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Attachment 1.

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 21/5/87

Principal Investigator DR. ZIA U. AHMED Trainee Investigator (if any) — 9

Application No. 87-011 Supporting Agency (if Non ICDDR,B) Shigella proj (US AID fund)

Title of Study ISOLATION OF ATTENUATED MUTANTS OF SHIGELLA AND EVALUATION OF THEIR ~~SAFETY AND THEIR ABILITY TO~~ STIMULATE IMMUNE PROTECTION IN RABBITS ~~AND MONKEYS~~

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). N/A

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: NO
   (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: N/A
   (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 N/A
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.

No human subjects are involved in the study.

(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

MAY 25 1987

Trainee

A PROTOCOL UNDER THE SHIGELLA PROJECT

TITLE: ISOLATION OF ATTENUATED MUTANTS OF SHIGELLA AND EVALUATION OF THEIR SAFETY AND THEIR ABILITY TO STIMULATE IMMUNE PROTECTION IN RABBITS AND MONKEYS

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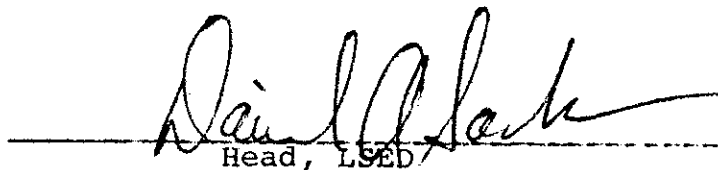
SECTION I - RESEARCH PROTOCOL

1.	TITLE	: Isolation of attenuated mutants of shigella and evaluation of their safety and their ability to stimulate immune protection in rabbits and monkeys.
2.	PRINCIPAL INVESTIGATOR	: Dr. Zia Uddin Ahmed (100%)
	CO-INVESTIGATOR	: Mr. Mahfuz R. Sarker (100%) Mrs. K. Haider (50%) Dr. Khorshed A. Chowdhury (25%)
3.	CONSULTANT	: Dr. David A. Sack Dr. Ivan Ciznar
4.	STARTING DATE	: July 01, 1987
5.	COMPLETION DATE	: June 30, 1990
6.	TOTAL DIRECT COST	: US$222,942.00
7.	SCIENTIFIC DIVISION HEAD	: Dr. David A. Sack

This protocol has been approved by Epidemiology and Laboratory Sciences Division.



Head, LSED

Date: May 17, 1987

8. ABSTRACT SUMMARY:

The major objective of the study is to isolate a set of attenuated mutants of Shigella. Attenuations that will be attempted include:

i) mutation that would make the strain unable to grow at 37° - 39° C;

ii) mutation in the galactose epimerase (galE) gene and in the pathway leading to diaminopimelic acid synthesis, both mutations with a "suicidal" consequence in that cell lysis occurs in the presence of galactose and in the absence diaminopimelic acid respectively;

iii) introduction of mutation that would make the strain dependent on the supply of high levels of exogenous thymine.

Stable strains, which will be at least a double mutant for two independent blocks, will be tested for stability, that is, non-revertibility when a culture is grown under restrictive conditions.

These strains will be tested in a rabbit infection model and in monkeys for:

a) safety - that is, whether a heavy inoculum of 10^{11} cfu should causes disease or histopathological lesions;

b) protective potential - that is, whether the strain is able to protect the animal from disease and death after a virulent challenge.

c) immunogenecity- that is, whether the strain produces antibody against major antigenic components in amounts comparable to that produced by its wild type parent strain.

SECTION II -- RESEARCH PLAN

A. INTRODUCTION:

1. Objective:

Objective of the study is to isolate a set of stable attenuated mutants of *Shigella* and to test their safety, immunogenecity and protective potential in an adult rabbit colonization model. These strains would constitute a group of candidate vaccines for further testing.

2. Background:

(a) Types of attenuated mutants being considered in this study.

Considerable interest has developed in recent years in developing live oral vaccines against enteric diseases by using genetically attenuated strains. Various methods of attenuation are being discussed. The choice would depend on the pathobiology of the organism. For an invasive pathogen like *Shigella* of which neither the pre-invasion biology is well understood nor the involvement of a toxin in disease development is clear, one approach merits consideration. That is, allowing the strain to retain its invasive potential but making it so crippled that it would fail to proliferate and maintain itself for very long. Such crippling could be brought about by introducing blocks in certain metabolic pathways resulting in a "suicidal" consequence and/or making the strain sensitive to growth at the temperature of the body.

