Attachment 1. 208 Date

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Khaleda Haider
Application No. 86-011
Title of Study Study of the antigenic composition of OM components of Sh.dyst.I strains in relation to their plasmid profile

Trainee Investigator (if any)
Supporting Agency (if Non-ICDDR,B)

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

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

1. Source of Population:
   (a) Ill subjects Yes No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve:
   (a) Physical risks to the subjects Yes No
   (b) Social Risks Yes No
   (c) Psychological risks to subjects Yes No
   (d) Discomfort to subjects Yes No
   (e) Invasion of privacy Yes No
   (f) Disclosure of information damaging to subject or others Yes No
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) Yes No
   (b) Use of fetal tissue or abortus Yes No
   (c) Use of organs or body fluids Yes No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study Yes No
   (b) Procedures to be followed including alternatives used Yes No
   (c) Physical risks Yes No
   (d) Sensitive questions Yes No
   (e) Benefits to be derived Yes No
   (f) Right to refuse to participate or to withdraw from study Yes No
   (g) Confidential handling of data Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No
5. Will signed consent form be required:
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) Yes No
6. Will precautions be taken to protect anonymity of subjects Yes No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

(PTO)

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

Khaleda Haider
Principal Investigator

Trainee

86-071.
3/3/86

SECTION I - RESEARCH PROTOCOL

1. Title : Study of the antigenic composition of outermembrane components of Shigella dysenteriae type 1 strains in relation to their plasmid profile

2. Principal Investigator : Khaleda Haider

   Consultant : Dr. Ivan Ciznar

3. Starting Date : April 1986

4. Completion Date : March 1987

5. Total Direct Cost : $ 17,790

6. Scientific Programme Head :

   This protocol has been approved by the Host Defense Working Group

   Signature of the Scientific Program Head Ivan Ciznar

   Date 26.2.1986

7. Abstract Summary :

The composition of outer membrane components of the Shigella dysenteriae type 1 strains with altered plasmid profiles that have been obtained in our laboratory will be studied. These strains have distinct plasmid profiles involving a set of four major plasmids in the size range 140 to 1.94 Mdal. The antisera will be raised against the whole cells as well as isolated outermembrane (OM) of individual mutants. Polyacrylamide gel electrophoresis, Cross immunoelectrophoresis and Western blotting will be applied for

the analysis. Invasiveness of the mutants will be studied by Sereny's test and on Hela cells. Correlation will be studied between the plasmid profile of the strains, invasiveness and antigenicity of the outer membrane components.

After completion of the study we hope to be able

a. To correlate plasmid profile of S. dysenteriae 1 with its invasiveness and to the antigenicity of OM components.

b. To identify antigens related to invasiveness of Shigella dysenteriae type 1.

8. Reviews :

a. Ethical Review Committee : _____

b. Research Review Committee : _____

c. Director : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objectives

   a. To study plasmid profiles of Shigella dysenteriae type 1, in relation to its invasiveness, antigenicity and composition of the outer membrane components.

2. BACKGROUND

Cell envelope of gram negative bacteria is an immunochemically complex structure composed of three morphologically distinct layers(1,2), namely, the cytoplasmic membrane; a rigid peptidoglycan layer, and a second membranous structure, the outer or L-membrane. The outer membrane contains substantial amounts of protein, phospholipid and lipopolysaccharide (LPS), aclass of antigens referred to as an "O" antigen.

To manifest pathogenecity Shigella must

1. Posses smooth LPS-O antigen
2. Have genes encoding the ability to invade epithelial cells and proliferate therein, and,
3. Elaborate a toxin during the infective process

The importance of smooth LPS as one of the virulence properties of Shigella is best illustrated by the phase variation exhibited by Shigella sonnei (3). Phase I S. sonnei possesses a smooth, complete LPS structure with 2-amino 2-deoxy-L, altruonic acid as a component of

the oligosaccharide repeat unit; phase I organisms are usually virulent. Phase II S. sonnei are rough, do not manifest the O-repeat polymer, and are non-pathogenic. A loss of the 120 Mdal plasmid is associated with the transition from phase I to Phase II (3). Reintroduction of this large plasmid into phase II S. sonnei restores them to phase I and regenerates their epithelial cell invasiveness.

Shigella dysenteriae 1 and S. flexneri are known to exhibit colonial variation wherein some translucent, virulent colonies become opaque and avirulent (4). This phenomenon is distinct from phase I variation seen in S. sonnei since both the translucent and opaque colonies manifest smooth complete LPS.

