 Date 2/5/91

Attachment 1.
(FACE SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator ZIA UDDIN AHMED

Application No. 91-004

Title of Study "DEVELOPMENT OF A VACCINE AGAINST SHIGELLOSIS"

Trainee Investigator (if any) _____

Supporting Agency (if Non-ICDDR,B) _____

Project status:
(x) 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: 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: (NA)
   (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 (NA)
7. Check documents being submitted herewith to Committee:
   NA 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)
   N/A 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.

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

A-032073

91-004
3/5/91

APPLICATION FOR PROJECT GRANT

1. Principal investigator : Zia Uddin Ahmed, Ph.D.

2. Other investigators : M. Musharraf Ashraf, M.Sc.

   Two Research Officer to be recruited.

3. Title of projects : Development of a vaccine against shigellosis.

4. Starting date : July 1, 1991

5. Date of completion : 31 June 1994

6. Funding source : Not known

7. Total budget requested : US$ 396,830

8. Head of programme : _____

   Acting Head
   Laboratory Sciences Division

9. Aims of Project

a) General aim

To contribute towards the development of a vaccine against shigellosis.

b) Specific aims

i. to define the *thyA* mutation in *S. flexneri* strain TSF21,

ii. to create *thyA*$^-$ Tox$^-$ mutants of *S. dysenteriae* 1.

iii. to test safety, immunogenicity and protective potential of the mutants in animals.

c) Significance

See general aim.

10. Ethical implications : Not applicable

11. Abstract summary

Attenuating effect of a *thyA* mutant and its ability to confer immune protection in monkeys has been demonstrated by us by the construction and study of the candidate vaccine *Shigella flexneri* strain TSF21 (ref: Ahmed *et al.*, *Vaccine* 8, 153-158; 1990). Although genetic evidence suggests that the *thyA* mutation is possibly a deletion, we have not demonstrated this formally by sequencing the *thyA* gene in strain TSF21. Thus, we propose to do this through this project proposal. The mutant TSF21 provides cross-protection against the two other common *S. flexneri*

serotypes, that is, serotype 2a and 3a; it does not, however, provide protection against *S. dysenteriae* 1 (unpublished results). We thus propose to create *thyA* Tox$^-$ mutants of *S. dysenteriae* 1 for its possible use as a vaccine component. The mutant will be studied for safety, immunogenicity and protective efficacy in monkeys.

12. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

A. BACKGROUND

Types of vaccines

Shigellosis appears to be an "immunizing" disease, that is, natural infection confers immunity to reinfection. This is also evident in human volunteer studies and studies in monkeys. Hence, the disease is potentially amenable to control by the use of a suitable vaccine.

Vaccines of various types have been tested in the control of shigellosis. To date, however, no practical vaccine with consistent results on safety and protection is available. A summary of the previous vacccines is presented in Table I.

Table I

Vaccines tested against shigellosis and results obtained

Vaccine/type	Results	Reference
Parenteral vaccines		
Parenteral heat-killed shigella	No protection against experimental challenge in humans	Formal and Levine, 1984 Shaughnessy *et al.* 1946
Parenteral (subcutaneous) live *S. flexneri* 2a	No protection in monkeys	Formal *et al.* 1967
Oral live vaccines		
a. E. coli/Shigella hybrid vaccines		
i) xyl^+ - rha^+ region *E. coli* K-12 transferred to *S. flexneri* 2a	Good protection in monkeys	Formal *et al.* 1965
ii) *E. coli* K-12 expressing *Shigella flexneri* O-antigens	No protection in humans	Levine *et al.* 1977
iii) *E. coli* containing the invasive plasmid of *S. flexneri* and expressing *S. flexneri* 2a somatic antigen	No protection in humans	Formal *et al.* 1984
b. *Salmonella* - *Shigella* hybrid vaccine		
i. *S. typhi* Ty21a carrying *Shigella sonnei* O-antigen genes in a plasmid	Safe in volunteers. No consistent protection	Formal *et al.* 1981 Black *et al.* 1987

Table 1 continued ...

Vaccine/type		Results	Reference
c. Attenuated *Shigella* vaccines			
i.	Non-invasive mutant of *S. flexneri* 2a	Reactogenic in volunteers unstable	Formal *et al.* 1965 DuPont *et al.* 1972
ii.	Passage-avirulent *S. flexneri* 2a	Safe, unsatisfactory protection	Meitert *et al.* 1984
iii.	Streptomycin-dependent *Shigella flexneri* and *Shigella sonnei*	Good serotype-specific protection in field trials	Mel *et al.* 1965 Mel *et al.* 1971
		Inadequate immunogenicity and revertants recovered from subjects in custodial institutions	Levine *et al.* 1974 Levine *et al.* 1975
iv.	Aromatic-dependent *S. flexneri* Y mutant	Safe and protective in monkeys	Lindberg *et al.* 1988
v.	*S. flexneri* 5a mutant with poor intracellular spead and growth	Safe and protective in monkeys	Sansonetti and Arondel 1989
vi.	Purine-requiring and streptomycin-resistant *S. flexneri* 5a and *S. sonnei*	Safe and immunogenic in volunteers	Linde *et al.* 1990 Dentchev *et al.* 1990
vii.	Thy^- Ts^- double-mutant of *S. flexneri* Y	Safe and protective in a small number of rabbits and monkeys	Ahmed *et al.* 1990

Failure of killed parenteral vaccines and those of live hybrid vaccines and the fact that the disease is immunizing, led to a general acceptance of the view that an attenuated mutant of the organism is likely to provide better immune protection. This realization is reflected in the series of reports that appeared over the past two years or so on the development of different types of attenuated mutants, as outlined in Table I.

Nature of attenuation

A mutation leading to reduced proliferation of bacteria can be potentially attenuating. However, the mutation should not affect invasive ability of bacteria, but should allow limited intracellular growth. Invasion is important for protection; non-invasive mutants of *Shigella* with reduced proliferative activity provided no protection (Formal *et al.* 1965; Meitert *et al.* 1984). One way to attenuation is to make the bacteria dependent on a metabolite which is not available in sufficient amounts inside cells of the intestinal epithelium. Two categories of mutants have been reported:

i. Auxotrophic mutants: These include the aromatic metabolite dependent Aro$^-$ mutants (Lindberg *et al.* 1988), purine-requiring Pur$^-$ mutants (Linde *et al.* 1990), and thymine-requiring Thy$^-$ mutants (Ahmed *et al.* 1990).

ii. Mutants with impaired intracellular spread: In this category is included aerobactin-negative (*iuc*) mutants causing impaired intercellular growth and the *icsA* mutants with reduced intracellular spread (Sansonetti and Arondel, 1990).

Attenuated mutants should be well-defined genetically and should be stable so that the chance of revertants being produced is infinitely small. Thus, a deletion in the mutant gene is desirable. Mutant genes have been identified in three recently developed vaccine strains:

a. *aroD* gene in *S. flexneri* Y (Lindberg *et al.* 1988).

b. *icsA iuc* genes in *S. flexneri* 5a (Sansonetti and Aroundel, 1989).

c. *thyA* gene in *S. flexneri* Y (Ahmed *et al.* 1990).

All of these mutants have been demonstrated to be stable, but to the best of our knowledge, demonstration of a deletion or other non-revertible changes has not been reported by sequencing the gene.

Attenuated mutant strain TSF21

The *thyA* mutant described by us (Ahmed *et al.* 1990) has been shown to be stable. It is likely to be a deletion type of mutation, but the possible deletion has not been demonstrated by isolation and sequence determination of the *thyA* gene. This is proposed to be done in this study.

Our major emphasis so far has been on the development of attenuated mutants of *Shigella flexneri* Y for use as a live oral vaccine against shigellosis. Towards this end, a super-high-level thymine-requiring mutant with a defect in the *thyA* gene which codes for the essential DNA metabolic enzyme thymidylate synthetase, has been developed. The *thyA* mutation is of a non-reverting type; revertants *in vitro* could not be detected in approximately 10^{10} cells tested. This is suggestive of a deletion of the gene. The possibility, however, has not been formally demonstrated by determining the mucleotide sequence of the gene. The theoretical safety margin of the strain has been further enhanced by the introduction of a second mutation, a temperature-sensitive mutation, which is "leaky" with a reversible frequency of $\sim 10^{-6}$ at 39°C. The resulting strain designated as *Shigella flexneri* strain TSF21 has been studied in *vitro* and in rabbits and monkeys for safety and protective potential. The strain has many of the properties desired in a vaccine. These are outlined below:

- It is avirulent, safe and non-reactogenic as tests using over 40 rabbits and 10 monkeys have demonstrated, each animal after being fed with at least 1×10^{11} viable bacteria.
- Because the vaccine strain carries two independent mutations, it has been calculated that no revertains with the parental level of virulence would be produced in about 1×10^{15} cells.

- The defective gene responsible for thymine-dependence of the strain has been identified. It is the *thyA* gene which determines the primary structure of an essential enzyme for DNA synthesis, thymidylate synthetase.

- Protective ability of the vaccine has been demonstrated both in rabbits and monkeys after oral immunization and highly virulent experimental challenge.

- The vaccine mounts a strong immune response (both humoral and possibly cell-mediated) after oral immunization.

- The mutant is sensitive to most of the commonly used antibiotics, except tetracycline and trimethoprim.

- Being of serotype Y which has the basic O-antigen structure shared by a number of other predominant serotypes found in this part of the world, the vaccine thus bears the potential of providing cross-protection. Indeed cross-protection has been demonstrated after challenging with *S. flexneri* 2a and 3a, but not with *S. dysenteriae* 1.

- Blood-picture and serum biochemical profile after oral administration of the vaccine at a dose of 1×10^{11} revealed no evidence of toxicity in monkeys.

[Reference: Vaccine 8, 153-158, 1990 and results of a vaccine trial in monkeys at NII, New Delhi (manuscript in preparation)].

