JOURNAL OF BACTERIOLOGY, July, 1965
Vol. 90, No. 1

Protection of Monkeys Against Experimental Shigellosis with Attenuated Vaccines

SAMUEL B. FORMAL, E. H. LABREC, AMOS PALMER, AND STANLEY FALKOW

Walter Reed Army Institute of Research, Washington, D.C., and the Division of Research Services, National Institutes of Health, Bethesda, Maryland

Received for publication 6 February 1965

ABSTRACT

FORMAL, SAMUEL B. (Walter Reed Army Institute of Research, Washington, D.C.), E. H. LABREC, AMOS PALMER, AND STANLEY FALKOW. Protection of monkeys against experimental shigellosis with attenuated vaccines. J. Bacteriol. **90**:63-68. 1965.—Two *Shigella flexneri* 2a strains of reduced virulence were used as oral vaccines to protect monkeys against experimental challenge. One strain, a spontaneous mutant, had lost its ability to cause disease and was unable to penetrate the intestinal epithelium and reach the lamina propria. The other strain was a hybrid obtained by mating virulent *S. flexneri* 2a with *Escherichia coli*. This hybrid strain retained the capacity to penetrate the intestinal epithelium but was not able to maintain itself in the lamina propria. Five oral doses of the nonpenetrating mutant strain were required to render monkeys resistant to experimental challenge, but a single dose of the hybrid strain sufficed to protect the animals. There was some evidence that a degree of specificity was involved in the induced resistance, although neither vaccine evoked a consistent serum antibody or a detectable coproantibody response.

Attempts to control bacillary dysentery have met with varying degrees of success in different parts of the world. In areas where the disease is no longer of significant importance, public health procedures probably have been responsible for the decrease in morbidity. The necessary public health facilities and skills will soon be available in many countries; however, in others, they will be achieved only after many years, and, during this time, bacillary dysentery will continue to remain a significant problem.

Immunization, if it could be attained, would be a logical way to control bacillary dysentery in areas with inadequate public health facilities. Efforts in this direction, however, have not been successful (Hardy, DeCapito, and Halbert, 1948; Higgins, Floyd, and Kadar, 1955). There is some evidence that individuals, after having recovered from bacillary dysentery, are resistant for an unknown period of time to subsequent attacks by an organism of the same serotype (Hardy and Watt, 1944; Cruickshank, 1963). In view of this, the use of orally administered living vaccines might be considered, and the question of what characteristics such preparations should possess necessarily arises.

We have already identified the sequence of events which occur in the pathogenesis of a severe dysentery infection (LaBrec et al., 1964; Formal et. al., 1965). These steps consist of penetration of the intestinal epithelial cell by the dysentery bacillus, entrance into the lamina propria, followed by a period of intensive bacterial multiplication which generally leads to ulcer formation. Any decrease in the infecting organism's ability to complete any one of these steps must render it less virulent. Mutant strains have been isolated which have lost the capacity to penetrate the intestinal epithelium. When these strains are fed to starved guinea pigs or monkeys, the animals react no differently than if they had received nonpathogenic strains of *Escherichia coli* (LaBrec et al., 1964). Presently, we consider such mutant strains to be avirulent. *Shigella flexneri* strains which have incorporated the *rha*$^+$ *xyl*$^+$ region of the *E. coli* chromosome can penetrate the intestinal epithelium and enter the lamina propria, but are unable to undergo further multiplication. Although these hybrid strains fail to kill starved guinea pigs, they do cause an inflammatory reaction of the intestinal mucosa which, however, does not proceed to ulceration and subsides in approximately 4 days. When fed to monkeys, the hybrid strains fail to cause any apparent symptoms but are capable of producing a mild intestinal inflammation (Formal et al., 1965). We consider these hybrid strains to be attenuated. The purpose of this communica-

tion is to present preliminary results of an investigation in which two such strains of *S. flexneri* 2a were used as orally administered living vaccines to protect monkeys against an experimental infection.

MATERIALS AND METHODS

The pertinent characteristics of the bacterial strains used in this investigation are summarized in Table 1. *S. flexneri* 2a strain 2457T is a typical virulent culture which has been used extensively in previous investigations at this laboratory to infect guinea pigs and monkeys. *S. flexneri* 2457O is a spontaneous colonial variant of strain 2457T; it is avirulent for guinea pigs and monkeys (LaBrec et al., 1964). Strain X-16 was obtained by mating strain 2457T with *E. coli* K-12 Hfr strain W1895; it has incorporated the *E. coli* markers *ara*$^+$ *rha*$^+$ *mal*$^+$ *xyl*$^+$ into its genome. This hybrid fails to produce any obvious symptoms when fed to guinea pigs or monkeys, but it does cause a histologically detectable, acute, inflammatory reaction in the intestinal mucosa of both these animals (Formal et al., 1965). *S. flexneri* 1b strain 1Z and *S. flexneri* 6 strain CCHO 60 were isolated from human patients with classical bacillary dysentery.

