ment 1. 28 Date JULY 17, 1980

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. T. BUTLER

Application No. 80-032

Title of Study EXPERIMENTAL HEMOLYTIC-UREMIC SYNDROME

Trainee Investigator (if any) _____

Supporting Agency (if Non-ICDDR,B) _____

Project status:
(✓) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects — Yes (No)
   (b) Non-ill subjects — Yes (No)
   (c) Minors or persons under guardianship — Yes (No)
2. Does the study involve:
   (a) Physical risks to the subjects — Yes (No)
   (b) Social Risks — Yes (No)
   (c) Psychological risks to subjects — Yes (No)
   (d) Discomfort to subjects — Yes (No)
   (e) Invasion of privacy — Yes (No)
   (f) Disclosure of information damaging to subject or others — Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) — Yes (No)
   (b) Use of fetal tissue or abortus — Yes (No)
   (c) Use of organs or body fluids — Yes (No)
4. Are subjects clearly informed about: NA
   (a) Nature and purposes of study — Yes No
   (b) Procedures to be followed including alternatives used — Yes No
   (c) Physical risks — Yes No
   (d) Sensitive questions — Yes No
   (e) Benefits to be derived — Yes No
   (f) Right to refuse to participate or to withdraw from study — Yes No
   (g) Confidential handling of data — Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes No
5. Will signed consent form be required:
   (a) From subjects — Yes (No)
   (b) From parent or guardian (if subjects are minors) — Yes (No)
6. Will precautions be taken to protect anonymity of subjects NA — 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 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.

Thomas Butler
Principal Investigator

Trainee

80-032
rec'd 4/8/80

SECTION I - RESEARCH PROTOCOL

1. Title: Experimental Hemolytic-Uremic Syndrome

2. Principal Investigator: Dr. Thomas Butler

   Associate Investigators: Dr. Majid Molla, Dr. Mujibur Rahman, Dr. Mahmud.

3. Starting Date: July 1980

4. Completion Date: June 1981

5. Total Direct Cost:

6. Scientific Program Head:

   This protocol has been approved by the Pathogenesis and Therapy Working Group.

   Signature of Scientific Program Head: W.D. [signature]

   Date: 24/7/80

7. Abstract Summary:

The purpose of this pilot study is to develop an experimental animal model of the hemolytic-uremic syndrome (HUS) in shigellosis. We will test the hypothesis that the HUS is a generalized Shwartzman reaction by injecting rabbits with whole shiga bacilli and endotoxin extracted from Shiga bacilli or other Gram-negative bacteria and looking for intravascular coagulation, hemolysis, and deposition of fibrin thrombi in the renal glomeruli. The colon as the site of introducing endotoxin will be examined by injecting endotoxin into the colon as well as into the skin and intravenously. In this animal model the participations of leukocytosis, prostacyclin, adrenal corticosteroids, and complement will be tested by assays of blood or selective pretreatments.

of rabbits. Pending preliminary results of these studies, further experiments will be planned to modify the HUS by means including anti-endotoxin treatment, anticoagulation, and hormonal treatment.

8. Reviews:

a. Research Involving Human Subjects: _____

b. Research Review Committee: _____

c. Director: _____

d. BMRC: _____

e. Controller/Administrator: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective: The purpose of this work is to define the mechanism of the HUS in shigellosis by developing an experimental animal model in rabbits. Both the sensitizing bacterial products and the constituents of the host response to these bacterial products can be modified experimentally to determine the necessary components of the agent-host interaction that produce the HUS.

2. Background: In 1975, Rahaman and co-workers at the ICDDR,B described a devastating complication of shigellosis in Bangladeshi children, now called the hemolytic - uremic syndrome after shigellosis (1). It occurred in about 10% of children admitted to the hospital with shigellosis. Patients with the HUS differed from other patients by being usually less than 2 years old, having a severe grade of colitis by proctoscopy, having leukemoid reactions (WBC >50,000 per cumm), and having stool cultures positive for shigella dysenteriae, Type 1 (shiga bacillus). The complication occurred most often in the second week of illness when the patients were afebrile and their diarrhea was diminishing in intensity. It was marked by a falling hematocrit and platelet count, erythrocyte fragmentation and reticulocytosis, oliguria, and a rising blood urea nitrogen. More than half of these children died.

Investigations by Koster and co-workers at the ICDDR,B showed that most of these patients had endotoxemia (as defined by the limulus test), intravascular coagulation, and circulating immune complexes (2), and postmortem examinations of the kidneys revealed depositions of fibrin in renal glomeruli and renal artieries (3). Thus, a role for endotoxin in causing the HUS was strongly suggested by 1) endotoxemia being found significantly more frequently in HUS cases than in the non-HUS shigellosis cases, 2) the temporal association of endotoxemia and onset of hemolysis, and 3) the autopsy finding of renal glomerular thrombosis which is the hallmark of the generalized Shwartzman reaction, that is produced experimentally in rabbits by two intravenous injections of endotoxin spaced about 24 hours apart.