Creation of attenuated mutants of *S. dysenteriae* 1

Although *S. flexneri* Y has the basic O-antigen structure shared by other common serotypes and one may therefore expect cross-protection, efforts have been intensified in recent years to create attenuated mutants in various other serotypes. Following creation of mutants in *S. flexneri* 5a (Sansonetti and Arondel, 1990),. these workers recently reported the creation mutants in *S. flexneri* and *S. dysenteriae* 1 (Fontaine *et al.*, 1990). A. Lindberg's group described in 1988 a mutant of *S. flexneri* Y. Very recently, a mutant in *S. flexneri* 2a has been created (Verna and Lindberg, 1991). All these mutants are possible candidates for an anti-shigella vaccine.

Preliminary results in monkeys obtained with our mutant TSF21 (*S. flexneri* Y) indicate that significant cross-protection is possible against *S. flexneri* serotypes 2a and 3a, but not against *S. dysenteriae* 1. We thus consider it worthwhile to undertake research towards the construction of an attenuated mutant of *S. dysenteriae* 1, a *thyA* Tox$^-$ strain.

B. RESEARCH PLAN

Materials and Methods

Some preliminary experiments will be done for indications of possible sequence differences within the *thyA* gene of the wild type and mutant strain. These will include restriction-fragment

length analysis of the *thyA* gene. Total DNA will be digested with a set of restriction enzymes. Southern blots will be probed using the nick translation product of *E. coli thyA* gene. The hybridization profiles will then be comparted. Regions of suspected sequence changes will be amplified by the polymerase chain reaction (Mullis *et al.*, 1986), and sequenced by the dideoxy method (Sanger *et al.*, 1977).

However, cloning and sequencing the entire structural gene will probably be necessary in order to establish the ORFs and compare the two sequences.

I. Cloning of thyA gene from strain TSF21

Two approaches will be followed to first isolate the gene, which will then be sequenced.

(i) Cloning of the *thyA* gene in a plasmid-based mini-bank.

Total DNA of strain TSF21 will be digested with *EcoRI* or *HindIII* and the restriction fragments will be separated by agarose gel electrophoresis. We will assume that the *thyA* gene of strain TSF21 is similar to the *thyA* gene of *E. coli* K-12. The latter gene has been cloned in plasmid pBTAH (Belfort *et al.*, 1983) as a 1 kb fragment. This gene does not have internal *EcoRI* or *HindIII* site. We do have the plasmid in our collection and we routinely isolate the *thyA* gene as a 1 kb *HindIII* fragment. A "Southern" blot of TSF21 DNA will be prepared and hybridized with the wild type *thyA* gene of *E. coli*. The low-molecular weight region of the gel

where the *thyA* probe will hybridize will be noted. From a preparative agarose gel made by using the DNA digest, DNA fragments occuring in the similar low molecular weight area will be eluted which will likely contain the *thyA* gene of strain TSF21.

The DNA fragments will then the cloned in the plasmid vector pUC18 using standard procedures (Maniatis, 1984). Briefly, pUC18 will be digested with either *EcoRI* or *Hind*III to make it linear. The linearized plasmid will be mixed with the eluted DNA fragments. A ligation reaction will be carried out using T4 DNA ligase. The ligation mixture will be tested for successful ligation and transformed into *E. coli*. The cells will be spread onto X-gal plates containing ampicillin. Ampicillin-resistant white colonies on the plate are the transformats which carry pUC18 with an insert at the *EcoRI* or *Hind*III site. A collection of such colonies will make the mini-bank.

Plasmid DNA from each clone will be extracted and probed with the *thyA* fragment. Positive clones will be analysed by restriction digestion of the plasmid. By subcloning, the insert fragment size carrying the entire *thyA* gene will be isolated for sequencing.

II Isolation of thyA gene from strain TSF21 by using polymerase chain reaction (PCR).

This approach will involve the isolation by ampilification the *thyA* gene using the polymerase chain reaction (Mullis *et al.*, 1986) and its cloning and sequencing by the dideoxy method.

In analysing the *thyA* gene of *Shigella* we will assume that the *thyA* gene is highly conserved and the primary structure of the gene in *Shigella* is the same as it is in *E. coli* K-12. The $thyA^+$ gene of *E. coli* K-12 has been cloned and sequenced (Belfort *et al.*, 1983). We have also shown that the $thyA^+$ gene of *Shigella* complements the function in a $thyA^-$ strain of *E. coli* K-12 (Ahmed *et al.*, 1990). Primers for the PCR reaction will be based on the structure of the *thyA* gene of *E. coli* reported by Belfort *et al.*, (1983). Comparison of the sequence in strain TSF21 with that of the wild type strain will indicate the major changes, if any.

III Isolation of thyA Tox^- mutant of Shigella dysenteriae 1.

(i) Isolation of *thyA* mutant

We have tried for a long time to isolate a *thyA* mutant of *S. dysenteriae* 1 by the thymine-trimethoprim selection procedure, but without success.

To obtain defined *thyA* mutants in *S. dysenteriae* 1, the construction *in vitro* of a *thyA* gene inactivated with a defined insert would be necessary. We wish to construct such a gene using the *thyA* gene of *E. coli* which has been cloned and sequenced (Belfort *et al.*, 1983). The inactivated *thyA* gene will then be transferred to *Shigella* chromosome replacing the wild type gene by a marker-exchange recombination event.

The rationale of using the *E. coli thyA* gene is as follows. The *thyA* gene is the structural gene for the primary metabolic enzyme thymidylate synthase, essential for DNA replication. The gene is thus likely to be highly conserved within bacterial species. Thus, the *thyA* gene of *Shigella* and that of *E. coli* will have a high degree of DNA sequence homology and will be functionally identical. The latter assumption has been validated by us by a complementation test (Ahmed *et al.*, 1990).

Following is an outline of the procedure that will be followed to generate an inactivated *thyA* gene of *E. coli* and exchange it with the wild type gene of *Shigella*.

1. A cloned copy of the wild type *thyA* gene of *E. coli* is carried by the plasmid pBTAH2 (Belfort *et al.*,1983).

2. The plasmid will be isolated and, at the *Pvu*II site within the *thyA* gene, a piece of foreign DNA will be

inserted. The DNA will be an "interposon" (Fellay *et al.*, 1987).

3. The inactivated *thyA* gene with the interposon insert will be isolated by *Hind*III digestion and inserted at the *Hind*III site of the suicidal plasmid pJM703.1 to produce a hybrid plasmid . The suicidal plasmid pMJ703.1 is unstable in *Shigella* strains because the plasmid needs the λ *pir* function for replication, which the *Shigella* strains cannot provide. The hybrid plasmid will thus be maintained in *E. coli* strain SM *pir* after transformation. As apparent, this plasmid contains the inactivated *thyA* gene and codes for resistance to mercury and ampicillin.

4. The hybrid plasmid will be transformed in *Shigella*, selecting clones resistant to ampicillin and the interposon-mediated agent. A culture of *Shigella* carrying this plasmid will be grown with or without a selection. The plasmid will be lost at a high frequency (we have found this to be >90% in *S. dysenteriae* 1). Among the unselected cells and those obtained after interposon-selection, we will look for the product of the desired marker-exchange recombination event.

The product will be the desired strain - that is, a strain with a *thyA* gene which has been inactivated by the insertion

of a piece of exogenous DNA at a defined position within the gene.

If this hybrid plasmid proves inefficient in transferring the inactivated *thrA* gene of *E. coli* to *Shigella*, we will test other suicidal plasmid vectors.

(ii) Isolation of Tox$^-$ mutants

A Tox$^-$ mutant will be isolated by insertional inactivation of the Shiga-toxin gene. The strategy will be as follows:

A class of toxin produced by *Shigella* and some pathogenic *E. coli* are known to have very similar genetic structure at the level of DNA sequence. These include the Shiga-toxin, Shiga-like toxin (SLT) of which the two variants have been designated as SLT I and SLT II. Each toxin consists of one A-polypeptide and five B-polypeptides. A plant toxin isolated from *Ricinus communis* called ricin has also a structure (Halling *et al.* 1985) similar to Shiga-toxin and Shiga-like toxins. The structural genes for the A and B subunits have been cloned by different workers. Relevant information on these is presented in the appendix.

The procedure to obtain an insertionally inactivated Shiga-toxin gene is similar to one descriged above for an inactivated *thyA* gene and comprises of several steps. The first step is to insert a selectin cartridge such as an interposon (Fellay *et al.* 1987) into a cloned gene which can be any one of the following:

a. Intact SLT gene or the A or B-subunit gene.

b. Intact ricin gene or the A or B subunit gene.

The resulting structure will be cloned into a plasmid which is not stably maintained by *Shigella*. The plasmid will be maintained in a suitable *E. coli* host strain.

When necessary, the plasmid will be introduced in *Shigella*. The two halves of the ricin gene, for example, flanking the interposon will provide the required targets of homology for recombination to occur with the chromosomal Shiga-toxin sequence. A derivative resulting from a double cross-over event will be selected which will represent an inactivated Shiga-toxin gene through the insertion of an interposon.

A stable derivative will be tested for toxicity and its safety, immunogenicity and protective efficacy will be studied in monkeys. The experiments that will be performed and the methodology are described in the following manuscript:

Potential of *Shigella flexneri* Y strain TSF21 as a candidate vaccine against shigellosis: safety, immunogenicity and protective efficacy in bonnet monkeys.

