INTERNATIONAL CENTRE FOR
DIARRHOEAL DISEASE
RESEARCH, BANGLADESH

Memorandum

To: Dr. Demisse Habte, MD
Chairman, RRC

From: Tasnim Azim, LSD Tasnim Azim

Date: 31. 10. 95

Subj: Modifications of the protocol "A prospective study of reactive arthritis triggered by *Shigella*" (#95-026).

According to the suggestion of the RRC, enrollment of volunteers will be based on standard epidemiological procedures which is shown on pg.12. In addition, as was discussed in the RRC, although not included in the final recommendations, I have added collection of a second sample of blood from non-ReA dysentery controls (pg.12). On further deliberation amongst ourselves (the investigators), we have decided to collect 15mls of blood instead of 10mls because several assays are planned all of which may not be possible with 10mls of blood. Our earlier decision to collect 10mls of blood was based on the hope that there will be enough cases so that all assays may not have to be done on all cases. As a concern regarding the number of cases has been expressed by the RRC, we consider it safest to plan for all assays on all cases. Appropriate changes have been made in the consent forms.

I hope these changes meet with your approval.

Thank you.

Approved Dem
8/11/95

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

Memorandum

To : Dr. Tasnim Azim, LSD

October 21,1995

From : Demissie Habte, MD
Chairman, RRC

Subject : Protocol No.95-026 entitled "A prospective study of reactive arthritis triggered by Shigella".

The Research Review Committee (RRC) met on Wednesday, the 11th October 1995 to consider your protocol referred to above.

The Committee expressed concern that sufficient number of cases might not be recruited. Hence it suggested that shigella patients with a known history of reactive arthritis be included in the study.

A more serious concern is the observations that recruitment of volunteers was not based on standard epidemiological procedures. Accordingly, the Committee request that this be incorporated in a revised protocol.

Thank you.

CC: Acting Division Director, LSD

DH:zbmb

Attachment 1.
(FACE SHEET)

Date 6·10·95

ETHICAL REVIEW COMMITTEE; ICDDR,B.

Principal Investigator JASNIM AZIM

Trainee Investigator (if any) _____

Application No. # 95-026

Supporting Agency (if Non-ICDDR,B) _____

Title of Study A PROSPECTIVE STUDY OF REACTIVE ARTHRITIS TRIGGERED BY SHIGELLA

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 NA
   (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.

Jasnim Azim
Principal Investigator

Trainee

CHECK-LIST FOR SUBMISSION OF PROPOSALS
TO THE RESEARCH REVIEW COMMITTEE (RRC)
[Please tick (✓) the appropriate box]

1. Has the proposal been reviewed, discussed and cleared at the Division level ?

Yes ☑

No ☐

If the answer is 'NO', please clarify the reasons: _____

_____

_____

2. Has the proposal been peer-reviewed externally ?

Yes ☑

No ☐

If the answer is 'NO', please explain the reasons: _____

_____

_____

3. Does the proposal address gender issues ?

Yes ☐

No ☑

If the answer is 'NO', Please give the reasons.

_____

Reactive arthritis (ReA) will be diagnosed in all patients with shigella that are enrolled. However during analyses frequency of ReA in the sexes will be determined.

4. Has a funding source been identified ?

Yes ☑

No ☐

If the answer is 'YES', please indicate the name of the donor: _____

An application has been submitted to the European Union.

Contd... /

5. Whether the proposal is a collaborative one ?

Yes [✓]

No [ ]

If the answer is 'YES', the type of collaboration, name and address of the institution and name of the collaborating investigator be indicated:

Collaboration is with Prof. N.A. Mitchison and Dr. J. Siegen from Deutsches Rheuma Forschungszentrum and Klinikum Steglitz, Berlin. Collaboration involves training and intense involvement with the lab technique

6. Has the budget been cleared by Finance Division ?

Yes [✓]

No [ ]

If the answer is 'NO', reasons thereof be indicated: _____

_____

_____

7. Does the study involve any procedure employing hazardous materials, or equipments ?

Yes [✓]

No [ ]

If the answer is 'YES', fill the necessary form.

6.10.95
Date

Jasmin Ayim
Signature of the
Principal Investigator

ASSURANCE ON *HAZARDOUS PROCEDURE* FORM

DECLARATION BY THE PRINCIPAL INVESTIGATORS

Name of the Principal Investigator : TASNIM AZIM

Title of the Project : A PROSPECTIVE STUDY OF REACTIVE ARTHRITIS TRIGGERED BY SHIGELLA

I declare that (check) :

☐ The above mentioned protocol does not involve any procedure relevent to "Safety/Environmental" hazard.
(P.I. don't fill up the form, if this is the response)

☑ The above mentioned protocol involves procedure(s) with potentials to cause "Safety/Environmental" hazard, and relevant informations are provided below :

(The following portion to be filled in for only those protocols which need to handle the hazards mentioned and defined in this form)

The nature of hazards (check as many entries as appropriate) :

☐ Biologicals
☐ Chemicals
☐ Non-ionizing radiation
☑ Radioactive materials
☐ Ionizing radiation machine
☐ Other (specify) : _____

1.0 Important information :

1.1 Brief description of the objective of the study, relevance and procedure of using hazardous materials, methods of personnel protection, and budget code to cover for the procurement of safety supplies:

To determine the role of human leucocyte antigens (HLAs) and T cell cytokines in the development of reactive arthritis. Procedure is PCR followed by hybridization. Personnel are already and will be trained further in safety procedures. Budget code will be that of the study.