Shigella colitis without the HUS resembles histologically the local Shwartzman reaction by showing vascular thrombois and hemorrhagic necrosis, and the shigella HUS resembles the generalized Shwartzman reaction with the generalized intravascular coagulation and thrombosis in renal vessels producing renal cortical necrosis. Both Shwartzman reactions are produced in the rabbit by two injections of endotoxin. In the local Shwartzman reaction 5-10 micrograms of endotoxin is injected intracutaneously into a skin site; 18-24 hr later a sublethal dose of endotoxin, 10-50µg, is injected

intravenously. Within 6 hours the prepared skin site develops grossly visible hemorrhagic necrosis. In the generalized Shwartzman reaction, both injections of endotoxin are given intravenously, 10-100μg, and the rabbits are sacrificed for autopsy 6 hours after the second (provoking) dose of endotoxin. The kidneys show the characteristic vascular thrombosis and necrosis (4,5).

In uncomplicated shigellosis, the colon may be the site of a local Shwartzman reaction. In the HUS the local reaction may be converted to a generalized reaction by the absorption of excessive endotoxin from the infected colon into the systemic circulation to sensitize the renal vasculature or by more direct lymphatic or vascular connections between the colon and kidney that permit sensitization of renal vessels by bacterial products in colonic tissue.

In addition to endotoxin, or as alternatives to endotoxin as initiators of the HUS in shigellosis, there may be the participation of other host mechanisms in mediating this complication. One important factor appears to white blood cells. Nearly all patients with the HUS have a preceding or concomitant leukemoid reaction. Whether the exaggerated leukocytosis in these children is only coincidental or necessary in the pathogenesis of the HUS is unknown. It is possible that white blood cells, especially monocytes, which

contain enzymes like thromboplastin that are capable of initiating intravascular coagulation, are responsible for mediating the DIC of the HUS. This hypothesis can be tested in the animal model by rendering rabbits leukopenic with cytotoxic drugs.

Another possible mechanism for operative in the HUS is depletion of vascular prostacyclin by the infective process. Since prostacyclin may be important as an inhibitor of platelet aggregation in preventing intravascular thrombosis and has been shown to be increased in the bleeding diathesis of uremia (6), its depletion in the HUS might contribute to the formation of intravascular thrombosis.

Adrenal corticosteroids may also play a role in provoking the HUS. In most septic states, the stress of the febrile illness results in an increase in circulating cortisol. In fact, injections of cortisol cause leukocytosis. In the Shwartzman reaction in the rabbit, treatment of the rabbits with steroid hormones can allow a generalized Shwartzman reaction to proceed with only one injection of endotoxin, instead the usual two injections spaced apart by 24 hours. This suggests that cortisol may act synergistically with other endotixin - mediated events to

give expression to the pathology attributed to endotoxin. In our rabbit model of the HUS, the role of steroids can be assessed by pre-treatment of animals with steroids and by adrenalectomy before inducing Shwartzman reactions.

3. Rationale: The development of an animal model of the HUS in shigellosis will enable us to understand better the mechanism of this most serious complication of an intestinal infection. The resemblance of the HUS and Shwartzman reaction in the rabbit produced by endotoxin injections suggests that a model can be produced in rabbits by injections of Shigellae and endotoxin. Then, the conditions for provoking the HUS, both of the provoking agent and host response, can be defined. Finally, the animals can be modified by drug treatment and surgery in an attempt to ameliorate or prevent the HUS.

6. SPECIFIC AIMS:

1. To develop an animal model of the HUS in shigellosis, patterned on the Shwartzman reaction in the rabbit, by serial parenteral injections of Shigella bacteria and endotoxin.

2. To define the requirements of the provoking agent in regard to dosage of bacteria and/or endotoxin, the routes of injection, and the time course of injections.

3. To characterize the requirements of the host response with attention to the white cell response, intravascular coagulation, hemolysis, renal function, adrenal corticosteroid hormones, and prostacyclin synthesis.

4. To seek to modify and prevent the HUS by anti-endotoxin modalities and by blunting the host response by anti-coagulation, hormone treatment, and cytotoxic drugs.

