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Rcd. 21 July 1977

77-009

SECTION I - RESEARCH PROTOCOL

1) Title: Immunity and Amebiasis

2) Principal Investigator: R. Gilman

3) Starting Date: July 15, 1977

4) Completion Date: July 1, 1978

5) Total Direct Cost: $59,613.50

6) Abstract Summary:

Adult male patients with ulcerative amebiasis will be compared with age-matched patients suffering from non-ulcerative amebic dysentery, shigellosis and watery diarrhea. Parameters studied will be dinitrochlorobenzene (DNCB) skin response, T&B cells and lymphocytic blastic transformation to PHA, and E.h. antigen. Gastric acid, protein loss, and resolution of disease will also be studied. Serial rectal biopsies will be taken and mucin and immunoglobulin producing cells identified.

7) Reviews:

a) Research Involving Human Subjects: _____

b) Research Committee: _____

c) Director: _____

d) BMRC: _____

e) Controller/Administrator: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective: The objective of this study is to understand the mechanism by which amebic dysentery can produce severe necrotic invasive disease in one individual and relatively mild circumscribed disease in another individual.

2. Background: Studies performed by myself and others in Malaysia correlating the histological findings with the proctoscopic appearance in amebic dysentery patients showed that deep tissue invasion by E. histolytica (E.h.) trophozoites only occured in patients with necrotic ulcers. Last year we were able to show that patients with necrotic ulcers on proctoscopic examination had more severe disease, a shorter duration of illness, had a poorer prognosis, developed more complications and had decreased delay hypersensitivity skin reactions to several antigens including PPD. On the other hand these patients had no depression in their ability to respond to amebic antigen with antibody production

when compared to patients with amebic dysentery and no necrotic ulceration. In the last two years there have been several studies on immunity in amebiasis in either experimental animals or in human hosts. A study by Drupp showed that guinea pigs injected with amebic antigen tended to be protected from developing dysentery after challenge with amebic trophozoites. Savanat in Thailand used the lymphoblastic transformation test to define cellular immunity to amebiasis. He found that patients ill with amebic dysentery or amamebic liver abcess had a lymphoblastic transformation response to E.h. antigens. In contrast healthy controls or patients with other diseases had no transformation of lymphocytes in the presence of endamebae histolytica antigen. Harris and Bray from Zambia used a similar type test of lymphoblastic transformation response to E.h. antigen. They studied patients with amebic hepatitis, amebic dysentery carriers and healthy controls. Like Savanat they also found that lymphocytes of patients with amebic dysentery or amebic liver abcess underwent blastic transformation in the presence of E.h.

antigen. Control patients did not. They also studied the reaction of lymphocytes to phytohemagglutin (PHA) a non-specific mitogen. They found no difference between controls and patients with amebic dysentery but did find that patients with liver abcesses had suppression of blastic transformation to PHA. In none of these studies did patients have proctoscopic examination reported. It is therefore, impossible to know the degree of severity of amebic colitis in the patients in whom lymphoblastic transformation has been reported. In our studies delayed hypersensitivity was decreased only in patients in whom necrotic ulcers were seen. Suppression of delayed hypersensitivity to a specific antigen, like amebic antigen, may only occur in patients with necrotic ulcerations.

Early studies have shown that amebic colitis appears to have changes similar to those seen with ulcerative colitis. All cell types are increased. IgA still is predominant but IgG cells are greatly increased and are more common than IgM cell. They also tend to be located close to the surface epithelium. Further

studies on the cell ration of IgA, IgG and on IgM will help us delineate mechanisms by which the host responds to colonic infection. In addition, cryostat sections will be studied in an attempt to determine the presence of specific E.h. antibody producing cells.

The mucus producing cells on the surface of gastric mucosa produce neutral mucins, whereas the goblet cells in the glands of small and large bowel mostly produce acid mucins. However, the goblet cells in the lower two-thirds of the crypts in the intestinal mucosa produce neutral mucins. Strongly acid mucins are observed in the basal and intermediate parts of the glands in colonic mucosa, whereas weakly acidic mucins are present in the neck parts of glands and in the goblet cells on the luminal surface. It is assumed that the mucin producing cells initially produce neutral mucins and as they mature and migrate towards the surface, they first produce strongly acidic mucins and then weakly acidic mucins.