(The above hazards are defined in the reverse page)

DEFINITION OF HAZARDS

1. Chemical hazards are those which include extremely toxic, carcinogenic, flammable and reactive chemicals.

2. Reactive chemicals are those which cause fire and explosion during uncontrolled use.

3. Radioactive materials include an unstable isotope of an element that decays or disintegrates spontaneously, emitting radiation i.e. α, β or γ particles; and those materials contaminated with radioisotope or produce radiotoxicity.

4. Radiotoxicity is a term referring to the potential of an isotope to cause damage to living tissue by absorption of energy from the disintegration of the radioactive material introduced into the body.

1.2 Summary description of specific training and experience of the Principal Investigator in using hazardous material(s) under consideration.

The PI has work with radioactivity for the last 10 yrs.

1.3 Expected average quantity of waste generated (in kg./month)

This will depend on the number of samples. These assays will be done on selected samples towards the end of the study when approx. 250μCi of radioactive material will be used per month.

Radioactive [✓] Chemicals [ ] Biochemicals [ ]

2.0 Declaration

I agree to provide the Office of the *Occupational Safety and Environment Programme* (OSEPP) of ICDDR,B with appropriate information related to the study, and to comply with all applicable regulations of the OSEPP (ICDDR,B), other appropriate agencies, scholarly organizations or recognized professional groups. I also agree my participation and participation of all persons involved in my study in safety related training.

Jasmin Ayen — 6.10.95

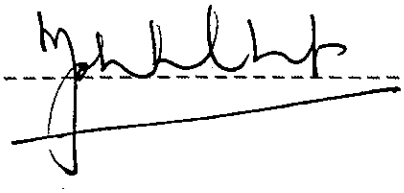
Signature of the Principal Investigator — Date

[signature] — 6.10.95

Signature of the ~~Division~~/Department Head — Date

APPLICATION FOR PROJECT GRANT

2.	PRINCIPAL INVESTIGATORS	:	Tasnim Azim, ICDDR,B N. Avrion Mitchison, Berlin Joachim Sieper, Berlin
	COINVESTIGATORS	:	M. Abdus Salam, ICDDR,B M. John Albert, ICDDR,B Narinder K. Mehra, New Delhi Dominique Charron, Paris
	CONSULTANTS	:	Andreas Krause, Berlin Angela Zink, Berlin Jerry Lanchbury, London Gabrielle Kinksley, London Philippe J. Sansonetti, Paris Antoine Toubert, Paris
1.	TITLE OF PROJECT	:	A prospective study of reactive arthritis triggered by *Shigella* dysentery
3.	STARTING DATE	:	As soon as possible
4.	COMPLETION DATE	:	3 years from start
5.	TOTAL BUDGET REQUESTED	:	US$ 434,200
6.	FUNDING SOURCE	:	European Commission
7.	HEAD OF PROGRAMME	:	Division Director Laboratory Sciences Division ICDDR,B



8. ABSTRACT SUMMARY

Reactive arthritis (ReA) is a pauciarticular inflammatory joint disease triggered by a range of intracellular microbes. Although not a major health problem of the dimensions of dysentery itself, it is possible that most cases of tropical arthritis and a significant proportion of those of anterior uveitis are related to this disease. It occurs both in developed and developing countries. In the past it has been characterised only on a retrospective basis, because of the rarity of the triggering infections in developed countries. This will be a prospective study of ReA following shigellosis in Bangladesh where 2500 patients (all adults) with shigellosis will be followed-up. The expected number of cases of ReA from this group of patients is 30-80. Patients with ReA, non-ReA shigellosis controls and healthy adults (without infections or arthritis) will be characterised in terms of microbial strain variation, immunogenetics (HLA B27 and other HLA types), cytokine profile and T-cell reactivity. The purpose is to identify the factors which control the onset of ReA, and in this way gain a deeper understanding of aetiological mechanisms. The overall aim of the study is (i) to assess the importance of ReA as a health problem in Bangladesh, and (ii) to elucidate the disease mechanism.

1) Only adults >15 years of age who have consented to participate will be studied.

2) The risks of the study are minimal and include the possibility of bruising which sometimes occurs with drawing blood and, that of synovial fluid aspiration. The risks will be minimised by taking aseptic precautions. Moreover, the doctors participating in the study will be extensively trained.

3) All standard aseptic precautions will be taken before these procedures.

4) Anonymity will be maintained by using only identification numbers during analysis.

5) a) A signed consent form, in Bengali, will be obtained

b) Information will not be withheld

c) This has been stated in the consent form

6) Clinical interview will be carried out for clinical history and clinical examination which will take approximately 20 mins. During follow-up visits, clinical interviews regarding the development of signs of ReA will be taken, and, where indicated, clinical examination of the joints will be conducted. This will vary between 15-30 mins.