M. M. Ashraf, D.K. Giri, H.V. Batra, P. Khandekar
Zia U. Ahmed and G.P. Talwar.

SUMMARY

A thymine-requiring and temperature-sensitive mutant of *Shigella flexneri* Y was tested in bonnet monkeys for safety, immunogenicity and protective efficacy. A dose of 10^{11} cells when fed orally mimicked natural infection in having invaded epithelial cells, but was otherwise clinically non-reactgenic. Animals immunized with two oral doses, each dose consisting of $1x10^{11}$ mutant bacteria were fully prtected when challenged, with respect to the lack of any clinical symptons or detectable histological abnormalities in the intestinal mucosa. Unimmunized animals when similarly challenged developed frank dysentery and the intestinal mucosa shoved severe histological abnormalities. Titres of serum ahtibodies increased by about 11-fold of the base level in animals immunized with a dose of 10^{11} cells, but not with lower doses. The challenge bacteria appeared to be phagocytised by macrophages. An apparent hypersensitivity reaction manifested as congestive patches in the stomach was seen in bonnet monkeys (both immunized and control) after challenging with the virulent parent strain. The extent and severity of this reaction was more pronounced in immunized animals. However, this type of hypersensitivity reaction could not be detected in rhesus monkeys after feeding and challenging in an identical manner. The vaccine strain, however, did not produce any such lesions either in bonnet monkeys or in rhesus monkeys. The fact that the post-challenge stomach lesions were seen in both immunized and control groups of bonnet monkeys would suggest that the lesions do not reflect any significant reactgenicity of the vaccine strain.

Molecular characterization of the mutant will be undertaken if the mutant shows some potential. That will be the basis of a separate study and may not be undertaken in the present protocol.

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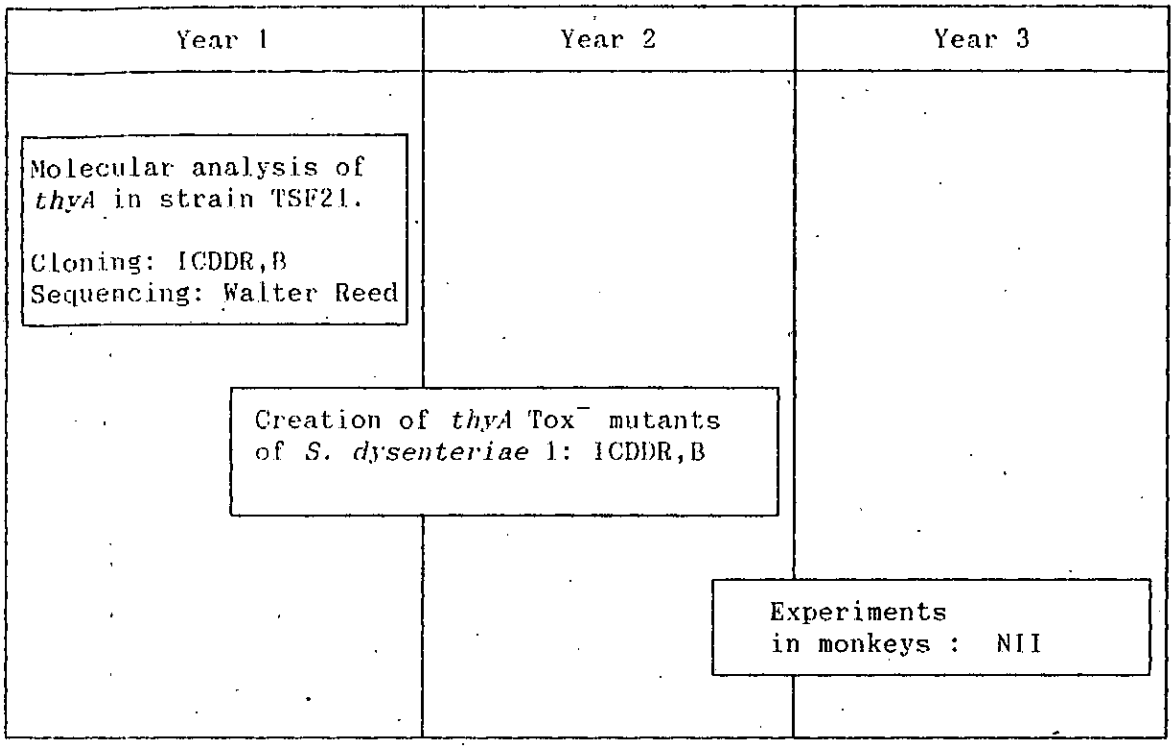
13. WORK-PLAN



14. Collaboration/specific tasks of investigators.

The protocol is conceived as a project involving scientific collaboration between ICDDR,B, the National Institute of Immunology (NII), New Delhi and with Dr. D.J. Kepecko of Walter Reed Army Institute of Research, Washington.

The participating institutions will share with us the scientific results and will absorb the bulk of the experimental cost. To give an example, at NII we have used about 60 monkeys in the vaccine trial work so far which we have not costed but the amount involved will be quite substantial. This sort of arrangement will continue with

NII. The Director of NII has assured continued support (c.c. enclosed).

With Walter Reed, our collaboration will involve PCR amplification of gene and sequencing of the PCR products or the cloned gene. The work will represent about 10% of the strain construction work and about 5% of the total work of the protocol. The two techniques of PCR amplification and gene sequencing could be introduced at ICDDR,B. We do have the necessary expertise. However, introduction of these techniques for a specific project and for a small number of experiments will not be cost-effective. On the other hand, collaboration with an external laboratory for such experiments where these techniques are in routine use will be both cost-effective and intellectually stimulating.

Dr. Kopecko has agreed to collaborate. The details of the arrangement will have to be worked out. A visit is being awaited for this purpose. Cloned SLT sequences are available at Walter Reed and are expected to be acquired as part of our collaboration.

15. BUDGET

Y E A R 1

Personnel (Local Salary)			
Bacterial Geneticist (P.I.)	NO.E	....	$ 29,000
Sr Res Officer (1)	GS.6	....	$ 8,000
Res. Officer (1)	GS.5	....	$ 6,000
Lab attendant (1)	GS.1	....	$ 1,000
Operating Cost			
(a) Material, supplies, interdepartmental (Stock and non-stock items, animals)		....	$ 40,000
(b) Contractual services		....	$ 2,000
(c) International travel (NII)		....	$ 2,000
			88,000

Y E A R 2

Add over yr 1:	(a) One extra GS5 (b) $5,000 International travel (c) 10% increase	....	102,300

Y E A R 3

Add over yr 2:	10% increase	....	112,530
Indirect cost		....	94,000
GRAND TOTAL	....	....	$ 396,830

APPENDIX

Cloned Shiga-toxin, Shiga-like Toxin and Ricin genes.

SLT I and II from phage 933J
==========

pJN37-19 SLT I
1142 bp
Carries 98% A subunit of SLT I
100% B subunit of SLT I

John Newland
Walter Reed Army Institute
Washington D.C. 20307, USA.

pNN111-19 SLT II
842 bp
Carries 95% of A subunit of SLT II

Reference: Newland, N.W. and R.J. Neill. 1988. DNA probes from Shiga-like toxins I and II and from toxin-converting bacteriophages. J. Clin. Microbiol. 26, 1292-1297.

SLT I and II from phage 933J
==========

pJN21) Carries B subunit of SLT (~2.5 kb insert)
pJN26)

pJN28 A subunit of SLT (>2 kb insert)
pJN25 A and B subunits of SLT (~3 kb insert)
Nancy Strockbine

Reference: Newland, J.W., N.A. Strockbine, S.F. Miller, Alison D. O'Brien and R.K. Holmes, 1988. Cloning of Shiga-like toxin structural genes from a toxin converting phage of *Escherichia coli*. Scinece 230, 179-181.

From Phage 11819
==========

pSC2 SLT A (1.5 kb insert) Contains ~left half of A-gene
pSC4 SLT B (1.4 kb insert) Contains the right half of A-gene and intact B-gene.

G. Keusch

Reference: Calderwood, S.B., F. Auclair, A. Donahue-Rolfe, G.T. Keusch and J.J. Mekalanos. 1987. Nucleaotide sequence of the Shiga-like toxin genes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA, 84, 4364-4368.

SLT from phage H30
==========

pSLT318. Carries a 3kb Kpn1 fragment inserted at polylinker site of pUC18 with the A and B genes.

Reference: Kozlov, Yu. V., A.A. Kabishev, E.V. Lukyanov, and A.A. Bayev. 1988. The primary sturcture of the operons coding for *Shigella dysenteriae* 1 toxin and temperate phage H30 Shiga-like toxin. Gene 67, 213-221.

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The USSR Academy of Sciences
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USSR.

Shiga-toxin from *S. dysenteriae* 1
==========

1. pNAS10 Carries complete A and B genes with some flanking DNA in a 3300 bp insert.

   Nancy Strockbine
   Department of Microbiology
   Univormed Services University of the Health Sciences
   4301 Jones Bridge Road
   Bathesda, Maryland 20814-4799.

Reference: Strockbine, N.A., M.P. Jackson, L.M. Sung, R.K. Holmes and A.D. O'Brien, 1988. Cloning and sequencing of the genes for Shiga-toxin from *Shigella dysenteriae* type 1. J. Bacteriol. 170, 1116-1122.

2. pSHT23 carries a EcoRI fragment of about 4 kb containing the A and B genes. pSHT23 is a pUC18 derivative.

Reference: The same as pSLT318.

Ricin from *Ricinues communis*
==========

Ricin gene sequence is similar to SLT I.

Reference: Halling, K.C., A.C. Halling, E.E. Muray, B.F. Ladin, L.L. Houston and R.F. Weaver. 1985. Cloning and characterization of a ricin gene from *Ricinus communis*. Nucleic Acids Res. 13, 8019-8033.

REVIEWERS' COMMENTS

Reviewer 1

The construction of an effective Shigella vaccine strain which can be given orally is an important objective. The PI in this grant has made considerable progress in developing a vaccine strain by showing that TSF21, a *thyA*, temperature-sensitive mutant is protective and avirulent when tested in animal models.

This grant proposes to expand on this work by identifying the molecular basis for the mutations in TSF21, and using this knowledge to construct a Shigella dysenteriae vaccine. In addition they plan to delete the *tox* gene from S. dysentariae.

