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Small-Animal Model To Measure Efficacy and Immunogenicity of *Shigella* Vaccine Strains

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The development of a small-animal model to test the protective efficacy and immunogenicity of a vaccine strain against shigellosis would greatly facilitate the evaluation of potential vaccine candidates. In guinea pigs, the ability of shigellae to invade and multiply within the corneal epithelium, causing keratoconjunctivitis, closely mimics the invasion process in the intestinal epithelium (B. Sereny, Acta Microbiol. Acad. Sci. Hung. 4:367–376, 1957). The serum response of animals recovering from a *Shigella* keratoconjunctival infection was determined and found to be consistent with that shown by convalescent humans and primates. This model was used to test the efficacy of two vaccine candidates, and the immune response of the guinea pigs to the vaccine strains was examined. Both vaccine strains demonstrated significant protection against challenge by homologous virulent *Shigella* strains, and the results were comparable with results obtained in trials with monkeys. The guinea pig model also provides a rapid and inexpensive means of evaluating different immunization regimens as well as of testing other variables such as length of protection against disease.

Attempts to develop a successful vaccine against shigellosis have led to the testing of a wide variety of vaccine candidates, including both parenteral and orally administered vaccines (2, 8, 10, 15, 17, 19–21, 23–26, 30, 36). Initial testing requires immunization and subsequent challenge of primates and/or human subjects in safety and efficacy trials to determine whether a vaccine candidate warrants further development and use in large-scale field trials. Since these studies are both difficult to perform and costly, the development of a small-animal model that allows reliable and efficient characterization of the protective efficacy and immunogenicity of potential vaccine strains would greatly facilitate the evaluation process and would allow the examination of a larger number of candidates.

Most of the current vaccine candidates are administered orally to simulate the route of a natural infection in which the shigellae invade the epithelial cells of the human colonic mucosa. Vaccination by the oral route is also necessary to produce the protective local immunity thought to be critical in preventing shigellosis (20, 34). The ability of the *Shigella* organism to invade the corneal epithelia of guinea pigs, rabbits, and mice and to spread to contiguous cells, causing keratoconjunctivitis, provides a model system that mimics the invasive process occurring in the mucosal epithelium (22, 31). The development of keratoconjunctivitis is most reproducible in the eyes of guinea pigs (22), and this model has been used extensively to test the virulence of *Shigella* strains and to test potential vaccine candidates for attenuation (8, 20, 21, 23, 30, 36). However, protection against keratoconjunctivitis shigellosa induced by immunization has only been used occasionally to test the efficacy of vaccine strains or antigen preparations used for immunization (1, 16, 21, 33, 36). Some of these experiments have employed subcutaneous injections as the route of immunization (1, 33), while most recently Linde et al. (21) and Verma and Lindberg (36) have reported the use of the Sereny test in estimating the efficacy of vaccine candidate strains. Since immunization in the conjunctival sacs of guinea pigs with vaccine candidate strains more closely approximates the site of delivery of orally administered vaccines, we examined the usefulness of this model in determining protective efficacy and immunogenicity of two *Shigella* vaccine strains. Since it has been reported that levels of serum antibodies to *Shigella* lipopolysaccharide (LPS) (2–4) and possibly antibodies to outer membrane proteins (1) are associated with protection, we monitored the serum response of immunized animals to these strains. In addition, we examined the usefulness of the model for rapid testing of a variety of parameters, such as immunization regimens, which are important to the success of a particular vaccine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Two vaccine strains were used to immunize guinea pigs. One strain, Sfl 124, is a live attenuated *Shigella flexneri* Y strain derived from Sfl 114, which was constructed by making the virulent parent strain, Sfl 1, an *aroD* mutant, thus rendering it dependent on aromatic metabolites not available in mammalian tissues (19, 20). The tetracycline resistance properties of Sfl 114 were removed, and the resulting vaccine strain was designated Sfl 124. Sfl 114 elicited homologous protection when administered orally to macaque monkeys and was well tolerated by the monkeys (19). The other strain, EcSf2a-2, is an *aroD* mutant hybrid *Escherichia coli-S. flexneri* strain that expresses the *S. flexneri* 2a somatic antigen (27). This strain was derived from EC104 and EcSf2a-1 (8, 11) and is an *E. coli* K-12 derivative with the His, Pro, and Arg markers from *S. flexneri* 2a and the virulence plasmid from *S. flexneri* 5. EC104 has been previously tested in monkeys (8), and the *aroD* mutant, EcSf2a-2, is currently being tested in monkeys and humans (11). *S. flexneri* 2a 2457T is a virulent strain from the Walter Reed Army Institute of Research collection and was used as a challenge strain. Other virulent strains included in this study are *S. flexneri* 5 strain M90T-W and