7) The subject will receive free treatment for shigellosis even after discharge and will receive free treatment for ReA if that develops. This study will provide information regarding the frequency of ReA in Bangladesh, the HLA profile of our population (which is relevant to many diseases) and will provide further understanding of the pathogenesis of chronic inflammation following an infection.

8) The study will use hospital records, blood and synovial fluid.

9. AIMS OF THE PROJECT

a) General Aim

To assess the importance of reactive arthritis (ReA) as a health problem in Bangladesh where there is a high incidence of shigellosis. In addition, to understand how the immune response to *Shigella* generates chronic inflammation.

b) Specific Aims

1) To determine the frequency of ReA in Bangladesh following infection with *Shigella*.

2) To ascertain why only a minority of individuals infected with *Shigella* go on to develop ReA by investigating the role of (i) the immune response of the host - the role of human leucocyte antigens (HLAs) (especially B27), T cell cytokines (especially Th1/Th2 balance) and antigen specific T cell reactivity, and (ii) the type of infecting organism - the role of *Shigella* species other than *Shigella flexneri*, which has been shown to be associated with reactive arthritis, in precipitating reactive arthritis as well as the possible role of arthritogenic plasmid(s) in the development of ReA.

3) To define a host/parasite hypersensitivity-susceptibility profile.

c) Rationale

The incidence of ReA in Bangladesh is not known. Although it is not expected to be a major health problem of the dimensions of dysentery itself, it is possible that a large number of cases of tropical arthritis and of those with anterior uveitis are forms of this sequelae. Most of the information about the epidemiology of ReA has come from retrospective studies which provide little information about the predisposing factors. As ReA is an example of a hypersensitivity reaction following an infection, this prospective study will provide a better understanding of the path from infection to hypersensitivity

and may therefore help to identify a host/parasite hypersensitivity-susceptibility profile.

d) Significance

The study will increase the understanding of the causes of hypersensitivity/autoimmunity following infection and may delineate a host/parasite hypersensitivity-susceptibility profile. This increased understanding will call for new modalities of treatment such as treatment with cytokines or anti-cytokines, gene therapy with cytokines, which are already beginning to be evaluated in Europe.

10. ETHICAL IMPLICATIONS

Patients >15 years of age, coming to the Clinical Research and Service Centre (CRSC) of the ICDDR,B with a history of passage of bloody mucoid stools, will be enrolled in the study and admitted to the study ward. Age matched healthy adults will be included as controls.

The ethical implications of the study are outlined below:

a) The study will not interfere with the management and treatment of the patients and none of the procedures will be harmful.

b) Fifteen ml of venous blood will be drawn at initial enrollment from all patients and healthy controls. Another 15 ml of blood will be taken from only those patients who develop ReA at the time of readmission into the CRSC of ICDDR,B. This volume of blood will not be detrimental to the patient.

c) Synovial fluid will be aspirated from the affected joint of patients with ReA to exclude purulent arthritis. This is a safe procedure when undertaken with aseptic precautions. Moreover, the doctors participating in the study will be trained in the diagnosis and management of ReA.

d) The nature and purpose of the study including the procedures involved will be explained to the patients and controls and written consent will be obtained.

e) All patients will be provided with the full course of antibiotic therapy free of charge.

11. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) BACKGROUND

Reactive arthritis is a chronic inflammatory disease of the joints (reviewed in ref. 4) triggered by certain bacterial infections including those of the urogenital tract with *Chlamydia trachomatis*, of the respiratory tract with *Chlamydia pneumoniae* (2) or of the gut with *Yersinia*, *Salmonella*, *Campylobacter* and *Shigella* (14). Arthritis is not a common outcome of these infections and in most cases the arthritis is brief and not chronic. The disease can follow various patterns: (i) short and self-limiting, (ii) recurrent episodes of arthritis and (iii) continuous, unremitting. In long-term follow-up studies, 20-50% of the patients have recurrent episodes of arthritis and some have long-term disability. ReA following *Shigella* infection shows a more chronic course.

Reactive arthritis is an example of how the immune response to a microbe can generate chronic inflammation and other forms of immunopathology. The role of infection in generating hypersensitivity and immunopathology is a central theme of contemporary immunology. Ideas about the pathway from infection to hypersensitivity divide into two groups: (i) T cell epitopes and molecular mimicry and (ii) cytokines. The concept of molecular mimicry assumes that foreign peptide sequences, e.g. in a microbial heat shock protein, occasionally mimic sequences in the body, in this case the corresponding heat shock protein (6). Once T cells respond to one of these sequences, epitope-spreading can take over and disease ensues (16). The second hypothesis is that there is an imbalance in the pattern of cytokine secretion by CD4 T cells (Th1/Th2 imbalance) (24). Th1 cells secrete interferon gamma and interleukin 2 (IL2) and are required for an effective cellular immune response against intracellular bacteria while Th2 cells (secreting IL4, IL5 and IL10) are required for the humoral immune response, they are elevated in allergic diseases and can inhibit the Th1 response (19). The Th1/Th2 balance is therefore likely to influence the outcome of an infection. This study will critically test the hypothesis that reactive arthritis develops as a result of cytokine imbalance. In its simplest form, this hypothesis attributes the arthritis to a failure of the normal T-cell mediated defense against intracellular microbial infection, brought about by excessive Th2 activity (24).

