigella was initially referred to as a nuerotoxin because of it caused paresis in animals, it is not clear if this is at all related to the frequent neurologic abnormalities that we see in humans who are infected with shigella.

Previous assay systems for shigella toxin have been cumbersome and unsuitable for clinical samples. (2) The most widely used assay system was a quantitative assay using detachement of HeLa cells from a preformed monolayer.(2) The rabbit illeal loop and animal paresis or death bioassays have also been used. All of these assay systems are relatively cumbersome, and because many biologic materials can elicit the same endpoint, specimens from patients, as opposed to supernatants from cell culture, have never been tested directly to determine whether toxin is present.

The recent efforts to obtain more highly purified toxin (6,7) have made possible the production of a mouse monoclonal antibody to shigella toxin, (8) which is the essential component of what will hopefully be a sensitive and specific ELISA assay method. An ELISA utilizing this antibody as the capture antibody has been shown to be sensitive in detecting sub-nanogram amounts of purified toxin, either when the toxin was assayed directly, or after it had been added to stool specimens of patients without clinical disease. Our next step is to evaluate and refine this test for use with clinical specimens, and to see if toxin levels in various body fluids (stool, serum, spinal fluid) correlate with disease manifestations.

METHODS

1. SHIGELLA TOXIN ASSAY

The reagents for the shigella toxin assay consist of the following:

A. Antisera: mouse monclonal antibody, with specificity against the B subunit of the toxin

rabbit polyclonal antibody against toxin

B. Purified toxin, for use as positive control

The above three items have been provided free of charge by the laboratory of Dr. Gerald Keusch, who developed the reagents and initial procedure.

The following reagents are commercially available:

A. Carrier surface, 96 well microtitier, either Cooke microtitier 129 A or B disposable polystyrene microhemagglutination plates, or disposable polyvinyl microhemagglutination , M29, from Dynatech Laboratories.

B. Conjugate: Either goat antirabbit IgG alkaline phosphatase, or goat antirabbit IgG horseradish-peroxidase

C. Common stock reagents (Na_2CO_3, $NaHCO_3$, NaN_3, NaCl, KH_2PO_4, KCl, Tween 20,) for preperation of the coating buffer and PBS Tween for washing.

D. Fetal calf serum, or bovine serum albumin.

The procedure for the ELISA is now taken directly from the chapter by Voller et al in Rose and Freidman, Manual of Clinical Immunology, American Society for Microbiology, Washington, D. C., which is the current standard guide for this procedure. In brief it is:

1. Dilute mouse monoclonal antibody to a 10 microgram/ml concentration in the appropriate coating buffer.

2. Coat overnight all 96 wells of the plate with 0.2 ml of monoclonal antibody solution.

3. Wash plates twice with PBS tween.

4. To block unbound sites, add .2 ml of PBS Tween with 5% BSA and incubate for an hour.

5. Wash twice with PBS Tween.

6. Add .2 ml of test samples to duplicate wells.

7. Prepare 10, 5, 1, .50, .25 and .125 concentrations of purified toxin and add to duplicate wells, results to be used to construct a standard curve.

8. Leave two wells blank in order to obtain zero reading.

9. Let all samples incubate overnight at 4 degrees centrigrade.

10. Wash five times with PBS tween.

11. Add .2ml of rabbit polyclonal antitoxin diluted 1/5,000 in PBS Tween, and incubate for two hours at room temperature.

12. Wash five times with PBS Tween.

13. Add .2ml of goat anti-rabbit conjugate diluted 1/1,000 in PBS Tween, and incubate for two hours.

14. Wash five times with PBS Tween.

15. Add .2 ml of p-nitrophenyl substrate at 1mg/ml concentration with pH 9.8 diethanolamine buffer.

16. Read at appropriate wavelength two hours after substrate is added.

Our major objective now, with the procedure, is to see how well it works under field conditions, and to see if we can shorten the procedure, primarily by reducing the time for the four different incubation steps. Another possible problem is to see if stool gives us any non-specific activity, and to test methods for eliminating this. We currently have all the reagents except for the conjugates. Using varying duplicate dilutions of purified toxin and stool spiked with purified toxin, we will attempt to vary a number of the steps in the listed procedure and see if it has any effect on the results. By spiking stool we will also get an idea if there is either non-specific activity, or if there might instead be some neutralizing activity.

CLINICAL STUDIES

In order to determine whether the toxin can be found in clinical specimens, we will obtain stool from 300 inpatients hospitalized with a variety of types of diarrhea, and see if toxin is detectable in their stools. We will attempt to correlate the toxin level with infecting organism, (species of shigella, other pathogen, or no pathogen), and a few (at least initially) clinical indicators of disease, including height of fever on day the specimen was obtained, number of weight cells in the the stool, prescence or absence of blood in the stool, and white cell count. In addition we will attempt to identify 10 patients with the hemolytic uremic syndrome and obtain stool, urine and a small amount of blood (.1ml) for detection of toxin. We will also attempt to identify 10 patients with invasive diarrhea and seizures, and will likewise obtain stool, serum (.1 ml), and a small amount of CSF (.1ml).

