29

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date Dec 18, 1985

19.12.85

Principal Investigator Zia U. Ahmed

Trainee Investigator (if any) _____

Application No. 85-040

Supporting Agency (if Non-ICDDR,B) WHO

New study

Title: Isolation of attenuated strains of Shigelle dysenteriae 1 susceptible to bacteriolysis as a consequence of induced genetic block and evaluation of their protective potential in a rabbit model.

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.

This protocol does not involve human subjects for experimentation at any stage. Hence, most questions do not apply.

Zia U. Ahmed (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.

Zia U. Ahmed

Principal Investigator

Trainee

85-040
19.12.85

SECTION I - RESEARCH PROTOCOL

1. TITLE : Isolation of attenuated strains of Shigella dysenteriae 1 susceptible to bacteriolysis as a consequence of induced genetic block and evaluation of their protective potential in a rabbit model.

2. PRINCIPAL INVESTIGATOR : Zia Uddin Ahmed, Ph. D.

Co-invetigators : Mahfuzur Rahman Sarker and three other workers to be recruited later.

Consultant : David A. Sack, M.D.

3. STARTING DATE : July 1986

4. COMPLETIOIN DATE : June 1989

5. TOTAL DIRECT COST : US$75,254

6. SCIENTIFIC PROGRAM HEAD :

This protocol has been approved by the Disease Transmission Working Group.

David A. Sack

PROGRAMME HEAD

Dec 1, 1985
Date

7. ABSTRACT SUMMARY

Recently considerable interest and optimism have grown over the prospects of immunological control of shigellosis by using live oral vaccines consisting of attenuated mutant strains of the pathogen. Various methods of genetic attenuation are being tested with promising results which, together with the enhanced knowledge on some key aspects of the pathobiology of shigella, would make it possible to adopt an attenuation strategy that may be both safe and effective. For an invasive pathogen such as shigella, of which neither the details of the pre-invasion biology nor the role of a toxin in the disease is clear, the following strategy is considered worthwhile. That is, isolating strains that would invade but would destroy themselves soon after invasion. Such a "suicidal" consequence can be introduced into a strain by genetic blocks in metabolic pathways.

The protocol aims at isolating strains of Shigella dysenteriae 1 with two independent suicidal blocks so that the reversion frequency will be at a low level. One of these blocks will be in the galactose utilization pathway leading to galactose-induced bacteriolysis. Diaminopimelic acid (DAP) auxotrophy would be the second block. Since the distributioin of DAP is restricted to the cell-wall, any impairment in its biosynthesis would lead to a weak cell-wall. As a result, cell growth will trigger cell lysis.

The attenuated mutant strains would then be tested in an adult rabbit model for their colonizing and protective potential.

SECTIOIN II - RESEARCH PLAN

A. INTRODUCTIOIN

1. Objective

Our overall aim is to develop mutant strains of Shigella dysenteriae 1 which when taken orally will induce a local immune response but which will not cause disease nor will revert to become virulent. These S. dysenteriae 1 strains would constitute a group of candidate vaccines for further testing.

2. Background

(a) The pathobiology of shigella and genetics of invasiveness:

Shigellosis is a prototype of invasive diarrhoea. The pathogenic pathway begins with the ingestion of a small number of virulent organisms. The disease appears to have two phases - the watery phase and the dysenteric phase. The former may involve some kind of preinvasion colonization (such as transient mucosal adherence) and production of toxin. The dysenteric phase follows invasion and intracellular multiplication.

The mechanism of pre-invasion colonization is not well understood. Among the four strains of shigella that were examined many years back by Duguid and Gillies (1956),

only S. flexneri had adhesive surface appendages (fimbriae). Adherence of the non-fimbriate strains may thus be mediated by O-antigens and/or host factors. The major event triggering the dysenteric phase of the disease is invasion.