All the bacteria causing ReA are either obligate or facultative intracellular organisms which may enable them to escape the immune system. Antigens of *Chlamydia* (13), *Yersinia* (10), *Salmonella* (9) and *Shigella* (8), have been detected in the joint in patients with ReA which could be instrumental in

driving the local immune response. There is evidence to suggest that local expansion of T cells in the synovial fluid takes place which may be in response to persisting bacterial antigen. There is more proliferation of T cells from the synovial fluid rather than from the peripheral blood of patients with ReA, in response to *Chlamydia trachomatis* and *C. pneumoniae* (20), *Yersinia enterocolitica* (7,21), *Salmonella* (21) and *Shigella* (23). In addition, CD4 T cells recognising antigens of the triggering bacteria accumulate in synovial fluid and there are fewer of the bacteria-recognising T cells in peripheral blood (20). The exact role of these cells in the aetiology of the disease is poorly understood. One of the mechanisms could be increased IL4 secretion which has been detected in the joints of patients with ReA compared to those with rheumatoid arthritis (24) and the IL4 was secreted by CD4 T cells. It has been hypothesised that IL4 may prevent elimination of the triggering bacteria and lead to ongoing inflammation and eventually to tissue damage. In this study we will examine the T-cell reactivity from peripheral blood (as well as synovial fluid of patients with ReA) to *Shigella* antigens. It is hoped that a set of immunodominant antigens may be revealed (by the Paris group) which will be used to study the T-cell reactivity. This study will attempt to see whether the set of antigens defined by T cells taken from ReA patients is different from that taken from non-ReA dysentery patients.

There is a strong association between HLA-B27 and ReA (3) and, interestingly, the association of HLA-B27 with *Shigella* induced ReA is the highest, at 80-90% (4). Although there has been no thorough study of HLA typing in Bangladesh; studies from India have shown an approximate frequency of HLA-B27 at 9% (5). Similar frequencies can be expected from Bangladesh; however, this information

will emerge from this study. The mechanism whereby the HLA-B27 molecule, present on all nucleated cells, confers disease susceptibility is unknown but several possibilities have been suggested: (i) HLA-B27 presents an unidentified arthritogenic self-peptide, (ii) it presents a bacterial peptide which mimics an arthritogenic self-peptide, (iii) it itself shares peptide sequences in common with the triggering bacteria, (iv) it presents a bacterial peptide important for defense against the triggering bacteria, (v) it presents a clonotypic (TCR-derived) peptide important in disease regulation, or (vi) it itself mimics an important clonotypic peptide. These possibilities have been reviewed extensively (4).

The microbiology of the triggering bacteria is likely to influence disease severity and incidence. Eight separate *S. flexneri* strains, all of which were arthritogenic, were shown to contain a 2 Mdal plasmid (25,26). This plasmid contains an 8-amino acid sequence with strong, but not complete, homology to sequences present in HLA-B27 and the related B7 molecule, which may trigger arthritis via molecular mimicry. The large number of patients studied prospectively here, will enable us to confirm whether the previously observed association of the 2-Mdal plasmid with ReA is real. Moreover, if a few B27-negative cases of ReA shows up, it will argue against the mimicry hypothesis. Another important question regarding the ReA triggering bacteria is whether *Shigella* species other than *flexneri* (*S. dysenteriae* type 1, *S. boydii* and *S. sonnei*) can trigger arthritis is not known (12,15) which we will address here by enrolling, not only patients with *S. flexneri* infection, but also with other *Shigella* species.

Information about ReA has relied on retrospective studies done mostly in developed countries. As such information from developing countries is

limited, supplementary information will be obtained by retrospectively examining 25-30 ReA cases in Indians. This will be done in Delhi.

b) RESEARCH PLAN

Patient population

Patients, >15 years of age, attending the Clinical Research and Service Centre (CRSC) of the ICDDR,B, with a history of passage of bloody, mucoid stools will initially be enrolled in the study. Extrapolation from the surveillance data of ICDDR,B, (in which stool culture of every 25th patient is performed) (27), shows that the number of patients with shigellosis attending the CRSC per year is from 1500-3,000 of whom most are infected with *S. flexneri* followed by *S. dysenteriae* type 1 (1). Our aim is to enroll 2,500 patients with shigellosis so that with a follow-up success rate of ~80% we should have 2,000 completed follow-ups within 2 years. However, the duration of the study will be adjusted according to our rate of success in enrolling 2,000 patients. Frequencies of ReA, as reported from the USA and Europe, vary between 1.5-4%. On the basis of these figures, of the 2,000 patients with shigellosis, 30-80 may develop ReA.