Temperature-sensitivity:

Temperature sensitivity can provide effective attenuation. Strains can be isolated that are unable to grow at the body temperature (non-permissive temperature) but can be grown in the laboratory at a lower temperature (permissive temperature). There are many advantages of temperature-sensitive mutations. For example, nutritional conditions are not likely to have any effect on the expression of temperature sensitivity and surface antigens are likely to remain unaltered. Simple manipulations involving introduction in a particular strain of *Shigella* of a point mutation by mutagenesis followed by conjugational transfer of a second temperature-sensitive mutation from *E. coli* can generate a stable double mutant with a low reversion frequency.

Mutants susceptible to lysis:

Two types of mutants are known that can be put into category. One is exemplified by mutation in the galactose epimerase gene that predisposes the cell to undergo lysis when allowed to grow in the presence of galactose. An example is the *Salmonella typhi* strain Ty21a (Germanier and Furer, 1975). The *gal*E mutant has a block in (UDP)-galactose-4-epimerase and cannot synthesize (when grown in the presence of glucose, but not galactose) UDP-galactose, a precursor for the incorporation of galactose into the LPS (Germainer and Furer, 1971). The mutant is thus able to synthesize only an incomplete LPS (rough) in the absence of galactose but when grown in the presence of galactose, smooth immunologically active LPS is synthesized. In such a strain, attenuation is provided by the pronounced bacterial lysis which occurs when the culture is grown in the presence of galactose because of the accumulation of toxic levels of the phosphorylated

sugar, uridine-diphosphate-galactose (UDP). In *Shigella flexneri* the O-antigen does not contain galactose but the core structure contains one galactose moiety (Carlin *et al.* 1984). In *Shigella dysenteriae* 1 the third sugar of the O-antigen is galactose (Dmitriev *et al.* 1976). Theoretically, therefore, it should be possible in both the species to isolate *gal*E mutants. These will be rough if grown in the absence of galactose but will be both smooth and suicidal when grown in the presence of galactose, similar to what happens in *Salmonella typhi* Ty21a. Whether or not a mutation causing a defect in the core LPS structure (as it may be the case in *S. flexneri*) will be lethal and hence cannot be isolated, is a matter that cannot be predicted.

The other suicidal mutation is a mutation in the pathway that leads to the synthesis of diaminopimelic acid (DAP) in *E. coli* (Davis, 1952; Rhuland, 1957; Meadow *et al.*, 1957). Diaminopimelic acid is localized exclusively in the cell wall of many gram negative bacteria including *Shigella* (Work and Dewey, 1953). A mutant unable to synthesis DAP (DAP^- mutant) makes a weak cell wall in the absence of DAP and as a consequence suffers cell lysis.

Mutants dependent on high levels of thymine:

In *E. coli*, mutation in the *thy*A gene makes the cell dependent on exogenous supply of thymine (Miller, 1972).

Many *thy*A mutants have been isolated that require high levels of thymine for growth. These high thymine requiring mutants are likely to suffer a severe thymine starvation in the intestine.

The above three categories of mutants could thus provide

significant genetic attenuation and could also be safe for humans because conditions in the human intestine would limit their proliferative potential. Temperature-sensitive mutants would not grow in the non-permissive temperature, *galE* and *dap* mutants will lyse because of the abundance of galactose and lack of DAP in the intestine. Thymine-dependent mutants will likewise have limited proliferative ability because of thymine deprivation. Stability of an attenuated mutant can be substantially enhanced by isolating a double or tripple mutant carrying two or more such attenuating mutations.

(b) An infection model for testing safety and immunogenecity of strains.

To assess safety and immunogenecity of strains it is necessary to have an experimental infection model. An adult rabbit infection model has been used successfully with *Shigella flexneri* 6 (David A. Sack, private communication). Tetracycline-fed rabbits were given intravenous cimetidine prior to orgastric administration of bacterial inoculum to produce successful colonization and death of the animal. The infection model has been adopted by us for use with *Shigella dysenteriae* 1, *Shigella flexneri*, *Shigella sonnei* and *Plesiomonas shigelloides* (see next section for some results).

(c) Results of some studies obtained to-date:

(i) Attempt to transfer the *galE* gene of *Salmonella* (carrying an internal deletion), into *Shigella*.

The *galE* gene of *Salmonella* carrying an internal deletion of about 0.4 kb has been cloned by Professor Rowley's group at Adelaide into plasmid pADE161. An attempt was made to transfer the

galE from pADE161 to *Shigella* chromosome. Plasmid pADE161 which carries spectinomycin resistance was transformed into *Shigella*. In a transformant, the *galE* gene could be expected to replace the wild-type *galE* gene in the chromosome at a certain frequency by homologous combination. Therefore, from a culture which has been subjected to plasmid carrying condition, one can expect to recover galactose-sensitive cells resulting from interaction of *galE* gene into the chromosome and the loss of the wild-type *galE* gene from the cell along with the lost plasmid.

