INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH
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CENTRE
FOR HEALTH AND
POPULATION RESEARCH

LSD
2000

Memorandum

4 July 2000

To : Dr. Rashidul Haque
Laboratory Sciences Division

From : Advocate UM Habibunnesa Habiba
Co-chairperson, Ethical Review Committee

5/7/2000

Sub : Approval of protocol # 2000-018

This has reference to your memo of 3rd July 2000 submitting a modified copy of your protocol # 2000-018 entitled "Mechanism of acquired immunity to *E-histolytica* infection and disease in Bangladeshi children". I am pleased to inform you that the protocol is hereby approved upon your appropriate addressing of the issues raised by the Ethical Review Committee in its meeting held on 28th June 2000.

Thanking you and wishing you success in running the said study.

cc: Division Director
Laboratory Sciences Division

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CENTRE
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POPULATION RESEARCH

Memorandum

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CENTRE
FOR HEALTH AND
POPULATION RESEARCH

Memorandum

2 July 2000

To : Dr. Rashidul Haque
Laboratory Sciences Division

From : Advocate UM Habibunnesa Habiba
Co-chairperson
Ethical Review Committee

2-7-00

Sub : Protocol # 2000-018

The Ethical Review Committee in its meeting held on 28th June 2000 reviewed your protocol # 2000-018 entitled "Mechanism of acquired immunity to *E-histolytica* infection and disease in Bangladeshi children". After thorough review and discussion, the committee made the following observations.

(a) the 'Methodology Section' should be revised to make it more understandable.

(b) in the consent form, the technical words such as '*E. histolytica*' should be replaced by commonly used terms such Ameobic dysentery (অ্যামোবিক আমাশা)

(c) the Bangla consent form should be revised in the line of the English version.

(d) the number of deaths due to *E. histolytica* has been described as 50,000 on page 1 whereas in page 3, it has been described as 100,000. The PI should clarify which one is correct.

(e) item nos. 1(a) of the face sheet should be marked "YES".

(f) in the English consent form, the PI has indicated that blood samples will be taken four items whereas in the Bangla consent form, he has mentioned that blood samples will be collected twice. The PI should clarify which one is correct and revise the consent forms accordingly. In the 'Abstract Summary' also there is discrepancy about the quantity of blood to be taken from the children.

(g) it is indicated in the 'Objective Section' that conc. of serum IgA, IgM, class antibodies, IFN-gamma, TNP-alpha and sIgA will be determined in saliva and stool samples and will measure mucosal and cell-mediated immunity in the children. But in the 'Methodology Section', the PI has not described how it will be determined and measured.

You are, therefore, advised to modify the protocol and resubmit a modified copy for consideration of the Chair.

Thank you.

cc: Division Director
Laboratory Sciences Division

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH
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CENTRE
FOR HEALTH AND
POPULATION RESEARCH

July 3, 2000

Respected Co-chairperson,
I have gone through the revised protocol and response to reviewer. I think the protocol may be considered for approval.
Thanks.
Dr. R. Haque

To: Co-chairperson
Ethical Review Committee

From: Dr. Rashidul Haque Rashidul Haque

Sub: Protocol # 2000-018

Thank you very much for reviewing my protocol as mentioned above. I have taken into consideration almost all of your comments and corrected appropriately in the proposal. My response to your comments are the following:

a) We have had limitation to write up the methodology section in more details. The donor (Howard Hughes Medical Institute) has limitation for space for every section of the proposal. The proposal has been submitted to the RRC and ERC as it has been submitted to the Howard Hughes Medical Institute.

b) In the consent form, the technical word such as *'E. histolytica'* has been replaced by the commonly used term such as " Amebic dysentery" (আমাশয়)

c) The Bangla consent form has been revised in the line of English version.

d) According to Dr. Julia Walsh (Walsh J.A. 1986, Rev. Infect Dis; 8:228-238) the number of deaths due to *E. histolytica* is 50,000 - this is the most widely cited reference for mortality due to *E. histolytica*. According to the WHO's recent estimate the number of deaths due to *E. histolytica* is up to 100,000 (Weekly Epidemiological Record, WHO, 1997, 72:97-100). So, both of them are correct and estimate only. To remove your confusion we have now used 100,000 deaths both in pages 1 and 3 of the proposal.

e) We have marked item nos. 1(a) of the face sheet as "YES"

f) We have revised the Bangla consent form. Now there is no discrepancy between the Bangla and English consent form. We have also corrected the " Abstract Summary" and now there is no discrepancy about the quantity of blood that will be taken from the children.

g) As I have mentioned above that due to limitation of space we did not describe how the anti-lectin IgA and IgG, IFN-gama, TNF-alpha will be determined in saliva ,stool and blood. Those tests will be carried out according to the established methods in our laboratory as well as using the commercially available kits according to maufracturer's instruction and published methods in the literature (J *Infect Dis* 1996, 174:157-162; *Clin Exp Immunol* 1999 etc115:189-195).

Thank you very much.

cc: Division Director
Laboratory Sciences Division

Attachment I

APPROVED COPY

Date: 14-6-2000

(FACE SHEET)

RESEARCH REVIEW COMMITTEE, ICDDR,B.

Principal Investigator: Rashidul Haque

Application No. 2000-018

Title of Study: Mechanism of acquired immunity to E. histolytica infection and disease in Bangladeshi children

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 the 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) Minor or persons under guardianship — (Yes) No

2. Does the Study Involve:
   (a) Physical risk to the subjects — (Yes) No
   (b) Social risk — 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 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 the study — (Yes) No
   (b) Procedures to be followed including alternatives used — (Yes) No
   (c) Physical risk — (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 parents or guardian (if subjects are minor) — (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 with 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. Example 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 Committee 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.

Rashidul Haque
Principal Investigator

_____
Trainee

RESEARCH PROTOCOL

Protocol No.:

FOR OFFICE USE ONLY

RRC Approval: Yes/ No Date:

ERC Approval: Yes/No Date:

AEEC Approval: Yes/No Date:

Project Title: Mechanism of acquired immunity to E. histolytica infection and disease in Bangladeshi children.