Genetic hybrids of Shigella and E. coli in which genes encoding production of E. coli 08 or 025 LPS were transferred to S. flexneri 2a recipients were prepared by Gemski et al (5). The transconjugant Shigella sp strains expressing E. coli 08 smooth LPS surface antigen were all avirulent. In contrast, in some Shigella sp. transconjugants bearing E coli 025 LPS remained virulent, invaded guinea pig intestinal mucosa, and caused keratoconjunctivitis when inoculated into the conjunctival sac. The immunodominant repeating sugar of the 08 LPS is D-mannose. This is distinct from LPS of S. flexneri 2a wherein the structure providing type specificity involves attachment of glucosyl secondary side chains to rhamnose of an N-acetylgluosamine-rhamnose-rhamnose repeat unit. Although the precise chemical identity of E. coli 025 LPS has not yet been elucidated it is recognized that rhamnose is present in its 0 repeat unit. These obervations emphasized the role of specific LPS as one of the

virulence properties of Shigella spp. and suggest that the chemical structure of the O-repeat sugar unit is one factor in Shigella sp-host cell interactions.

The role of smooth LPS in the pathogenesis of Shigella sp infection has been further studied by Okamura et al (6). These workers noted that some rough mutants derived from a virulent smooth S. flexneri 2a strain can invade Hela cell but were incapable of causing keratoconjunctivitis but when O-antigen genes from a smooth avirulent S. flexneri strain were transferred to the rough mutant, the transconjugants regained the ability to cause keratoconjunctivitis.

A number of genes appear to encode properties of epithelial cell invasiveness, ability to proliferate within the epithelial cells, and the capacity to cause keratoconjunctivitis in guinea pig's eye. Formal et al (7) identified one locus near the purine E region of the Shigella sp. chromosome that represents a gene locus associated with the ability to provoke keratoconjunctivitis in the a guinea pig eye. Virulent S. flexneri also possess large 120 to 140 Mdal plasmids that, according to Sansonetti and co-workers (8), contain genetic materials relating to invasiveness for epithelial cells. Loss of this plasmid results in avirulence. Genetic transfer of the 140 Mdal plasmid back into avirulent, plasmid free strains resulted in reacquisition of epithelial cell invasiveness by the recipient strains. Silva et al (9) have documented the presence of such large plasmids in all species of Shigella and have shown that only strains possessing the large plasmid can evoke keratoconjunctivitis in the guinea pig eye test. Recently a small 6 Mdal plasmid has been found to be involved in 'O' antigen production in S. dysenteriae I and to be essential for virulence (10).

Hale et al (11) has shown that the 140 Mdal plasmid in S. flexneri encodes the production of certain outer membrane proteins. It appears that these proteins are involved in the process by which Shigella organisms invade epithelial cells. Shigella minicells which harbour the 140 Mdal plasmid were found to be able to invade Hela cells, whereas syngenetic plasmid-free mini cells could not. Hale et al (11) have hypothesized that the OMPs encoded by the virulence plasmid represent bacterial adhesins which bind to some as yet undetermined receptors on the surface of host enterocytes. Expression of these peptidis is thermo-regulated, as is expression of the invasive phenotype itself (12).

Some years ago Formal et al (13) and Falkow et al (14) noted that genes in the Xylose-rhamnose region of the Shigella sp. chromosome appear to be involved with the ability of Shigella organisms to be involved with the ability of Shigella organisms to proliferate within epithelial cells after cell invasion. Recently, Sansonetti et al (15) were able, by the stepwise conjugal transfer of a large plasmid and three chromosomal segments from S. flexneri 2a to E. coli K-12 to construct a strain possessing the prerequisites for expression of full virulence. Recently Maurelli et al (16) have shown that a 37 Kilobase gene sequence in a large plasmid Shigella sp. is the minimum sequence necessary for invasion of Hela cells.

New knowledge of the pathogenic step involved in invasion of epithelial cells by Shigella strain and of the role of large plasmids has been applied to the development of experimental vaccines Adamur et al (17) for example, immunized guinea pigs and rabbits subcutaneously with OMP of Shigella flexneri 3a and S. sonnei phase I. Immunized

animals were challenged by inoculation into the conjunctival sac of 5×10^8 to 1×10^9 pathogenic organisms but were completely protected from keratoconjunctivitis from a challenge with S. flexneri 3a. These priliminary results in animal models suggest that the OMPs of pathogenic Shigella strains may indeed be important immunogens to be found in vaccines.

3. Rationale

We have obtained 6 strains of S. dysenteriae 1 with altered plasmid make-up, by using different chemical and physical treatments. These strains thus appear to be suitable for a preliminary study of the possible correlation between the plasmid profile, invasiveness and the antigenicity of outermembrane components.

B. SPECIFIC AIMS

1. To study the involvement of the 140 and 6 Mdal plasmid in invasiveness by using mutant strains, which are negative in the Sereny test.

2. To compare the plasmid profile, the outer membrane protein profile and antigenicity of outermembrane components of the study strains.

3. To see whether loss of plasmid(s) correlates with the change of 'O' antigen pattern.

C. MATERIALS AND METHODS

1. Bacterial strains: Total 6 mutants and two wild strains of *S. dysenteriae* I strain will be studied.

2. Immunization : Antisera will be raised against the whole cells as well as outer membranes of the individual mutants and their parent strains.