All enrolled patients will be admitted to the study ward of the CRSC for at least 24 hrs. Their stools will be examined microscopically for the presence of white blood cells, red blood cells and parasites. Stools will also be cultured for enteric pathogens. Colonies of *Escherichia coli* will be collected and stored for future identification. This will be done to see whether other enteric pathogens are also associated with ReA. All patients with *Shigella* positive cultures will, on discharge, be followed weekly for eight weeks initially (during the training period of health assistants) and

then for six weeks. The health assistants will make a provisional diagnosis of ReA if the patient meets the following criteria:

- arthritis occurring <6 weeks after the onset of diarrhoea
- asymmetric arthritis involving predominantly the lower limbs
- swelling of at least one joint
- no history of another disease with a similar pattern of arthritis (chronic arthritis)

Such patients will be referred to the CRSC of ICDDR,B and admitted to the study ward for further investigation and confirmation of diagnosis by the doctors involved in the study, who will be trained by rheumatologists from Europe. The doctors will look for the following extra- and para-articular manifestations:

- dactylitis
- enthesitis
- urethritis
- inflammation of the eye
- inflammation of the skin (keratoderma blennorrhagica)
- inflammation of the mucosa (circinate balanitis, painless mouth ulcers)

Synovial fluid will be aspirated, where appropriate, to exclude septic arthritis and it will also be used for immunological studies for ReA. Appropriate treatment will be given to all patients. If services of specialists are required, they will be consulted from other hospitals.

A 15 ml sample of blood will be drawn by venepuncture from all patients on enrollment. A further 15 ml of blood will be collected from patients who

develop ReA when they are readmitted to the CRSC and at similar times from two non-ReA dysentery controls.

Healthy adults, >15 yrs of age, of either sex, of the same socioeconomic background, free of any infection and, without a history of arthritis, will be included as controls. The number of controls will match the number of ReA cases (30-80) and will be taken whenever we get a case and from the same community as the case. From these healthy controls a stool sample will be collected for microscopic examination of cells and parasites and for culture. In addition, a single 15 ml sample of blood will be drawn by venepuncture.

Laboratory tests

Tests will be carried out on: (i) peripheral blood, (ii) synovial fluid, and (iii) strains of *Shigella*.

Peripheral blood: Peripheral blood will be used for experiments on plasma, mononuclear cells and granulocytes.

From the first sample of blood, 2 mls will be used for an abbreviated serological typing for HLA from all 2500 patients enrolled. The remaining 13 mls of blood will be separated and plasma, mononuclear cells and granulocytes will be stored at $-70^{o}C$ (for plasma) and $-150^{o}C$ (for cells). From the second sample of blood, from ReA patients, separation will be carried out on all 15 mls and the cell fractions as well as plasma stored as before.

Intensive work will be carried out on the stored samples from only patients who develop ReA, non-ReA dysentery controls and healthy controls, (for each case two non-ReA dysentery controls and one healthy control).

The following will be done on stored samples:

1) Mononuclear cells will be analysed for cytokine expression. For this purpose, RNA will be extracted, and 2 µg (obtained from a 6 x 10^6 aliquot) reverse transcribed. The amounts of cytokine expression will be determined by semiquantitative PCR with the use of a panel of primer pairs specific for IL2, IL12 and IFN gamma (Th1 cytokines) and IL4 and IL10 (Th2 cytokines) as described previously (11).

   Remaining mononuclear cells will be used for preparing T cell lines and autologous B cell lines by Epstein-Barr virus transformation. T cell lines will be used for proliferation assays to different *Shigella* antigens while autologous B cell lines will be used for antigen presentation. These will be done using an established procedure (17). Further investigations on T cells will be done in the following order of priority:

   a) Detailed analysis of immunodominant *Shigella* antigens recognised by CD4 T cells by means of proliferation assays.

   b) Analysis of CD8 T cell reactivity by means of cell lines and by cytotoxicity.

2) Granulocytes will be used for extracting DNA which will be used for a more complete HLA typing: (a) B27+ samples will be subtyped, and (b) generic typing of HLA-DRB alleles will be performed. Typing will be done by PCR in combination with sequence-specific oligonucleotide probes as described previously (18).

3) Plasma will be used for the following:

- IgE levels by ELISA
- soluble CD23 (Fc-epsilon R) levels by ELISA (CD23 is related to IgE levels)
- IgG, IgA and IgM levels to *Shigella* specific antigens by ELISA
- IgG subtypes by ELISA

Synovial fluid. Synovial fluid, which will be aspirated from patients with ReA, will be used (in addition to excluding purulent arthritis) for sedimenting cells on which cytokine expression and generation of T cell lines for proliferation assays will be carried out as for peripheral blood mononuclear cells.

Bacterial strains. *Shigella* strains will be stored at -70°C and investigations of plasmids, particularly of the 2 Mdal plasmid associated with ReA, will be carried out. Associations of the plasmid with HLA-B27 will be looked for and a search for other arthritogenic plasmids having sequence homology with B27/7 molecules will be conducted. All the work described here will be conducted in Berlin and Paris.