Theme: *(Check all that apply)*

- ☐ Nutrition
- ☑ Emerging and Re-emerging Infectious Diseases
- ☐ Population Dynamics
- ☐ Reproductive Health
- ☐ Vaccine evaluation
- ☐ Environmental Health
- ☐ Health Services
- ☑ Child Health
- ☐ Clinical Case Management
- ☐ Social and Behavioural Sciences

Key words: E. histolytica, immunity

Principal Investigator: Dr. Rashidul Haque **Division:** LSD **Phone:** 2411

Address: **Email:** rhaque@icddrb.org

Co-Principal Investigator(s): Dr. Dilara Islam

Co-Investigator(s): Dr. Hamidur Rahman
Dr. Sazzad Hasan

Student Investigator/Intern: Department of Medicine
University of Virginia

Collaborating Institute(s):

Population: Inclusion of special groups *(Check all that apply)*:

Gender
- ☑ Male
- ☑ Females

Age
- ☑ 0 – 5 years
- ☑ 5 – 9 years
- ☐ 10 – 19 years
- ☐ 20 +
- ☐ > 65

- ☐ Pregnant Women
- ☐ Fetuses
- ☐ Prisoners
- ☐ Destitutes
- ☐ Service providers
- ☐ Cognitively Impaired
- ☐ CSW
- ☐ Others (specify _____)
- ☐ Animal

Project / study Site *(Check all the apply)*:

- ☐ Dhaka Hospital
- ☐ Matlab Hospital
- ☐ Matlab DSS area
- ☐ Matlab non-DSS area
- ☐ Mirzapur
- ☐ Dhaka Community
- ☐ Chakaria
- ☐ Abhoynagar
- ☐ Mirsarai
- ☐ Patyia
- ☐ Other areas in Bangladesh Mirpur, Dhaka
- ☐ Outside Bangladesh
  name of country: _____
- ☐ Multi centre trial
  (Name other countries involved)

Revised on: 24 May 2000

Type of Study *(Check all that apply)*:

- ☐ Case Control study
- ☐ Community based trial / intervention
- ☐ Program Project (Umbrella)
- ☐ Secondary Data Analysis
- ☐ Clinical Trial (Hospital/Clinic)
- ☐ Family follow-up study
- ☐ Cross sectional survey
- ☑ Longitudinal Study (cohort or follow-up)
- ☐ Record Review
- ☐ Prophylactic trial
- ☐ Surveillance / monitoring
- ☐ Others

Targeted Population *(Check all that apply)*:

- ☐ No ethnic selection (Bangladeshi)
- ☐ Bangalee
- ☐ Tribal groups
- ☐ Expatriates
- ☐ Immigrants
- ☑ Refugee

Consent Process *(Check all that apply)*:

- ☑ Written
- ☐ Oral
- ☐ None
- ☐ Bengali language
- ☐ English language

Proposed Sample size:

Total sample size: 280 ☐

Sub-group I 140 ☐

II 140 ☐

Determination of Risk: Does the Research Involve *(Check all that apply)*:

- ☐ Human exposure to radioactive agents?
- ☐ Fetal tissue or abortus?
- ☐ Investigational new device?
  (specify_____)
- ☐ Existing data available from Co-investigator
- ☐ Human exposure to infectious agents?
- ☐ Investigational new drug
- ☐ Existing data available via public archives/source
- ☑ Pathological or diagnostic clinical specimen only
- ☐ Observation of public behavior
- ☐ New treatment regime

Yes	No	
☐	☐	Is the information recorded in such a manner that subjects can be identified from information provided directly or through identifiers linked to the subjects?
☐	☐	Does the research deal with sensitive aspects of the subject's behavior: sexual behavior, alcohol use or illegal conduct such as drug use?
		Could the information recorded about the individual if it became known outside of the research:
☐	☐	a. place the subject at risk of criminal or civil liability?
☐	☐	b. damage the subject's financial standing, reputation or employability; social rejection, lead to stigma, divorce etc.

Do you consider this research *(Check one)*:

- ☐ greater than minimal risk
- ☐ no risk
- ☑ no more than minimal risk
- ☐ only part of the diagnostic test

Minimal Risk is "a risk where the probability and magnitude of harm or discomfort anticipated in the proposed research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical, psychological examinations or tests. For example, the risk of drawing a small amount of blood from a healthy individual for research purposes is no greater than the risk of doing so as a part of routine physical examination".

Yes/No

☒ ☐ Is the proposal funded?

If yes, sponsor Name: Howard Hughes Medical Institute, USA (HHMI)

Yes/No

☐ ☐ Is the proposal being submitted for funding ?

If yes, name of funding agency: (1) _____

(2) _____

Do any of the participating investigators and/or their immediate families have an equity relationship (e.g. stockholder) with the sponsor of the project or manufacturer and/or owner of the test product or device to be studied or serve as a consultant to any of the above?

IF YES, submit a written statement of disclosure to the Director.

Dates of Proposed Period of Support

(Day, Month, Year - DD/MM/YY)

Beginning date 1-9-2000

End date _____

Cost Required for the Budget Period ($)

a.	*Ist Year*	*2nd Year*	*3rd Year*	*Other years*
	45,000	45,000	45,000	90,000

b. *Direct Cost :* US$202,500 *Total Cost :* 225,000

Approval of the Project by the Division Director of the Applicant

The above-mentioned project has been discussed and reviewed at the Division level as well by the external reviewers. The protocol has been revised according to the reviewer's comments and is approved.

Prof. V.I. Mathan
Name of the Division Director

Signature

11/06/2000
Date of Approval

Certification by the Principal Investigator

I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

Signature of PI Rashidul Hoque

Date: 11.6.2000

Name of Contact Person (if applicable)
Dr. Rashidul Hoque

SUMMARY

TITLE OF PROJECT:

Mechanisms of Acquired Immunity to *E. histolytica* Infection and Disease in Bangladesh Children

Hypothesis: Immunity is acquired by prior *E. histolytica* infection. The prime mediator of immunity to infection is hypothesized to be a mucosal secretory IgA antibody response that neutralizes the parasite Gal/GalNAc adherence lectin, while the prime mediator of protection from disease is hypothesized to be a cell mediated immune response that activates macrophages to kill the parasite.

