53

...l Investigator ... Trainee Investigator (if any)

...tion No. 85-137 Supporting ...

...Study Isolation of temperature-sensitive mutants of Shigella dysenteriae 1 Evaluation of their colonizing and ...ective potential in adult rabbit

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

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

Source of Population:

(a) Ill subjects	Yes	No
(b) Non-ill subjects	Yes	No
(c) Minors or persons, under guardianship	Yes	No

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

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

Are subjects clearly informed about:

(a) Nature and purposes of study	Yes	No
(b) Procedures to be followed including alternatives used	Yes	No
(c) Physical risks	Yes	No
(d) Sensitive questions	Yes	No
(e) Benefits to be derived	Yes	No
(f) Right to refuse to participate or to withdraw from study	Yes	No
(g) Confidential handling of data	Yes	No
(h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure	Yes	No

5. Will signed consent form be required:

(a) From subjects	Yes	No
(b) From parent or guardian (if subjects are minors)	Yes	No

6. Will precautions be taken to protect anonymity of subjects Yes No

7. Check documents being submitted herewith to Committee:

___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
___ Protocol (Required)
✓ Abstract Summary (Required)
___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
___ Informed consent form for subjects
___ Informed consent form for parent or guardian
___ Procedure for maintaining confidentiality
___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:

1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

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

(PTO)

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

...

...ncipal Investigator

Trainee

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective:

Objective of the study is to isolate temperature-sensitive (ts) mutants of Shigella dysenteriae 1 and select a set of mutants with the ability to induce protection in an adult rabbit model.

2. Background:

Considerable interest and optimism have grown over recent years in developing live oral vaccine against enteric pathogens by using genetically attenuated strains. Various methods of attenuation are being examined with increasing hope of success. Attenuation can be achieved in a variety of ways but the choice would depend greatly 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 an extracellular product (toxin) in the disease is clear, one approach merits special consideration. That is, allowing the strain to invade but making it so crippled that it fails to proliferate and maintain itself in the body for very long. Such crippling could be brought about by introducing "suicidal blocks" and/or temperature-sensitive (ts) mutations.

Suicidal blocks currently being considered are (a) block in the galactose utilization pathway as exemplified by

Salmonella typhi Ty21a (Germainer and Furer, 1975) and (b) diaminopimelic acid (DAP) requiring mutants as reported in E. coli (Davis, 1952; Rhuland, 1957; Meadow et al., 1957). Both of these blocks make growth 'suicidal' that is, rapid cell lysis occurs. In the former case, the mutant accumulates toxic amounts of galactose-1-phosphate and uridine diphosphate galactose resulting in cell lysis. DAP auxotrophy causes the synthesis of a weak cell-wall because the component has a restricted distribution and is localized in the cell-wall. However, since DAP auxotrophy does not interfere with cellular growth, the mutation causes rapid cell lysis.

Likewise, temperature sensitivity can provide effective attenuation. Strains can be isolated that are unable to grow at the body temperature of the host (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 transformation can be used to introduce several ts mutations in one strain thus rendering reversion frequency negligible (Hooke et al., 1985).

To the best of our knowledge, isolation of temperature-sensitive mutants of shigella has not been reported. Methods

of conventional genetics can be used fairly easily to isolate such mutants. But for these mutants to have any vaccine potential it is necessary that they retain the ability to partially colonize the gut which, in the case of shigella, probably means invasion. Invasiveness is likely necessary for the strain to optimally stimulate the local immune system.

Ability of an attenuated strain of Shigella to colonize the gut is thus an important contingency on which would depend in a major way the vaccine potential of such strains. Colonization ability is, therefore, an aspect which, we believe, merits investigation. It is in this context that we wish to study the colonizing and protective attributes of a range of Shigella dysenteriae 1 temperature-sensitive mutants.

3. Rationale:

Temperature - sensitivity offers an important attenuation mechanism in pathogenic bacteria. Since several independent temperature-sensitive mutations can be combined into one strain, the method is capable of generating highly stable and safe strains for use as a live vaccine. However, a vaccine strain must retain in the process some important characteristics that would enable it to trigger immunity. For invasive enteric pathogens such as Shigella, a ts mutant should be able to colonize the gut at the restrictive temperature for a limited period of time.

We presume that it is possible to isolate ts mutants which will colonize the gut for a period of time long enough to stimulate local immunity before the strain is cleared from the system. Such strains are also expected to be protective. The present protocol is designed to test this conjecture.

We have selected S. dysenteriae 1 for this study because of its virulence and epidemic potential and because vaccine development against this strain is identified by ICDDR,B and WHO as a high priority area.

B. SPECIFIC AIM

The specific aim is to isolate temperature-sensitive (ts) mutants of Shigella dysenteriae 1 and select a set of mutants with the ability to induce protection in an adult rabbit model.

C. EXPERIMENTAL

(i) Isolation of ts mutants:

Temperature-sensitive mutants will be isolated by mutagenizing cultures of Shigella dysenteriae 1 with N-methyl-N-nitro-N-Nitrosoguanidine and subjecting the mutagenized culture to cycles of penicillin and cycloserine enrichment. Details of the procedure will be similar to those followed for the isolation of ts mutants of E. coli (Hooke et al., 1978). On the basis of the response of the isolates to restrictive temperature, the mutants will be classified as (a) "tight" - that is, complete cessation of

growth immediately after transfer to the nonpermissive temperature and (b) "Coasting" strains capable of limited proliferation after transfer to nonpermissive temperature following which, there is complete cessation of growth. These mutants will then be tested for their ability to colonize rabbit intestine.

Plasmid profile of these strains will be examined in order to determine if the strains still carry the large plasmid (M.W. about 140 Mdal) which is believed to carry determinants of invasiveness.