The rationale behind this work is sound. There are however some problems with the experimental design. Ideally, a vaccine strain should be constructed with defined mutations which cannot revert The *thyA* mutation in TSF21 was obtained by trimethoprim resistance. The molecular nature of these lesions is unknown. The temperature-sensitive mutation on TSF21 was obtained by UV radiation. During this process other mutations may well have occurred. For these reasons, the PI would be better off abandoning the strain he has reconstructed and remaking a *ThyA* mutant using a more clearly defined method of mutagenesis.

There are many genetic tools available for using transposon mutagenesis to generate mutations in Shigella. Yoshikawa et al (Molecular Microbiology, 1991, 5, (9), 187-195) and Timmis (Infectional and Immunity, 43:391-396) contain useful protocols for making such mutants. Yoshikawa in fact has generated a *Thya* mutant in this manner. Transposon mutations are easily generated, mapped and cloned and could provide a good basis for constructing a deletion mutant. Alternatively the PI could generate a S flexneri vaccine strain using a cloning strategy similar to the one described for S. dysentariae. Constructing a new S. flexneri strain with a defined lesion would be a better investment of time than characterizing TSF21.

The collaboration with Denis Kopecko's laboratory at Walter Reed enhances this proposal: this group should be able to provide expertise lacking in the PI's group.

Project title: Development of a vaccine against shigellosis.
.....
.....

Principal Investigator(s): .....

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	✓		
Adequacy of Project Design		✓	
Suitability of Methodology		✓	
Feasibility within time period	✓		
Appropriateness of Budget	✓		
Potential value to field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification:
- on technical grounds ☑
- on level of financial support ☐

I do not support the application ☐

Dr. Moyenul Islam
Laboratory Sciences Division
Internation Centre for Diarrheal Disease Research
GPO Box 128
Dhaka - 1000
Bangladesh

Dear Dr. Islam:

This letter concerns my impressions of a project grant proposal entitled "Development of a Vaccine Against Shigellosis".

Your laboratory has made impressive progress in the development of a shigella vaccine, and the work should be supported. A budget of less than $100,000 a year is very modest indeed.

I have a few comments. Please take into account that I am an old man, and what I say may not be correct.

a. I assume that once the laboratory tests are done, one would want to be able to assess the vaccine in human beings. Therefore, I would take care in selecting the parent strain from which the vaccine is constructed. The S. flexneri Y strain which is described in tetracycline-resistant (probably transmissable) and trimethoprim-resistant (page 9). I doubt that such a strain could be used as a vaccine in humans in the United States. It is something to be considered when constructing the Shiga vaccine.

b. Thy A probes are available and one should be able to determine whether or not a deletion in the gene has occurred. Nevertheless, anything that is learned about the vaccine strain, including sequencing of the thy A gene, is of value.

c. I can find nothing in the published paper or in this proposal where any attempt is made to determine whether the vaccine strain is or is not invasive. Just to state that the strain has the 140 Mdal plasmid is not sufficient to show invasiveness. Certainly the vaccine can be grown at 30° C and then incubated at 37° C. Samples can be taken and tested in HeLa cells for invasiveness and in Western blots for the expression of invasion proteins. Without such data, it could be argued that a non-invasive strain might give protection under the conditions of assay. Then it would not be necessary to make the strain temperature sensitive and thy A; one would just need a deletion of the invasive genes.

d. One comment on the Shiga toxin genes which is noted on page 27: unless you have P-3 facilities in your laboratory, it would not be possible for you to obtain this clone from Nancy Stockbine.

e. My last comment has to do with the accuracy of Table I. E. coli Shigella hybrid vaccines with the E. coli xyl + - rha+ region gave excellent protection in monkeys.

RESPONSE TO REVIEWERS' COMMENTS

I wish to respond to the two reviews that have been made available to me on my protocol entitled "Development of a vaccine against shigellosis". One reviewer has used the check-list. To his review I assign the number 1; to the other, number 2.

REVIEWER 1

The major comments of this reviewer has been presented in paragraph 2 and 3. The reviewer seem to have supported the strategy proposed by us to develop defined Tox$^-$ *thyA* mutants of *S. dysenteriae* 1. Concerning the strain TSF21 his view is that this strain should be abandoned in favour of making a new more defined mutant. The former suggestion is based on two points -- (i) the strain was subjected to one round of UV mutagenesis and hence it may have in it other undetected mutations, and (ii) apparent difficulty in sequencing the *thyA* gene. Both of these points are important. No auxotrophic mutations other than thymine-auxotrophy has been found in this strain. Despite the possibility of undetected mutations being present in the strain, we have found the strain to provide consistent protection and has proved to be highly immunogenic and avirulent. If the proposed sequencing work demonstrates a deletion in the gene, criticism about the 'undefined' nature of this mutant would largely be alleviated.

Thus, while we propose not to abandon the strain because so much data are now in our hands on safety and efficacy of the strain, we will simultanously work towards generating deletion mutants by transposon mutagenesis.

REVIEWER 2

This reviewer has supported the proposed analysis of the *thyA* gene in strain TSF21 by sequencing and using DNA probes.

One of his concerns is on resistance of the strain to tetracycline and trimethoprim which is quite valid. While it may not be possible to remove trimethoprim resistance from the strain, we will try to obtain a tetracycline-sensitive derivative by ampicillin enrichment. Tetracycline-resistance of this strain as to its transmissibility has not been tested by us. We appreciate this observation and propose to do the test.

Regarding proof of invasiveness of the vaccine strain, we should draw attention to the following paper which is now in press and where we have shown that the strain is invasive *in vivo*:

> M.M. ASHRAF, D.K. GIRI, H.V. PATRA, P. KHANDEKAR, ZIA U. AHMED AND G.P. TALWAR., 1991. POTENTIALS OF *SHIGELLA FLEXNERI* STRAIN TSF21 AS A CANDIDATE VACCINE AGAINST SHIGELLOSIS: SAFETY, IMMUNOGENICITY AND PROTECTIVE EFFICACY IN BONNET MONKEYS. FEMS MICRIBIOL. IMMUNOL.

To the comment on Shiga-toxin gene and use of P-3 facilities, we wish to indicate that intact functional Shiga-toxin gene will not be used. Rather, only a sub-unit of the Shiga-like toxin which does not require a P-3 facility because in this case one is not dealing with a functional toxin gene, will be used.

Vaccine

Protection of adult rabbits and monkeys from lethal shigellosis by oral immunization with a thymine-requiring and temperature-sensitive mutant of *Shigella flexneri* Y

Zia U. Ahmed, Mahfuzur R. Sarker and David A. Sack*

As an approach to the development of attenuated strains, thymine-requiring (Thy⁻) and temperature-sensitive (Ts⁻) single and double mutants of Shigella flexneri *Y have been isolated by u.v. mutagenesis and selection. The mutants retained the 140 MDa plasmid, were Sereny-negative and avirulent in 'conditioned' rabbits and in monkeys. Rabbits and monkeys immunized orally with two doses of the mutants were solidly protected against disease and lethality when challenged with the homologous virulent strain. The immunized animals had higher levels of antibodies reactive to the lipopolysaccharide of the wild type strain and also a shorter duration of shedding of the challenge bacteria. It was concluded that Thy⁻ and Ts⁻ mutants of* Shigella *spp. are highly attenuated and immunogenic to be considered for further studies towards the development of vaccine candidates.*

Keywords: *Shigella flexneri*; attenuation; thymine-auxotrophy

Introduction

It is generally believed that immune protection against enteric infections is likely to be better achieved by oral live vaccines. Attempts to develop a live vaccine against shigellosis have, in general, proceeded along two paths. One is the carrier strain approach in which the genes from *Shigella* spp. that code for antigen production were transferred to non-pathogenic *E. coli* K-12 strains or to the *Salmonella typhi* strain Ty21a. The other approach involves the genetic attenuation of the *Shigella* organism such that the strain is no longer virulent but colonizes the intestine sufficiently to induce a protective local immune response.

Examples of the first approach have been documented in the literature[1,2] and some vaccines developed by this approach have shown promise in animal and volunteer studies. To date, however, these have not been found to induce sufficiently consistent protection to proceed to a field trial. The second approach has also been used. The streptomycin-dependent mutants of *Shigella* spp. used by Mel and co-workers[3,4] established that protection was possible by such attenuated strains. Recently, a more defined attenuated mutant, an aromatic metabolite dependent *aroD* mutant of *Shigella flexneri* Y has been tested in monkeys for safety and protective potential[5], with promising results.

Bacterial Genetics Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), GPO Box 128, Dhaka 1000, Bangladesh. *Present address: Division of Geographic Medicine, The Johns Hopkins University of Hygiene and Public Health, Baltimore, MD 21205, USA.
(Received 25 May 1989; revised 4 July 1989; accepted 11 July 1989)

0264-410X/90/020153-06

Attenuating effects of mutations leading to a requirement for adenine (*purA* mutation) or thymine (*thyA* mutation) have been studied in *Salmonella* spp.[6,7]. It is not known whether *thyA* mutations would cause significant attenuation in *Shigella* spp. The *purE* mutation in *S. flexneri* 2a did not cause attenuation[8]. Thymine-requiring (*thyA*) mutants of *Shigella flexneri* Y have been isolated by the 'thymine-trimethoprim' selection[9]. A *thyA* mutant expressing temperature-sensitive growth was also developed. These mutants were studied for safety and their ability to confer protection in a rabbit model of experimental shigellosis and in a small number of monkeys. The results of the study are reported in this communication.