3. Biochemical and immunological tests

   a. Double diffusion technique (Ouchterlony, 1964) will be used for detection an semi-quantitative estimation of antigen(18).

   b. LPS will be extracted by phenol water procedure (Westphal, Jann, 1965) (19).

   c. Outer membrane protein will be isolated by the method described by Johnston K.H. and Gotschlich E.C. (1974). (20).

   d. SDS-PAGE analysis of outer membrane protein will be carried out by standard methods of Laemmili (1970) (21).

   e. Cross immuno electrophoresis will be performed to analyse the antigenic pattern of the organism by the method described by Kroll, J. (22).

   f. Western blotting will be performed according to the methods described by H. Towbin (23).

4. Tests for invasiveness : The invasiveness will be assayed by both Sereny test (24) and by the infection of cultured HeLa cells (25).

D. SIGNIFICANCE

It is not fully understood what is the role of the outermembrane proteins and LPS of Shigella dysenteriae type 1 strains in the pathogenicity of the organism and the hosts immune response against it. Some of these outermembrane components in Shigella generally are mediated directly or indirectly by plasmid(s). In the case of S. dysenteriae type 1 the situation appears to be similar.

It has been shown that the invasive ability of strains of Shigella may be mediated by plasmids of size ranging from 6 to 140 Mdal.

This protocol will enable us to identify the plasmid dependent immunogenic OM components. Some of these components might help in the invasion process and some in the protection of the host against the organisms.

Identification of such plasmid(s) will open up the possibility of developing a vaccine for Shigella by cloning the protective functions in a suitable carrier bacterium, which would be the continuation of this work.

This protocol will help us to study the relation of plasmids of S. dysenteriae 1 to antigenic composition of the outermembrane components.

E. FACILITIES REQUIRED

ICDDR,B Genetics Laboratory, Immunology laboratory and the Animal House facilities will be shared.

F. COLLABORATIVE ARRANGEMENTS - None

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Escherichia coli K-12 after transfer of plasmid and chromosomal genes from Shigella flexneri. Infect. Immun. 39:1392-1402.

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ICDDRB BUDGET PROPOSAL--1986

Program Name: HDWG

Project/Protocol/Branch: ANTIGENS OF SHIGELLA OMP

Principle Investigator: K. HAIDER

Budget Code:

Protocol No:

SUMMARY BUDGET

0 Local Salary	US$	5320
International Salary		0
Consultants		0
Travel Local		0
Travel International		2500
Supplies		5070
Other costs		700
Inter-departmental		2200
Total Direct Operating		15790
Capital Expenditure		2000
TOTAL DIRECT COST	US$	17790

PERSONNEL REQUIREMENT (Local)

	No/Pos	Man mon	Amount
A Staff	3	16.2	4000
B Recrt	1	12	1320
TOTAL	4	28.2	5320

LOCAL STAFF ON 1.1.86

Job	No	Man mo	$/mo	Amount
P.I.	1	6	300	1800
RES OFFICER	1	4	220	880
RES OFFICER	1	6	220	1320
TOTAL	3	16.2		4000

NEW RECRUITS

Job	No	Man mo	$/mo	Amount
LAB ATTENDANT	1	12	110	1320
TOTAL	1	12		1320

TRAVEL PLANS--INTERNATIONAL

(calculate on form and enter total)

TOTAL: 2500

SUPPLIES AND MATERIALS

A/C	ITEMS	AMOUNT
3701	Drugs	
3702	Glassware	500
3703	Hosp suppl	
3704	Stationary	250
3705	Chem, media	
3706	Uniform	50
3707	Fuel	
3708	Lab suppl	1000
3709	Housekeep	50
3710	Janitorial	50
3711	Tool&spares	
3712	Non stock	2000
	SUBTOTAL	3900
3713	FREIGHT	1170
	TOTAL	5070

OTHER COSTS

A/C	ITEMS	Amount
3800	Maintenance	50
3900	Rent,comm,util	50
4100	Bank chrges	
4200	Legal	
4300	Print, pub	450
4400	Entertainment	50
4500	Service chrges	100
4600	Staff dev	
	TOTAL	700

INTERDEPARTMENTAL SERVICES

A/C	ITEMS	Amount
4801	Computer	
4802	Trans, Dhaka	
4803	Trans,Matlab	
4804	Water Trans	
4805	Trans,Teknaf	
4806	Xerox	100
4807	Pathology	
4808	Microbiology	
4809	Biochemistry	
4810	X-ray	
4811	I.V.	
4812	Media	1000
4813	Patient hosp	
4814	Animal	600
4815	Med Illust	500
4817	Telex	
4818	Outpt care	
4830	Trans sub	
	TOTAL	2200

CAPITAL EXPENSES

Item	Amount
TRANSBLOT	1000