Greco et al have observed in cases of ulcerative colitis alterations in the distribution of goblet cells with neutral mucins which increase in number and extend more towards the surface. In advanced ulcerative colitis there is marked reduction and eventually disappearance of goblet cells with neutral mucins. Only weakly acidic goblet cells are observed (due to possible slowing down of cellular turnover due to destruction of the crypts).

Whether or not similar changes occur in amebic colitis, Shigellosis and other colitidis, is the aim of this study with the application of the differential stains for mucins mentioned above.

The mechanism of the reduction of mucin production in early stages of amebic colitis will also be studied by these techniques.

In this study proctoscopy will be used to classify patients into two groups. Those with gross ulcers visible upon proctoscopy and those in whom no gross ulcers can be seen. In this latter group the

proctoscopic changes are limited to edema, hyperfriability, loss of folds, thickening of the mucosa and increased mucus production.

A simple non-radio active method useful in the estimation of intestinal protein loss has been described. This method takes advantage of alpha 1 antitrypsin resistance to proteolytic degradation. By determination of the serum to lyophilized stool, ratio, a semi-quantitative indicator of protein loss can be measured. This would prove an extremely useful marker of active versus non-active disease and could be used to define endpoints of disease. Differences in the three groups of patients with invasive disease could be studied.

Although studies in volunteers have shown that patients given bicarbonate are more susceptible to shigellosis, no description of the status of gastric acid in patients with shigellosis or amebiasis has been given. Patients with cholera may have a decrease in gastric acid production. Patients with achlorhydria or gastrectomy are more suscept-

ible to get infected with cholera.

In this study intestinal tubes will be dropped the morning after admission and 50 ug/Kg of histalogue will be used to study differences in gastric acid in the groups both on admission and if possible at seven and twenty-one days.

3. Rationale: The rationale behind these studies is to use these two separate gradings to study the immune response of patients with amebic dysentery. immuno and histological studies on the rectal mucosa will be studied. Using a new non-invasive technique protein loss will be determined by the Crossley method. In some cases gastric acid will be measured before and after a histalogue stimulation test.

B SPECIFIC AIMS

1. Correlation of delayed hypersensitivity depression with amebic ulceration.

2. To look at changes in so ratios of IgA, IgG and IgM cells using flourescent antibody or immuno-peroxidase technique in rectal biopsy mucosa.

3. Persistence of antigen after therapy.

4. Specific antibody producing cells to E.h. in the rectal mucosa as determined on cryostat sections.

5. The histology of recovery of amebic ulceration in the rectal mucosa.

6. The changes in intestinal mucin patterns during acute disease and convalescence.

7. The relationship of strongyloides to disease severity in amebiasis.

8. The degree of protein loss in amebic dysentery, shigellosis and E.coli diarrhea.

9. Gastric Acid - Differences in patients with dysentery, cholera and E.coli diarrhea.

C. METHODS OF PROCEDURE

Adult male patients with amebic dysentery will be admitted to the study ward of the hospital. Patients between the

ages of 15 and 35 will be excluded from the study. These patients rarely have necrotic amebic dysentery. For each patient with amebic dysentery an age match control with shigellosis and watery diarrhea caused by either E.coli or cholera will be entered into the study. On admission all patients will have hematocratic, white count differential, specific gravity, sedimentation rate and platelet count. Blood will be taken for BUN, creatinine, SGOT, electrolyte, and specific gravity determinations. Urine will be analysed microscopically and sent for culture. Patients will have an initial rectal swab, a rectal swab on day four, day seven and if necessary day 14 and day 21. Initial rectal swabs will have 10 lactose fermentors picked, pooled, and tested for LT and ST. Studies will be drawn on the morning following admission with patients in a fasted state for the following test.

1) T and B cells 2 cc of heparinizezed blood 2) lymphoblastic transformation determination to both E.h. and PHA (12 cc). All patients will also have a small bore tube swallowed and basal and histamine stimulated gastric

acid measured. Serum for Ferritin levels will also be drawn.

