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Development of an *Aotus nancymaae* Model for *Shigella* Vaccine Immunogenicity and Efficacy Studies

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Several animal models exist to evaluate the immunogenicity and protective efficacy of candidate *Shigella* vaccines. The two most widely used nonprimate models for vaccine development include a murine pulmonary challenge model and a guinea pig keratoconjunctivitis model. Nonhuman primate models exhibit clinical features and gross and microscopic colonic lesions that mimic those induced in human shigellosis. Challenge models for enterotoxigenic *Escherichia coli* (ETEC) and *Campylobacter* spp. have been successfully developed with *Aotus nancymaae*, and the addition of a *Shigella-Aotus* challenge model would facilitate the testing of combination vaccines. A series of experiments were designed to identify the dose of *Shigella flexneri* 2a strain 2457T that induces an attack rate of 75% in the *Aotus* monkey. After primary challenge, the dose required to induce an attack rate of 75% was calculated to be 1×10^{11} CFU. *Shigella*-specific immune responses were low after primary challenge and subsequently boosted upon rechallenge. However, preexisting immunity derived from the primary challenge was insufficient to protect against the homologous *Shigella* serotype. A successive study in *A. nancymaae* evaluated the ability of multiple oral immunizations with live-attenuated *Shigella* vaccine strain SC602 to protect against challenge. After three oral immunizations, animals were challenged with *S. flexneri* 2a 2457T. A 70% attack rate was demonstrated in control animals, whereas animals immunized with vaccine strain SC602 were protected from challenge (efficacy of 80%; $P = 0.05$). The overall study results indicate that the *Shigella-Aotus nancymaae* challenge model may be a valuable tool for evaluating vaccine efficacy and investigating immune correlates of protection.

Shigellosis, or bacillary dysentery, resulted in more than 100,000 deaths globally in 2010, mostly in developing countries (1). Although shigellosis is considered a disease of developing countries, over 14,000 laboratory-confirmed cases are reported to occur in the United States annually (2). In the United States, *Shigella* infections constitute the third most common cause of gastroenteritis, after *Campylobacter* and *Salmonella* infections. Populations particularly susceptible are children in day care centers, migrant workers, travelers to developing countries, and homosexual men (3–6). The low infectious dose, the fecal-oral route of transmission, and the emergence of resistance to multiple antibiotics among *Shigella* isolates pose a major public health problem throughout the developing world and necessitate the development of a safe, efficacious vaccine.

There are several animal models to investigate pathogenic mechanisms utilized by *Shigella* spp. and to evaluate the immunogenicity and protective efficacy of candidate vaccines. The two most widely used models for vaccine development include a murine pulmonary challenge model (7), which is useful for preliminary screening of vaccine candidates, and a guinea pig keratoconjunctivitis model (8). The ability of *Shigella* to invade the corneal epithelium of guinea pigs and spread to contiguous cells, causing keratoconjunctivitis, provides a model system that mimics the invasive process that occurs in the mucosal epithelium. Recently, a guinea pig rectocolitis model has been described (9) that induces bloody, mucoidal stools. Adaptations to the published protocol have facilitated use of the rectocolitis model in vaccination/efficacy studies in larger and older guinea pigs (R. W. Kaminski and E. V. Oaks, unpublished data).

Nonhuman primate models also exist for shigellosis and have been used to better understand pathogenesis (10) and to evaluate vaccine immunogenicity and efficacy (11). In the rhesus monkey model, oral challenge doses are administered at levels of 1×10^{10} to 1×10^{11} CFU, and the animals are given bicarbonate solution to neutralize stomach acidity. The clinical features combined with gross and microscopic colonic lesions induced by wild-type shigellae in monkeys are similar to those induced in human shigellosis (12). The similar disease courses and pathologies of human and monkey shigellosis provide an excellent model to study shigellosis. Despite the similarities, several differences remain between the pathology associated with human and monkey shigellosis. For example, gastric mucosal lesions have been observed in rhesus monkeys after experimental or natural infection with shigellae (10), whereas in humans, lesions are limited to the colonic epithelium (13). Oral feeding of rhesus monkeys with *Shigella flexneri* 2a induces an inflammatory reaction in the gastric mucosa that is similar to that in the gut. The gastric lesions could be a result of the high level of bacteria (10^{10} CFU) needed for challenge or differences in rhesus monkey physiology compared to human physiology.