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Shigella sonnei 53G, both obtained from the Walter Reed Army Institute of Research collection. Vaccine lots of Sfl 124 and EcSf2a-2 were prepared by the Department of Biologics Research, Walter Reed Army Institute of Research; one lot of EcSf2a-2 (lot 5) was prepared by the Salk Institute (Swift Water, Pa.). Both vaccine strains were grown in tryptone-yeast extract medium, centrifuged, resuspended in 7.5% lactose-phosphate-glutamate medium (pH 7.1), and lyophilized. The vaccine stock prepared at the Salk Institute was resuspended in 7.5% lactose-phosphate-glutamate medium with 10% dextran 10 added. All vaccine vials were rehydrated from the lyophilized state with distilled water and diluted with phosphate-buffered saline (PBS), pH 7.4, when required. In some cases, the vaccine strain was spread from the lyophilized vial onto Congo red plates consisting of 0.01% Congo red dye (Sigma Chemical Co., St. Louis, Mo.) in Trypticase soy agar (Difco Laboratories, Detroit, Mich.). Colonies which bound the dye were then spread for growth on Trypticase soy agar plates and grown overnight at 37°C. Virulent strains used for challenge were first streaked on Congo red plates; positive colonies were then spread for growth on Trypticase soy agar plates and grown overnight at 37°C. Plates were then harvested with 10 ml of PBS, and the suspension was used for immunization. Plate counts were done for each rehydrated vial or harvested culture. Strains used in immunoblots were the *Shigella* strains described above and recombinant clones containing genes for *Shigella* invasion plasmid antigens (Ipa proteins). The recombinant strains used were pWR390 (DH5α), a pUC12 recombinant containing a 2.9-kb *Eco*RI insert with the *ipaH* structural gene (13); pHC17 (DH5α), a pUC18 recombinant containing a 4.7-kb *Hin*dIII insert with the *ipaB* and *ipaC* structural genes (35); and pUC19 (DH5α), used as a control.

Immunizations and Sereny tests. Male Hartley guinea pigs were used for Sereny tests (31) and immunization experiments with the vaccine strains. Control animals used to confirm the virulence of the challenge organisms were the same age as the test animals. The number of vaccine organisms used in each inoculation depended on the particular vaccine lot and dilution factor used and are given for each experiment. Inoculation with virulent organisms was done with 3×10^8 to 5×10^8 organisms harvested from overnight growth plates. Approximately 0.05 ml of cell suspension was deposited into the conjunctival sac of each eye by dropper, and the lids of the eyes were massaged lightly to ensure that the inoculum was distributed over the entire eye. For vaccine strains, immunization occurred on days 0, 1, 14, and 15 unless otherwise specified. Following challenge with the virulent strain, animals were inspected daily for the development of keratoconjunctivitis. The degree of keratoconjunctivitis was rated on the basis of time of development of symptoms, severity of clinical symptoms, and rate of clearing of symptoms (see Table 2 for rating scheme). Significance of protection was assessed by using the Fisher exact probability test.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for Care and Use of Laboratory Animals* (26a).

ELISA. An enzyme-linked immunosorbent assay (ELISA) was used to quantitate the serum antibody against LPS from *S. flexneri* 2a strain 2457T in the case of animals immunized with EcSf2a-2 or 2457T. The LPS antigen was prepared by the method of Westphal and Jann (37) and was generously provided by Samuel B. Formal. The ELISA used to measure LPS antibodies has been described previously (8) and is described here briefly. The LPS antigen was diluted at a concentration of 30 μg/ml in coating buffer (20 mM Na_2CO_3, pH 9.6), and 25 μl was used per well to coat 96-well microtiter plates (Nunc, Roskilde, Denmark) overnight at 4°C. The plates were aspirated and washed three times with washing buffer (PBS with 0.05% Tween 20). Plates were then covered with filler solution (20 g of casein [Sigma Chemical Co.], 2.0 g of sodium azide per liter of PBS, pH 7.2 to 7.4) and incubated at 37°C for 30 min. The filler was aspirated, and the plates were washed with washing buffer three times. Guinea pig sera, serially diluted in filler buffer, were then added to the wells and incubated for 2 h at 25°C. Following three washes with washing buffer, the plates were incubated with 25 μl of horseradish peroxidase-conjugated goat anti-guinea pig immunoglobulin G (IgG) (heavy and light chains; Kirkegaard & Perry, Gaithersburg, Md.) per well, diluted 1:500, for 2 h at 25°C. After being washed three times, plates were developed with TMB (3,3',5,5'-tetramethylbenzidine) peroxidase substrate (Kirkegaard & Perry) and read at 450 nm on a microplate reader (model MR600; Dynatech Laboratories, Inc., Alexandria, Va.). ELISAs were also run with alkaline phosphatase-conjugated protein A (Sigma Chemical Co.), diluted 1:200. Protein A was used so that specific IgG antibodies to LPS would be detected. After the plates were washed, 100 μl of 1 M diethanolamine buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) containing 1 mg of *p*-nitrophenyl phosphate per ml was added to each well. The plates were incubated for 30 min, and the reaction was then stopped by the addition of 50 μl of 3 M NaOH per well. The plates were read at 405 nm. Endpoint titers were defined as the last dilution having an optical density of 0.1 or greater. The endpoint titers were reproducible to plus or minus 1 dilution. Values given are the averages of 2 to 3 replicates.

Immunoblotting. Whole-cell lysates of bacteria were prepared in the following manner. Cells were grown overnight at 37°C in Luria-Bertani medium and were adjusted to an optical density at 600 nm of 1. One hundred microliters of each sample was spun down, resuspended in 10 μl of sample buffer (0.0625 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 12.5% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue), and heated at 100°C for 5 min. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 13% polyacrylamide gels and transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) in a Tris-glycine-methanol buffer (28) for 2 h at 800 ma. The nitrocellulose sheets were blocked with the casein filler solution described in "ELISA" for 1 h at 25°C. The blots were then incubated for at least 2 h at 25°C with guinea pig sera diluted 1:100 in the casein filler solution. After being washed three times with PBS, the blots were incubated with protein A-alkaline phosphatase conjugate (Sigma Chemical Co.) diluted 1:200 with casein filler for 2 h at 25°C. Blots were also incubated with anti-guinea pig IgG conjugated to alkaline phosphatase (Sigma Chemical Co.); the results obtained by this method were identical to those obtained by using the protein A conjugate. The blots were washed with 50 mM Tris-saline (pH 8.0), 50 mM Tris-saline with 0.05% Tween 20, and 50 mM Tris-HCl (pH 8.0) and then incubated with the substrate (100 mg of Fast Red TR salt [Sigma Chemical Co.], 50 mg of naphthol AS-MX phosphate [Sigma Chemical Co.] in 50 ml of 50 mM Tris [pH 8.0]) for 30 min.