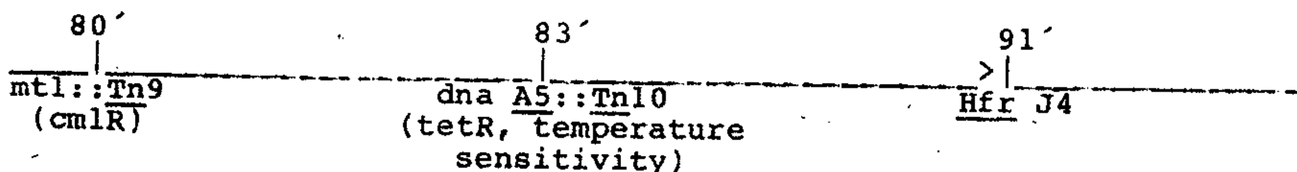
Transformant *Shigella flexneri* (strain PR-1) and *Shigella dysenteriae* 1 (ES-2) were plated on rich medium and replica plated onto plate containing galactose. About 34,000 colonies of the former and 16,000 colonies of the latter were tested. Of the 3 colonies of PR-1 that failed to replicate on galactose containing plate, 2 of them failed to grow and one reverted to wild-type after 3-4 transfers of single colonies on nutrient agar. Similarly, the colony of strain ES-2 that failed to replicate on galactose medium was lost after 3 transfers.

An alternative to such laborious screening is the use of rough and smooth specific phages. Integration of *galE* into the chromosome is likely to produce a rough LPS. When lysate of a smooth specific phage is mixed with the bacterial culture and plated, rough cells will escape lysis and form a colony which could be tested for galactose sensitivity. Obviously, phage selection is extremely powerful. Unfortunately, none of the strains of the four *Shigella* species that we tested were sensitive to smooth specific phage U3 and a range of rough specific phages such as Fo, Fpl, C21, 65R and Br10.

Failure to isolate stable galactose-sensitive strains might be due to restriction barrier and poor recombination between the heterologous Shigella and Salmonella DNA.

(ii) Isolation of temperature-sensitive mutants. Two methods have been successfully used for the isolation of a number of temperature-sensitive mutants in strains of Shigella dysenteriae and Shigella flexneri. The first one involved UV mutagenesis, ampicillin enrichment and selection. The second one involved the conjugational transfer of the dnaA5 temperature-sensitive gene from E. coli to Shigella.

For conjugational transfer of the E. coli temperature-sensitive mutation, the following strain construction was carried out. Total DNA from E. coli strain E528 (dnaA5 Zi::Tn10, F$^+$, polA1 endA1 str^R) was prepared and used to transform E. coli strain RM172 (Hfr J4, mtl::Tn9). Tetracycline resistant colonies were purified and tested for temperature sensitivity. In this manner, strain PR-E3 was isolated. It is resistant to chloramphenicol and tetracycline and is temperature-sensitive. It´s presumed genetic structure is:



This strain is expected to transfer the 83´ region early and would allow the selection of Tn9 or Tn10 in the exconjugants. The exconjugants could then be tested for temperature sensitivity.

Table 1 shows origin and characteristics of some temperature-sensitive

mutants of *Shigella dysenteriae* and *Shigella flexneri* obtained by mutagenesis and by the conjugational transfer of temperature sensitive genes from *E. coli*.

Table 1

Some temperature-sensitive mutants of *Shigella*.

Strain	Method of isolation	Number screened	Temperature-sensitivity and other characters
S. dysenteriae 1 strain TS-9	EMS mutagenesis	5000 colonies of an enriched culture	Grows slowly at 30 °C on agar, no growth at 41 C. Restricted growth at 39 C.
S. flexneri strain TSF-26	UV (254nm)	"	Grows well at 30 °C in plate and broth but not at 41 C. Restricted growth at 39 C.
S. dysenteriae 2 strains TSD 1-10 (10 independent isolates)	Product of conjugation with *E. coli* strain PR-E3	About 300 selected exconjugants tested	Grow well at 30 °C but not at 41 C. Possibly carries dna A5::*Tn10* and mtl::*Tn9*.

(iii) Results on rabbit infection model studies:

Conditioned rabbits were challenged with bacterial inoculum. The animals were observed for one week for disease development and death. Some results are shown in Table 2.

Some studies on protection in rabbits were carried out using a strain of *Shigella sonnei*. The inoculum corresponding to LD 50/7 days was estimated to be 10^{10} cfu. A group of 5 rabbits were immunized by giving two doses of 10^{9} cfu each at a week interval. The animals were challenged 7 days after the second immunizing dose with the same strain and disease or death was noted. Results are shown in Table 3.