In recent years, oral challenge models have been developed in *Aotus nancymaae* monkeys for both *Campylobacter jejuni* and en-

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terotoxigenic *Escherichia coli* (ETEC). Both *Aotus* challenge models result in reproducible attack rates of ≥70% and are characterized by colonization of the gastrointestinal tract and the induction of diarrhea (14, 15). The addition of a *Shigella-Aotus* challenge model would enable the testing of potential combination vaccines against the three most common enteric bacterial pathogens responsible for traveler's diarrhea.

To that end, the research described herein focuses on determining a dose of *S. flexneri* 2a strain 2457T that reproducibly achieved an attack rate of ≥75%. Once the challenge dose was established, the immunogenicity and protective efficacy of a well-characterized, live-attenuated *Shigella flexneri* 2a vaccine strain, SC602, were investigated in the *Aotus* model.

MATERIALS AND METHODS

Animal use and welfare. Captive-bred *Aotus nancymaae* monkeys were purchased from the Facultad de Medicina Veterinaria de la Universidad Nacional Mayor de San Marcos, Lima, Peru, and shipped to U.S. Naval Medical Research Unit No. 6 (NAMRU-6) in Lima, Peru, for the study. The animals had not previously been used in a *Shigella* study. The study was conducted in an Association for the Assessment and Accreditation of Laboratory Animal Care, International, accredited vivarium with local approval by the NAMRU-6 Institutional Animal Care and Use Committee (IACUC), second-level approval from the U.S. Navy Bureau of Medicine and Surgery, and was approved by the Peruvian Dirección General Forestal y de Fauna Silvestre (resolution number 0023-2011-AG-DGFFS-DGEFFS). 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 the principles stated in the *Guide for Care and Use of Laboratory Animals* (16).

The animals were identified by unique tattoo numbers on their abdomens and were maintained in pairs when not required to be individually housed for sample collection. Prior to inclusion in the study, animals were screened by stool cultures for existing infection with *Shigella* spp. and for prior *Shigella* exposure by enzyme-linked immunosorbent assay (ELISA) for anti-*S. flexneri* 2a lipopolysaccharide (LPS) serum IgG titers. Those animals meeting the inclusion criteria (negative stool cultures and IgG titers of ≤20) were randomized to the various treatment groups. *Aotus* monkeys used in the challenge dose-finding study had a mean (± standard deviation [SD]) weight of 840 ± 66 g and a mean age of 19 ± 3 months on day 0 of the study. *Aotus* monkeys used in the vaccine immunogenicity and efficacy study had a mean weight of 868 ± 86 g and a mean age of 20 ± 5 months on day 0 of the study.

Preparation and administration of *Shigella* vaccine and challenge inoculums. *Shigella flexneri* 2a strain 2457T is a wild-type *Shigella* strain that is Sereny test positive (17), pathogenic to monkeys (11, 18), and virulent in humans (19, 20). A vial of current good manufacturing practice (cGMP) *S. flexneri* 2a 2457T was reconstituted in saline, serially diluted, and plated for isolation on Trypticase soy agar (TSA) with 0.01% Congo red dye. After overnight incubation at 37°C, three small, smooth, Congo red-positive colonies were used to inoculate TSA plates (without Congo red) for confluent growth. The plates were harvested with 3.0 ml of cold phosphate-buffered saline (PBS), and the suspension was diluted based on a standardized OD_{600} (optical density at 600 nm) value. *Shigella flexneri* 2a vaccine strain SC602 has deletions in *virG* (*icsA*) and *iuc* (encoding aerobactin) genes (20). Strain SC602 is Congo red positive, indicating retention of the virulence plasmid, and unable to cause keratoconjunctivitis in the guinea pig eye (Sereny test negative) (21). *S. flexneri* 2a strain SC602 (20) was propagated using the same procedures used for *S. flexneri* 2a strain 2457T.