Materials and methods

Organisms and culture methods

An isolate of *Shigella flexneri* SH-4 obtained from the Clinical Research Centre of ICDDR,B was used to derive the mutants. The strain of *S. dysenteriae* 1 (AC 9624) was obtained from ICDDR,B Culture Collection. The strain SH-4 is a serological variant of type Y, as determined with monoclonal antibodies[10]. It is resistant to tetracycline but sensitive to ampicillin, chloramphenicol, penicillin, nalidixic acid, trimethoprim, gentamicin and kanamycin. The strain required 0.01% yeast extract for rapid growth in a minimal medium (MA)[11]. Unless otherwise indicated, the MA medium was supplemented with 0.01% yeast extract. Trypticase soy agar (TSA) or broth (TSB) obtained from Gibco Laboratories, Inc. were supplemented with 0.6% yeast extract and used for routine cultivation of bacteria. All media were supplemented with 200 $\mu g\,ml^{-1}$ thymine to grow the Thy⁻

Table 1 Strains used in the study with their source or derivation

Strain	Source/Derivation
Shigella flexneri Y	
SH-4 (Wild type)	ICDDR,B culture collection
SH-7 (Thy⁻)	Derived from SH-4 by 'thymine-trimethoprim' selection
TSF-26A (Ts⁻)	Derived from SH-4 by u.v. mutagenesis and ampicillin enrichment
TSF-21 (Thy⁻Ts⁻)	Derivative of TSF-26A selected by 'thymine-trimethoprim' selection
S. dysenteriae 1	
AC 9624	ICDDR,B culture collection
E. coli K-12	
P 3140 (pULB113; Amp' Kan' Tet')	J. Hackett, University of Adelaide
χ 2913 (ΔthyA)	R. Curtiss III, University of Washington

strains. Bacteria used in feeding animals were cultivated in brain heart infusion (BHI) agar plates. Strains used in the study and their derivation are shown in *Table 1*.

Isolation of Thy⁻ and Ts⁻ mutants

Thy⁻ mutants were isolated by plating cells grown overnight in TSB (with 200 $\mu g\,ml^{-1}$ thymine) on to TSA plates containing 200 $\mu g\,ml^{-1}$ thymine and 30 $\mu g\,ml^{-1}$ trimethoprim. Trimethoprim-resistant colonies (frequency $\approx 10^{-6}$) were isolated, purified and tested for thymine requirement using a minimal medium. About 10% of the 200 trimethoprim-resistant colonies tested proved to be Thy⁻. The Thy⁻ single-mutant (SH-7) was isolated as above from the wild type strain SH-4. To isolate the Thy⁻Ts⁻ double mutant, the wild type strain was mutagenized with u.v. light and subjected to two cycles of ampicillin-enrichment. A 5 ml suspension of cells (washed in phosphate-buffered saline, PBS, pH 7.4) of strain SH-4 (10^8 c.f.u. ml^{-1}) was irradiated with a short wave (254 nm) u.v. light (model UVG-54, Ultraviolet Product Inc., USA) for 2 min from a distance of 35 cm, to kill about 95% cells. About 10^7 surviving cells were propagated overnight at 30°C in TSB. Of the resulting culture 100 μl was diluted 100-fold in TSB and shaken at the restrictive temperature of 39°C for 2–3 h. Ampicillin, 50 $\mu g\,ml^{-1}$, was then added and the culture shaken until clearing lysis occurred. The surviving cells were collected by centrifugation, washed twice in PBS and propagated overnight in TSB at 30°C. Cells from the resulting culture were subjected to a second ampicillin enrichment, following which a culture was developed at 30°C. Cells were spread on TSA plates, (about 200 c.f.u. per plate) and incubated at 30°C for 8 h and transferred to 39°C overnight. The colonies that developed were marked and the plates returned to 30°C. After overnight incubation, those colonies that developed fresh were isolated as potential Ts⁻ clones and purified. From the Ts⁻ mutant TSF-26A, a Thy⁻ derivative, the double mutant TSF-21, was isolated.

Adult rabbit model of experimental shigellosis

An adult rabbit model developed for *Vibrio cholerae* and enterotoxigenic *E. coli*[12] has been successfully adapted to *Shigella flexneri*[1] by following the modifications[13] introduced for oral inoculation of the bacteria[14]. Briefly, the procedure is as follows: Adult rabbits weighing about 2 kg were starved for 36–40 h during which period they were provided with water containing tetracycline (1 $mg\,ml^{-1}$). On an average, the animals consumed 100 ml of the water over the period of starvation. Then, at time 0, cimetidine 50 mg (kg body weight)$^{-1}$ was administered intravenously. At 15 and 30 min, 15 ml of a solution of 5% $NaHCO_3$ in water was administered by a gastric tube. Immediately after the second $NaHCO_3$ dose, the bacteria mixed with BHI broth (total volume 15 ml) were given by gastric tube followed by i.p. administration of 2 ml of a tincture of opium. The animals were returned to a normal diet and observed for 7 days for the development of disease or death. Animals prepared in this manner are referred to as 'conditioned' rabbits.

Immunization and challenge of rabbits

Conditioned rabbits and monkeys were 'immunized' by feeding at least 1×10^{11} cells of the mutant strains. After 1 week, a booster dose of the same strain was administered. Two weeks later (3 weeks in the case of monkeys), the animals were challenged by oral administration of at least 1×10^{11} cells of the virulent parent strain SH-4.

Other methods

Plasmid DNA was extracted by a rapid alkaline extraction method[15]. Lipopolysaccharide (LPS) was isolated by phenol–water extraction and purified by ultracentrifugation[16]. Salt aggregation test (SAT) was performed by the method of Rozgonyi *et al.*[17]. Strains were tested for cytotoxins in the polymyxin B extracts using a HeLa cell assay and for Shiga-toxin using ELISA[18].

A microtitre ELISA was performed to detect antibodies reactive to LPS in serum samples. The sera were pre-adsorbed to an *E. coli* isolate obtained from a rabbit before immunization.

Results and discussion

Characterization of the mutants

All the strains of *S. flexneri* Y used in the study carried the 140 MDa plasmid and seven other plasmids (36, 6, 4, 2.5, 1.9, 1.5 and 1.0 MDa). The strains appeared 'smooth' in their colony morphology. In a salt aggregation test[17], the lowest molarity of ammonium sulphate that caused aggregation was relatively high (2 M) for all the strains. The LPS profiles of the wild type strain and the mutants (*Figure 1*) as seen in a silver-stained polyacrylamide gel were identical indicating a similarity in carbohydrate O-side chains.

The Thy⁻ mutants (SH-7 and TSF-21) did not grow in minimal medium containing 0.01% yeast extract (in which the parent strain grew) unless the medium was also supplemented with thymine. This would suggest that the

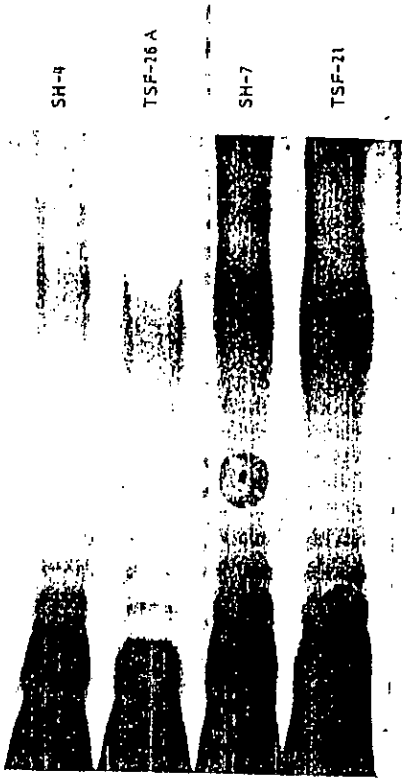

Figure 1 LPS profiles of the *Shigella* strains as seen in a silver stained SDS-polyacrylamide gel

strain TSF-21, which was subjected to u.v. mutagenesis and whose nutritional requirement was entirely satisfied by thymine, did not accumulate additional auxotrophic mutation(s) as a result of mutagenesis.

The genetic lesion responsible for the Ts$^-$ phenotype has not been identified. The Thy$^-$ mutants, however, are due to defects in the *thy*A gene. This was determined in the following way. The plasmid pULB113 (36 MDa; Amp^r, Kan^r, Tet^r), which is an RP4::mini-Mu element, is known to promote chromosome transfer and formation of R-prime elements[19]. The plasmid from *E. coli* strain P 3140 was introduced to a wild type *Shigella dysenteriae* 1 strain (AC 9624) by conjugation. An exconjugant was selected and purified. It was shaken at 37°C until mid-log phase of growth and transferred to 43°C for 2 h to induce the Mu functions. The culture was then mated with the thymine-requiring *E. coli* K-12 strain χ2913 carrying the gene Δ*thy*A 752. The mating mixture was plated on to MA plates without yeast extract (to counter select the *Shigella* parent) and containing ampicillin, kanamycin and tetracycline. All the 50 exconjugants tested were thymine-dependent. These Thy$^+$ derivatives carried a plasmid (presumed to be *thy*A$^+$ RP4::mini-Mu) similar in size to pULB113 (results not shown) which was then transferred by conjugation to the thymine-requiring strains of *Shigella*, strains SH-7 and TSF-21, selecting thymine-independent derivatives. These derivatives also acquired, as expected, a plasmid similar to pULB113 (results not shown).

Stability of the mutants

The Ts$^-$ mutant TSF-26A was relatively unstable. It produced revertants at an appreciable frequency when grown at elevated temperatures. The Thy$^-$ mutant SH-7 was, however, highly stable and so also was the Thy$^-$Ts$^-$ double mutant TSF-21 (*Table 2*).

In order to examine whether mutagenesis would enhance the frequency of revertants, cultures were mutagenized with u.v. light. Approximately 5×10^6 cells, suspended in 5 ml of PBS were u.v. mutagenized. About 5×10^4 surviving cells were propagated overnight in TSB under appropriate conditions of thymine supplementation and incubation temperature. The frequency of revertants in this culture was determined. The thymine-requiring mutant SH-7 failed to produce any revertants in approximately 10^7 cells tested. The double mutant TSF-21 was mutagenized as above and plated on minimal agar plates containing thymine and incubated at 39°C to score Ts$^+$ revertants or on minimal agar plates lacking thymine and incubated at 30°C to score Thy$^+$ revertants. Ts$^+$ revertants appeared at a frequency of 10^{-5} (same as that of the parent strain TSF-26A), while no Thy$^+$ revertants were detected in 10^9 cells tested.