Specific Aim 1 will test whether acquired immunity to *E. histolytica* infection and/or disease exists via a prospective study of 280 children.

Specific Aim 2 will examine the roles of the human mucosal and systemic immune responses to *E. histolytica* in protection from *E. histolytica* infection and disease. This will include prospective surveillance for *E. histolytica* infection in children from Aim 1 with and without serum and/or mucosal anti-Gal/GalNAc lectin antibodies, and measuring cell-mediated immune responses against the Gal/GalNAc lectin in immune and non-immune individuals.

RESEARCH OBJECTIVE:

Infection with the parasite *E. histolytica* is responsible for about 40-50 million cases of diseases and 100, 000 deaths annually worldwide. It is not known whether immunity is acquired to *E. histolytica* intestinal colonization. Successful completion of this will prove the existence of acquired immunity in human to amebiasis, and will correlate mucosal and systemic immune responses against the *E. histolytica* Gal/GalNAc lectin with protection from colonization and/or disease. The information acquired will be essential for the rational design of an amebiasis vaccine, and will serve as a model for understanding protective immunity against intestinal eukaryotic pathogens.

RESEARCH PLAN

INTRODUCTION AND HYPOTHESIS

The World Health Organization in 1997 (WHO, 1997) identified the study of human immunity as a priority research need, "essential for evaluating the feasibility of developing an *E. histolytica* vaccine". Our development of rapid means to screen large populations for *E. histolytica* infection, plus the availability of preliminary data from our ongoing human field study funded by the National Institute of Health (NIH), USA, make this an opportune time to pursue fundamental studies of human immunity to amebiasis.

It is not known if humans acquire immunity against colonization or invasion by *E. histolytica*. If human immunity to *E. histolytica* infection exists then what are the mechanisms - these are the questions to be answered before any vaccine trial for *E. histolytica* can take place. This proposal originates from a long-term collaboration between the laboratories of Rashidul Haque at the International Center for Diarrhoeal Disease Research Bangladesh (ICDDR,B) and Bill Petri's lab at the University of Virginia (UVA). This collaboration has to date: (1) resulted in the development of rapid fecal antigen detection tests for *E. histolytica*; (2) yielded age-specific prevalence rates of *E. histolytica* infection and other enteric pathogens in children in Bangladesh; (3) established an ongoing prospective study of *E. histolytica* infection in local children; and (4) most recently demonstrated a correlation in humans of the presence of intestinal sIgA anti-Gal/GalNAc lectin antibodies with the absence of *E. histolytica* infection.

Hypothesis: Immunity is acquired by prior *E. histolytica* infection. We also hypothesize that the prime mediator of immunity to <u>infection</u> is a mucosal sIgA antibody response that neutralizes the parasite galactose or N-acetyl-D-galactosamine-specific adherence lectin (Gal/GalNAc lectin), while the prime mediator of protection from <u>disease</u> is a cell mediated immune response that activates macrophages to kill the parasite.

Specific Aim 1 : Test whether acquired immunity to *E. histolytica* infection and/or disease exists. This will include the prospective examination of infection and disease severity in 280 children with and without evidence of prior amebiasis, and the testing of whether immunity is parasite strain-specific.

Specific Aim 2: Examine the roles of the human mucosal and systemic immune responses to *E. histolytica* in protection from *E. histolytica* infection and disease. This will include prospective surveillance for *E. histolytica* infection in the subset of children from Aim 1 with and without stool sIgA anti-lectin antibodies (based on our data demonstrating an inverse correlation between stool sIgA anti-lectin antibodies and *E. histolytica* infection). The "immune" and "non-immune" individuals defined in Specific Aim 1 will also be compared for systemic humoral and cell-mediated immune responses against the Gal/GalNAc lectin, including interferon gamma and TNF-alpha production (known to activate macrophages in vitro to kill *E. histolytica*).

SUCCESSFUL COMPLETION of this work will prove the existence of acquired immunity in humans to amebiasis, and will correlate mucosal and systemic immune responses against the *E. histolytica* Gal/GalNAc lectin with protection from colonization and/or disease. Additionally, the prospective surveillance will enable a more accurate estimate the impact of amebiasis on child health, and will provide a well-defined field site for future testing of amebiasis vaccines.

BACKGROUND AND SIGNIFICANCE

Importance of the disease amebiasis. There are an estimated 40 to 50 million cases of amebic colitis and liver abscess and 100,000 deaths from *E. histolytica* infection each year (WHO, 1997). The preponderance of *E. histolytica* infection, morbidity and mortality is experienced in Central and South America, the Indian sub-continent and Africa. In Bangladesh , where we propose to do these studies, diarrheal diseases are the leading cause of death in children under the age of 5 years. We have found *E. histolytica* infection in 7-8% of grade school aged children with diarrhea seen at the ICDDR,B hospital (Haque et al 1997). *E. histolytica* was identified in 4.2% of all patients, making it the fifth leading enteropathogen identified.

Re-description of *E. histolytica* as pathogenic *E. histolytica* and nonpathogenic *E. dispar*.

Entamoeba histolytica has recently been redescribed as:

E. histolytica (formerly called the pathogenic zymodemes of *E. histolytica*), and

E. dispar (formerly called the nonpathogenic zymodeme of *E. histolytica*)

The two species are morphologically identical. They can be differentiated by isoenzyme analysis, antigen detection using monoclonal antibodies to the Gal/GalNAc lectin (Haque et al. 1997), sequences of single copy genes, and small subunit ribosomal RNA sequences (Diamond & Clark 1993). *E. dispar* has never been documented to cause colitis or liver abscess. *E. histolytica* is the cause of amebic colitis and liver abscess, and can also asymptomatically colonize the large bowel. This nomenclature was recently formalized in a joint statement by WHO, PAHO, and UNESCO (WHO, 1997). *Entamoeba histolytica* can be further divided into two groups by isoenzyme analysis into "pathogenic zymodemes" II and XIV, and by PCR methods into multiple genetically distinct isolates (Clark & Diamond 1993).