As seen in Table 2, none of the strains of *S. dysenteriae* 1 tested produced illness or death whereas *S. flexneri* and *S. Sonnei* strains proved relatively virulent in this animal model. To test whether *S. dysenteriae* 1 does nevertheless cause an inflammatory response in the gut, a histopathological examination of the ileum was carried out. Four of the five rabbits in the high dose group (10^{10} cells per animal) were characterized by mild to severe infiltration of PMNs in the Lamina propria near the tip of villi, superficial erosion and vacuolization of luminal epitheliulm of the tip of villi with occasional purulent exudate in the lumen. Payer patches were moderately reactive and infiltrated with PMNs and there was edema in the lamina propria. These lesions indicated an inflammatoiry reaction. Control animals and those that had received lower doses (10^{8} and 10^{9} cells) did not show this inflammatory response.

Table 2

Ability of different strains of *Shigella* to cause illness or death in conditioned adult rabbit

Strain	Large plasmid	Inoculum used	No. of animals challenged	Results
Shigella dysenteriae 1				
AA 1237	+	10^{10}	5	No death or illness
AA 19780	+	10^{10}	5	"
AA 20686	+	10^{10}	5	"
AA 20956	+	10^{10}	5	"
Shigella flexneri				
AA 21714	+	10^{10}	5	2 died within 18h and 1 after 36h.
AA 20538	+	10^{10}	5	3 died within 18h.
G - 11	-	10^{10}	5	No death or illness
Shigella sonnei				
AB 3998	ND	$5x10^{9}$	6	2 died in 48h.
		$2x10^{11}$	6	4 died in 48h.

Table 3

Immunization and challenge of rabbits with *Shigella sonnei* strain AB 3998

No. of animals used	Immunizing dose	Challenge dose	Immunization and challenge interval	Result
6	0 (control)	$2x10^{11}$	-	4 animals died within 48h.
5	10^{9}	$2x10^{11}$	7d after second dose	No illness or death occurred to any of the rabbits.

It is thus seen that *Shigella flexneri* and *Shigella sonnei* are able to produce illness and cause death to rabbits but *Shigella dysenteriae* 1 clearly lacks this ability. We therefore thought it of interest to examine whether the latter would nonetheless produce histopathological lesions in the intestine. Preliminary results indicated some inflammatory response as stated.

(iv) Other studies:

Defining a minimal medium

Most strains of *Shigella* do not grow in any of the commonly used minimal media. We have formulated a combination of three supplements, methionine, niacin and tryptophan, which when added to minimal salts would support good growth of most isolates of *Shigella* spp. in a broth and plate culture.

Transformation in *Shigella*:

Strains of *Shigella* are difficult to transform. We are studying plasmid transformation (pADE161 and pBR322) in different species of *Shigella*. Under standard conditions of transformation (used in *E. coli*), efficiency of transformation varies widely between species of *Shigella*. Using a standard protocol for transformation and keeping all the parameters constant, we are trying to determine the relative efficiency of transformation in strains of *Shigella*. Results indicate that *Shigella flexneri* is most difficult to transform. Strains of *S. sonnei* and *S. boydii* transform better than *S. flexneri*. The highest efficiency of transformation has been detected in *S. dysenteriae* 1 where, in addition, the existence of an apparent restriction modification phenomenon has been noted. Plasmid pBR322

cultivated in _E. coli_ transformed into _Shigella dysenteriae_ 1 with a low efficiency. The efficiency increased about a thousand fold when the plasmid was cultivated in _S. dysenteriae_ 1 and used to transform the homologous strain. Similar improvements of transformation efficiency could not be detected in other species of _Shigella_.

(v) Isolation of a double mutants:

The UV-induced temperature-sensitive mutant of _S. flexnari_, strain TSF-26 was selected to introduce a second attenuating mutation. A cultulre was grown overnight in TSB containing 200 ug/ml thymine. About 20 resistant colonies that developed were isolated, streaked for single colony isolation. A derivative was isolated which is both temperature-sensitive and thymine-dependent. This derivative is designated as TSF-21. A faster growing thymine requiring derivative of TSF-26 has also been isolated. It is designated as TSF-22. A temperature-sensitive thymine requiring double mutant of _S. dysenteriae_ 1 has been isolated which is designated as PSD-5.

3. Rationale of the study proposed in this protocol:

A live vaccine seems to bear good immunoprotective potential against shigellosis. A form of live vaccine that is being discussed with interest is the one that would consist of an attenuated form of the pathogen that will be sufficiently crippled so as not to be able to produce disease, but will retain the ability to develop a protective immune response.