On admission all patients will have the following skin test applied: dinitrochlorobezine will be applied 50 microgram and 2,000 microgram doses using a 0.1m. dose to the upper arm. Challenge will consist of 50 microgram dose of DNCB applied to the forearm on day 8. If the patient does not react, this dose will be reapplied on day 12. Serum will also be taken for determination of amebic antibody. On admission, one day, four days, seven days and 14 days the first stool in the morning will be frozen and then lyophilized. The degree of protein loss will be determined using the alpha antitrypsin method of Crossley. Patients on the evening of admission will have a string capsule placed and this capsule will then be pulled next morning and the presence or absence of strongyloides larvae noted. This study will be done to analyze whether or not the presence of strongyloides influences the type of disease in the host. Proctoscopy will be performed on all patients on admission, and some will have biopsies on either the first, fourth, seventh, fourteenth or twenty-first day. Patients who have watery diarrhea will be proctoscoped

only one time on the first day after admission. All patients during proctoscopy after a culture is taken will have a rectal biopsy. One portion of the rectal biopsy will be placed in formaldehyde. The other will be spotfrozen either in liquid nitrogen or on the Microtome. Another rectal biopsy will be used for wash out techniques in which the biopsy will first be put in phosphate buffered saline for 24 hours at 4°C.

Rectal biopsies will be received in (a) formalin, (b) frozen immediately for cryostat sections or snap frozen in liquid nitrogen, (c) phosphate buffered saline for wash-out technique at 4°C for 24 hours, followed by fixation in in formalin and/or ethanol, (d) Lillies Fixative.

Cryostat sections will be used for fluorescent antibody techniques.

Formalin and/or ethanol or Lillies fixed tissue will be processed for paraffin sections and the following stains will be performed on each specimen:

1) Hematoxylin and eosin.

2) P.A.S. (Periodic Acid - Schiff's).

3) Massons trichorome.

4) P.T.A.H. (Phosphotungstic Acid - Hematoxylin).

5) Immunoperoxidase method (peroxidase-antiperoxidase) for:

   (a) Localisation of antibody producing cells in rectal mucosa.

   (b) Specific identification of E.histolytica in rectal biopsy.

   (c) Possible application of PAP method for specific identification of other antigens in rectal mucosa.

6) Histochemical studies on the changing pattern of mucin production in healing amebic ulcerations in rectal mucosa as well as in other colitidis by applying:

   (a) PAS method for neutral mucins.

   (b) Alcian Blue and Alcian Green methods for acid and sulphated mucopolysaccharides.

   (c) Fluorescence microscopy on sections stained with Ferric Ion-Acridine Orange.

7) Metachromatic stains to study distribution of mast cells in colitis.

In the case of amebic dysentery this will mean 45 mg/kg of metronidazole given in three divided dosages for a period of five days.

In Shigellosis ampicillin at 100 mg/kg will be given in four divided dosages for a period of five days. In cholera or E.coli diarrhea, tetracycline will be given in four divided dosages with a total dose of 2 gms.

Patients' symptoms and signs will be assessed daily. CBC and stools will be examined every four days and venous follow-up bloods will be performed on day seven and fourteen. Preliminary results have shown that the disappearance of pus cells in the stool appear to correlate highly with barium enema resolution of disease. Patients in whom cells are still present or in whom diarrhea is present at the end of two weeks after institution of therapy will have a barium enema performed. Ten barium enemas will also be performed at the end of one week in non-ulcerated amebic dysentery patients and in adults with shigella dysentery.

Twenty-five necrotic and 25 non-ulcerated amebic dysentery patients over the age of 35 will be studied. Similarly 25 shigella dysentery patients and 25 patients with watery diarrhea will also be studied.

All patients will be hospitalized for 14 days excepting those with necrotic ulceration who will be hospitalized for at least 21 days.

Statistical test will be Chi Square and T-test analysis and will take approximately one month. Problems which we think will arise in the method period will be determination of IgG, IgM and IgA cell types. The background of gamma globulin in the inflamed rectum made it impossible for us to use fluorescent antibody analysis in ethanol fixed and paraffin embedded sections to analyse plasma cell population. We are currently experimenting with the possibility of using the immunoperoxidase method in

combination with the wash-out procedure of Brandzieg. Data obtained will enable us to correlate T-cell changes with disease severity and skin test depression. Similarly the same data will be available for the specific blastogenic response to E.h. antigen. The controls of shigella allow us to observe another invasive disease and the other controls allow us to examine a non-chronic non-invasive form of diarrhea.

D. SIGNIFICANCE

Significance of this work is an attempt to explore differences in host reaction in dysentery.