Gal/GalNAc lectin of *E. histolytica*. A parasite Gal/GalNAc lectin is responsible for the contact-dependent cytotoxicity for which *E. histolytica* was named. The Gal/GalNAc lectin is a heterodimer consisting of heavy (170-kDa) and light (35- or 31-kDa) subunits (Petri et al 1989; McCoy et al 1993). Attachment of *E. histolytica* trophozoites in vitro to colonic epithelial cells and colonic mucins is mediated by the 170-kDa subunit (Petri et al 1987a; Chadee et al 1987). Adherence of trophozoites to mucins in vitro can be inhibited by monoclonal antibodies directed to the lectin 170 kDA subunit (Petri et al 1990), suggesting that a mucosal antibody response may prevent parasite attachment and subsequent invasive disease.

The lectin is antigenically conserved in every isolate of *E. histolytica* tested, including hundreds of independent isolates from Bangladesh, South Africa, Egypt, Mexico, Brazil, the United States, Thailand and India (Petri et al 1990; Haque et al 1993, 1995; Abd-Alla et al 1993). Anti-lectin antibodies were detected in 99% of South African patients with amebic liver abscess, and 100% of individuals colonized with *E. histolytica* (Haque et al. 1999; Ravdin et al 1990).

The ability of the native lectin to elicit a protective immune response has been demonstrated in a gerbil model of amebic liver abscess. Complete protection from liver abscess formation was seen in the majority of the immunized animals (Petri & Ravdin 1991). Protection from liver abscess has also been demonstrated when animals have been immunized with the recombinant cysteine-rich region of the heavy subunit (Zhang & Stanley 1994; Mann et al. 1997; Soong et al. 1995; Lotter et al. 1997). Protection from infection has been passively transferred with antibodies against the lectin carbohydrate recognition domain (Lotter et al. 1997; Dodson et al. 1999).

Evidence in humans for immunity to *E. histolytica*. It is not known if acquired immunity to *E. histolytica* infection exists. Longitudinal studies of individuals asymptomatically infected with *E. histolytica* (all of whom have serum anti-amebic antibodies) have show that most spontaneously clear the infection in 3-9 months. However 10-14% of these *E. histolytica*-infected individuals have been shown to progress to invasive amebiasis, demonstrating that immunity to luminal infection, if it exists, is incomplete (Gathiram & Jackson 1985, 1987; Irusen et al. 1992; Haque et al. 1999). Case-series of patients with amebic colitis in Natal, South Africa (Gathiram & Jackson 1985) and in Dhaka, Bangladesh (Wanke et al. 1988; Haque et al. - preliminary studies) have demonstrated a decline in incidence above age 14, with a second peak of infection in adults > 40 years old. This could be interpreted as evidence of immunity acquired in childhood that wanes in the elderly. The only other evidence for immunity to invasive amebiasis is the retrospective patient chart review by De Leon (1970) which found a low incidence of patients being re-admitted to the hospital with liver abscess. Because DeLeon's study was retrospective and lacked a control group, it is impossible to know if the apparently low rate of hospital readmissions with amebic liver abscess was due to acquired immunity, a low prevalence of *E. histolytica* reinfection, or loss of patients to follow-up. We are left with a situation where there is no human "real world" data that acquired immunity exists. Development of a vaccine against amebiasis cannot optimally occur in a vacuum of knowledge of naturally acquired immunity.

In vitro or animal model evidence of potentially protective humoral and cell-mediated immune responses against *E. histolytica*. Immunization of animals with several *E. histolytica* antigens provides protection from an intra-hepatic challenge with *E. histolytica* (reviewed by Stanely 1997). These antigens include recombinant DNA prokaryotic expressed serine-rich and cystine-rich proteins and the Gal/GalNAc adherence lectin. Support for a role for antibodies in immunization-mediated protection has come from the demonstration that passive transfer of antibodies (prior to infectious challenge) against whole *E. histolytica* proteins, the serine rich proteins, or the cysteine-rich domain of the lectin, resulted in faster resolution of amebic liver abscess (Zhang et al 1994; Lotter et al 1997). The lack of severe amebiasis in patients with the acquired immunodeficiency syndrome suggests a less than stringent requirement of CD4 (+) T cells for protective immunity. However, macrophages and neutrophils, activated with INF-γ and TNF-α, are endowed with the capability of killing *E. histolytica* trophozoites, while in the absence of INF-γ, these effector cells were killed by the amebae (Salata et al 1985, 1986; Denis & Chadee 1989; Lin & Chadee 1992). In murine macrophages TNF-α was shown to play a central role in activating macrophages for nitric oxide-dependent cytotoxicity against *E. histolytica* (Denis & Chadee 1989; Lin & Chadee 1992; Lin et al 1994).

SUMMARY OF BACKGROUND AND SIGNIFICANCE. Both humoral and cellular immune responses are evident in humans with *E. histolytica* infection. The ability of this acquired immune response to protect against subsequent colonization or disease has yet to be examined. Animal and in vitro models of amebic infection support a role for antibody in preventing infection, and a role for interferon gamma and TNF alpha-mediated activation of macrophages in clearing existing infection.

SPECIFIC GAPS IN OUR KNOWLEDGE OF THE IMMUNE RESPONSE TO *E. HISTOLYTICA* TO BE ADDRESSED BY THIS PROPOSAL:

- Is immunity acquired to *E. histolytica* intestinal colonization?
- Is immunity acquired to amebic colitis?
- If immunity exists, does it correlate with systemic or secretory antibody or cell-mediated immune responses against the Gal/GalNAc lectin?