TABLE 1. Protection against homologous challenge following conjunctival infection with virulent *Shigella* strains

Original infection[a]	Time[b] (wks)	Challenge strain	Attack rate[c]	Protection[d] (%)
Sfl 1	4	Sfl 1	0/16	100
Sfl 1, Cr^-	4	Sfl 1	3/4	25
Sfl 1	7	Sfl 1	1/8	87
None		Sfl 1	6/8	25
2457T	4	2457T	0/4	100
2457T	7	2457T	2/12	83
2457T	13	2457T	1/10	90
None		2457T	6/6	0
53G	4	53G	0/12	100
53G	7	53G	0/6	100
None		53G	22/24	8

[a] Strains are as follows: 2457T, *S. flexneri* 2a strain 2457T; Sfl 1, *S. flexneri* Y strain 1; 53G, *S. sonnei* strain 53G. Both *S. flexneri* strains are virulent and bind Congo red dye. Sfl 1, Cr^- represents the avirulent variant of Sfl 1, which does not bind Congo red dye. For studies involving 53G, form I colonies were selected for the original infection and challenge.

[b] Challenge with 4.0×10^8 to 6.0×10^8 CFU of homologous virulent *Shigella* strains was given 4, 7, or 13 weeks following the first infection.

[c] Attack rate is defined as the number of eyes having mild to fully developed keratoconjunctivitis divided by the total number of eyes challenged with the virulent strain.

[d] Protection is defined as the number of eyes having no symptoms of keratoconjunctival disease divided by the total number of eyes challenged with the virulent strain.

RESULTS

Immunity conferred by keratoconjunctivitis shigellosa. Previous studies have reported that a positive keratoconjunctival infection in an animal confers a degree of protective immunity against further homologous challenge to the eye (32). Our initial experiments were designed to establish that the *Shigella* strains in this study provide protection against further homologous challenge to the eye. We also wanted to determine whether the immune response of the infected animals was comparable to that found in convalescent humans and primates.

After initial infection with one of the virulent strains, the animals were rechallenged with the same strain 4, 7, or 13 weeks after infection. The attack rate following previous infection for any of the strains was less than 20%, indicating that homologous protection (more than 80%) occurred after the initial infection with the virulent strain (Table 1). Protection was still more than 80% 7 weeks postinfection for all strains and 13 weeks postinfection for the strain tested at that time point (2457T). However, when animals were initially infected with an avirulent Congo red-negative strain of Sfl 1 which did not produce any signs of keratoconjunctivitis, the attack rate was identical to that found in the control animals (75%), showing that no protection was conferred by avirulent Sfl 1 organisms (Table 1).

To examine the serum antibody response of animals infected with virulent strains, animals were bled prior to infection and 10 to 14 days after infection. The sera were reacted against *Shigella* whole-cell lysates and against recombinant clones expressing Ipa proteins in a Western blot (immunoblot) assay (Fig. 1). As seen in Fig. 1, sera obtained from guinea pigs which have been infected with *S. flexneri* 2a strain 2457T recognized the gene products of *ipaH*, *ipaB*, and *ipaC* as expressed by recombinant clones. The sera also reacted with these and other antigens in lysates of *Shigella* species. Sera obtained prior to infection with a virulent strain did not react with any of the Ipa proteins (data not shown).