The various genetic modifications intended to be introduced in strains of _Shigella_ during the course of the present study are expected to generate strains that will be stably attenuated so that

these will not be able to undertake a course of prolonged proliferation in the lumen and the epithelial tissue of the intestine and will thus be unable to produce disease. However, because of limited contact with the intestine, these may nevertheless, be able to stimulate protective immunity.

Under the present protocol, attenuated strains will be developed and tested for stability in the laboratory. The strains will be then fed to conditioned rabbits to test their safety, their ability to trigger an immune response and to protect the animal against a virulent challenge. In this way a set of strains comprising of *S. dysenteriae* 1 and *S. flexneri* will be developed that would then be similarly tested in monkeys with the aim of eventually subjecting some strains to human volunteer testing.

B. SPECIFIC AIM:

i) To isolate attenuated mutants of *S. dysenteriae* 1, and *S. flexneri* by introducing mutations such as (i) temperature-sensitivity, (ii) sensitivity to galactose and to diaminopimelate and (iii) mutation conferring a high level of thymine-dependence.

ii) Testing safety, 'immunogenecity' and protective potential of such attenuated strains in a rabbit infection model.

C. MATERIALS AND METHODS:

(1) Materials:

Shigella strains will be obtained from ICDDR,B hospital. These will include *S. flexneri*, *S. dysenteriae* 1 and *S. sonnei*. Present *E. coli* stock in our laboratory represents a collection from Yale University Genetics Stock Centre, the University of Adelaide and

the University of Edinburgh, mainly representing an assortment of strains with various Hfr's and Tn10 insertions at different locations in the chromosome. Some temperature-sensitive mutations and thyA mutations are also included in the stock.

TABLE-4

Strain	Characteristics of interest	Source
CV-2	Hfr, transferring early the temperature-sensitive adk2 gene at 11′	Yale
BW 7623	Hfr, Tn10 at 12′, transferring clockwise	Y
BW 7620	Hfr, Tn10, transferring ~22′ early	Y
BW 5660	Hfr, Tn10 at 58′, transferring ~66′ early	Y
BW 6159	Hfr, Tn10 at 85′, transferring ~66′ early	Y
BW 6175	Hfr, Tn10 near 90′, transfers ~85′ early	Y
NK 6051	Hfr, Tn10 near 12′, transfers ~97′ early	Y
BW 7261	Hfr, Tn10 near 2′ transfers ~12′ early	Y
BW 6163	Hfr, Tn10, transfers ~60′ early	Y
BW 6169	Hfr, Tn10 near 61′, transfers ~84′ early	Y
BW 6166	Hfr, Tn10, transfers ~90′ early	Y
TST-1	F^-, Tn10 near 92′	Y
PC-1	F^-, thyA	Y
NK 6066	Hfr, Tn9 near 90′, transfers ~90′ early	Y
X 2913	F^- thyA 752	Y
X 2904	F^- thyA 748::Tn10	Y
E 486	F^-, carrying dna 486 temperature-sensitive mutation	Y
SJ-16	F^-, Tn10 near 3′	Y
Hfr 3000	Hfr H, Thi$^-$, n$^-$, transfers ~98′ early	Y

Y= Yale University Genetic Stock Centre

Table-4 (continued)

Strain	Characteristics of interest	Source
AB 4134	F^-, carrying the temperature-sensitive mutation gly53	Y
S 1228	F^-, Tn10 near 80´	Y
RK 4349	F^-, Tn10 near 86´	Y
ST 222	F^-, carries temperature-sensitive mutation near C3 at ˜2´	Y
ND 40	Hfr, Tn10 near 16´, transfers ˜6´ region early anticlockwise	Edinburgh
PR-E-3		
P 770	Hfr, transfers clockwise ˜30´ early	Adelaide
P 2218	F^-, Tn10-5 at 15´	Adelaide
P 1173	Hfr, transfers 10´ early anticlockwise	Adelaide
G 11	Hfr, transfers anticlockwise ˜70´ early	Y
E 917	Km^R Str^R	Adelaide (Manning)
P 525	E. coli K-12, $btoB^-$ (resistant to phage BF 23)	Adelaide
P 109	E. coli K-12, $btoB^+$ contains receptor of BF23 and hence sensitive to the phage	Adelaide
RM 172	Hfr, Tn9 at 80´ transfers anticlockwise from about 90´	Adelaide
P 400	E. coli K-12, propagating strain for phages P1, 43, C21	Adelaide
E 528	F^+, Tn10 near dnaA at 83´	Adelaide (Manning)
PR-E-3	Hfr, Tn9 at 80´, dnaA::Tn10, transfers clockwise ˜90´ early	ICDDR,P
C 600	F´ts::Tn10	Univ. Leicester
ED 478	F´ts::Tn1725	Univ. Edinburgh

(2) Methods:

(i) Selection of mutants by mutagenesis and ampicillin enrichment.