The role of toxin in shigella pathogenesis is controversial. Strains of shigella manifest three toxin activities: neurotoxic activity in mice, cytotoxicity in cultured animal cells and enterotoxicity in rabbit ileal loop. Recent evidence suggests that a single peptide-toxin is responsible for all the three activities (Eiklid and Olsnes, 1983). The facts that non-toxigenic (later proved to be actually hypotoxigenic) strains produce disease manifestations similar to the toxigenic strains and that substantial quantities of toxin can be detected in patient stool by using a highly sensitive ELISA method (M. Bennish, personal communication) point to the necessity of isolating true toxin - negative mutants for further studies.

Among the virulence factors, invasiveness has received relatively more attention and the genetic determination of this trait is better understood. A large 140 Mdal plasmid is present in nearly all isolates of shigella. Its role in the production of form I cell surface antigen has been studied. Strains of S. sonnei that apparently had lost the plasmid became non-virulent and

also lost the ability to produce characteristic antigen (Sansonetti et al., 1981). A large plasmid is also implicated in the invasiveness of S. flexneri (Sansonetti et al., 1982). Evidence to the contrary is also known. For example, nalidixic acid resistant mutants of S. flexneri 6 carrying the 140 Mdal plasmid were non-invasive in the Sereny test (D.A. Sack, personal communication).

In S. dysenteriae 1 there are several plasmids including the large 140 Mdal plasmid. Here again, its apparent loss correlates with non-invasiveness (K. Haider, personal communication).

(b) Past shigella vaccines:

Orally delivered vaccines are at present considered to be the most promising immunizing agents against intestinal infections. The vaccines could be nonliving antigens, attenuated strains of the pathogen or harmless carrier strains expressing protective antigens of the pathogen. Being an invasive organism, shigella presents problems when applied to live vaccine development. Thorne and Gorbach (1977) highlighted some of these problems. A good colonizer which, in the case of shigella means a good invader, is likely to be a good vaccinator (Dr. Jekyll). But an invader has a greater potential to revert to virulence (Mr. Hyde).

In shigella three approaches based on conventional genetics were adopted in the past to develop a live vaccine.

(i) Strains of shigella that became, through a single step mutation, streptomycin dependent and were unable to proliferate indefinitely in the intestine were used as live oral vaccines. Also used were avirulent colonial variants. These so-called "first generation" vaccines had poor growth in the intestine and required very large inoculum and multiple doses. Some strains also underwent genetic reversion

(ii) The second generation of vaccine strains are the mutant-hybrid (MH) strains in which avirulent shigella mutants received, through genetic hybridization, the xylose-rhamnose region of E. coli K12 chromosome. The presence of the xyl-rha region in the shigella chromosome made the strain unable to maintain itself in the intestine and was thus considered safe. The Shigella flexneri 2a MH strain, although safe, failed to multiply in the intestine. These MH strains thus also failed to produce a successful vaccine.

For references to the original work on these two categories of tested vaccines see Levine et al. (1977).

(iii) The third generation of vaccine consisted of a hybrid E. coli strain in which Shigella flexneri 2a surface antigenic determinants we transferred via

conjugation (Levine et al., 1977). This vaccine was also unsuccessful. Recently, the plasmid-borne Shigella sonnei form I surface antigen determinant was transferred to the typhoid vaccine strain Salmonella typhi Ty21a. This strain protected mice against Shigella sonnei challenge (Formal et al., 1981) when administered through intraperitoneal or subcutaneous routes. It is not known whether the strain is effective in human intestine after oral administration.

The strain, however, is very safe; an inoculum of 1010 viable cells produced no untoward effects in human volunteers. This candidate oral vacccine may be scheduled for a field trial soon (D.J. Kopecko, personal communication).

(c) Current activities:

Current avtivities in shigella vaccine development generally involve the following approaches:

(i) The search for suitable carrier strains that could contain and express shigella antigens has continued. Recently, scientists working at the Walter Reed Army Institute of Research have successfully transferred S. flexneri 2a O-antigen genes into Salmonella typhi Ty21a and also isolated a strain of E. coli K12 which carries the S. flexneri invasive plasmid and the O-antigenic determinants of S. sonnei, S. flexneri 2a, S. flexneri 3

and S. dysenteriae 1 (D.J. Kopecko, personal communication). These strains appear promising but these are yet to be tested in humans.