Each of the above strains was maintained in the lyophilized state. For each experiment, a new ampoule of the strain was reconstituted and grown on meat-extract agar.

Protection tests. Male and female rhesus monkeys (*Macaca mulata*) weighing from 4 to 8 lb (1.8 to 3.6 kg) were used in all studies. They were housed in individual cages and observed for at least 1 week for diarrheal symptoms. During this time, fresh fecal specimens were examined at least three times for the presence of dysentery bacilli, and animals found to be carriers were not used. Approximately 15% of the monkeys were found to be carriers of *S. flexneri* type 3 or type 4.

Vaccines were administered through a stomach tube; a single dose consisted of approximately 5×10^{10} viable cells suspended in 20 ml of Brain Heart Infusion (BHI) broth (Difco). In one experiment, the vaccine consisted of 30 mg (dry wt) of acetone-killed and dried cells of strain 2457T suspended in 20 ml of BHI broth. During the period of vaccine administration, fecal cultures of the animals were made daily. In most cases, the vaccine strain was not shed for more than 2 days after the final dose, and in no instance was it recovered later than 4 days after the final dose. In all experiments, the treated animals together with the proper untreated controls were challenged 10 days after the last vaccine dose by being fed approximately 5×10^{10} virulent viable cells suspended in 20 ml of BHI broth. They were then observed for 1 week. Animals were considered to have developed symptoms if they exhibited a severe propulsive diarrhea or classical dysentery with blood in the diarrheal stool. In most of the experiments in which the hybrid strain was used as a vaccine, antibiotics were administered to the animal immediately after the onset of symptoms. We calculated exact probabilities for a 2×2 contingency table using the factorial method, as described by Batson (1956).

Serological tests. The latex particle agglutination test was used to detect antibody in serum and in stool specimens. A 10% dilution of latex particles (Difco) was prepared in saline. Somatic antigen, prepared from *S. flexneri* 2457T by the method of Goebel, Binkley, and Perlman (1945), was added to the latex suspension at a final concentration of 20 μg/ml, and this suspension was incubated for 1 hr at 37 C. The sensitized latex particles were then washed three times in saline and diluted to yield a 1:40 dilution of the original commercial preparation. Agglutination tests were performed by adding 0.1 ml of sensitized latex particles to 0.9 ml of twofold serial dilutions of serum or stool extract. The agglutination tests were incubated at 52 C for 1 hr, followed by incubation at room temperature overnight.

For coproantibody determinations, monkeys were placed in restraining chairs and fed 7.5 g of $MgSO_4$ dissolved in 20 ml of tap water (Freter, 1962*b*). Within 2 to 3 hr after this treatment, the animals developed a voluminous diarrhea. Fresh diarrheal stool was collected and centrifuged at 4 C; the supernatant fluid was removed, frozen, and stored at −20 C. In this manner, pre- and postvaccine stools were collected and were subquently assayed for antibody at the same time.

RESULTS

Preliminary experiments with small groups of animals indicated that 28 daily doses of the

TABLE 1. *Characteristics of strains of Shigella flexneri*

Organism	Causes death when fed to starved guinea pigs	Causes intestinal lesions in starved guinea pigs	Causes keratoconjunctivitis	Invades and multiplies within HeLa cells	Causes dysentery when fed to monkeys
Virulent wild-type *S. flexneri* strains*	Yes	Yes	Yes	Yes	Yes
Avirulent mutant *S. flexneri* 2a strain 2457O	No	No	No	No	No
Attenuated *S. flexneri* 2a-*Escherichia coli* hybrid strain X-16	No	Yes	Yes	Yes	No

* *S. flexneri* 2a strain 2457T, *S. flexneri* 1b strain 1Z, *S. flexneri* 6 strain CCHO 60.