PRELIMINARY STUDIES

Specific identification of *E. histolytica* in stool using an antigen detection test. A rapid and simple approach to the diagnosis of *E. histolytica* infection is an antigen-detection ELISA based on the antigenic differences in the Gal/GalNAc lectin in *E. histolytica* and *E. dispar* (Petri et al 1990). This assay has been applied to single stool specimens from over a thousand patients with diarrhea or dysentery in Dhaka, Bangladesh (Haque et al 1993, 1994, 1995, 1997). Compared to culture plus zymodeme analysis, differentiation of *E. dispar* from *E. histolytica* was 95% sensitive and 93% specific. The *E. histolytica* test (which detects only *E. histolytica*) and the *Entamoeba* test (which detects both *E. histolytica* and *E. dispar* in stool) have both received FDA 510k approval for in vitro diagnosis of amebiasis in humans. We have also developed a PCR technique for detection of *E. histolytica* in stool, and demonstrated that it has excellent correlation (93%) with culture and the TechLab *E. histolytica*-specific antigen detection test. The ability of these tests to rapidly and specifically identify *E. histolytica* in stool will be an important part of the proposed studies.

Identification of the Mirpur community in Dhaka, Bangladesh as an optimal location for the study of *E. histolytica* infection. Mirpur, a suburb of Dhaka, Bangladesh, is an urban slum. The majority of the inhabitants are of Bihari ethnic origin, who settled in Mirpur after the war with Pakistan in 1971. The area is a densely populated yet stable community, and is located 15 minutes away from the ICDDR,B, with a population of approximately fifty thousand.

***E. histolytica* infection is prevalent in Mirpur.** *Entamoeba histolytica* infection was present in 5%, and *E. dispar* in 13%, of asymptomatic 2-5 year old children from Mirpur. *E. dispar*-infected children were no more likely than uninfected children to have anti-lectin serum antibodies. In contrast, all children with asymptomatic *E. histolytica* had anti-lectin serum antibodies. This anti-lectin response included antibodies against the carbohydrate recognition domain, which have been demonstrated in animal models to confer passive protection from amebiasis (Dodson et al 1999).

Asymptomatic colonization with *E. histolytica* is self-limited. Seventeen asymptomatic *E. histolytica*-infected children were re-examined for *E. histolytica* infection at 6 and 12 months. Fifteen of 17 children had no detectable infection at 6 months (2 after having received metronidazole for dysentery), with the remaining two children clearing the infection without specific treatment at 12 months. We concluded that the self-limited nature of *E. histolytica* infection is consistent with the development of an immune response effective at preventing invasive amebiasis, and at eradicating colonization (Haque et al 1999).

Evidence of a correlation of intestinal anti-lectin sIgA with lack of *E. histolytica* colonization. Examination of stool sIgA antibodies in the Mirpur children has led to two interesting pieces of data. First, we have found that only 23% of children positive for anti-lectin antibody in serum were positive for anti-lectin IgA in the stool sample. This indicates that the mucosal immune response to the lectin may be shorter-lived than the systemic immune response. Second, we have found a correlation between anti-lectin IgA in stool and the absence of *E. histolytica* infection (Haque et al. unpublished; **Table 1**). This is the first correlation ever made between a human immune response and the presence or absence of *E. histolytica* infection. We conclude that this data is consistent with an anti-lectin sIgA response protecting from colonization by neutralizing the adherence lectin.

Table 1. A mucosal anti-lectin sIgA antibody response is associated with absence of *E. histolytica* intestinal colonization.

	Anti-lectin Antibodies:	
E. histolytica Intestinal Infection*[1]	Mucosal sIgA*[2]	Serum IgG
Yes	0/19 (0%)	19/19 (100%)
No	33/282 (12%)	141/282 (50%)

*[1]Determined by culture and antigen detection

*[2]Anti-lectin sIgA measured by the method of Kelsall et al. (Am. J. Trop. Med. Hyg. 1994; 51:454-9)

Establishment of a prospective study of amebiasis in Mirpur. This year we have initiated a study in Mirpur, Bangladesh to determine if children who have been infected with *E. histolytica* are less likely to be re-infected with *E. histolytica*. We have enrolled 280 children ages 2-5 years for a 3 year prospective study. Half of the 280 children enrolled are seropositive (serologic evidence of prior infection), while the second half are seronegative (without serologic evidence of prior infection). Our expectation is that children with evidence of prior infection will exhibit some degree of immunity to subsequent infection and/or invasive disease. The study is entering its first year of observation of the children for *E. histolytica* infection.

SUMMARY OF PRELIMINARY STUDIES:

•Antigen detection, PCR, and culture methods for the detection of *E. histolytica* infection have been field tested. The three methods are complementary, sensitive, and specific means to detect *E. histolytica* infection.

•Children in Mirpur, Bangladesh have a high prevalence of *E. histolytica* infection.

•Asymptomatic *E. histolytica* infection is self-limited and associated with a systemic immune response against the adherence lectin.

•A stool sIgA response against the Gal/GalNAc lectin is associated with absence of *E. histolytica* intestinal infection.

•A prospective study to test for acquired immunity to *E. histolytica* in Mirpur has begun.

RESEARCH DESIGN AND METHODS

Specific Aim 1 : Test whether acquired immunity to *E. histolytica* infection and/or disease exists. This will include the prospective examination of infection and disease severity in 280 children with and without evidence of prior amebiasis, and the testing of whether immunity is parasite strain-specific.

Rationale: The design of these experiments is simple: individuals with and without evidence of prior *E. histolytica* infection will be compared for the rate of new *E. histolytica* infection or disease. Based on the experience with other enteropathogens such as rotavirus and *Vibrio cholera,* it is a reasonable expectation that acquired immunity exists to amebiasis. Acquired immunity could include immunity to infection, or immunity only to severe disease.

Enrollment of children:

• Mirpur children ages 2-5

• Cohort of children with prior *E. histolytica* infection: prior infection defined as a positive serum or stool antibody test for anti-lectin antibodies.

• Cohort of children without evidence of prior infection: negative serum antibody response for anti-lectin antibodies.

• Cohorts matched for age, sex, family size. The parents of each child will be questioned about the child's symptoms of intestinal and extraintestinal amebiasis, dysentery, fever, and history of drug ingestion, and the child will be examined for hepatomegaly prior to enrollment. Subjects with a recent (< 1 month) history of anti-amebic medication use will be excluded from the study.

Outcomes to be analyzed:

- Incidence of *E. histolytica* infection in the "immune" and naive cohorts of children.
- Severity of *E. histolytica* associated diarrhea in the two cohorts.
- Incidence of *E. histolytica* associated dysentery in the two cohorts.
- Whether immunity is parasite strain-specific.