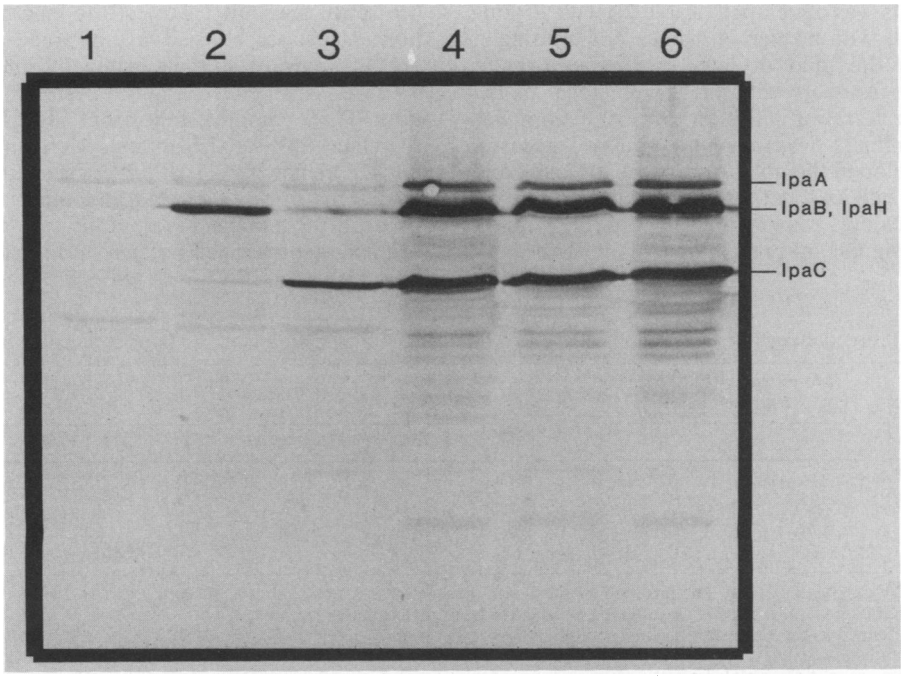

FIG. 1. Immunoblot showing response of serum obtained from a guinea pig infected with virulent *S. flexneri* 2a strain 2457T to *Shigella* antigens. Lysates of recombinant clones expressing Ipa proteins and of virulent *Shigella* strains were run on SDS-PAGE and blotted onto nitrocellulose as described in Materials and Methods. Lanes: 1, pUC19 (DH5α) control; 2, pWR390 (DH5α) expressing IpaH; 3, pHC17 (DH5α) expressing IpaB and IpaC; 4, *S. flexneri* Y strain Sfl 1; 5, *S. flexneri* 5 strain M90T-W; 6, *S. flexneri* 2a strain 2457T. Ipa proteins IpaA, IpaB, IpaC, and IpaH are identified to the right of the blot.

TABLE 2. Protection against homologous and heterologous challenge[a] following immunization with vaccine strain Sfl 124

Vaccine source[b]	Immunizing dose[c] (CFU)	Challenge strain	No. of eyes with the following reaction rating[d]:				Protection (%)[e]		
			0	1	2	3	Full	Partial	Combined
Vial	4×10^7	Sfl 1	12	4	2	0	67	22	89
Plate	5.5×10^8	Sfl 1	15	3	0	0	83	17	100
None		Sfl 1	5	0	19	0	21	0	21
Plate	5.5×10^8	2457T	0	0	0	10	0	0	0
None		2457T	0	0	0	10	0	0	0

[a] Challenge dose was 5.0×10^8 CFU, given 2 weeks after the last immunization. Homologous challenge was done with *S. flexneri* Y strain Sfl 1, and heterologous challenge was done with *S. flexneri* 2a strain 2457T.

[b] Vaccine organisms obtained from vials were prepared in liquid culture, aliquoted in bottles, and lyophilized as described in Materials and Methods. Vaccine organisms obtained from plates were cultured overnight on Trypticase soy agar plates and harvested with PBS.

[c] Immunization on days 0, 1, 14, 15. Doses given are averages of four immunizing doses.

[d] Reaction ratings are defined as follows: 0, no Sereny reaction or mild irritation; 1, mild keratoconjunctivitis or late development and/or rapid clearing; 2, keratoconjunctivitis, but not purulent; 3, fully developed keratoconjunctivitis with purulence.

[e] Protection is determined as follows: full, number of eyes with reaction rating of 0 divided by the total number of eyes challenged; partial, number of eyes with reaction rating of 1 divided by the total number of eyes challenged; combined, sum of eyes with full and partial protection (reaction ratings 0 and 1) divided by the total number of eyes challenged.

Efficacy trials with vaccine strain Sfl 124. Efficacy trials were conducted with the vaccine strain Sfl 124 obtained either from lyophilized vials or from plates as described in Materials and Methods. When these trials were first initiated, several immunization schedules were tested. Preliminary studies indicated that a boosting dose 14 days after the primary immunization gave better protection than a primary immunization alone (29). Therefore, in subsequent experiments, animals were given a primary immunization on days 0 and 1 and a boosting immunization on days 14 and 15. While these trials were conducted, it was evident that protection was sometimes manifested by the development of a mild form of keratoconjunctivitis in the immunized animals or by late development and/or rapid clearing of the disease. Thus, a rating scheme for the degree of keratoconjunctivitis present in the animals was developed (Table 2), with a rating of 0 given for animals with no sign of disease and a rating of 1 for animals with the mild or late-developing form of keratoconjunctivitis. Animals with the severe form of the disease were given a rating of 2 or 3. Protection was classified as full (animals developed no signs of disease), partial (animals developed the mild form of the disease), and combined (sum of full and partial protection). When Sfl 124-vaccinated guinea pigs were challenged with the virulent homologous strain, significant protection ($P < 0.01$ for both full and combined protection) was conferred by organisms obtained either from lyophilized vials or from plate harvests (Table 2). There was no protection, partial or full, against a heterologous challenge with 2457T.

Efficacy trials with vaccine strain EcSf2a-2. Efficacy of the *aroD* mutant *E. coli-S. flexneri* 2a hybrid vaccine strain was tested with material obtained either from lyophilized vials or from harvested plates. Since the viable plate count of the material obtained from lyophilized vials decreased with storage time, the results are shown in Table 3 according to the immunizing dose. Significant protection ($P < 0.01$ for both full and combined protection) against challenge with the homologous virulent strain 2457T was observed with all doses.