TABLE 2. *Diarrheal symptoms in monkeys immunized orally with either one or two doses of an attenuated Escherichia coli K-12–Shigella flexneri 2a hybrid strain and challenged with virulent S. flexneri 2a*

Expt	Group	Treatment[a]	No. with symptoms/total tested[b]
M35[c]	1	Two doses	1/14
	2	Control	13/18
M34[d]	1	One dose	1/20
	2	Control	7/19
M37[e]	1	One dose	1/15
	2	Two doses	0/10
	3	Control	12/24

[a] The two doses of vaccine were fed at an interval of 3 days, and animals (test and control) were challenged 10 days after the last vaccine dose was administered.

[b] Symptoms consisted of either a severe propulsive diarrheal or classical bacillary dysentery with blood in the diarrheal stool.

[c] $P = 0.0003$.

[d] $P = 0.02$.

[e] Group 1 versus group 2, $P = 0.6$; groups 1 and 2 versus group 3, $P = 0.0003$.

avirulent mutant strain 2457O rendered animals resistant to infection with the virulent 2457T strain. In subsequent experiments, 22 monkeys were immunized orally with five doses of a mutant, avirulent strain of *S. flexneri* 2a; 21 uninoculated monkeys were used as controls. The doses were administered to the test monkeys at 3-day intervals, and, 10 days after the last dose, both groups of animals were challenged with the virulent, parental strain of *S. flexneri* 2a. None of the 22 immunized monkeys exhibited symptoms consisting of severe, propulsive diarrhea or classical dysentery with blood in the diarrheal stool, whereas 7 of the 21 control monkeys did exhibit such symptoms (2 of these 7 died). These results indicate that, under the prescribed conditions, significant ($P = 0.004$) protection can be achieved against clinical symptoms. The administration of the vaccine did not appear to affect the viability of the challenge strain in the bowel, because isolation of the virulent *S. flexneri* 2a challenge was made with equal frequency in both the immunized and the control animals. When three doses of the vaccine were administered to six monkeys at 3-day intervals, and the animals were challenged 10 days after the last dose, evidence of protection was not obtained. Three of the six monkeys exhibited the symptoms described above and eventually died. During this experiment, another five-dose regimen was tested in five monkeys, and, as in the previous experiments, none of the monkeys exhibited clinical symptoms.

We have not yet conducted an experiment in which the three-dose regimen of vaccine is administered over the same time span (ca. 2 weeks) as the five-dose regimen.

Experiments similar to those just described were also conducted with the *E. coli* K-12–*S. flexneri* 2a hybrid strain as a vaccine. In the first experiment, the effect of five oral doses of the hybrid-strain vaccine or five oral doses of acetone-killed and dried (AKD) cells (30 mg per dose) was investigated. The immunizing doses were fed at 3-day intervals, and the animals were challenged 10 days after the last vaccine dose with the virulent strain of *S. flexneri* 2a. None of the eight monkeys given the five-dose regimen of the hybrid-strain vaccine developed clinical symptoms, whereas three of the eight monkeys given five doses of AKD cells did exhibit severe symptoms. Of nine monkeys in an uninoculated control group, five exhibited symptoms, and two of these five eventually died (hybrid-strain vaccine group versus control group, $P = 0.02$). This experiment demonstrated that the hybrid vaccine conferred a significant degree of resistance on those animals receiving it.

Three experiments were conducted to determine whether only one or two doses of the hybrid vaccine was sufficient to render animals resistant to challenge with the virulent strain of *S. flexneri* 2a. The results (Table 2) demonstrate that animals receiving even one dose of the hybrid vaccine were significantly more resistant to challenge than were control animals. Moreover, there was no significant difference in the response of challenged animals which received either one or two doses of the hybrid vaccine. The results of all tests in which monkeys were fed either five doses of the mutant or one or more doses of the hybrid strain of *S. flexneri* 2a, and then challenged with virulent *S. flexneri* 2a, may be summarized as follows. Of the 94 monkeys that received vaccine, only 3 exhibited clinical symptoms, whereas 46 of 97 uninoculated control-group monkeys did develop these symptoms ($P = 0.00001$).

Two experiments were carried out to test the specificity of the induced resistance. In the first experiment, two groups of five monkeys each were fed five doses of strain 2457O vaccine and, together with control animals, were challenged with either the virulent 2457T strain of *S. flexneri* 2a or a heterologous, but serologically related, virulent strain of *S. flexneri* 1b. In the second experiment, two groups of animals which had received two doses of the hybrid-strain vaccine, together with controls, were challenged with the virulent strain of either *S. flexneri* 2a or *S. flexneri* 6.