Statistical evaluation:

The assumptions that underlie this aim are as follows: First we estimate that 5% of the children at any one time are infected with *E. histolytica* and that the average duration of asymptomatic infection with *E. histolytica* is 3 months. Therefor a 5% prevalence of *E. histolytica* infection represents a yearly incidence of infection of approximately 20%. Second, we estimate that 10% of the children will be lost to follow-up each year. The power of this study to detect a 50% difference in infection rates between naive and immune individuals over 3 years is 99.9% (Bayer 1988). We have chosen a 50% level of protection as a estimate based on other enteric diseases. For example prior rotavirus infection provides 77% protection from diarrhea and 38% protection from re-infection (Velazquez et al 1996) and vibriocidal serum antibody titers $\geq$20 are associated with 50% protection from infection and illness (Glass et al 1985).

Prospective analysis:

Health care workers will interview the parents and child every other day. Parents will be asked about the child's eating patterns, stool frequency, consistency and presence of blood, and fever. For all children, stool specimens will be collected every month for detection of *E. histolytica* and *E. dispar* infection by antigen capture. Blood samples will be collected at the time of enrollment and at the end of the study, as well as at the beginning and at the end of the first *E. histolytica* infection.

Work-up of children who develop diarrhea:

Children with diarrhea will be detected either by the every other day visits of the health care workers, or through the parents contacting project personnel at the field office. Parents will be instructed to contact the field office in Mirpur whenever their child has a diarrheal or gastrointestinal illness. There are no telephones available to the families in Mirpur, so the every other day visits to the families and the proximity of the field office to the subjects is key. When diarrheal disease is detected, a stool sample will be collected and examined for enteropathogens and the child will be examined. Samples will be kept on ice and transported to the laboratory for processing within 2-4 hours. Parents will be instructed in the use of oral rehydration solution, and antibiotics administered or the patient seen or hospitalized at the ICDDR,B when appropriate.

Definitions: Standard WHO definitions for Diarrhea, Dysentery, Amebic colitis, Amebic liver abscess will be followed. If *E. histolytica* is detected in stools but diarrhea is not present and no evidence exists for extra-intestinal amebiasis will be regarded as a case of subclinical-*E. histolytica* infection. Severity of diarrhea will be assessed by a modification of the point system of Ruuska et al (1990).

E. histolytica isolate identification by gene polymorphisms. Identification of polymorphisms in the serine rich antigen and the strain specific gene will be by the method of Clark and Diamond (1993).

Specific Aim 2: Examine the roles of the human mucosal and systemic immune responses to *E. histolytica* in protection from *E. histolytica* infection and disease.

Rationale:

- In our preliminary studies we have demonstrated that there is a correlation between a mucosal anti-lectin IgA response and absence of *E. histolytica* infection in the gut.
- IFN-γ and TNF-α play an important role in activating macrophages to kill *E. histolytica*.

If our hypothesis is correct, we would predict that clearance and immunity to *E. histolytica* infection will be correlated with a mucosal antibody response that neutralizes the parasite's Gal/GalNAc lectin, and that in addition protection from invasive disease (colitis) is associated with antigen-specific IFN-γ and TNF-α production.

This Aim will include prospective surveillance for *E. histolytica* infection in the subset of children from Aim 1 with and without stool sIgA anti-lectin antibodies (based on our data demonstrating an inverse correlation between stool sIgA anti-lectin antibodies and *E. histolytica* infection). The "immune" and "non-immune" individuals defined in Specific Aim 1 will also be compared for systemic humoral and cell-mediated immune responses against the Gal/GalNAc lectin, including IFN-γ and TNF-α production (known to activate macrophages in vitro to kill *E. histolytica*). Also, comparisons will be made of sIgA and cytokine responses during immune-mediated clearance of asymptomatic infection with *E. histolytica*.

Stool, saliva and blood samples will be collected from the subjects and stored at 4^0C for transport to the laboratory where they will either immediately tested or stored at -70^0C for future assays. Serum will be examined for IgG and IgA antibodies against the lectin. Saliva and stool samples will also be tested for sIgA antibodies against the lectin. Neutralization of lectin-mediated adherence by antibodies will be measured by CHO cell adherence assays (Dodson et al. 1999). Gal/GalNAc lectin-stimulated production of IFN-γ and TNF-α by peripheral blood mononuclear cells will be measured by commercially available kits and/or by ELISPOT. The methods for these tests are well established in the laboratories of the ICDDR,B.

Data analysis. All the data will be collected in pre-coded standard forms. The forms will be pre-tested before being used in the study. All data collected in the study will routinely entered into the microcomputer. The database created at the beginning of the study will be continuously updated with field surveillance data and laboratory results. Data from the prospective surveillance will be used to estimate: (i) Natural history of colonization - how long does it persist, how many children develop symptoms/signs of invasive amebiasis who are colonized, (ii) The role of mucosal and systemic immune responses to *E. histolytica* with protection from infection/disease, and (iii) correlation of immune responses and disease with specific parasite isolates.

Comparisons of data between groups and different time points will be done using nonparametric statistical methods. As necessary data will also be analyzed by multiple analysis of variance for repeated measures with Bonferroni/Dunn statistics at 5% significance level using appropriate statistical softwares.

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ABSTRACT SUMMARY FOR ERC:

It is now generally accepted that what was earlier known as *E. histolytica* actually comprises of two genetically distinct but morphologically indistinguishable species, *E. histolytica* and *E. dispar*. The confirmation of these two distinct species of *Entamoeba* is perhaps the major accomplishment in the field of amebiasis research during the last decade. We have to throw out almost all of the data that have been collected over the years on the epidemiology and immunology of *E. histolytica*.