Usually a standard protocol will be followed. Briefly, it is as follows. Cells from a fresh broth culture will be washed in phosphate buffered saline (PBS) and subjected to UV light (254 nm) or ethylmethane sulfonate treatment such that about 90% cells are killed. Mutagenized cells will be washed in PBS and grown overnight in appropriate medium that would allow overgrowth of the desired mutant. Cells will then be washed and now be grown in a broth where the desired mutant cells will not be able to grow but will stay in a quiescent state. When the culture will be at about mid-log phase, ampicillin will be added. Ampicillin inhibits cell wall synthesis but does not affect cell growth. Thus, growing cells will undergo lysis because of a weak cell wall. On the other hand, quiescent cells will survive the treatment. After a clearing lysis has occurred (about 2h), the lysate will be centrifuged at 10,000 rpm in a Sorvall centrifuge (SS-34 rotor), the pellet will be washed once to remove ampicillin and transferred to a broth and incubated under conditions that would allow an overgrowth of the mutant cells. After overnight growth, the culture will be subjected to another similar ampicillin treatment to further enrich the culture with mutant cells.

Details of how the different mutants will be isolated by cycles of the growth and ampicillin lysis schedule is outlined below:

(a) Temperature-sensitive mutants:

Mutagenized culture will be grown at 25-30 ^{o}C for overgrowth of temperature-sensitive (TS) cells. Ampicillin lysis will be carried out at 38 ^{o}C. After two enrichments, cells will be plated on TSA, incubated at 30 ^{o}C for 8h to allow the TS cells to establish a microcolony. Plates will then be transferred to 38 ^{o}C overnight. Small colonies will be marked and plates will be returned to 30 ^{o}C. Those small colonies that will now grow at 30° but not at 38 ^{o}C will be picked up as TS candidates for further testing.

(b) Galactose-sensitive mutants:

Mutagenized cells will be grown in TSB overnight at 37 ^{o}C. Ampicillin lysis will be carried out in TSB. After two enrichments, cells will be plated on TSA and replica plated onto TSA containing 0.5% galactose. This procedure assumes that TSB contains traces of galactose which would kill both highly sensitive cells and also the highly resistant cells, thus enriching the culture with cells having an intermediate level of galactose sensitivity.

If mutants are not recovered by the above procedure, minimal medium with glucose will be used for both growth and enrichment of galactose-sensitive cells.

(c) Diaminopimelate (DAP) requiring mutants:

Mutants requiring DAP may have varying degrees of impairment in DAP biosynthesis. A class of dap^{-} mutants of E. coli will lyse if lysine, not DAP, is present in the medium. These mutants are the ones we intend to isolate as these are likely to lyse in complex media such as LB, TSB, NB etc. because these media contain lysine but not DAP.

These mutants can, however, be isolated and grown in medium containing both lysine and DAP.

Mutagenized culture will be grown in minimal medium (MM) containing lysine and DAP. Cells will be washed and starved in MM for a few hours in order to arrest growth of the DAP requiring cells so that on subsequent transfer to TSB for ampicillin enrichment they escape lysis. After two ampicillin enrichments, cells will be plated on TSA containing DAP and replica plated on TSA to detect DAP-sensitive colonies.

(d) High levels of thymine-requiring mutants:

Cells, mutagenized or non-mutagenized, will be grown overnight in TSB with thymine (50-200 ug/ml) and about 10^7 cells will be plated onto TSA plates containing 10-30 ug/ml trimethoprim plus 200 ug/ml thymine. Resistant colonies will be picked and purified and their thymine dependence and stability will be determined.

No enrichment is anticipated for isolating this type of mutants because normally the thymine-trimethoprim selection is very specific and can select efficiently the thyA mutants in E. coli.

(ii) Conjugational transfer of genes from E. coli to Shigella:

Mutations conferring genetic attenuation such as temperature sensitivity galE, dap, thyA are available in E. coli. Appropriate Hfr strains which could efficiently transfer these genes may already be existing in different laboratories. We are trying to locate such strains. Suitable Hfrs will be conjugated with Shigella to transfer these mutant genes.

(iii) Transposon mutagenesis

A number of transposable drug resistance elements (transposons) are known. Some of these elements are highly transposable and have been cloned in temperature-sensitive plasmid vectors to allow their use as tools of mutagenesis.