One possible problem with carrier strains such as Salmonella typhi or E. coli K12 pertains to the question of colonization of the intestine by these carriers. It is clear that for triggering local immunity a live vaccine must be able to colonize the gut, an attribute which these carrier strains perhaps do not possess. Selection of strains with the ability to colonize the gut efficiently is thus an area that merits active investigation. Successful vaccine of this category will probably depend on this important contingency.

(ii) One way to obviate the necessity of finding a good colonizer (to carry antigens of the pathogen) is to capitalize on the invasiveness of the pathogen itself. That is to say, the pathogen should be modified such that its invasive ability is retained but it looses its virulence. There are very few choices left for making an invasive strain avirulent after it has been allowed to invade the mucosa. The most promising and logical approach is to see that the strain looses the capacity to multiply within the intestine and is consequently cleared rapidly, allowing only enough time to trigger local immunity.

Therefore, one currently favoured approach is to isolate what may be called "suicidal" mutants of the pathogen. An example of such a strain is Salmonella typhi Ty21a. The strain has a block in the galactose utilization pathway which causes the accumulation of toxic amounts of galactose-1-phosphate and uridine-diphosphate-galactose resulting in cell lysis (Germainer and Furer, 1975). In a like manner, other "suicidal blocks" can be introduced to ensure a low reversion rate and enhance safety margin.

(iii) Recently, interest has generated in cloning protective shigella genes. It involves the identification of the protective shigella antigens and cloning the corresponding genes in a safe carrier bacterium. In cloning shigella antigenic determinants the choice of the carrier strain may again present problems. However, these efforts promise to lead shigella vaccine development activities to more defined levels - -to those of single peptide vaccines and synthetic peptide vaccines.

3. Rationale:

We have selected S. dysenteriae 1 for a live vaccine strain because the serotype is unique in its virulence, epidemic potential and antibiotic resistance. We believe that development of attenuated vaccine strain is likely to be successful even though past attempts have not resulted in a practical vaccine. This is because of enhanced knowledge of

some of the key events in the pathobiology of shigella and on the genetics of virulence. It will be considerably aided by an animal model being developed at present. We also believe that attenuated shigella is a worthwhile choice for a live vaccine rather than E. coli or Salmonella carry antigenic determinants of shigella. The reason for this is that the nature of colonization of shigella in its intestinal ecological niche is likely to be related to the development of local immune response and this attribute may not be readily offered by a carrier strain.

Isolation of "suicidal' mutants in shigella has not been reported, to the best of our knowledge. However, attempts are being made to obtain by recombinant DNA methods mutants in shigella with deletion in the galactose operon (D. Kopecko, personal communication). Such deletion mutants would be quite stable. But our proposal of double mutants with two independent blocks may also make the strain quite safe.

B. SPECIFIC AIM:

(a) To isolate strains of Shigella dysenteriae 1 with two "suicidal" blocks -- one in the galactose utilization pathway and the other in the biosynthesis of diaminopimelic acid.

(b) Testing an adult rabbit animal model the strains' ability to colonize the gut and induce protection .

C. MATERIALS AND METHODS:

(a) Plasmid-mediated invasiveness:

There is strong evidence that a large plasmid (over 100 Mdal) carries determinants of invasiveness in the shigellae. We wish to examine whether the 140 Mdal plasmid detected in S. dysenteriae 1 determines invasiveness. Plasmidless strains will be isolated by subjecting cultures to curing conditions and isolates will be screened for plasmid loss. A high degree of correlation between plasmid loss and loss of invasiveness will be taken as evidence that the plasmid determines invasiveness. This information is necessary because after a strain has been crippled there will remain no easy way to monitor its invasive ability in experimental systems. Presence of this plasmid may thus indicate that the strain is potentially invasive.