Data on the acquisition of immunity following infection with *E. histolytica* are limited. It is not known whether humans acquire immunity against colonization or invasion by *E. histolytica*. If human immunity to *E. histolytica* infection exists then what are the mechanisms - these are the questions to be answered before any vaccine trial for *E. histolytica* can take place. More studies are required to understand the human immunity to amebiasis. The present study is being planned to test whether acquired immunity to *E. histolytica* infection and/or disease exists. This will include the prospective examination of infection and disease severity in 280 children aged 2-5 years with and without evidence of prior amebiasis. The study will also examine the roles of the human mucosal and systemic immune responses to *E. histolytica* in protection from *E. histolytica* infection and disease. The "immune" and "non-immune" individuals defined in Specific Aim 1 will also be compared for systemic humoral and cell-mediated immune responses against the Gal/GalNAc lectin, including interferon gamma and TNF-alpha production (known to activate macrophages in vitro to kill *E. histolytica*). At present we are conducting a study similar to this present study and this study will now add up few more things on it.

1. Preschool and School aged children are at the greatest risk for *E. histolytica* infection.

2. There is no major potential risk – 2 ml of blood and stool samples will be collected from the study subjects. Subjects will not be hospitalized and no other invasive techniques will be used that will cause physical, psychological, social, legal risk.

3. Disposable syringes and needles will be used for collecting blood for protecting or minimizing any potential risk.

4. Confidentiality will be strictly maintained by coding each subject.

5. a) Signed informed consents will be obtained from the parents/legal guardian of the subjects.

   b) No information will be withheld from the guardians.

   c) There is no potential risk to the subject or privacy of individual is involved. Recruited children will be under active surveillance and treatment will be available for any illness.

6. A short interview will be taken regarding child's family at home that will not require more than 5 minutes. Then the children will be followed by the Community Health Workers (CHW) for diarrhoea surveillance using a pre-coded form.

7. Subjects that will participate will be benefited from the study since they get free treatment of their minor illness (cough, fever, infections etc.) Children infected with heavy helminthic infections will get anti-helmintic therapy. There is direct benefit for the community that out weigh the risks.

8. We shall not use any records. However, we shall obtain blood and collect stool.

Project: Mechanisms of Acquired Immunity to *E. histolytica* Infection and Disease in Bangladeshi Children.

CONSENT FORM

(To be read out to parents/legal guardian whose children are taking part in the study)

International Center for Diarrheal Diseases Research, Bangladesh (ICDDR,B) and the University of Virginia will undertake a research project as mentioned above. The purpose of this study will be to understand the mechanisms of acquired immunity to amebic dysentery in Bangladeshi children. More knowledge and information are required to control the diarrhea and dysentery due to *E. histolytica* infection.

Participation of your child is voluntary. There is no obligation to take part in this study. Also, if you want to take out your child from the study you can do so any time during the study. Confidentiality will be strictly maintained.

If you allow your child to participate in the study, the following procedures will be carried out during the 3 years study period:

We shall collect 2 ml of blood sample by venipuncture of anti-cubital vein at the time of enrollment. Saliva sample will also be collected at that time. Stool samples will be collected every month during the study period. A second blood sample of 2 ml will be drawn when the first *E. histolytica* infection will be detected, while a third blood sample will collected when the *E. histolytica* will be cleared as detected by the examination of stool. If your child is recruited into the study he/she will be under active surveillance. Health Assistants will be visiting twice a week. At each visit, they will collect information on diarrhea and dysentery of the child. If your child develops any signs and symptoms of diarrhea and dysentery detail investigations will be carried out and appropriate treatment will be given. The child will be given standard anti-amebic treatment if he has any symptoms of invasive amebiasis. After 3 years of surveillance a fourth blood sample (2-ml blood) will be collected again.

Your child will be directly benefited from the participation in this study, as we will be providing free medical treatment for common pediatric illnesses. If you agree that your child can participate in this study, please sign below or put your thumb impression.

Signature of Investigator:
Date:

Signature or left thumb impression
of child's parent/legal guardian:
Complete address:

Date:

Signature of witness:
Complete address:

Date:

বাংলাদেশী শিশুদের অ্যামোবিক আমাশয়ের বিরুদ্ধে অর্জিত রোগপ্রতিরোধী ক্ষমতার উপর গবেষণা

সম্মতিপত্র

(গবেষণায় অংশগ্রহনকারী শিশুর পিতা-মাতা/আইনত: অভিভাবককে পড়ে শোনানো হবে।)

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র, বাংলাদেশ (আই.সি.ডি.ডি.আর,বি) এবং জার্কিনিয়া বিশ্ববিদ্যালয় যৌথভাবে অ্যামোবিক আমাশয়ের উপর একটি গবেষণা কার্যক্রম পরিচালনা করবে। এই গবেষণার উদ্দেশ্য হবে অ্যামোবিক আমাশয়ের রোগ প্রতিরোধী ক্ষমতা সম্বন্ধে জানা। অ্যামোবিক আমাশয় প্রতিরোধে আমাদের অনেক নতুন তথ্য ও জ্ঞান দরকার।

গবেষণায় আপনার শিশুর অন্তর্ভুক্ত হওয়া সম্পূর্ণরূপে আপনার ইচ্ছাধীন। এতে কোন বাধ্য-বাধকতা নেই। গবেষণা চলাকালীন সময়েও আপনি আপনার সম্মতি প্রত্যাহার করতে পারবেন। এ ব্যাপারে গোপনীয়তা সম্পূর্ণরূপে বজায় রাখা হবে।

আপনি যদি আপনার শিশুকে এই গবেষণায় অন্তর্ভুক্ত করতে অনুমতি প্রদান করেন তবে নিম্নোক্ত নিয়মাবলী তিন বছর পর্যন্ত অনুসরণ করতে হবে।