Data analyses ,

Data analyses will be conducted to assess (i) the epidemiology of ReA, and (ii) the relationship of host and bacterial factors with the development of ReA. Incidence rates for reactive arthritis among patients with shigellosis will be calculated. Specific incidence rates for sex and HLA-B27 will be estimated. The frequency of HLA-B27 subtypes in different groups of patients will also be estimated. Logistic regression analysis will be used for these purposes. The immunological parameters of three study groups (ReA cases, non-ReA cases with shigellosis and healthy controls) will be compared by ANOVA

(parametric data) or Kruskal-Wallis (non-parametric data) which will be followed by 2 group analysis using Student-Newman-Keuls method (parametric data) or Dunn's test (non-parametric data). For categorical data, Fisher's exact test will be used. Paired samples will be compared using Wilcoxon's signed rank sum test.

c) BIBLIOGRAPHY

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4. Burmester GR, A Daser, T Kamradt, A Krause, NA Mitchison, J Sieper, N Wolf. 1995. Immunology of reactive arthritides. Ann Rev Immunol 13:229.

5. Chopra A, D Raghunath, A Singh. 1990. Spectrum of seronegative spondarthritides (SSA) with special reference to HLA profiles. J Assoc Physicians India 38:351.

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12. PUBLICATIONS OF PRINCIPAL INVESTIGATOR (LAST FIVE YEARS)

Tasnim Azim

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5. Qadri F, **Azim** T, Hossain A, Choudhury A, Albert MJ. 1994. Monoclonal antibodies specific for *Shigella dysenteriae* type 13. Diagn Microbial Infect Dis 18:145-149.

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10. **Azim T**, Halder RC, Sarker MS, Ahmed S, Hamadani J, Chowdhury A, Qadri F, Salam MA, Sack RB, Albert MJ. 1995. Cytokines in the stools of children with complicated shigellosis. Clin Diag Lab Immunol 2:492-495.

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13. FLOW CHART

The project will be carried out in the following five phases:

1) Setting up organization, hiring personnel, ordering and delivery of equipment and supplies. Excepted duration: 3 months.

2) Preliminary exploration. Excepted duration: 4-6 months.

During this phase enrollment will begin, and a sufficient number of patients will be followed up to make two key estimates; one of the frequency of HLA-B27+ individuals, and the other of the frequency with which such individuals develop ReA in Dhaka.

During this phase two important pieces of training and coordination will be completed: training of Dr. Azim in HLA typing in London, Berlin and Delhi, and training of the Dhaka health assistants and doctors in handling ReA (diagnosis, sampling synovial fluid).

3) The main project. Excepted duration: two years.

Enrollment and follow up of 2500 cases of shigellosis, as described above. If ReA develops more frequently than excepted (note that the

expectation is based only on European/USA figures) then the enrollment will be curtailed and the study shortened.

4) Laboratory studies. Excepted duration: two years, but these will start as early as the start of phase 2 above.

5) Break-point. The main project will have a break-point at half time, i.e. in the middle of year 2. At that point decisions would be taken concerning which types of *Shigella* infection to drop from the study, whether to extend the T-cell studies, and others as appropriate.

6) Analysis of data and writing-up for publications. Excepted duration: 6 months, starting towards the end of phase 3.

PHASE	YEAR 1	YEAR 2	YEAR 3
1. Setting up	xxx		
2. Preliminary exploration	xxxxx		
3. Main project	xxxx	xxxxxxxxxxxx	xxxxxxxx
4. Laboratory studies		xxxxxxxxxxxx	xxxxxxxxxxxx
5. Writing up			xxxxxx

14. ITEMISED SPECIFIC TASKS FOR EACH LISTED INVESTIGATOR

ICDDR,B Group:

Dr. Tasnim Azim will supervise the work in the laboratory in Dhaka. She will conduct HLA typing and carry out RT-PCRs for cytokines after receiving training from Berlin, London and New Delhi. She will coordinate with physicians regarding patient enrollment, follow-up and

sample collection. She will be involved in data analysis in collaboration with Berlin.

Dr. M. Abdus Salam will supervise the clinical aspects of the study and will participate in the training on arthritis by doctors from Europe.

Dr. M. John Albert will ensure smooth running of the study and help with its microbiological aspects.

Berlin Group:

Prof. N. Avrion Mitchison will be responsible for project coordination.

Dr. J. Sieper will take joint responsibility with Prof. N.A. Mitchison in project coordination. He will be a medical consultant to the project and visit Dhaka to train the doctors and health assistants.

Dr. Krause will also be a medical consultant and visit Dhaka to train doctors and health assistants.

Dr. A. Zink will supervise the epidemiological aspects of the study and take responsibility for statistical analysis in collaboration with ICDDR,B.

Delhi Group:

Prof. N.K. Mehra will supervise the work in Delhi which will involve carrying out retrospective immunogenetics studies on patients with ReA in New Delhi and train Dr. T. Azim in HLA typing.

London Group:

They will provide reagents (primers) for HLA typing and, together with the Paris group, examine further genetic markers (described below).

Paris Group:

They will carry out further molecular typing of DNA using biotinylated probes and search for associations with other markers including (i) TNF alpha, LMP, TAC and *MIC* genes, (ii) polymorphism within the MHC promoter regions, and iii) with any future microsatellite markers associated with ankylosing spondylitis (this will be then transferred to the Berlin group). They will also study T-cell reactivity to *Shigella* antigens along with the Berlin group.