In no case will we obtain these specimens solely for the purpose of this study. We will only obtain a very small aliquot of either serum or spinal fluid when a patient is having blood drawn or a spinal tap done in the process of their routine clinical care in the hospital.

The information from the toxin assay and clinical findings on patients will be entered into a data based program for a micro-computer (STAT-PAC adapted for the KAYPRO) and analyzed using this system, which has both a data base and statistical analysis component.

SIGNIFICANCE

Shigella is the major identifiable cause of morbidity and mortality in patients admitted to our hospital. By attempting to see if toxin is present in patients infected with shigella, and to see if the level of toxin correlates with any of the clinical features of the disease, we will help to finally establish if the toxin has a role in the pathogenesis of shigella. In addition if we can simplify and shorten the assay procedure, it might serve as a sameday screening test for shigella infection, which would give us a considerable advantage when compared to the minimum of 48 hours that it now takes us to identify shigella infections using routine microbiologic methods.

COLLABORATIVE ARRANGEMENTS

This study will be done in collaboration with Professor Gerald Keusch of Tufts University in Boston, U.S.A, who has developed the essential reagents for the ELISA.

ABSTRACT SUMMARY

1. The population studied will be ill patients admitted to the Dhaka station hospital of the ICDDR,B.

2. As no tests will be done on any of these patients in addition to what is already being done, the study does not pose any risk to the patient.

3. Not applicable.

4. Stool, blood and CSF samples will be processed according to patient number. No confidential information will be used.

5. As there is no risk to the subject, and the issue of privacy is not involved, informed consent will not be obtained.

6. No interview will be performed.

7. There will be no direct benefit to the individual. Society will benefit

as we will possibly gain a better understanding of the mechanism of Shigella infections, which are a major cause of morbidity and mortality in the less developed world.

8. Hospital records will be used, as will stool specimens, serum, urine and spinal fluid. No sampling will be done solely for the purpose of this study. We will use aliqots of body fluid that are being obtained for clinical care of the patient.

BIBLIOGRAPHY

1. Levine, MH. Bacillary dysentery, mechanisms and treatment. Medical Clinics of North America, 66:623-38, 1982

2. Keusch, GT, Grady, GF, Takeuchi A, Sprinz H. The pathogenesis of shigella diarrhea. II. Enterotoxin induced acute enteritis in the rabbit ileum. JID 126: 92-5, 1972

3. Keusch GT, Jacewicz M, Hirschman SZ. Quantitative Microassay in Cell Culture for enterotoxin of Shigella dysenteriae 1. JID, 125: 539-41, 1972

4. Keusch, GT, Grady GF, Mata LJ, McIver J. The pathogenesis of Shigella Diarrhea. I Enterotoxin production by Shigella dysenteriae 1. Journal of Clinical Investigation, 51, 1212-1218, 1972

5. Barrett-Conner E, Connor JD. Extraintestinal manifestations of Shigellosis. American Journal of Gastroenterology, 53: 234, 1970

6. O'Brien AD, et al. Characterization of Shigella dysenteriae 1 (Shiga) Toxin Purified by Anti-Shiga Toxin Affinity Chromatography. Infection and

Immunity, 30:170-179, 1980

7. Brown JE et al. Purification and Biological Characterization of Shiga Toxin from Shigella dysenteriae 1. Infection and Immunity 36: 996-1005, 1982

8. Gentry, DE, Gentry MK, Brown JE. Isolation and Characterization of Monoclonal Antibodies to Shiga Toxin. Infection and Immunity 41: 430-433, 1983

DETAILED BUDGET

1. Personnel services	Not applicable (pilot protocol)
2. Supplies and materials	
Microtiter plates	$150
Alkaline phosphatase and immuno-peroxidase conjugates	$250
Other reagents	$200
Glassware	$150
Stool collection containers to be used to centrifuse and collect specimens	$150
Miscellaneous supplies (pencils, pen, paper)	$50

Total	$950
3. EQUIPMENT	No cost
4. PATIENT HOSPITALIZATION	No cost
5. OUTPATIENT CARE	No cost
6. ICDDR,B TRANSPORT	No cost
7. TRAVEL AND TRANSPORTATION OF PERSONS	No cost
8. TRANSPORTATION OF THINGS	$750
9. RENT, COMMUNICATION AND UTILITIES	No cost
10. INFORMATION SERVICES	No cost
11. PRINTING AND REPRODUCTION	No cost
12. OTHER CONTRACTURAL SERVICES	No cost
13. CONSTRUCTION, RENOVATION, ALTERATION	No cost
TOTAL COST	US$1,700,oo