Use of the keratoconjunctivitis model to measure duration of protective immunity. To test the length of time that protection against shigellosis was sustained after a natural infection, a group of animals originally infected with *S. sonnei* 53G were challenged 3 and 6 months after the initial infection. Three months following an infection with virulent shigellae, 90% of the animals were fully protected against further challenge (11 of 12 eyes challenged developed no symptoms of disease), but at 6 months postinfection, only 10% of the animals were fully protected (1 of 10 eyes challenged developed no sign of disease). However, 60% of

TABLE 3. Protection against homologous challenge[a] following immunization with the hybrid vaccine strain EcSf2a-2

Vaccine source	Immunizing dose[b] (CFU)	No. of eyes with the following reaction rating:				Protection (%)			Serum titer[c] on day:			
		0	1	2	3	Full	Partial	Combined	0	12	26	38
Plate	8.0×10^8	21	3	0	0	88	12	100	5	25	183 (150–200)	ND
Vial	2.7×10^8	24	10	2	2	63	26	89	5	25	370 (50–800)	1,600[d]
Vial	6.8×10^7	17	9	0	2	61	32	93	ND	50	733 (600–867)	ND
None		3	0	0	45	6	0	6	10	NA	NA	333

[a] Challenge was given 2 weeks after the last immunization (day 28) with a dose of 5.0×10^8 CFU of *S. flexneri* 2a strain 2457T.

[b] Immunization was given on days 0, 1, 14, 15. Doses given are the averages of four immunizations.

[c] Serum ELISA titer is defined as the reciprocal of the last dilution having an optical density at 450 nm of 0.100 or greater. The ELISAs were run against *S. flexneri* 2a LPS with horseradish peroxidase-conjugated goat anti-guinea pig IgG (see Materials and Methods). Serum samples were collected from three animals from the plate immunization group, five animals from the group immunized with a vial dose of 2.7×10^8, two animals from the group receiving a vial dose of 6.8×10^7, and two animals from the control group. Two to three replicates were run for each serum sample. Numbers given are the average of values for the animals in each group; the range of values within the group is given in parentheses where applicable. Animals were bled on day 0 (preimmunization), day 12 (post-primary immunization), day 26 (post-boosting immunization), and day 38 (postchallenge). Serum samples were collected from three animals from the control group 10 days postchallenge (day 38 of the experiment). ND, not done; NA not applicable.

[d] For this time point, serum samples were collected from only two of the five animals in this group.

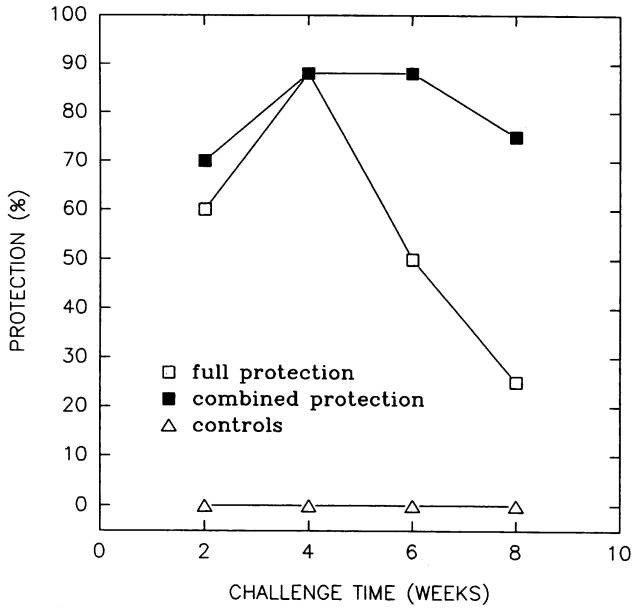

FIG. 2. Duration of protective immunity conferred by vaccine strain EcSf2a-2. Animals were immunized on days 0, 1, 14, and 15 with 3.5×10^8 CFU of vaccine strain EcSf2a-2 obtained from lyophilized vials and were challenged with 5.0×10^8 CFU of *S. flexneri* 2a strain 2457T 2, 4, 6, and 8 weeks after the last immunization. Protection figures are calculated as described in Table 2 and are based on a total of 10 eyes for the 2-week challenge group and a total of 8 eyes for all other groups.

the eyes challenged in the 6-months group developed only a mild form of keratoconjunctivitis (rating of 1), giving 70% combined protection (full and partial) against disease. Thus, although full protection against subsequent homologous challenge following infection drops off somewhere between 3 and 6 months following the initial infection, partial protection against disease drops off less rapidly. To determine whether this model could be used to monitor the length of time that protection was sustained following immunization with a vaccine strain, guinea pigs were immunized on days 0, 1, 14, and 15 with EcSf2a-2 and challenged with the homologous virulent strain 2457T at intervals of 2 weeks (2, 4, 6, and 8 weeks following the immunization on day 14). Results from this experiment (Fig. 2) indicated that there was a peak in full protection at a challenge time of 4 weeks, after which the full protection dropped off. However, the partial protection at the later time points increased, indicating that although full protection had declined 4 weeks after immunization, combined protection (full plus partial) still existed at about the same level 6 and 8 weeks after the last immunization.

Serum response of vaccinated animals. Animals immunized with Sfl 124 and EcSf2a-2 vaccines were bled preimmunization and following the primary immunization and the boosting dose. The sera were reacted with immunoblots of lysates of virulent *S. flexneri* strains Sfl 1 (serotype Y), M90T-W (serotype 5), and 2457T (serotype 2a) and of recombinant clones expressing IpaB, IpaC, and IpaH (Fig. 3). Seven of eight samples obtained from animals immunized with Sfl 124 reacted to some degree with the Ipa proteins and showed no reaction to the pUC19 control. In contrast, none of the sera obtained from guinea pigs immunized with the EcSf2a-2 vaccine strain showed any response to the Ipa proteins. This was true whether protein A conjugate or anti-guinea pig IgG (whole molecule, which should react to light chains of IgM and IgA molecules, although possibly less efficiently) was used as a second antibody, indicating that no Ig isotype reacting to Ipa proteins was produced by the EcSf2a-2 vaccine. Sera obtained from these animals reacted strongly against a ladder of LPS antigens in lysates obtained from *S. flexneri* 2a strain 2457T (Fig. 3); there was only a slight reaction to these bands in lysates of heterologous strains Sfl 1 (serotype Y) and M90T-W (serotype 5). This slight cross-reactivity is attributed to the shared 3,4-group specificity of serotypes Y, 2a, and 5 (12). It was also noted that sera obtained following the primary immunization showed only a slight reaction to Ipa proteins or to LPS, while sera obtained from animals following the boost showed a marked increase in antibody response to Ipa proteins (in the case of Sfl 124) and to LPS antigens.