E. FACILITIES REQUIRED

1. Office Space: One office is required for Dr Gilman and another office will be required for the study doctors, Dr Rabbani and Dr Asma. The second office should be preferably in the hospital in the study ward office.

2. Laboratory Space

200 cubic feet for Laboratory and
200 cubic feet for Immunology Lab. for one year.

3. Hospital Resources:

15 Study Ward Beds - 9 months.

4. 4. Infant Mice: 200

5. Logistical Support:

Vehicle - Outpatient follow up + one run daily NRU

6. Major Items of Equipment:

Histological supplies as mentioned in F.buski protocol

a) F.buski Protocol

b) Revco-1/3

c) Refrigerator - ½

d) Ward Refrigerator - ½

e) Proctoscopic Room

F. COLLABORATIVE ARRANGEMENTS

An informal collaborative arrangement between Dr. T. Jackson and myself has been going on. We will supply tritiated thymidine and other miscellaneous supplies.

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CONSENT FORM

I realize that I am taking part in a study of intestinal diarrhea and my ability to react to disease. In this study I will have an examination by tube of my rectum and have a small piece of tissue taken for examination on three to four occasions, The only possible hazard to this examination is the possibility that I may have some bleeding following the snipping of this tissue from my rectum. This has not been a problem in over four-hundred patients examined at the Cholera Research Laboratory. If it does however, in the rare instance produce bleeding I may require a blood transfusion. Blood will be taken from my vein on admission and periodically after that for up to four to five times. Twice before going home I will have repeat examination of my rectum and repeat piece of tissue sample taken. I will have several injections in my skin and some materials will be placed on my skin. These materials may produce itching and redness of the skin. I will swallow a capsule which has a string and will have the string pulled up next morning. The string will be used to look for worms. I may receive an enema with barium and five to six X-ray pictures taken. I will also have a tube put down my stomach and the gastric power of my stomach tested. I also realize that I can refuse to be in this study and this refusal will in no way interfere or change my regular treatment that I will obtain at the Cholera Research Hospital. I also understand that I can withdraw my participation from this study at

any time without incurring any penalties or without change in the routine form of therapy for my disease.

_____
Signature

_____
Date

Witness:

_____

_____

ABSTRACT SUMMARY

This is a study comparing the immune response in freely consenting adults who are suffering from amebic dysentery, watery diarrhea, and Shigellosis.

Rectal biopsies will be taken between 3-4 times in each patient. The risks on this are very low. In a series of 100 children in the U.S.A., no risks were found on rectal biopsy. As we do the biopsy through an anoscope, there is no danger of perforation. Bleeding, if it occurs (we have had no problem in over 300 biopsies), can be controlled by local measures such as cautery.

Skin tests will be performed. Occasionally, large reactions may produce some mild discomfort. This is controllable using steroid cream.

Barium enemas will be performed, these will be done on patients after the first 5 days of disease so that the hazards, extremely low that they are, will be further reduced.

All patients will be given a hospital number and their records cited in this fashion.

Patients will have proctoscopy and grading. This will enable us to give accurate prognosis in dysentery cases. As severe amebiasis needs more chronic care the extent of the disease definition of complications and their treatment will all be to the benefit of the patient. Similarly in Shigellosis.

Blood and stool will be saved and the histology of rectal biopsies determined.

SCHEDULES

Admission Day	Amebic Ulcer	Non-Ulcer	Watery	Shigella
CBC	+	+	+	+
M.E. Stool	+	+	+	+
Chem & Serum	+	+	+	+
Chest X-ray	+	+	+	+
R.S.	+	+	+	+
E.coli Pick 10 pool			+	
Stool anti	+	+	+	+
P.M.				
Enterocap	+	+	+	+
PPD	+	+	+	+
DNCB	+	+	+	+
Derm	+	+	+	+
Bx	+	+		+
Day 1 - A.M.				
Hep. Blood	+	+	+	+
NRU 14 cc				
Reading P.M.	+	+	+	+
Stool anti-trypsin	+	+	+	+
Assay				
Bx	+¼	+¼	+	+(¼)