(ii) Determination of virulence and colonization potential.

Virulence of the ts mutants will be determined by using the Sereny test and by feeding conditioned rabbits (see below) an inoculum of size equal to an inoculum of the virulent parent that would kill 50-100% of the animals.

Colonization potential of the strains will be assessed in an Adult Rabbit Model. The rabbit colonization model (RCM) was developed for Shigella flexneri 6 (D.A. Sack, personal communication). The procedure was as follows. Rabbits are fed with at least 25 mg of tetracycline over a 36h period prior to administration of bacterial inoculum. Then, at time 0, cimetine (50 mg/kg body wt) is administered intravenously. At 15 and 30 min, 15 ml of a solution of 5% $NaHCO_3$ is fed. Immediately after the second dose, 15 ml of bacterial suspension is fed which is followed by i.p. injection of 2 ml

of a tincture of opium. The animals are sacrificed 18 hour and bacteria in the intestine are quantitated (Gray et al., 1983). We also intend to carry out a histological examination of the small and large intestine for evidence of colonization and pathological conditions.

The adult rabbit model is currently being studied by us with Shigella dysenteriae 1 and will be used in the present study.

(iii) Protection test.

A preliminary evaluation of the protective ability of the ts mutants will be carried out in the following manner. Rabbits will be given two inocula of the ts mutant containing 10^{10} CFU, each separated by a time interval of 21 days. They will then be challenged with two virulent homologous inocula - one equivalent to the LD_{50} dose and the other one log higher than LD_{50} dose. Death occurring within 7 days will be recorded.

(iv) Time Scale:

Mutant isolation:

November 1985 to February 1987.

Mutant testing:

March 1986 to June 1987.

D. SIGNIFICANCE

Temperature-sensitive mutation represents a potent route to achieving highly effective genetic attenuation and thus promises to be useful in the isolation of live vaccine strains. The present study is expected to answer whether ts mutants, despite the attenuation, nevertheless retain the ability to partially colonize the gut in experimental rabbit and also afford protection. The investigation thus would provide an indication as to whether this approach has a potential for generating live vaccine strains of Shigella.

E. FACILITIES REQUIRED

See Budget.

REFERENCES:

Cray, W.C. Jr., Tokunaga, E. and Pierce, N.F. 1983. Successful colonization and immunization of adult rabbits by oral inoculation with Vibrio cholerae 01. Infect. Immun. 41, 735-741.

Davis, B., 1952. Biosynthetic interrelations of lysine, diaminopimelic acid and threonine in mutants of Escherichia coli. Nature 169, 534-536.

Germainer, R. and Furer, E., 1975. Isolation and characterization of Gal E mutant Ty21a of Salmonella typhi: A candidate strain for a live, oral typhoid vaccine. J. Infect. Dis. 131, 553-558.

Meadow, P., Hoare, d.s. and Work, E., 1957. Interrelationship between lysine and -- diaminopimelic acid and their derivatives and analogues in mutants of Escherichia coli, Biochem. J. 66, 270-282.

Hooke, A.M., Bellanti, J.A. and Oeschger, M.P. 1985. Live attenuated bacterial vaccines: new approaches for safety and efficacy. Lancet, I, 1472-1474.

Hooke, A.M., Oeschger, M.P., Zeligs, B.J. and Bellanti, J.A., 1978. Ideal target organism for quantitative bactericidal assays. Infect. Immun. 20, 406-411.

Rhuland, L.E., 1957. Role of diaminopimelic acid in the cellular integrity of Escherichia coli. J. Bacteriol. 73, 778-783.

SECTION III - BUDGET - year 1
(November 1985 to October 1986)

1. PERSONNEL SERVICES:

Name	Position, time effort %		Cost, $
Zia Uddin Ahmed	P.I.	50%	2,351
David, A. Sack	Consultant		0
	Co-Investigator (pathologist)	25%	1,250
To be recruited	Research Officer, level 6	100%	2,500
	Research Officer, level 4	100%	1,800
	Lab. attendant level 1	100%	800
			8,701

2. SUPPLIES & MATERIALS:

Item		
a)	Media, chemicals, biochemicals ...	5,000
b)	Glassware, dispensable plastic ...	5,000
c)	Rabbits, 200 @ $12 per animal ...	2,400
		12,400

3. EQUIPMENT:

Name	Cost
Refrigerated Incubator (including freight)	2,700
4. XEROX, PUBLICATION	1,000
	24,801

BUDGET - Year 2
(November 1986 to October 1987)

		Cost, $
1.	PERSONNEL (with 15% salary increase)	10,006
2.	SUPPLIES	13,400
3.	EQUIPMENT	-
4.	XEROX, PUBLICATION	1,000
		24,406
	Cost	
	Direct	49,207
	Indirect	15,254
	TOTAL COST	64,461

5. JUSTIFICATION OF BUDGET ITEMS:

Salary:

A Senior Technician will provide working support to the animal model work and a Research Officer to the mutant isolation work. The P.I. will be responsible for training these workers, planning experiments and ensuring efficient operation of the laboratory. Dr. David A. Sack will advise on the animal model work. The laboratory attendant will be mainly involved in washing glassware.

We have incorporated a senior level local position with a time input of 25% intended to be filled by a trained pathologist who will participate with the animal studies and will study the histology of the rabbit intestine following oral administration of bacteria.

Supplies:

We aticipate a sizable input in acquiring standard laboratory glassware and other routine items because we are in the initial phase of organizing and equipping the Bacterial Genetics Laboratory.

Refrigerated Incubator:

Genetics Laboratory has a small refrigerated incubator (6 cft) which is both old and overburdened. Screening for temperature-sensitive mutants would require a larger incubation space. Hence this item will be very useful.