To confirm the LPS serum response observed in the immunoblots, the antibody titer to serotype 2a LPS of sera collected from animals immunized with EcSf2a-2 was quantitated by an ELISA. Serum samples from 10 animals were obtained prior to immunization and following the primary dose and the boost. These samples were run in an ELISA with *S. flexneri* 2a strain 2457T LPS as antigen (Table 3). All sera showed significant titer increases (greater than fourfold increases) against the *S. flexneri* 2a LPS following the boost. Two immunized animals were tested for postchallenge titer, and both showed significant titer increases over the post-boost titer. The mean postchallenge titer of serum samples obtained from two animals infected with 2457T was 333, which is 2 to 3 dilutions lower than the postchallenge titer for the immunized animals.

Effect of boosting dose on efficacy and serum response of immunized animals. In the initial experiments determining optimum immunization regimens, results suggested that a boosting dose increased the protection of vaccine strain Sfl 124 (29). To determine the effect of a boosting dose on both the protection conferred by a vaccine strain and the serum response of the immunized animals, we used four different regimens to vaccinate guinea pigs with strain EcSf2a-2 (Table 4). Groups 1 and 2, which received a boosting dose, were compared with two groups receiving only a primary immunization. Groups 1, 2, and 3 were challenged 28 days after the initial immunization. However, since there was a longer period between final immunization and challenge for group 3, we also compared groups 1 and 2 with group 4. In this case, challenge was made 14 days after the first dose of the final immunization, making the time between the final immunization and challenge the same for these three groups. Serum samples were collected from two animals from each group and tested for reactivity against *S. flexneri* 2a LPS (Table 4). The full protection observed in the two groups which had received a boosting dose (groups 1 and 2) was at least two times higher than that observed in the control group. In both groups, the combined protection was significantly different from that observed for the controls ($P < 0.01$). In contrast, the groups that received immunizing doses on days 0, 2, and 4 with no boosting dose (groups 3 and 4) did not differ significantly in combined protection from the control group, and there was little difference in full protection between the immunized group and the control group. The differences in combined protection between groups 1 and 3, groups 2 and 3, groups 1 and 4, and groups 2 and 4 were significant ($P < 0.01$, $P < 0.02$, $P < 0.01$, and $P < 0.05$, respectively). There was no significant difference in protection observed for groups 3 and 4. Thus, the protection