TA:mh/T10:REA:PRT

BUDGET PROPOSAL

PROJECT TITLE: Reactive Arthritis triggered by Shigella

PROJECT DURATION : 3 years from starting

NAME OF P. I. : Dr T Azim

STARTING DATE :

RRC APPROVAL DATE :

CLOSING DATE :

ERC APPROVAL DATE :

Amount in US Dollar

Line item	Ist year	2nd year	3rd year	TOTAL	TOTAL ECU
PERSONNEL LOCAL: SALARIES *	34,206	36,601	39,163	109,970	98,973
-Local Employees Salaries and benefits					
INTERNATIONAL SALARIES	1,613	1,726	1,847	5,186	4,667
- Dr M J Albert (1% time)					
CONSULTATION FEES	200	200	200	600	540
LOCAL TRAVEL **: (General Transportation and patient follow up transportation cost)	10,000	20,000	15,000	45,000	40,500
INTERNATIONAL TRAVEL: (Ticket, Transportation etc)	4,600	4,600	3,000	12,200	10,980
SUPPLIES & MATERIALS					
-Drugs	5,000	6,000	5,000	16,000	14,400
-Lab. Supplies	15,000	15,000	15,000	45,000	40,500
Sub-Total	20,000	21,000	20,000	61,000	54,900
OTHER CONTRACTUAL SERVICES					
-Rent, Communication & Utilities (Postage,Telex, Fax, Phone etc.)	500	500	500	1,500	1,350
- Printing and Photocopies	200	200	100	500	450
Repairing & Maintenance etc	200	500	300	1,000	900
Sub-Total	900	1,200	900	3,000	2,700
INTER DEPARTMENTAL SERVICES					
-Patient Hospitalisation	13,000	26,000	14,000	53,000	47,700
-Medical Illustration	100	100	100	300	270
-Xerox, Library Service	100	100	100	300	270
-Lab. and Pathological test	9,000	10,000	9,000	28,000	25,200
Sub-Total	22,200	36,200	23,200	81,600	73,440
CAPITAL EXPENDITURE: Equipment, Furniture etc					
-20o C Freezer	600			600	540
- 70 o C Freezer	8,500			8,500	7,650
- 150 o C Freezer	29,178			29,178	26,260
- Personal Computer, Printer	5,000			5,000	4,500
Sub-Total	43,278	0	0	43,278	38,950
TOTAL OPERATING COST	136,997	121,527	103,310	361,833	325,650
Overhead (20% of Direct Cost)	27,399	24,305	20,662	72,367	65,130
TOTAL PROJECT COST	164,397	145,831	123,971	434,200	390,780

* DETAILS OF LOCAL SALARIES

Amount in US Dollar

Line item	Ist year	2nd year	3rd year	TOTAL	TOTAL ECU
PERSONNEL LOCAL: SALARIES					
Dr T. Azim (30% time)	3,494	3,739	4,000	11,233	10,110
Dr M A Salam (10% time)	1,852	1,982	2,121	5,955	5,359
Medical Officer	8,148	8,718	9,329	26,195	23,576
Research Officer (2 no)	9,096	9,733	10,414	29,243	26,318
Health Assistant (4 no)	11,616	12,429	13,299	37,344	33,610
TOTAL	34,206	36,601	39,163	109,970	98,973

** Local Travel has been calculated on the basis of 2500 patients US$ 1.5 per visit and weekly visit for 3 months (US$ 3,750 x 4 x 3 = 45,000).

Abdul Ahad
Budget & Cost Officer
ICDDR, B. Mohakhali
Dhaka-1212, Bangladesh

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR PATIENTS WITH SHIGELLOSIS

You have bloody diarrhoea caused by a germ called *Shigella* which can lead to arthritis with chronic joint pains and involvement of other organs such as the eyes, skin etc. We do not know how *Shigella* causes arthritis and in order to understand more about this illness, we are conducting a study which, in future, may help us prevent or treat it. We would like you to participate in this study.

For the purpose of the study, you will be hospitalized for at least 1 day. Before discharge, we will collect 15 mls of venous blood (approximately 3 teaspoonful) from your forearm for immunological tests. After discharge, we will visit you at home once every week for 6 weeks to see whether you develop pain or swelling in any of your joints. If you do, we will ask you to come back to ICDDR,B for confirmation of the diagnosis. If you have arthritis, you will be readmitted and appropriate treatment will be provided. In addition, we will aspirate fluid from your swollen joint and take 15 ml of venous blood from your forearm for more tests. You will be discharged from hospital on improvement. Even if you do not develop arthritis, we may ask you to return to ICDDR,B when we will take another 15 ml blood (3 teaspoonful) from your forearm. These procedures are safe. However, we will provide treatment for any untoward effect if it were to occur.

You will receive the same care and treatment that is normally provided whether you particiate in this study or not. You may withdraw your consent any time during the study without causing any effect on your treatment at the CRSC. All information obtained during the study will be confidential and if you wish to know the results, we will provide those to you on request, as they become available. Such information, however, are subject to review by the Institution/Centre's review boards.