TABLE 3. *Diarrheal symptoms in monkeys immunized orally with either the mutant- or the hybrid-strain vaccine and subsequently challenged with Shigella flexneri strains of the homologous or heterologous serotypes**

Expt	Treatment	Challenge	Symptoms	P
M25	Mutant vaccine	*S. flexneri* 2a	0/5	
	Control	*S. flexneri* 2a	2/6	
	Mutant vaccine	*S. flexneri* 1b	2/5	
	Control	*S. flexneri* 1b	4/6†	
M41	Hybrid vaccine	*S. flexneri* 2a	0/14	0.057
	Control	*S. flexneri* 2a	4/15	
	Hybrid vaccine	*S. flexneri* 6	5/14	>0.2
	Control	*S. flexneri* 6	6/15	
Pooled data of M25 and M41	Vaccine	Homologous (*S. flexneri* 2a)	0/19	<0.02
	Control		6/21	
	Vaccine	Heterologous (*S. flexneri* 1b or 6)	7/19	>0.2
	Control		10/21	

* Treatment and symptoms are the same as described in Table 2.
† Three of the four animals died.

The results of this study are summarized in Table 3. In both experiments, evidence for resistance to homologous challenge was obtained, but neither experiment yielded any evidence for heterologous protection. When the data from both experiments were pooled, statistically significant evidence was obtained that a degree of specificity was involved in the resistance conferred by the vaccines.

Serum and coproantibody studies were carried out on a limited number of animals receiving either five doses of the avirulent mutant or two doses of the attenuated hybrid vaccines. In each case, serum or diarrheal stool (obtained by use of orally administered magnesium sulfate) was collected before the vaccine was fed and 7 days after the last vaccine dose. The results of this study are presented in Table 4. All the monkeys tested had prevaccination serum antibodies. Three of eleven animals which received five doses of the mutant strain had greater than a one-tube rise in serum antibody titer. None of five animals fed two doses of the hybrid strain exhibited a rise in serum antibody titer. We were not able to detect a coproantibody response in any of the animals which received the vaccines.

TABLE 4. *Serum and coproantibodies in monkeys after oral immunization with either the mutant or the hybrid strain**

Strain	Animal no.	Serum		Coproantibody	
		Pre	Post	Pre	Post
Mutant	M27-5	40†	160	Neg‡	Neg
	M27-6	80	640	Neg	Neg
	M27-7	40	40	4	8
	M27-8	160	160	Neg	Neg
	M27-9	160	160		
	M27-10	320	320		
	M27-11	20	640		
	M27-13	320	160		
	M27-14	40	80		
	M27-15	80	160		
Hybrid	M33-11	40	20	Neg	Neg
	M33-12	20	10	Neg	Neg
	M33-13	20	40	Neg	Neg
	M33-14	40	80	Neg	Neg

* Animals received either five doses of the mutant or three doses of the hybrid strain, and material for titration was collected 7 days after the last dose.
† Reciprocal of highest dilution in which agglutination was observed.
‡ No agglutination in undiluted diarrheal stool.

DISCUSSION

The two strains used in this study possess the characteristics which we presently consider to be typical of avirulent and attenuated dysentery bacilli. The avirulent mutant causes neither symptoms nor histological changes in the bowel mucosa; the attenuated strain does not provoke diarrhea but does produce transient inflammatory changes in the bowel wall. Both strains induce significant resistance to homologous challenge when administered orally as vaccines, and our evidence indicates that there is a degree of specificity involved in this induced resistance. In

regard to protection tests, the major difference between the two vaccine organisms involved the number of doses of vaccine required to induce resistance. Five doses of the avirulent mutant were required, whereas a single dose of the attenuated hybrid strain sufficed. Evidence of protection was not observed when five doses of AKD cells were administered as an oral vaccine.

The mechanism of this induced resistance is not known at present. Neither of the vaccines produced consistent rises in serum antibody or detectable levels of coproantibody. Possibly, the latex particle agglutination test is not sufficiently sensitive to detect low levels of coproantibody. Freter (1962*b*) emphasized the difficulties of detecting antibody in feces and suggested the use of the Farr (1958) test for this purpose (Freter, 1962*a*). We attempted to prepare Farr-test antigen from *S. flexneri* 2a but were not able to obtain suitable preparations (LaBrec and Formal, *unpublished data*). Because of this, we must still consider the possibility that coproantibody plays a role in the induced resistance which was observed.