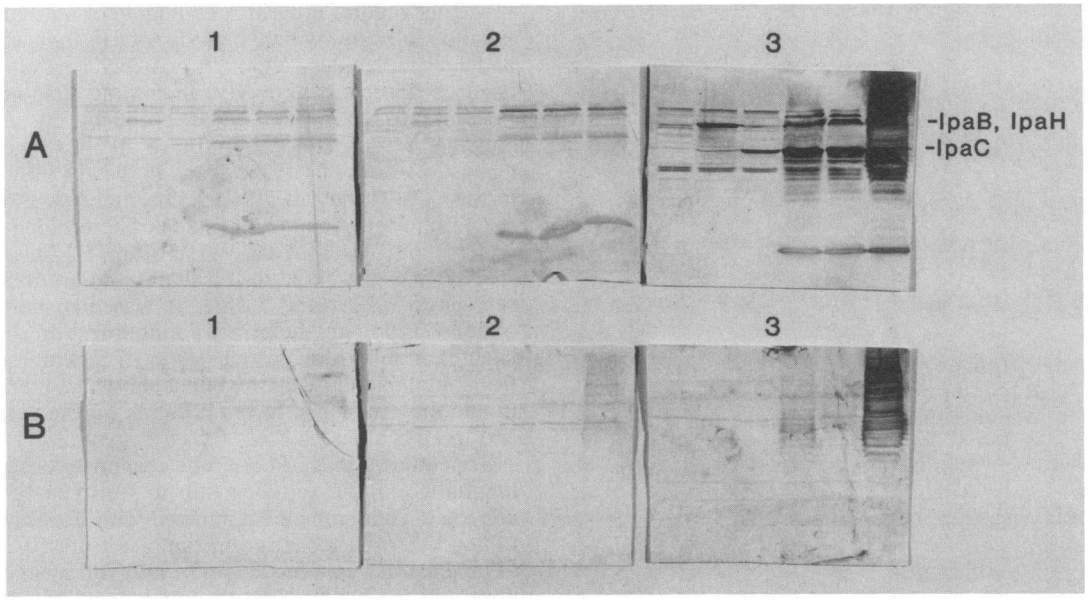

FIG. 3. Immunoblots of lysates of recombinant clones expressing Ipa proteins and of virulent *Shigella* strains reacted against sera obtained from guinea pigs immunized with Sfl 124 (A) and EcSf2a-2 (B). Lysates of recombinant clones and of virulent *Shigella* strains were run on SDS-PAGE and blotted onto nitrocellulose as described in Materials and Methods. All six immunoblots contain the same antigens, as follows. Lanes: 1, pUC19 (DH5α) control; 2, pWR390 (DH5α) expressing IpaH; 3, pHC17 (DH5α) expressing IpaB and IpaC; 4, *S. flexneri* Y strain Sfl 1; 5, *S. flexneri* 5 strain M90T-W; 6, *S. flexneri* 2a strain 2457T. Ipa proteins IpaB, IpaC, and IpaH are marked to the right of each panel. (A) Blots: 1, preimmunization serum, day 0; 2, serum collected post-primary immunization with Sfl 124, day 12; 3, serum collected post-boosting immunization with Sfl 124, day 26. (B) Blots: 1, preimmunization serum, day 0; 2, serum collected post-primary immunization with EcSf2a-2, day 12; 3, serum collected post-boosting immunization with EcSf2a-2, day 26.

observed for the animals receiving a boost was significantly higher than that observed for animals receiving only a primary immunization whether the challenge was 14 or 28 days after the first dose. The ELISA results indicated that the anti-LPS titer prior to challenge (day 26) was 3 dilutions higher for animals in groups 1 and 2 than that found for group 3 animals and 4 to 5 dilutions higher than that found for group 4 animals (day 12). The postchallenge titers for animals in groups 1 and 2 were also higher than those found for animals in groups 3 and 4, although the differences were not as pronounced.

DISCUSSION

Natural *Shigella* infections confer immunity and provide protection against subsequent infection with homologous virulent shigellae (5, 6, 14). Effective vaccines against shigellosis are therefore most likely to be live attenuated vaccines administered orally. Previously, screening these candidates has required immunization studies with primates and/or humans. Since these trials are difficult to perform and costly, a small-animal model to test the ability of a vaccine strain to confer protective immunity would facilitate screening and characterization of a larger number of vaccine candidates. Likely candidates for a small-animal model must meet several criteria. The bacterial invasion in the small-animal host must mimic the invasive process that occurs in the intestinal epithelium of the human or primate host and provide a period of protective immunity against further homologous challenge with virulent shigellae. Since the antigenic epitopes that provide protection against an attack by virulent *Shigella* organisms are not completely defined at the present time, an animal model to measure vaccine efficacy against shigellosis requires that infection of the animal elicit an immune response similar to that invoked in humans who have contracted the disease. In the guinea pig keratoconjunctivitis model, shigellae invade the corneal epithelium in a manner similar to that seen in the intestinal epithelium (22, 31), and, in his original experiments, Sereny found that a *Shigella* infection conferred protective immunity against further homologous challenge with virulent shigellae (31, 32). We therefore examined more extensively the protective immunity and serum immune response elicited in this model system and tested the usefulness of the keratoconjunctivitis model in assaying immunogenicity and efficacy of vaccine strains.

Our initial experiments (Table 1) found that a keratoconjunctival infection following challenge with a virulent *Shigella* strain conferred protective immunity against further homologous challenge in 80 to 100% of the animals for at least three months. These results are comparable to those found in human studies, in which it was shown that a shigellosis attack confers about 75% protection against subsequent homologous infection for several months following the initial infection (6). Evidence has suggested that the serotype-specific LPS surface antigens may play an important role in stimulating protective immunity against shigellosis (2–4, 9, 18). However, it has also been reported that an immune response to the outer membrane proteins might induce protection to both homologous and heterologous challenge (1). In fact, immune responses to both LPS and Ipa proteins are found in sera of convalescent humans and primates (6, 28). In the current study, immunoblot analysis of sera obtained from guinea pigs recovering from a keratoconjunctival infection showed a serum response to both Ipa proteins (Fig. 1) and LPS (Tables 3 and 4) comparable with that observed for humans and primates recovering from shigellosis (6, 12, 28, 30). Thus, the protective immunity and

TABLE 4. Effect of boosting immunization with EcSf2a-2 on protection and serum response to *S. flexneri* 2a LPS of immunized animals

Immunization schedule[a]	No. of eyes with the following reaction rating:				Protection (%)			Serum titer[b] on day:			
	0	1	2	3	Full	Partial	Combined	0	12	26	36
Group 1 (days 0, 2, 14, and 15)	5	5	0	0	50	50	100	5	10	400	3,200
Group 2 (days 0, 2, 4, and 14)	5	5	1	1	42	42	83	5	10	400	3,200
Group 3 (days 0, 2, and 4)	2	2	7	1	17	17	33	5	7	50	800
Group 4 (days 0, 2, and 4)	4	1	5	2	33	8	42	5	28	1,600[c]	NA
Controls (no immunization)	3	0	0	15	17	0	17	ND	NA	NA	267

[a] Immunization was done with an average dose of 3.8×10^8. In groups 1, 2, and 3, challenge was given 28 days after the first immunizing dose (14 days after the last dose for groups 1 and 2, and 28 days after the last dose for group 3). In group 4, challenge was given 14 days after the last immunizing dose.

[b] ELISA titer is defined as the reciprocal of the last dilution having an optical density at 405 nm of 0.100 or more. The ELISAs were run against *S. flexneri* 2a LPS using alkaline phosphatase-conjugated protein A (see Materials and Methods). Two to three replicates were run for each serum sample. Figures given are the averages of the values for the two animals in each group. Serum samples were collected from two animals from each immunization group on day 0 (preimmunization), day 12 (post-first immunization), day 26 (post-second immunization), and day 36 (postchallenge). Serum samples were collected from three animals from the control group on day 36 of the experiment. ND, not done; NA, not applicable (see footnote *c*).