Growth and the level of thymine requirement

Growth of the Ts$^-$ mutant TSF-26A in a broth at 30°C and the effect of a temperature up-shift on subsequent growth of the culture was studied by the procedure of Hooke *et al.*[20]. The up-shift from 30°C to 39°C caused growth to continue for about 3 h following which there was a complete cessation of growth (*Figure 2*). The strain thus displayed a 'coasting' phenotype[20] at the restrictive temperature. Growth at 30°C and that after an up-shift to 37°C was similar. Growth after up-shift to 38°C was not, however, tested.

The level of thymine that the *thy*A mutants (SH-7 and TSF-21) required for growth in a minimal medium was high. Addition of yeast extract to MA agar medium up to 0.1% (w/v) was not sufficient to support growth of these mutants without the concomitant addition of thymine to 50 μg ml^{-1} (395 μM). These mutants may thus be classified as super-high thymine-requiring mutants. It is known that from such strains low-thymine-requiring derivatives may develop[21]. The proportion of cells in a culture of strain SH-7 that were able to form colonies in minimal agar medium containing decreasing levels of thymine were determined. Thymine concentrations down

Table 2 The frequency of Ts$^+$ and Thy$^+$ revertants in the mutants

Strain	Conditions under which reversion was determined	Revertants scored	Reversion frequency
TSF-26A (Ts$^-$)	TSA plate at 39°C	Ts$^+$	10^{-5}
	TSA plate at 37°C	Ts$^+$	10^{-4}
SH-7 (Thy$^-$)	Minimal* agar without thymine at 37°C	Thy$^+$	$<5 \times 10^{-9}$
TSF-21 (Thy$^-$Ts$^-$)	(a) TSA + thymine at 39°C	Ts$^+$	10^{-5}
	(b) Minimal* agar without thymine at 30°C	Thy$^+$	$<10^{-9}$
	(c) Minimal* agar without thymine at 39°C	Thy$^+$Ts$^+$	$<5 \times 10^{-10}$

Cells obtained from overnight TSB cultures developed under permissive conditions (30°C for strain TSF-26A, 37°C with 200 μg ml^{-1} thymine for strain SH-7, 30°C with 200 μg ml^{-1} thymine for the Thy$^-$Ts$^-$ double mutant TSF-21) were washed in PBS and plated as indicated
*The minimal agar contained 0.01% yeast extract

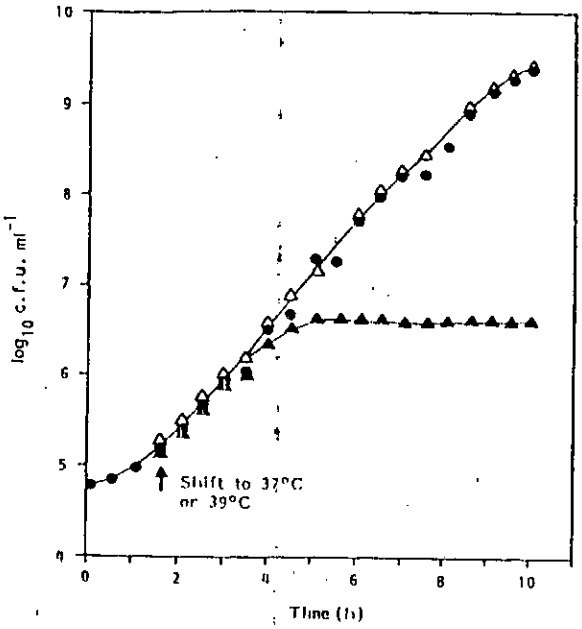

Figure 2 Growth of the Ts⁻ strain TSF-26A at 30°C and after a temperature up-shift to 37°C and 39°C. ●, 30°C; △, shift to 37°C; ▲, shift to 39°C

to 50 $\mu g\ ml^{-1}$ did not cause any reduction in the number of colonies recovered, but a variability in colony size was noted at this low level of thymine. At concentrations of 20 $\mu g\ ml^{-1}$ or less, no colonies developed in 10^6 cells tested.

Avirulence of the mutants

The mutants TSF-21 and SH-7 did not produce keratoconjunctivitis in the guinea-pigs in 7 days after 10^8 cells had been deposited in the eye while the wild-type strain produced the reaction within 48 h. Neither the wild type strain nor the mutants produced detectable Shiga-toxin as determined by a sensitive ELISA[18].

Safety of the strains in a rabbit model of experimental shigellosis was studied by oral administration of bacteria and observing the animals for disease or death. In animals challenged with the wild type strain, signs of illness involved an initial indisposition followed by watery or mucoid diarrhoea at about 18 h. Death usually occurred within 24–48 h, although some animals died without any diarrhoea. Results of an experiment in which the animals were fed with the wild type strain and the mutant derivatives are presented in *Table 3*. None of the animals fed with the mutant strains showed any signs of illness and no lethality occurred, but the wild type strain caused significant lethality. The mutant bacteria were shed, as seen in cultures of rectal swab samples, for 1–2 days and the wild type bacteria (by the surviving animals) for 5–7 days.

Monkeys were also fed with 10^{11} cells of strain TSF-21. None of the three monkeys tested produced any symptoms. Cultures of rectal swab samples obtained from these animals became free of the bacteria after 1 day.

Immune protection of rabbits and monkeys

The data, presented in *Table 4*, indicate that immunization with the Thy⁻ mutant SH-7 and the Thy⁻Ts⁻ double mutant TSF-21 provided solid protection. Antibody response in the serum of rabbits immunized with strain TSF-21 and the pattern of shedding of the challenge bacteria were examined. In five paired sera, IgG antibodies to LPS increased significantly. Mean absorbance values for the ELISA with a serum dilution of 1:25 were 0.11 for the pre-immunization and 0.40 for postimmunization samples (paired t-test, $p = 0.02$). There was no increase in IgM antibodies. The rectal swab cultures of animals immunized by each of strain SH-7 and TSF-21 became free of the challenge bacteria (SH-4) within 24 h while the unimmunized animals that survived the challenge shed the challenge bacteria for about 7 days.

A preliminary trial of the strain TSF-21 was carried out in a small number of monkeys for its protective potential. Six rhesus monkeys, each weighing about 4 kg, were caged separately. Rectal swab samples of each animal were cultured every week for 4 weeks prior to immunization. No *Shigella* organisms were detected in these animals. The monkeys were immunized by feeding 10^{11} cells of strain TSF-21 (suspended in 20 ml of BHI broth) following gastric acid neutralization by a prior oral administration of a solution of 5% sodium bicarbonate (20 ml). A booster dose of 10^{11} cells was similarly administered 1 week later. The control animals were identically treated except that sterile BHI broth was

Table 3 Effect of oral administration of cells of the mutant strains and the wild type parent strain on the survival of conditioned adult rabbits

Strain	Dose administered (c.f.u.)	No. of animals used	No. of animals that survived
SH-4 (Wild type)	10^{10}	32*	12
SH-7 (Thy⁻)	10^{11}	10	10
TSF-21 (Thy⁻Ts⁻)	2×10^{11}	12	12

Strains were grown in Brain Heart Infusion agar for 24 h (48 h for strain TSF-21), harvested by sterile cotton swab and suspended in PBS at room temperature to about 10^{11} c.f.u. ml^{-1}. Dilutions were plated for viable counts and the suspension was fed to the rabbits immediately
*Sum of five independent experiments. As a control in two of these experiments a group of rabbits, usually 3-5, were identically treated except that these rabbits were fed with only sterile BHI broth

Table 4 Protection of rabbits immunized by oral feeding of cells of the mutant strains from a virulent challenge of the wild type strain

Immunizing strain and dose used	No. animals used/ No. that survived challenge*		
	Immunized group	Control group	Test of significance*
SH-7 (Thy⁻) 7×10^{11} c.f.u.	13/13	8/3†	$p = 0.001$
TSF-21 (Thy⁻Ts⁻) 2×10^{11} c.f.u.	9/9‡	6/2	$p = 0.003$

*Challenged with at least 1×10^{11} c.f.u. of strain SH-4; *Immunized groups individually compared with the pooled control groups by a Fisher's exact test (one-tailed); †This value is from an experiment that had not been run in parallel with the immunized group; ‡One of these animals developed a mild watery diarrhoea for 1 day

fed instead of bacterial inoculum. Immunization did not produce any symptoms in any of the monkeys. Three weeks after the second feeding, all the six animals were challenged with a relatively massive dose (3×10^{11} cells) of the parent strain SH-4.

All the three unimmunized monkeys developed classical bacillary dysentery with blood and mucus in the stool within 24 h after challenge. Aneroxia, dehydration and vomiting were also conspicuous on the day following the challenge and these became severe after 48 h. At this time, treatment of these three animals with ampicillin injection was initiated and the animals soon recovered. In contrast, none of the three immunized animals showed any signs of illness, except that one animal had two semi-solid stools on the day after challenge.

The Thy^-Ts^- was shed in the stool for 1 day. The Aro^- mutant tested by Lindberg *et al.*[5] was shed for 2–4 days. The immunized monkeys had significantly higher titres of IgG antibodies in the serum (about tenfold higher than control animals), but showed no increase in IgM or IgA antibodies (unpublished data). The results are similar to those reported by Lindberg *et al.*[5] with the Aro^- mutant. However, in our study, the challenge strain was detected in the rectal swab cultures of the immunized animals for only 2 days, a duration considerably less than that (about 17 days) found by Lindberg *et al.*[5] after immunization with the Aro^- mutant.