C. METHODS OF PROCEDURE

1. Bacterial Strains: A human isolate of shigella dysenteriae I, will be grown overnight in trypticase soy broth. The number of viable bacteria per ml will be determined by serial dilutions and plate counts. The culture will be killed by adding formalin to a final concentration of 1 per cent and incubating at 37°C for 1 hour. The bacteria will be washed by centrifuging the culture at 12,000xG for one hour, suspending the pellet in normal saline, and repeating the wash once more. The bacterial will be made up to a concentration of 2×10^9 bacteria/ml.

2. Endotoxin: Lipopolysaccharide will be obtained from Sigma chemical Co. extracted from E. coli 026:B6 and from Shigella flexneri.

3. Rabbits: In the pilot studies, the following experiments will be carried out.

a. Generalized Shwartzman reactions. Five rabbits will each be prepared by intravenous injections of 10^7,10^8, or 10^9 whole bacteria in 0.5 ml saline. Twenty-one hours later, they will be provoked by the same dosage of intravenous bacteria. Six hours later, they will sacrificed, and rabbits showing renal cortical hemorrhage or necrosis will be counted as positive reactions. The fifty percent effective dose will be calculated by the method of Reed and Muench. Controls will be included which receive only the preparative and provoking injections of bacteria.

Using endotoxins, doses of 1, 10, and 100 ug will be given intravenously to determine effective doses of each preparation.

b. Local Shwartzman reactions. Skin sites on rabbits will be injected intracutaneously with 1, 10, and 100 ug of endotoxin or with 10^6, 10^7, or 10^8 whole bacteria in 0.25 l saline. The rabbits will be given an intravenous dose of 10 ug endotoxin or 10^8 bacteria 21 hours later. The presence of hemorrhage and necrosis at the skin sites 6 hours later will be recorded as positive reactions, and the effective doses calculated.

c. Hemolysis and uremia. Once the effective doses for the generalized and local Shwartzman reactions have been established, ten times the ED50 will be given to prepare rabbits for both generalized and local reactions. Before the provoking doses, blood will be drawn for determination of Hct, WBC, platelets, haptoglobin, fibrinogen degradation products, urea nitrogen, and creatinine. Six hours, 24 hours, and 48 hours after the provoking doses, these determinations will be repeated.

4. Modified Shwartzman reactions. After the classical reactions have been produced by Shigella organisms and endotoxins, modifications will be attempted to make the model more relevant to the HUS.

a. Intestinal local Shwartzman reaction. Instead of skin, the the colon will be prepared by injections of bacteria and endotoxin given intramurally in marked locations during laparotomy. Twenty-one hours later the rabbits will be given provoking doses of bacteria or endotoxin. Six hours later the rabbits will be sacrificed and the colonic injection sites examined for hemorrhage and necrosis. Biopsy specimens will be taken from the colon and the kidneys examined and blood taken for studies of DIC, hemolysis, and uremia.

b. Subacute Shwartzman reaction. The HUS in humans develops over a period of several days after the onset of diarrhea when the fever and dysentery are waning. Thus, we will modify the Shwartzman reaction by injecting endotoxin and bacteria repeatedly over several days. Subcutaneous injections of 10 µg LPS or 10^8 bacteria will be given daily for 5 days. Before each injection blood will be examined for hemolysis, DIC, and uremia. On the sixth day rabbits will be sacrificed and the kidneys inspected for hemorrhage and necrosis.

5. Endotoxin absorption. To examine whether endotoxin is absorbed from the colon into the blood and kidney, the colon will be injected with 10 µg LPS or 10^8 whole bacteria. Two hours later, samples of blood and urine will be collected for endotoxin by the limulus assay. Rabbits will be sacrificed and the kidneys obtained for homogenizing and endotoxin extraction for the limulus test. Levels of endotoxin in these fluids and tissues will be compared following colonic and cutaneous injections of the same doses of endotoxin or bacteria.

6. Roles of white cells, complement, cortisol, and prostacyclin in HUS. To examine the role of leukocytosis, rabbits will be rendered leukopenic by cytoxan treatment the week before the

experiment. Leukopenic rabbits will be tested for development of both generalized and local Shwartzman reactions.

To assess the participation of complement proteins, rabbits will be made hypocomplementemic by cobravenom before the Shwartzman reactions are elicited.

In some experiments plasma cortisol levels will be measured before and after provocation of the Shwartzman reactions. Rabbits will also be adrenalectomized before provocation of Shwartzman reactions and the effects of adrenalectomy noted on the frequency of reactions and measurements of WBC, hemolysis, DIC, and uremia.

The role of vascular prostacyclin in the HUS will be studied in collaboration with Dr. Donati in Italy, who will measure vascular prostacyclin levels in tissues obtained before and during the Shwartzman reactions.