The method involves mixed growth of the strain to be mutagenized with a transposon donor strain and by using appropriate drug-selection, selection of the recipient that has inherited the transposon in question and has thus become drug resistant. These are purified, shifted to a higher temperature ($42^{o}C$) where the vector cannot replicate and is thus lost. Among a sample of such drug resistant colonies one looks for the desired mutant type.

(iv) Stability of the attenuated mutants:

The frequency of reversion of the mutant strains will determined by growing the culture under restrictive condition and determining the appearence of wild type colonies. Approximately, 10^{7} cfu will be plated on individual agar plates. One hundred such plates will have used 10^{9} cells. A mutant (single mutation) will be considered stable if no revertant appears in about 10^{9} cells.

(v) Rabbit infection model:

(a) Conditioning of rabbits:

Adult rabbits will be conditioned as follows. The animals will be starved for 18h during which period they will be allowed to drink water containing tetracycline (1 mg/ml). Average consumption of water during this period varies from 100 to 200 ml which is equivalent to about 100 to 200 mg of tetracycline intake by an animal. Then, at

time 0, cimetidine (50 mg/kg body weight) will be ad intravenously. At 15 and 30 min, 15 ml of a solution of will be administered by gastric tube. Immediately after t $NaHCo3$ dose, 15 ml of bacterial inoculum is given by gas followed by i.p. administration of 2 ml of a tincture of opi

(b) Determination of LD_{50} :

The lethal-dose that kills 50% of the animals determined as follows. Inocula of sizes 10^6, 10^7, 10^8, 10^{11} will be administered to groups of five rabbits occurring within 7 days will be recorded. This range of in likely to be adequate for LD_{50} determination using *S. flexn* *sonnei* (see Table 2, P.11). Since, 10^{10} cfu of *S. dysente* not kill rabbits, we will try a dose one log higher (10^{11}) killing can nevertheless, be achieved.

(c) Determination of ´Colonization´:

After 18 h, the surviving rabbits will be sacrific cm segment of the ileum lying 10 cm distal to the ileo-caeca will be isolated from the rest of the intestine by ties and The segment will be opened by longitudinal cut and immersed in 20 ml of PBS. The wet weight of the segment will be dete bacteria with PBS will be quantitated in McConkey´s agar dilution. Colonization will be expressed as mean log of recovered in the wash fluid ± S.E. per gram of tissue.

(d) Protection of ´immunized´ rabbits:

Rabbits will be fed with inocula of 10^{10} and 10^{11} attenuated strain that is to be tested. The immunized rabbi

challenged with a range of inocula of the virulent homologous strain. Protection from death colonization and the development of intestinal lesions (see below) will be recorded in the case of S. flexneri and S. sonnei and protection from colonization and the development of intestinal lesion in the case of S. dysenteriae 1.

Attenuated mutant strains will be administered in doses of 10^{10} and 10^{11}. If death occurs to a significant number of animals then a standard LD_{50} determination will be undertaken in order to decide on the size of an immunizing inoculum.

(e) Examination of the ileum for histopathological lesions:

Histological specimens of the ileum prepared by standard methods will be examined for lesions.

Animal will be sacrificed 18h after the administration of an inoculum. Segment of ileum will be collected and placed in individual vials of 10% buffered neutral formalin for fixation. Well fixed tissues will be processed for routine histopathological examination. While doing histopathological examination inflammatory changes e.g. congestion, edema, necrosis, ulcers, exudates etc. will be recorded and graded according to their severity. For evaluation of these changes - a grading system of 0-4 (i.e. none, mild, moderate and severe) will be used to express severity of the changes observed. Groups of rabbits challenged with test bacteria can then be compared to the control group using the chi-square analysis.

(f) Safety of the mutant strains:

If an inoculum of 10^{11} cfu neither fails to cause any lethality nor does it produce significant histological damage to the intestine, the strain will be considered ´safe´.

(g) ´Immunogenecity´ of the attenuated mutants:

An attenuated mutant strain that would retain the ability to protect experimental animals from a virulent challenge would be a candidate for studies on its immunogenecity. "Immunogenecity" of a strain is defined here in a somewhat restricted sense. It is defined as the ability of a strain, when given orally, to stimulate the production of antibodies against major antigenic components of Shigella spp. It would be necessary to compare the levels of antibody stimulation caused by the mutant and the wild-type strain in a controlled comparative experiment to be able to make a statement that the mutant strain compares favourably in triggering antibody production to the wild type strain.