Monkeys were not allowed food overnight prior to administration of vaccine or challenge inoculums. Gastric acid production was inhibited with ranitidine (1.5 mg/kg of body weight) by intramuscular (i.m.) injection 90 min prior to inoculum delivery. Anesthetized animals (ketamine HCl; 50 mg/ml, 4 to 5 mg/kg, given i.m.) were orogastrically administered 5 ml of the rice-based buffer CeraVacx I (CeraProducts, Jessup, MD) using a single-use, sterilized, 5 Fr/Ch (1.7-mm) 16-in. (41-cm) feeding tube to neutralize the stomach contents. Immediately prior to inoculum delivery, the fluid content of the stomach was sampled and measured for pH. The challenge dose of *S. flexneri* 2a strain 2457T and the immunization dose of *S. flexneri* 2a strain SC602 were delivered orogastrically in a 5-ml volume using a new feeding tube.

Challenge dose-finding study design. Groups (9 animals/group) of *A. nancymaae* were orogastrically inoculated with increasing doses (5×10^9, 5×10^{10}, or 5×10^{11} CFU) of *Shigella flexneri* 2a 2457T. A fourth group of 10 animals was administered PBS. Nine weeks following primary challenge (day 63), all animals were rechallenged with 1×10^{11} CFU of *S. flexneri* 2a 2457T. Animals were observed for 10 days following each inoculation for symptoms of illness (described below) and then treated with enrofloxacin (5 mg/kg, i.m.) daily for 5 days.

Vaccine immunogenicity and efficacy study design. Groups of eight *A. nancymaae* were orogastrically immunized on study days 0, 14, and 42 with 1×10^{10} or 1×10^{11} CFU of the live-attenuated vaccine strain *Shigella flexneri* 2a strain SC602. Another group was immunized with a subclinical dose (1×10^9 CFU) of *S. flexneri* 2a strain 2457T. A control group (10 *Aotus* monkeys) was inoculated with PBS on the same schedule. On study day 70, all animals were orogastrically challenged with 1×10^{11} CFU of *S. flexneri* 2a 2457T as described above.

Observations after vaccination and challenge. Animals were observed for signs and symptoms of diarrhea twice daily prior to each vaccination or challenge, for 5 days after vaccination, and for 10 days after challenge. Observations included activity level, stool consistency, and the presence of blood observed in the feces. Activity level was scored on a scale of 0 to 3 as follows: 0, active and responsive; 1, reduced activity; 2, immobile; 3, recumbent. Fecal occult blood was determined by a hemoccult test (Hemoccult II SENSA; Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. Stools were graded daily as follows: grade 1 (formed, firm stool pellets), grade 2 (formed but soft stool pellets or droppings), grade 3 (loose, unformed feces), grade 4 (watery, nonclear feces), and grade 5 (watery, clear liquid stools). Stools graded 1 or 2 were considered normal, whereas stools graded 3, 4, or 5 were considered abnormal. The case definition of a diarrhea episode was defined as the passing of a grade 3 or higher stools for at least two consecutive days during the observation period. The duration of diarrhea was defined as the time between the first day of a diarrhea episode and the last day of diarrhea preceding two consecutive diarrhea-free days. Animals meeting the case definition of diarrhea prior to challenge were excluded from data analysis. Clinical symptoms of *Shigella*-induced gastroenteritis were defined as evidence of *Shigella* colonization (PCR or isolation) and either (i) an episode of diarrhea (as defined above), (ii) blood in the stool (occult, gross, or melena) for two consecutive days, or (iii) death.