). Significance

Successful development of an animal model of the HUS should increase our knowledge of this complication by clarifying the role of endotoxin from Shigella dysenteriae in producing the

syndrome. Similarly, the role of various responses to bacteria and endotoxin can be elucidated. With this information at hand, therapeutic measures to ameliorate or prevent the HUS can be rationally proposed.

E. Facilities Required:

1. Office space: space for the Principal Investigator
2. Laboratory space: no additional space than that available in Animal Resources.
3. Hospital resources: None
4. Animal resources: 100 rabbits
5. Logistical support: None
6. Equipment: None
7. Supplies: Syringes
   Test tubes
   Formalin
   Culture media
   Endotoxin of E.coli, Shigella flexneri (Sigma)
   Limulus lysate (Sigma)

F. Collaborative Arrangements: Not required

Abstract Summary

A pilot research project is proposed to develop an animal model of the hemolytic-uremic syndrome after Shigellosis. Rabbits will be used for serial injections of whole Shiga bacteria or endotoxin extracted from Shigella or E.coli in order to approximate the local and generalized Shwartzman reactions that have already been described. Blood will be obtained from rabbits before and after injections of bacteria and endotoxin for studies of hemolysis, intravascular coagulation, and renal failure. Rabbits will be sacrificed after the final injection and their kidneys examined for evidence of hemorrhage and necrosis.

Information from this study will help us understand better the cause of the hemolytic-uremic syndrome, which is a serious and fatal complication in the children of Bangladesh. After we know more about the cause from these studies in rabbits, clinical studies can be designed to confirm these findings and seek to prevent the syndrome.

This Project involves no interviews, physical, psychological, social, legal or any other risks. Rabbits will be handled gently with a minimum of pain and will be disposed of humanely at the end of the experiments. Personnel of the ICDDR,B will not be exposed to infections hazards or other risks.

BUDGET

	US DOLLAR	TAKA
INVESTIGATORS		
Dr. Thomas Butler (40%)	-	-
Dr. Abdullah Al-Mahmud (20%)	-	Tk. 16,600
Dr. A.M. Molla (10%)	-	Tk. 10,000
Dr. M.M. Rahaman	-	-
Technicians 2 (25%)	-	Tk. 12,500
ANIMALS		
Rabbits 100 x 120		Tk. 1,200
REAGENTS AND OTHERS		
Disposable Plastic Syringes : 700(10cc)	US$ 112.00	
Disposable Needles (20g) : 500	US$ 25.00	
Disposable Needles (25g) : 200	US$ 10.00	
Glass vials for haematology: 300	US$ 35.00	
Trypticase soy broth culture media(5 litres)	US$ 9.00	
Endotoxin of E.coli and Shigella flex	US$ 22.00	
Limulus Lysate	US$ 90.00	
Formalin 1 bottle		Tk. 150
Anaesthetics (Peztoberbital Sodium) 500 mls		Tk. 75
LABORATORY TESTS		
Urea Tk.1.50 x 300 =		Tk. 450
Creatinine Tk.1.50 x 300 =		Tk. 450
Hct Tk.2.60 x 300 =		Tk. 780
TC,DC Tk.1.60 x 300 =		Tk. 480
Platelets Tk.1.20 x 300 =		Tk. 360
Total :	US$ 303	Tk. 43,045
		4,545
Grand Total		Tk. 47,590

B. Budget Summary

Category	Taka	Dollars
1. Personnel	40,300	
2. Animals	1,200	
3. Reagents	3,570	
4. Laboratory tests	2,520	
5. Hospitalization	None	
6. Outpatients	None	
7. ICDDR,B transport	None	
8. Travel persons	None	
9. Transportation things	None	
10. Rent/Communication	None	
11. Printing/Reproduction	None	
	47,590	3,173

(Conversion $1 = 15 Taka)

REFERENCES

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2. Koster, F., Levin, J., Walker, L., Tung, K.S.C., Gilman, R.H., Rahaman, M.M., Majid, M.A., Islam, S., Williams, R.C. Hemolytic-uremic syndrome after Shigellosis. Relation to endotoxemia and circulating immune complexes. N. Engl. J. Med. 298: 927-933, 1978.

3. Koster, F., Boonpucknavig, V., Sujaho, S., Gilman, R., Rahaman, M.M. Renal histopathology in the hemolytic-uremic syndrome following shigellosis. In preparation. 1980.

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5. Kasper, D.L. Chemical and biological characterization of the lipopolysaccharide of Bacteriodes fragilis subspecies fragilis. J. Infect. Dis. 134:59-66, 1976.

6. Remuzzi, G., Cavenaghi, A.E., Mecca, G., Donati, M.B., de Gaetano, G. Prostacyclin-like activity and bleeding in renal failure. Lancet 2:1195-1197, 1977.