The results with the Thy^-Ts^- double mutant indicate that it was safe and protective both in rabbits and in monkeys. It is of interest to note that the temperature-sensitive mutation in the latter strain did not diminish its protective potential. This may seem paradoxical in view of the fact that at 30°C (the temperature at which the mutant is grown) virulence traits are not expressed well[22,23]. However, it is important to consider, several points in this regard. When fed, the temperature-sensitive bacteria probably undergo limited proliferation (due to 'coasting') in the gut (rectal temperature in rabbits is 37°C, that of the monkeys tested ranged from 38–40°C) during which key protective antigens are probably expressed. Also, there are often differences in the expression of genes *in vitro* and *in vivo*. For example, in *Vibrio cholerae* the *tcp*A gene is expressed well *in vitro*[24] only at a temperature below 30°C, but it is thought to be a colonizing factor for *V. cholerae*. Furthermore, differences in the conditions of growth *in vitro* and those existing *in vivo* can also be quite considerable and may influence bacterial proliferation. Thus, the fact that the mutant is temperature-sensitive and is to be grown at 30°C need not necessarily imply that it will be poorly immunogenic and will express diminished protective potential. In fact, our results do indicate that this is not the case with the strain TSF-21. The mechanism by which the strain offers significant protection can only be conjectured at the present time.

The Ts^- mutation in strain TSF-21 is leaky with a reversion frequency of 10^{-4} to 10^{-5} in the temperature range of 37–39°C. The *thy*A mutation, however, is stable and may represent a deletion in the gene, but this remains to be formally demonstrated. It is known that positive selection for the loss of functions such as the one used here for the isolation of the *thy*A mutants may often be associated with a deletion of chromosome regions[25,26].

Conclusion

The strain TSF-21 has a theoretical reversion frequency $< 10^{-14}$ ($10^{-5} \times 10^{-9}$) when grown under the restrictive conditions (at 39°C and thymine-deprivation). In the human intestine where the temperature is expected to be in the range of 37–38°C and intracellular levels of thymine low, the strain may not produce revertants at a frequency higher than 10^{-14}.

A stable Thy^- auxotroph (such as one with a deletion in the *thy*A gene) in which Thy^+ revertants cannot be recovered *in vitro* at the limit of detection, is to be considered safe. However, such a strain would be more attractive as a vaccine candidate if it carries an additional mutation that gives it, at least theoretically, an 'extra' margin of safety. Although, safety can be enhanced by two stable auxotrophic mutations, this may make the strain too crippled, adversely affecting its protective potential. A temperature-sensitive mutation which is often unstable, may provide additional safety without affecting protective potential, as our results with strain TSF-21 indicate. We believe that Thy^-Ts^- mutants of *Shigella* are sufficiently safe and immunogenic to be considered for further studies (which ought to include isolation of defined Δ*thy*A mutants) towards the development of live oral candidate vaccines against shigellosis.

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Potentials of *Shigella flexneri* Y strain TSF21 as a candidate vaccine against shigellosis: safety, immunogenicity and protective efficacy in Bonnet monkeys

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1. SUMMARY

A thymine-requiring and temperature-sensitive mutant of *Shigella flexneri* Y was tested in Bonnet monkeys for safety, immunogenicity and protective efficacy. A dose of 10^{11} cells when fed orally mimicked natural infection in having invaded epithelial cells, but was otherwise clinically non-reactogenic. Animals immunized with two oral doses, each dose consisting of 1×10^{11} mutant bacteria, were fully protected when challenged, with respect to the lack of any clinical symptoms or detectable histological abnormalities in the intestinal mucosa. Unimmunized animals when similarly challenged developed frank dysentery and the intestinal mucosa showed severe histological abnormalities. Titres of serum antibodies increased by about 11-fold of the base level in animals immunized with a dose of 10^{11} cells, but not with lower doses. The challenge bacteria appeared to be phagocytised by macrophages. In some monkeys of a particular group, congestive patches were seen in the stomach, but not in any other part of the gut, after the animals were fed with the virulent parent strain. The lesions were relatively severe in the immunized groups of animals.

2. INTRODUCTION

Shigellosis, caused by the invasive enteric pathogen *Shigella*, appears to be an immunizing disease, amenable to control and prevention by a suitable vaccine. Strategies considered useful in interrupting transmission of the disease such as improvements in sanitation and personal hygiene possibly cannot be rapidly implemented in the developing countries due to socio-economic constraints. Furthermore, the unusual propensity of *Shigella* to fast acquire resistance to antibiotics makes treatment and case management difficult. An effective and practical vaccine is thus consid-

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ered to be an attractive option. Based on the knowledge of the previous vaccines that have been tested, and the pathophysiology of the disease, it is generally believed that a live oral vaccine consisting of an attenuated mutant of the pathogen is likely to be effective.

We have recently reported [1] on the construction and characterization of thymine-requiring (*thyA*) and temperature-sensitive (Ts^-) single and double-mutant of *Shigella flexneri* Y together with results on safety and protection in conditioned adult rabbits. Both the *thyA* single-mutant (strain SH7) and *thyA* Ts^- double-mutant (strain TSF21) were found to be safe when fed orally and provided solid immune protection against a lethal challenge of the virulent parent strain. In monkeys, the *thyA* Ts^- double-mutant was, as in rabbits, safe and protective. The experiment with monkeys described in the above report was a limited examination of the symptomatic changes in a small number of monkeys. We have extended the trial of the mutant in larger number of monkeys and report the results in this communication.

3. MATERIALS AND METHODS

3.1. Organism

Derivation of the *Shigella flexneri* Y double-mutant (TSF21) from the wild-type parent strain SH4, its culture, maintenance, in vitro properties and method of preparing inocula for immunization and challenge have been described elsewhere [1].

3.2. Immunization and challenge

Bonnet monkeys (*Macaca radiata*) weighing 3.5–5 kg were used in the experiments. A total of 19 monkeys were used – 3 animals to study the reactogenicity of a dose of 1×10^{11} cells of strain TSF21 and 16 to determine immunogenicity and protective dose. Each monkey was separately caged. Rectal swabs from each monkey were cultured once a week for 4 weeks before experiments. Animals that were free of *Shigella* organisms were used. During feeding, monkeys were restrained in the cage. Gastric acid was neutralized by orally administering 15 ml of a solution of 5% (w/v) sodium bicarbonate by a syringe. Bacterial inoculum suspended in 15 ml brain heart infusion broth was administrated similarly, 10 min after gastric acid neutralization. After the first immunization, a booster dose was administered 7 days later. Control animals were identically treated except that instead of bacterial inoculum these animals were given 15 ml of sterile medium. Fourteen days after the booster dose, all monkeys were orally challenged each with approximately 10^{11} cells of the virulent parent strain SH4.

3.3. Coloscopy and histopathology

All monkeys were subjected to coloscopic examination and biopsy samples were collected from the proximal colon 48 h before challenge. Animals were sacrificed 7 days after challenge for an examination of the gastrointestinal tract. Portions of the colon, intestine and stomach were collected and stored under liquid nitrogen for histopathological studies. Tissue sections were stained by hematoxyline for histological examination.

3.4. Immunological methods

Antibodies (IgA and IgG) reactive to purified LPS of the wild-type strain were detected in the sera and gut washings by an ELISA. Titres are expressed as the reciprocal of the highest dilution of the test sample that gave an antigen binding equal to half that of a standard high titre hyperimmune serum.

Bacteria were visualized within tissue sections by peroxidase–antiperoxidase (PAP) and indirect immunofluorescence techniques using a monoclonal antibody (Y-5) specific for *Shigella flexneri* [2] and another monoclonal antibody (PA_2B10/6a) reacting to *Salmonella* and *Shigella* [3].

4. RESULTS

4.1. Invasive ability of the mutant

The mutant strain TSF21 does not produce a positive Sereny's reaction in the Guinea-pig's eye [1]. To test whether the strain is able to invade intestinal cells of monkeys, three animals were fed each with a single dose of 1×10^{11} cells. The animals were sacrificed 48 h after feeding. Ex-

Fig. 1. PAP-stained epithelial cells (using the monoclonal antibody $PA_2B10/6a$) obtained from the colon of a Bonnet monkey 48 h after oral administration of *S. flexneri* Y strain TSF21. Dark patches inside the cells (arrows) represent significant bacterial growth.

amination of tissue sections of the intestine after PAP staining revealed the presence of bacteria within the epithelial cells and in macrophages (Fig. 1). The monoclonal antibodies Y-5 and $PA_2B10/6a$ also produced strong immunofluorescence in the cells (results not shown), adding to the identity of the bacteria. No gross or microscopic abnormalities were detected in the gastrointestinal tract of these animals.

4.2. Safety and protection

Three groups of animals, four in each group, were immunized with different doses (10^7, 10^9 and 10^{11} cfu per animal) and observed for general symptomatic conditions such as diarrhoea, vomiting, fever, anorexia, depression and evidence of colic pain (hunched back posture). In none of the groups did any animal show any signs of indisposition. After challenge with the virulent parent strain, significant differences were observed between the three groups of immunized animals. Observations made over a period of 7 days after challenge are presented in Table 1. It is apparent that animals fed with a dose of 10^9 cells or lower reacted to the challenge in the same manner as did the control unimmunized animals and developed classical bacillary dysentery. Dysentery in these animals lasted for 3–4 days. Animals fed with a dose of 10^{11} bacteria, however, were solidly protected.

4.3. Pre-challenge and post-challenge histology of the intestine

Biopsy samples from the colon of all animals immunized with different doses of the mutant strain and from the control animals were obtained before challenge, that is, 12 days after the booster dose, using a coloscope and examined for any histopathological changes in the mucosa. All the animals had a normal mucosa except in one animal immunized with a dose of 10^{11} cells. In this animal, mild mononuclear cell infiltration in the mucosa and slight enlargement of a few crypts due to

Table 1

Symptomatic effects in immunized monkeys following a virulent challenge with 10^{11} cells observed over a period of 7 days

Immunization dose	No. of animals	Diarrhoea with blood/mucus [a]	Rectal temperature [b]	Anorexia, dullness, colic pain	Mortality
10^{11}	4	0	4 (102–103° F)	0	0
10^9	4	4	4 (102.2–103.8° F)	4	1 (4th day)
10^7	4	4	4 (102–103.8° F)	4	0
Control	4	3 [c]	4 (102–104° F)	3	0

[a] Symptoms lasted for 3–4 days.