[c] Animals in groups 1, 2, and 3 were bled 8 days postchallenge on day 36. Animals in group 4 were also bled 8 days postchallenge, which for this group was on day 22 of the experiment.

the immune response found in the guinea pig keratoconjunctivitis model closely correspond to these parameters in humans and primates.

One major advantage of a small-animal model for vaccine testing is the ability to examine a large number of potential vaccine strains without using the difficult and expensive primate trials that have been required for initial screening trials in the past. Previous exposure to *Shigella* species is an additional problem often encountered in primate studies, making evaluation of protection data difficult. To determine whether the results obtained from the keratoconjunctivitis model were consistent with results obtained from primate studies, we compared the results of efficacy studies with the two *aroD* mutant vaccine strains, Sfl 124 and EcSf2a-2, obtained from both animal models. When Lindberg et al. (20) immunized nine monkeys on days 0, 7, 14, and 39 with Sfl 114, the tetracycline-resistant precursor strain to Sfl 124, all monkeys were protected against challenge with the virulent parent strain, Sfl 1, 13 days following the last immunization. Similar levels of protection were obtained with the keratoconjunctivitis model after immunization with Sfl 124 and homologous challenge with Sfl 1 14 days after the boosting dose (Table 2). When 16 monkeys were immunized with three doses of vaccine strain EcSf2a-2 within a 7-day period, only 5 of the 16 developed symptoms of shigellosis, compared with 81% of the 18 controls, giving a 60% protection rate (7). Results obtained from the guinea pig model also showed significant protection ($P < 0.01$) with this vaccine (Tables 3 and 4 and Fig. 2). An increase in serum antibody titer to the serotype 2a LPS following immunization occurred in both animal models (11) (Tables 3 and 4). Since the protection data for the primate and guinea pig models appear to be comparable, we suggest that the guinea pig model can be used to evaluate the protective efficacy of candidate vaccine strains prior to testing in primates or humans. The finding that guinea pigs are not preexposed to *Shigella* infection is also an advantage of this model.

Previous *Shigella* vaccine trials have used a variety of immunization schedules, some with several immunizations spaced 1 to 2 days apart and some with one or more boosting immunizations 7 to 39 days following the primary immunization (8, 17, 19, 23–26, 30). Since it is possible that different vaccine strains might require different immunization schedules, testing of a variety of regimens for optimum protection is a critical task of a vaccine trial. An additional advantage of the keratoconjunctivitis model for the evaluation of a vaccine strain is the ability to easily test various parameters, such as immunization regimens and length of protection conferred by a vaccine strain. For example, in this study it was found that primary immunization followed by a boosting dose 14 days later produced the best protection against challenge with virulent *Shigella* strains (Table 4) as well as an optimal serum response (Tables 3 and 4 and Fig. 3). In fact, the postchallenge LPS titers of animals which had received a boosting dose was substantially higher than those of animals that had recovered from a natural infection. However, the immune response of the animals to vaccine strains Sfl 124 and EcSf2a-2 was different. Animals responded similarly to the Sfl 124 attenuated *Shigella* strain and to a virulent *S. flexneri* strain (Fig. 1 and 3A), i.e., there was a response to the Ipa proteins as well as to LPS. On the other hand, sera obtained from animals immunized with the hybrid EcSf2a-2 strain reacted strongly only to LPS. This difference in response is not due to an absence of the Ipa proteins in the EcSf2a-2 strain, since sera obtained from guinea pigs challenged with virulent *Shigella* strains recognize Ipa proteins in this vaccine strain (data not shown). It is possible that these proteins are not expressed in the vaccine organisms at levels high enough for a measurable immune response, but a serum response could possibly appear with further immunizations.

There has been little information generated concerning the length of protection conferred by either a natural infection or a particular vaccine strain. Earlier studies examining protective immunity following recovery from keratoconjunctivitis shigellosis suggested that resistance to reinfection lasted several months (22, 32). Mel et al. reported that in field trials with soldiers stationed in Yugoslavia who had been immunized with a live attenuated *Shigella* vaccine, protection endured for at least several months (24). The experiments reported in this article indicate that the keratoconjunctivitis model provides a means to monitor the length of protection

conferred by a natural infection and by immunization with live attenuated vaccine strains. In addition, it was noted that although full protection against disease dropped off following either a natural infection (after 3 months) or immunization with vaccine strain EcSf2-2 (after 4 weeks), partial protection against disease remained for an additional period of time. It is possible that additional boosting doses could be given to maintain adequate protection for a longer period of time; this possibility could be tested with the guinea pig model.

In summary, the data presented in this report indicate that the keratoconjunctivitis model provides a simple and cost-effective method to test the efficacy of vaccine candidate strains and to monitor the serum immune response to these strains. This model yields protection data comparable to those of primate studies with the vaccine strains examined in this study. In addition, it provides a means of testing a variety of immunization regimens as well as other variables important to the effectiveness of a vaccine strain, such as optimum time of protection following immunization, dosage effect on protection, and the serum immune response to different antigens. Therefore, we propose that the guinea pig keratoconjunctivitis model can be used for the initial evaluation of candidate vaccine strains in conjunction with safety studies and testing with primates and humans.

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