গবেষণায় অন্তর্ভুক্তির সময় শিশুর হাতের শিরা থেকে পরীক্ষার জন্য ২ মিলিলিটার (½ চা চামচ পরিমান) রক্ত নেয়া হবে। এ সময় শিশুর থুতুও নেয়া হবে। আপনার শিশুর পায়খানা প্রতিমাসে একবার করে গবেষণা চলাকালীন সময়ে নেয়া হবে। দ্বিতীয়বার ২ মিলিলিটার রক্ত নেয়া হবে যখন শিশু প্রথমবারের মত অ্যামোবিক আমাশয়ে আক্রান্ত হবে এবং তৃতীয়বার ২ মিলিলিটার রক্ত নেয়া হবে শিশু যখন অ্যামোবিক আমাশয় থেকে মুক্ত হবে। যখন থেকে আপনার শিশুকে আমাদের গবেষণায় অন্তর্ভুক্ত করা হবে তখন থেকেই সে আমাদের পরিদর্শনাধীন থাকবে। স্বাস্থ কর্মীরা সপ্তাহে দুইবার শিশুকে বাসায় দেখতে যাবে। প্রতি পরিদর্শনকালে আপনার শিশু ডায়েরিয়া বা আমাশয়ে আক্রান্ত হয়েছে কি-না সে বিষয়ে তথ্য সংগ্রহ করবে। যদি আপনার শিশুর ডায়েরিয়া বা আমাশয়ের লক্ষন দেখা যায় তবে তাকে উপযুক্ত চিকিৎসা দেয়া হবে। কোন প্রকার অন্ত্রভেদী অ্যামোবিক আমাশয় দেখা দিলে তাকে আমাশয়ের (এন্টি-অ্যামোবিক) চিকিৎসা দেয়া হবে। তিন বছর পর গবেষণার শেষে চতুর্থবার আরো ২ মিলিলিটার রক্ত নেয়া হবে।

আপনার শিশুকে এই গবেষণায় অংশগ্রহন করালে সে লাভবান হবে কারন আমরা তাকে সাধারন শিশু রোগের চিকিৎসা বিনামূল্যে প্রদান করবো। আপনি যদি এই গবেষণায় আপনার শিশুর অংশগ্রহনে রাজী থাকেন তবে অনুগ্রহপূর্বক নিম্নে স্বাক্ষর অথবা বৃদ্ধাঙ্গুলির ছাপ দিন।

গবেষকের স্বাক্ষর :

তারিখ :

পিতা-মাতা/আইনত: অভিভাবকের স্বাক্ষর অথবা বাম বৃদ্ধাঙ্গুলির ছাপ :

পুরো ঠিকানা :

তারিখ :

সাক্ষীর স্বাক্ষর :

পুরো ঠিকানা :

তারিখ :

HHMI Budget and Expense System

Institution (Award Year): International Center for Diarrheal Disease Research (2000)
Grant Number: 75301564901
Scholar: Rashidul Haque
Grant Term: 9/1/2000 - 8/31/2005
Web Submission Status: BIS, Budget Started

Year 1: 09/01/2000 - 08/31/2001
Year 2: 09/01/2001 - 08/31/2002
Year 3: 09/01/2002 - 08/31/2003
Year 4: 09/01/2003 - 08/31/2004
Year 5: 09/01/2004 - 08/31/2005

Enter Initial Budget

All amounts must be entered in US Dollars.

	Year 1 2001	Year 2 2002	Year 3 2003	Year 4 2004	Year 5 2005	Total
HHMI Payment	$45,000	$45,000	$45,000	$45,000	$45,000	$225,000
Direct Costs: Scholar's Research						
Personnel						
Research Scholar	2,000	2,000	2,500	2,500	2,500	11,500
Postdoctoral Fellows/Trainees	0	0	0	0	0	0
Graduate Students	0	0	0	0	0	0
Technical Salaries/Benefits	6,500	7,500	10,250	10,250	10,250	44,750
Other Personnel Salaries/Benefits	2,000	2,000	3,000	3,000	3,000	13,000
Subcomponent Subtotal	10,500	11,500	15,750	15,750	15,750	69,250
Equipment, Equipment Maintenance and Supplies						
Equipment Costing $5000 or More	0	6,000	0	0	0	6,000
Equipment Costing Less Than $5000	3,000	0	2,250	0	1,250	6,500
Equipment Maintenance	0	0	0	0	0	0
Computers	2,500	0	0	2,000	0	4,500
Supplies	12,000	11,000	11,000	10,750	12,000	56,750
Subcomponent Subtotal	17,500	17,000	13,250	12,750	13,250	73,750
Additional Research Expenses						
Travel and Meetings	2,500	3,000	2,500	3,000	2,500	13,500

Books, Periodicals, and Subscriptions	500	0	0	0	0	500
Publishing and Publicity	500	0	0	0	0	500
Subcomponent Subtotal	3,500	3,000	2,500	3,000	2,500	14,500
Component Subtotal	31,500	31,500	31,500	31,500	31,500	157,500
Direct Costs: Departmental Shared Resources						
Personnel						
Postdoctoral Fellows/Trainees	0	0	0	0	0	0
Graduate Students	0	0	0	0	0	0
Technical Salaries/Benefits	0	0	2,000	3,000	3,000	8,000
Other Personnel Salaries/Benefits	2,500	2,500	2,500	2,500	2,500	12,500
Subcomponent Subtotal	2,500	2,500	4,500	5,500	5,500	20,500
Equipment, Equipment Maintenance and Supplies						
Equipment Costing $5000 or More	6,000	5,000	0	0	0	11,000
Equipment Costing Less Than $5000	0	0	3,000	1,000	1,000	5,000
Equipment Maintenance	500	500	500	500	500	2,500
Computers	0	0	0	0	0	0
Supplies	0	0	0	1,000	1,000	2,000
Subcomponent Subtotal	6,500	5,500	3,500	2,500	2,500	20,500
Additional Research Expenses						
Travel and Meetings	0	0	0	0	0	0
Books, Periodicals, and Subscriptions	0	500	500	500	500	2,000
Publishing and Publicity	0	500	500	500	500	2,000
Subcomponent Subtotal	0	1,000	1,000	1,000	1,000	4,000
Component Subtotal	9,000	9,000	9,000	9,000	9,000	45,000
Indirect Costs						
Indirect Costs	4,500	4,500	4,500	4,500	4,500	22,500
Component Subtotal	4,500	4,500	4,500	4,500	4,500	22,500
Grand Total	$45,000	$45,000	$45,000	$45,000	$45,000	$225,000

SAVE & RETURN to the Main Menu

SAVE & REFRESH Screen

COA Glossary

CANCEL

S. Moin 11/June/2000

Shamima Moin
Controller, Budget & Costing