[b] Normal rectal temperature in these monkeys ranged between 100 and 101° F.

[c] One animal in this group which did not produce any symptoms after challenge had a high pre-challenge IgG (reactive to LPS of the challenge strain) titre in the serum.

Table 2

Histopathology of the gastrointestinal tract 7 days after challenging with 10^{11} cells of the virulent parent strain

Histological changes	Immunization dose (cfu)			
	10^{11}	10^{9}	10^{7}	Control
Surface epithelial erosion/ ulceration	0	+ + +	+ + +	+ + +
Epithelial hyperplasia	+	+ + +	+ + +	+ + +
Interstitial inflammation	+	+ + +	+ + +	+ + +
Inflammatory engorgement of crypts	+	+ + +	+ + +	+ + +

0 = absent; + = mild; + + + = severe.

accumulation of inflammatory exudate were observed. Biopsy specimens of the colon when similarly examined 48 h after challenge also showed normal mucosa in animals that were immunized with 10^{11} cells and were protected. Animals immunized with lower doses showed gross morphological changes. These included haemorrhagic ulceration, dilation of the crypts and massive leukocyte infiltration in the colon. Changes in the intestine included congestion, proliferative necrotic enteritis and engorgement of crypts.

The gastrointestinal tracts of the animals were examined for gross changes and microscopic abnormalities after sacrificing the animals 7 days after challenge. Mild to severe enteropathic changes were observed. A summary of histopathologic changes observed in colon seven days after a virulent challenge is presented in Table 2.

4.4. Immune response

ELISA of the serum samples revealed significant increase in serum IgG levels in monkeys immunized with a dose of 10^{11} bacteria when tested 14 days after the booster dose (Fig. 2). The level further increased (to about 11-fold of the base level) 7 days after challenge. In animals immunized with 10^{7} or 10^{9} cells, increase in IgG titre 14 days after the booster dose was not significant; it was about 2-fold. Also, there was only a slight increase in IgG titre in these animals following challenge. IgA levels in the serum was also tested. At a serum dilution of 1 : 50, detectable levels of the antibody were not found in any of the animals.

4.5. Post-challenge reactions in immunized animals

A number of post-challenge findings are of interest. With PAP staining, using the monoclonal antibodies Y-5 and $PA_2B10/6a$, many phagocytic cells of the mucosa were found positive, suggesting invasion of these cells by the challenge bacteria. The number of such cells appeared directly related to the immunization dose, increasing with the dose. By an indirect immunofluorescence technique using the above monoclonal antibodies, the challenge bacteria could also be localized within the phagocytes of the protected animals immunized with a dose of 10^{11} cells. These results may thus indicate the possibility of cell-mediated immunity playing a role in the protection of these immunized animals. It is generally believed that immune protection against enteric infections is largely mediated by intestinal secretory IgA. However, in these animals, the IgA levels in the sera were not elevated. Neither was there any significant rise of IgA titres in the gut washings. Gut washings obtained 7 days after challenge gave a

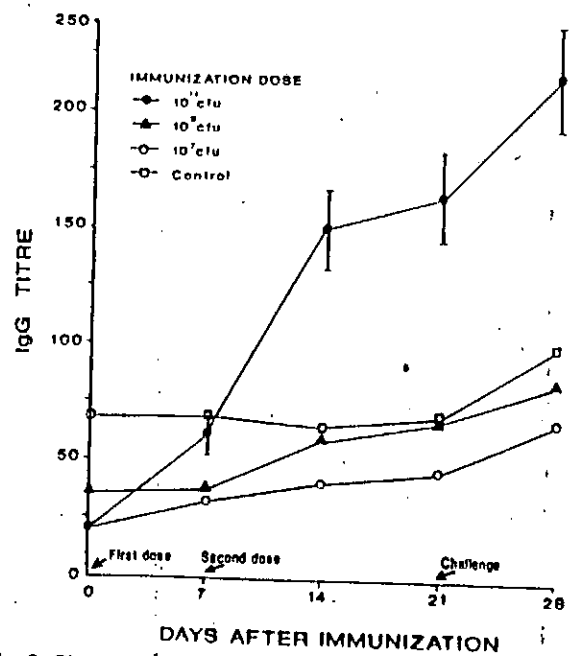

Fig. 2. Changes in serum IgG titres in Bonnet monkeys during the course of immunization and challenge. Titres of IgG reactive to the LPS of *S. flexneri* Y strain SH4 are expressed as reciprocal of the highest dilution of the test sample that gave an antigen binding equal to half that of a high-titre hyperimmune serum.

titre of 95 in the control animals, while the titres were 105, 125 and 136 in animals immunized with a dose of 10^7, 10^9 and 10^{11} bacteria, respectively.

A post-challenge reaction was seen in one group of monkeys. This involved a gross change in the stomach of the Bonnet monkeys (*Macaca radiata*) detected 7 days after a virulent challenge. This involved the development of relatively large congestive patches in the glandular parts of the stomach mucosa. In unimmunized animals the lesions were of much less intensity. Histologically, there were massive mononuclear cell infiltration, predominantly of lymphocytes and plasma cells in the mucosa and sub-mucosa, moderate congestion and edema. Bacterial invasion could not be demonstrated in sections of the stomach by histological assays using the monoclonal antibody Y-5. However, such lesions were not seen in any other part of the gut. Also, neither in a similarly treated group of 6 rhesus monkeys (*Macaca mulatta*) nor in a different group of 15 Bonnet monkeys did we find any evidence of such reaction (results not shown). Therefore, the nature of the congestive lesions which were seen in some Bonnet monkeys remains unexplained.

5. DISCUSSION

It is believed that a live vaccine is likely to be effective if it is able to mimic some aspects of natural infection. Invasion of epithelial cells by *Shigella* is an essential early step in the development of disease. An attenuated mutant may thus cause better immune stimulation if it retains the ability to invade the intestinal epithelium so that effective delivery of appropriate antigens to the intestinal lymphoid tissue is facilitated.

Formal et al. [4] reported that an attenuated *S. flexneri* 2a strain with spontaneous loss of virulence and loss of ability to invade the intestinal epithelium of monkeys offered poor protection against experimental challenge. A non-invasive *Escherichia coli–Shigella* hybrid also failed to protect when tested in human volunteers [5]. On the other hand, invasive hybrid derivatives (*E. coli* bearing somatic antigens of *Shigella*) were protective in monkeys [4]. In an efficacy trial in monkeys with another hybrid vaccine which was Sereny-negative but invaded HeLa cells, mild mucosal inflammation (suggestive of invasion) was detected and the vaccine provided significant protection [6]. Invasive ability of a live vaccine thus appears to be critical' for a protective immune response.

The high immunization dose required for protection of monkeys may not be unexpected because monkeys are much less susceptible to *Shigella* infection than humans. The infective dose in experimental monkeys is relatively high (at least 10^9 cells), while in human volunteers, significant infection can be produced with as little as 10^1–10^3 bacteria [7]. Lindberg et al. [8] tested an *aroD* mutant of *S. flexneri* Y in monkeys and reported solid protection after immunization with four doses, each consisting of about $2–3 \times 10^{10}$ bacteria. A mutant of *S. flexneri* 5 with impaired intracellular growth and spread, is also known to provide protection in monkeys after immunization with 3 doses, each consisting of 5×10^{10} cells [9].

The *thyA* Ts^- double-mutant of *S. flexneri* Y strain TSF21 is a potential vaccine candidate against shigellosis. Serotype Y carries the basic O-antigen polysaccharide structure common to the other five serotypes of *S. flexneri* [2] which thus bears the potential to provide cross-protection. Indeed, there are indications (unpublished results) that immunization with strain TSF21 may offer protection against the *S. flexneri* serotypes 2a and 3a. The strain TSF21 is sensitive to a range of commonly used antibiotics, has an LPS profile identical with that of the parent strain, and is Sereny-negative despite carrying the large invasion-mediating plasmid [1]. The degree of attenuation achieved in this strain appears to be largely due to thymine-auxotrophy. The Ts^- mutation with its high reversion rate is not likely to contribute significantly to the observed attenuation. Indeed, a Ts^- single-mutant when tested in conditioned rabbits proved to be as virulent as the parent strain (unpublished data). The Ts^- mutation with its reversion frequency of 10^{-5}, however, added an extra margin of safety to the double-mutant TSF21.

It is known that when wild-type cultures of *S. flexneri* are grown at 30°C, the cells loose the

ability to invade cultured cells and cannot produce a positive Sereny's reaction [10,11]. The mutant TSF21 with its permissive growth temperature of 30°C, however, expressed the important virulence trait of epithelial cell invasion and provided consistent immune protection in rabbits [1] and monkeys. The strain has a calculated frequency of $< 10^{-15}$ to revert to the wild-type level of virulence [1]. Although the lesion within the *thyA* gene has not been determined at the molecular level, it is probably a deletion as judged from genetic stability of the mutant. The candidate vaccine merits further studies towards defining the molecular lesion in the *thyA* gene before being considered for studies on its safety and immunogenicity in human subjects. The nature of the stomach lesions that were seen in the Bonnet monkeys remains unexplained. The fact that such lesions could not be detected in rhesus monkeys nor in a different lot of Bonnet monkeys tends to suggest that these do not reflect any reactogenicity of the vaccine strain.

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1. Which antibiotics it is sensitive to ?

2. Revert back ?

3. Stomach disinfections ?

4. Volunteer study ?

5 Monkey passage ...