The major emphasis will be placed on determining the levels of intestinal immunoglobulins, mainly secretory IgA, reactive to pure LPS antigen of Shigella strains. The experimental protocol will be as follows. Groups of rabbits will be given oral inocula of the wild type strain of S. flexneri containing 10^9 and 10^{10} viable cells. A total of three such feedings will be carried out at weekly intervals. Similar testing will be conducted using the mutant strains. Two to three weeks after the last feeding, the animals will be sacrificed and intestinal washings will be prepared and concentrated quantitatively. The material will then be subjected to ELISA using LPS as the antigen and alkaline phosphatase conjugates of swine anti-rabbit IgA, IgG and

IgM antibodies. LPS will be prepared by the hot phenol extraction procedure (Westphal *et al.*, 1952). ELISA will be carried out by following the method described by Lindberg *et al.*, (1984). The procedure would involve (i) demonstration of a lack of cross-reactivity of *Shigella* LPS with that of other enteropathogens, (ii) developing an appropriate method to determine and express the antibody titer and (iii) appropriate statistical analysis of the antibody titers to validate the comparison.

D. SIGNIFICANCE:

Reports on the isolation of attenuated mutants of Shigella are, to the best of our knowledge, scanty. The most well cited example is the streptomycin-dependant mutant of Shigella flexnari 2a which was used as a live oral vaccine (Mel et al, 1965, 1968, 1971) in field trials with not very clear results having been obtained on its safety and efficacy. Result with the live dysentery vaccine T-32 (Contacuzino Institute, Romania) are not widely reported and hence cannot be assessed.

We wish to isolate stable mutants of Shigella dysenteriae 1 and Shigella flexneri strains which will have restricted proliferative potential in the gut. These mutants are, therefore, expected to be avirulent. The mutuants would be tested in a rabbit infection model to evaluate their safety, their ability to trigger an immune response in the animal and protect the animal against a virulent challenge. The knowledge would contribute to an understaing of the protective potential of attenuated strains, nature of the local immune response elicited by these strains and may help identify protective antigens. Information in these areas are currently inadequate. Therefore, although restricted to animals, the study will help define some of the propoerties that a potential vaccine strain should retain. At the end of the study, some strains might appear promising for a volunteer trial after the completion of an intervening successful trial in the monkey.

E. BUDGET

BUDGET SUMMARY		YEAR 1 July 1, 1987 - June 30, 1988
9-3100	Local Salary	21,540
14-3700	Supplies	12,500
15-3800	Other costs	2,000
13-1600	Int. Travel	3,500
16-4800	Interdepartmental	19,500
21-	Direct Cost	59,040

Breakdown of Year 1 budget

57. Manpower allocated from other areas

Job	Level	Man mo	$ mo	Amount
Bacterial Geneticist	NO-E	12	800	9,600
Pathologist	NO-E	3	800	2,400
Bacterial Geneticist	NO-A	6	310	1,860
Sub-Total:				13,860

49. New Recruits:

Job	Level	No	Man mo	$ mo	Amount
Sr. Res. Officer	GS-6	1	12	260	3,120
Res. Officer	GS-5	1	12	190	2,280
Lab. Attendant	GS-2	2	12	95	2,280
Sub-Total					7,680

145. Int. Travel

	Amount
To attend ASM meeting, Florida, May 1988	3,500

151. Supplies and Materials:

Item	Amount
Glassware	2,000
Stationary	500
Non-Stock	10,000
Sub-Total	12,500

168. Other Costs:

189. Interdepartmental:

Printing & Publishing	2,000
Transport DK	500
Xerox	1,000
Media	8,000
Animals (rabbit, guinea pig monkey)	10,000
Total	19,500

204. Capital Expenses:

E. BUDGET

Budget Summary		Year 2 July 1, 1988 - June 30, 1989
9-3100	Local Salary	20,400
11-3300	Consultant	3,620
13-3600	Int. Travel	3,875
14-3700	Supplies	12,500
15-3800	Other costs	2,000
16-4800	Interdepartmental	18,500
	Total	60,895
Plus 15% increase		9,093
	Total Direct Cost:	69,988

Breakdown of Year 2 budget

49. New Recruits: Same as year 1

57. Manpower allocated from other areas: Same as year 1

Consultant:

Job	No. of days	Per diem Rate	Per diem Total	Travel	Amount
Mol. Biologist	14	80	1,120	2,500	3,620

145. Int. Travel:

To ASM Meeting to present paper	----	Travel	3,000
		Per diem (7 days)	875
			3,875

151. Supplies: Same as year 1

168. Other costs: Same as year 1

181. Interdepartmental: Same as year 1

BUDGET SUMMARY

YEAR 3
July 1, 1989 to June 30, 1990

Budget is the same as of year 2 plus 20% increase + $10,000 increase for animals (monkeys).

That is, year 2:		
	Total Direct Cost ----	69,988
	Add 20% increase ----	13,926
		83,914
		+ 10,000
	Total Direct Cost:	93,914

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