	Amebic Ulcer	Non-Ulcer	Watery	Shigella
Day 4 - CBC, RS, M & F	+	+	+	+
Stool anti-tryp	+	+	+	+
Bx	+½	+½		+½
Day 8 - CBC, RS, M & F	+	+	+	+
DNCB - 50	+	+	+	+
Stool anti-tryp				
Venous blood - 14 cc Hep, 2 cc Serum				
Bx	+¼	+¼		+¼
Day 12 - DNCB - 50 cc	+	+	+	+
Stool anti	+	+	+	+
Day 14 - DNCB				
50 mg - reading	+	+	+	+
Bx	+	+	+	+

SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

	Name	Position	% of effort	Annual Salary	Project Requirements TAKA	DOLLARS
1.	Dr R. Gilman		30%	$ 33,000	153,450.00	9,900.00
2.	Dr B. Seaton		5%	$ 18,907	14,652.92	945.35
3.	Dr Rabbani	Study Doctor	75%	Tk27,084	20,313.00	1,311.00
4.	Dr Asma or other	Study Doctor	70%	Tk27,084	18,959.00	1,223.00
5.	Dr M. Islam	Histo-pathologist	70%			
6.	Mrs Pashi	Histologist	80%	Tk21,309.60	17,047.68	1,099.85
7.		Histology Asst.	100%	Tk21,808	21,808.00	1,407.00
8.	Mr Joe Gomez	Serologist & Histology Asst.	70%	Tk15,927.60	11,149.32	719.31
9.		Bacteriologist	40%	Tk41,796	16,718.00	1,078.60
10.	Dr Ahmed		5%	Tk39,156	1,957.00	126.30
11.	Mr Henry Ghosh	Ward Clerk	75%	Tk 6,312	4,734.00	305.41
12.		Secretary	20%	Tk21,808	4,362.00	281.41
13.	Mrs Ghosh	Sr Staff Nurse	5%	Tk14,880	744.00	48.00
14.		Chemistry Lab Tech.	10%	Tk 8,668	866.00	56.00
15.	(Maybe used full time in other protocols - refer Dr M. Rahman)	Immunochemist	40%	Tk15,000	6,000.00	388.00
Overtime - Mr J. Gomez - 2 hours on Sat & Sunday				Annual	1,130.00	72.90
	Mrs Pashi - 8 hours every month				757.00	48.84
	Mr Henry Ghosh - 16 hours every month				581.76	37.53
				SUB TOTAL:	295,229.68	19,048.50

2. SUPPLIES & MATERIALS

Items	Amount Required
1. Immunological Reagents:	
a) Immunoplates	$ 1000.00
b) Peroxidase & FITC reagents	$ 1000.00
c) Syringes - 1000 - 20 cc	$ 125.20
d) Cultures - 750	$ 750.00
e) Enterocapsules - Donated	
f) Stencils	$ 10.00
g) Stains	$ 500.00
h) BUN-SGOT + Electrolytes - S.C.	$ 581.00
i) Lyophilization Vials - 1000	$ 528.00
j) Serum Vials - 1000	$ 130.00
k) Plaster Pippets	$ 59.00
l) E.h. antigen (ICN)	$ 200.00
2. Slides - 3,000	$ 58.35
3. Cover Slips - 3,000	$ 80.00
4. Needles: 21 guage - 700	$ 24.00
26 gauge - 500	$ 17.00
5. Syringes - 400 - 1 cc	$ 28.00
6. Skin Test Antigens	$ 150.00
7. Thin Levine Tube & Mercury	$ 50.00
8. Histology - Have Instruments - Listed previously	
9. Infant Mice	$ 48.00
10. Miscellaneous 15% of above	$ 800.00
SUB TOTAL.	$ 6138.55

3. EQUIPMENT

Items	Unit Cost	Amount Required
1. Histology Equipment		
2. Lyophilizer - Daily use		
3. Desks - 3		
4. Typewriter - Electric - 1		

4. PATIENT HOSPITALIZATION

1500 Hospital Days	Tk.202,500	$ 13,064.52

5. OUTPATIENT CARE

200 days	Tk. 10,000	$ 645.16

6. TRANSPORT

1500 Miles	Tk. 2,100	$ 135.48

7. OVERSEAS TRAVEL

England to Norway		$ 150.00
4 days per diem		$ 196.00

8. OUTSIDE TRANSPORT $ 500.00

9. RENT, COMMUNICATIONS & UTILITIES

1. Rent		$ 200.00
2. Printing		$ 400.00
3. Xerox		$ 150.00
	SUB TOTAL:	$ 750.00

10. CONSTRUCTION $ 150.00