(b) Isolation of "suicidal" mutants:

Galactose-sensitive mutants:

Attempts will be made to isolate mutants sensitive to galactose-induced bacteriolysis. The experiment will be generally modelled after that of Salmonella typhi Ty21a. Cultures will be mutagenized (UV light and MNNG), subjected to pencillin enrichment and screened for colonies on galactose-containing plate showing evidence of lysis. Prospective isolates will be purified by repeated transfer or single-colony isolation and will be studied in shaken cultures with

respect to growth, toxin production, nature and extent of bacteriolysis induced by galactose and influence of media and other sugars on cell lysis. Routine tests will be performed to study the frequency of reversion to galactose resistance and the nature of these resistance clones will be studied (levels of resistance, frequency etc.).

DAP auxotroph:

Diaminopimelic acid (DAP) is a cell wall constituent found in some bacteria. Its occurrence in strains of shigella has not been, to the best of our knowledge, reported. We wish to examine if shigella cell wall is substsantially enriched with DAP. The method we propose to follow is that of Fukasawa and Nikaido (1961).

We will assume for the present that shigella cell-wall contains DAP and proceed on to isolate mutants blocked in DAP synthesis. Cultures that will be used for this will be the stable galactose sensitive derivatives. These will be mutagenized and selected for DAP requirement. DAP requiring isolates will be grown in liquid culture to study cultural characteristics and physiology of lysis. These studies will be closely modelled after those reported for E. coli (Davis, 1952; Rhuland, 1957; Meadow et al., 1957).

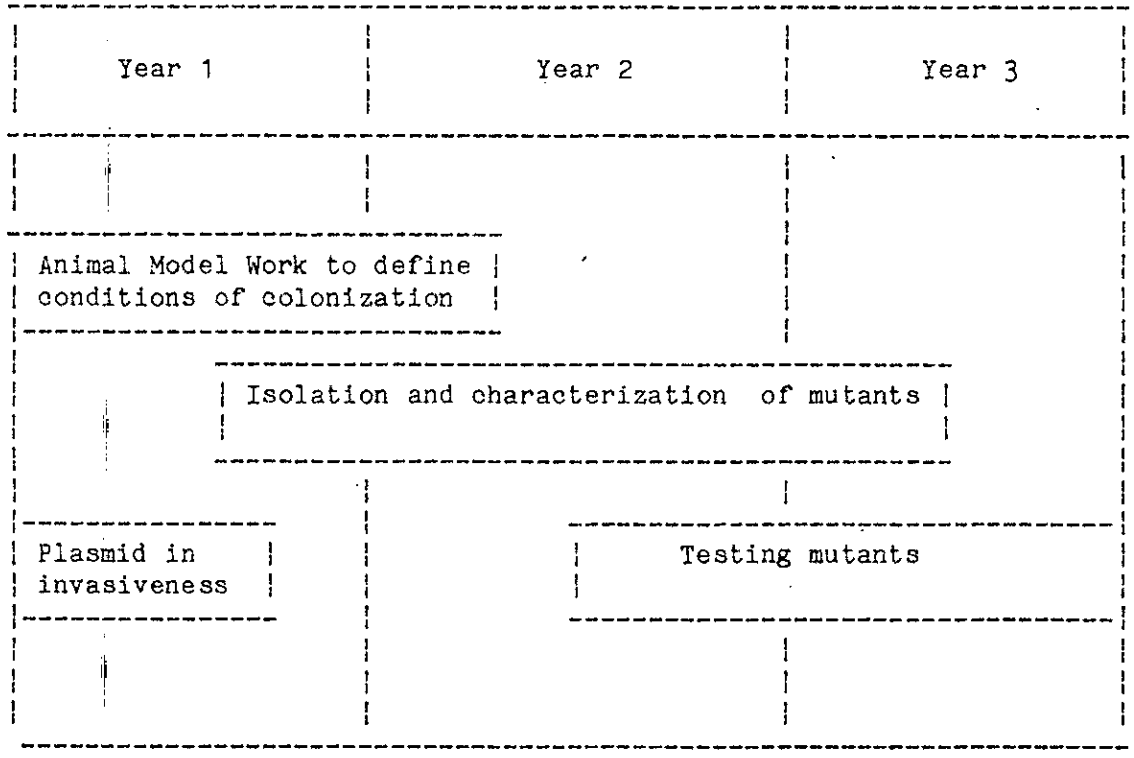
(c) Test of virulence and protection:

The mutant strains will be examined with respect to invasiveness in the Sereny test and virulence in an animal

model. A suitable animal model to test virulence and protective potential of strains would considerably aid the mutant isolation program. An adult rabbit model has been developed (Cray, et al. 1983) and adapted to Shigella flexneri 6 (D.A. Sack, private communication). Conditioned rabbits have been successfully colonized with this strain. This model will be further developed and adapted to S. dysenteriae type 1 to study colonization, virulence and protective ability of strains.

D. TIME SCALE:

The anticipated time scale for the work we propose to do is as follows:



11. REFERENCES:

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Levine, M.M., Woodward, W.E., Formal S.B., Gemski, Jr. P., DuPont,H.L., Hornick, R.B. and Snyder, M., 1977. Studies with a new generation of oral attenuated Shigella vaccine: Escherichia coli bearing surface antigens of Shigella flexneri. J. Infect. Dis., 136, 577-582.

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Thorne, G.M. and Gorbach, S.L., 1977. Shigella vaccines, shigella pathogens - Dr. Jekyll and Mr. Hyde. J. Infect. Dis. 136, 601-604.

SECTION III - BUDGET

YEAR 1
(July '86 - June '87)

1. PERSONNEL SERVICES

	Name	Position, % effort		Cost, US $
i)	Zia Uddin Ahmed	P.I., 50%	...	2,351
ii)	David A. Sack	Consultant	...	-
iii)	M.R. Sarker	Research Trainee	...	1,200
iv)		Pathologist 25%	...	1,200
v)	To be recruited	Research Officer, Level 5, 100%	...	1,703
vi)		Senior Technician Level 4, 100%	...	1,252
vii)		Lab attendant, Level 1, 100%	...	762
				8,468

2. EQUIPMENT

a)	Laminar Flow Hood, Vertical Air-flow, complete including air-freight	...	12,000
b)	Fume hood, 60", complete including air-freight	...	5,200

3. MATERIALS AND SUPPLIES

	Media, Chemicals, Biochemicals, Disposable plastic supplies, Glassware	... 5,000

4. XEROX, PRINTING AND PUBLICATION ... 1,000

Year 1 Total 31,668

BUDGET

YEAR 2
(July '87-June '88)

		Cost, US $
1. PERSONNEL SERVICES		
Same as year 1 + 20% increase	....	8,721
2. EQUIPMENT		
Spectronic 20 Spectrophotometer with freight	....	1,300
3. MATERIALS AND SUPPLIES		
Media, Chemicals and reagents, Glassware, Disposable plasticware	....	5,000
Animals (Rabbit and Guinea pig)	....	3,000
4. Building a darkroom for photomicrography fluorescent microscopy.	....	6,000
5. Xerox etc.	....	1,000
		25,021

BUDGET

YEAR 3
(July '88 - June '89)

1. PERSONNEL SERVICES		Cost US $
Same as year 2 + 20% increase	...	10.000
2. EQUIPMENT	...	-
3. MATERIALS AND SUPPLIES		
Media, reagents, disposable plasticware	...	5,000
Animals	...	3,000
4. Xerox Etc.	...	1,500
		19,965

COST

Direct cost year 1 - 3	...	75,254
Indirect cost (31%)	...	23,328
TOTAL COST		99,682