If you agree to participate in the study, please sign or put your-thumb impression below.

_____ _____

Signature or left thumb print impression of patient — Date

_____ _____

Signature of witness — Date

_____ _____

Signature of investigator — Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR HEALTHY VOLUNTEERS

We are conducting a study on adults with bloody dysentery caused by a bacteria called *Shigella* to see why some adults develop arthritis. In order to see immunological changes in adults with shigellosis, we will have to compare the results to those of adults without any infection. We would like you to participate in the study. For the purpose of this study, we will be required to draw 15 ml (3 teaspoon) of venous blood and stool from you once for special tests. These are safe procedures, but if any untoward effect does occur, we will provide the necessary treatment. Although the study will not benefit you directly, it will help us to better understand the disease and thereby prevent complications in future. All the information obtained during the study will be confidential. If you wish to know the results, we will provide those to you on request as they become available. Such information, however, are subject to review by the Institution/Centre's review boards. If you agree to the participate in this study, please sign or put your left thumb print impression below.

_____ _____

Signature or left thumb print impression of volunteer

Date

_____ _____

Signature of witness

Date

_____ _____

Signature of investigator

Date

RESPONSE TO REVIEWERS' COMMENTS

Reviewer # 1

No response is required to these comments.

Reviewer # 2

1) A provisional diagnosis of ReA will be made by health assistants during their follow-up visit. The criteria are stated on page 11.

2) As far as the cytokines are concerned, we will be assessing cytokine levels at the acute stage (on enrollment) and when ReA develops. As the reviewer himself points out, comparisons between synovial fluid and peripheral blood will be more important and this will be done in patients with ReA.

3) Some of the techniques involved are difficult but the best groups in the world are involved which will maximize our chances of success.

Reviewer #1

Institut für Immunologie
Universität München

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Mr
John **Albert**, Ph.D.
ICDDR, B
Acting Director
Laboratory Sciences Division
GPO Box 128

Dhaka-1000, Bangladesh

Copy to
Dr. T. Azim

3. Juli 1995

Research Proposal

This project is one of the first genuinely prospective studies on the epidemiology of reactive arthritis following shigellosis. The well-known association with the HLA-B27 genotype makes this study particularly interesting for understanding the general problem of how infections give rise to autoimmunity. As shigellosis represents a sizeable portion of infectious dysentery in Bangladesh the project also addresses a major health problem of this country. The particular attraction of the project lies in its scope as well as in the fashion it is planned to be executed:

1) A sound molecular HLA-typing of the patients is proposed with the involvement of two of the most prominent immunogenetic groups in London and Paris.

2) A well designed clinical follow-up is being guaranteed by the ICDDR, B which has already a unique network of epidemiological and clinical personnel in place

3) A highly original pathogenesis study of the TH_1 / TH_2 T lymphocyte balance is designed which is now assessable by studying lymphokine expression in PBL and synovial joint fluid. The Berlin centre has an outstanding record on exactly this topic (Mitchison, Sieper).

The ICDDR is a unique and the only place where such a study can be performed in a reasonably short time.

Prof. Dr. Gert Riethmüller

Dr. T. Azim

Reviewer #2

Title:"A prospective study of reactive arthritis triggered by Shigella dysnetery"

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	✓		
Adequacy of Project Design	✓		
Suitability of Methodology		✓	
Feasibility within time period	✓		
Appropriateness of budget	✓		
Potential value of field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☑

b) with qualification ☐

- on technical grounds ☐
- on level of financial support ☐

I do not support the application ☐

Name of Referee

Signature:....
Position:

Institution:

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Title: A Prospective Study of Reactive Arthritis triggered by *Shigella* Dysentery

PI:

Reviewer:

This is an ambitious proposal to look frequency of the active arthritis following *shigellosis* in a prospective study in a country where the magnitude of the problems are caused by arthritis is not well defined. The second aim is to discuss the understanding of etiological mechanisms.

The first aim is clearly worth pursuing in its own right but there may be problems with ascertainment of cases. It is presumably known how representative those cases of *shigellosis* coming to the CRSCR are compared to the population? What is not made clear is how cases of Reactive arthritis will be identified so that they do come back for re-admission. Some prospective screening for arthritic symptoms, or a specific request for any developing joint pains to come back might improve the ascertainment.

The inquiry into etiological mechanisms is asking questions with the potential to confirm previous European data on a larger scale - but they are not necessarily new questions. The relevance of TH1 vs TH2 type cytokines may vary according to the stage of the disease that they are looked at, since the systemic TH2 response may well be a protective one. However the difference between peripheral blood and synovial fluid in the same patient should be interesting. The analysis of specific T-cell responses may be easier to do in the synovial fluid mononuclears than in the peripheral blood, since such responses often appear to be depressed in the peripheral blood during the acute stage (although this may be a problem of presenting cells more than T-cells). In the same way most centres have found it harder to grow out CD8 T-cells than CD4. Nevertheless an impressive panel of Consultants has been assembled for this project, so that it stands a good chance of success. I therefore support this application.