It seems likely that antigen must pass through the intestinal epithelium to induce the resistance that we observed in the present investigation. In the case of the avirulent mutant, any antigenic material probably is absorbed in an inconsistent and unpredictable fashion, and repeated large doses of dead bacteria probably would produce the same effect (Freter and Gangarosa, 1963; Rauss and Kétyi, 1964). On the other hand, the hybrid strain invades the bowel mucosa, carrying with it antigenic material, and a single dose was sufficient to confer protection under the conditions of our test system.

When it is established satisfactorily that resistance can be induced, the risks involved in feeding the vaccine strains must be considered. Several experimental models are available to test the safety of vaccine strains. The ability to cause ulcerative lesions of the cornea or to penetrate HeLa cells seems to correlate well with the capacity of an organism to penetrate the intestinal epithelial cell. [Occasionally, strains which will not penetrate the intestinal epithelial cell will be observed to enter HeLa cells. These strains invariably possess fimbriae (as indicated by their ability to agglutinate guinea pig red blood cells; Duguid and Gilles. 1957) which allow the organism to stick to the HeLa cell and gain entrance to this cell by a process apparently different from that of virulent bacilli (LaBrec and Formal, *unpublished data*).] The inability of a strain to penetrate the intestinal epithelium can be confirmed further by feeding the organisms to monkeys and guinea pigs. Present tests for the safety of the attenuated hybrid strains depend on their inability to cause a fatal infection when fed to starved guinea pigs or to produce diarrheal symptoms in monkeys. These strains should penetrate HeLa cells; most retain the ability to cause keratoconjunctivitis, and all should produce inflammatory changes in the intestinal mucosa. As more experiments are carried out, other criteria for assessing vaccine safety undoubtedly will become available.

The present investigation is, of course, preliminary in nature. Several obvious investigations are presently under consideration: (i) use of a reduced number of bacteria per dose; (ii) length of the induced immunity; (iii) use of multiple serotypes in a single dose; (iv) efficacy of the vaccine under conditions of natural exposure.

ACKNOWLEDGMENTS

We wish to thank Smiley Austin, Giles White, Elmer L. Becker, and Edward L. Buescher for their excellent technical assistance.

LITERATURE CITED

BATSON, H. C. 1956. An introduction to statistics in medical sciences. Burgess Publishing Co., Minneapolis.

CRUICKSHANK, R. 1963. Acquired immunity: bacterial infections, p. 119. *In* R. Cruickshank [ed.], Modern trends in immunology. Butterworth Inc., Washington, D.C.

DUGUID, J. P., AND R. R. GILLIES. 1957. Fimbriae and adhesive properties in dysentery bacilli. J. Pathol. Bacteriol. **74**:397–411.

FARR, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I* BSA and antibody. J. Infect. Diseases **103**:239–262.

FORMAL, S. B., E. H. LABREC, T. H. KENT, AND S. FALKOW. 1965. Abortive intestinal infection with an *Escherichia coli-Shigella flexneri* hybrid strain. J. Bacteriol. **89**:1374–1382.

FRETER, R. 1962*a*. Influence of various antibody characteristics on the titration of O antibodies by different methods. J. Infect. Diseases **111**: 25–36.

FRETER, R. 1962*b*. Detection of coproantibody and its formation after parenteral and oral immunization of human volunteers. J. Infect. Diseases **111**:37–48.

FRETER, R., AND E. J. GANGAROSA. 1963. Oral immunization and production of coproantibody in human volunteers. J. Immunol. **91**:724–729.

GOEBEL, W. F., F. BINKLEY, AND E. PERLMAN. 1945. Studies on the Flexner group of dysentery bacilli. I. The somatic antigens of *Shigella* paradysenteriae (Flexner). J. Exptl. Med. **81**:315–330.

HARDY, A. V., T. DECAPITO, AND S. HALBERT. 1948. Studies of the acute diarrheal diseases. XIX. Immunization in shigellosis. Public Health Rept. U.S. **63**:685–688.

HARDY, A. V., AND J. WATT. 1944. The acute diarrheal diseases. J. Am. Med. Assoc. **124**:1173–1178.

HIGGINS, A. R., T. M. FLOYD, AND M. A. KADAR. 1955. Studies in shigellosis. III. A controlled evaluation of a monovalent Shigella vaccine in a highly endemic area. Am. J. Trop. Med. Hyg. **4**:281–288.

LABREC, E. H., H. SCHNEIDER, T. MAGNANI, AND S. B. FORMAL. 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.

RAUSS, K., AND I. KÉTYI. 1964. Serum and coproantibodies in mice, immunized perorally with *Shigella flexneri*. Z. Immunitaetsforsch. **127**: 37–50.