Clinical sample collection and processing. Blood samples were collected, and serum samples were stored at −80°C until assayed by ELISA. Blood samples were collected from individual animals on study days 0, 7, 14, 21, 49, 70, and 77 in the challenge dose-finding study. In the vaccine immunogenicity and efficacy study, blood samples were collected on study days 0, 21, 49, 70, 77, and 84. Stool samples were collected from cage drop pans before immunization or challenge and daily for 10 days after each vaccination or challenge.

***Shigella* colonization determination.** Colonization of *Aotus* after vaccination or challenge was determined as previously described for rhesus macaques (22). Briefly, stool was streaked onto Hektoen enteric agar plates. Suspected *Shigella* colonies were confirmed by slide agglutination with commercially available *S. flexneri* 2a antiserum (Denka Seiken Co.) or by colony immunoblotting with the anti-IpaB monoclonal antibody 2F1 (23). Stool samples testing negative for *Shigella* were subjected to PCR analysis targeting the *ipaH* gene (22).

Immunogenicity assessment. Serum antigen-specific antibody responses were assessed by an ELISA as previously described (24) with the

TABLE 1 Incidence of diarrhea and clinical symptoms after oral challenge of *Aotus nancymaae* with escalating doses of *S. flexneri* 2a strain 2457T and homologous rechallenge[a]

Group	Treatment (dose [CFU])	Primary challenge				Homologous rechallenge			
		No. of animals	Incidence (%) of diarrhea[b]	Incidence (%) of clinical symptoms[c]	*P* value[d]	No. of animals	Incidence (%) of diarrhea	Incidence (%) of clinical symptoms	*P* value
1	*S. flexneri* 2a 2457T (5×10^9)	8[e]	25	50	0.558	8[e]	25	38	0.145
2	*S. flexneri* 2a 2457T (5×10^{10})	9	56	56	0.057	7[e,f]	43	71	1.000
3	*S. flexneri* 2a 2457T (5×10^{11})	9	100	100	0.0001	8[f]	38	50	0.321
4	PBS	10	10	10		10	50	80	

[a] The groups of *Aotus nancymaae* monkeys were challenged with *S. flexneri* 2a strain 2457T, treated with 1×10^{11} CFU of *S. flexneri* 2a 2457T, and then rechallenged with a homologous strain.
[b] Diarrhea was defined as at least one loose-watery stool on at least two consecutive days during the observation period (10 days).
[c] Clinical symptoms of *Shigella*-induced gastroenteritis were defined as evidence of *Shigella* colonization (PCR or isolation) and either (i) an episode of diarrhea, (ii) blood in the stool (occult, gross, or melena) for two consecutive days, or (iii) death.
[d] The *P* values were calculated by Fisher's exact test compared to control group inoculated with PBS.
[e] One animal excluded from data analysis due to diarrhea for 2 days prior to challenge.
[f] One animal euthanized after the primary challenge.

following modifications: the antigen coating concentrations were 10 μg/ml for *S. flexneri* 2a LPS and 1 μg/ml for *S. flexneri* 2a Invaplex (25), and purified IpaB and IpaC were used in a total assay volume of 100 μl. Conjugated rabbit anti-*Aotus* IgG and anti-*Aotus* IgA secondary antibodies (Lampire Biological Labs Inc., Pipersville, PA) were used to detected antigen-bound serum antibodies. Seroconversion was defined as ≥4-fold increase in the titer over the baseline level.

Statistical analysis. Intragroup comparisons of clinical and immunologic outcomes were performed using nonparametric tests for continuous outcomes (Wilcoxon rank sum test for comparing the values for two groups; Kruskal-Wallis test for comparing the values for more than two groups) and Fisher's exact tests for nominal outcomes. All statistical tests were interpreted in a two-tailed fashion with acceptance of significance set at the $P < 0.05$ level.

RESULTS

Dose-finding and rechallenge study. (i) Clinical symptoms, microbiology, and challenge results. Results from a preliminary, pilot study indicated that oral challenge with 5×10^9 CFU of *S. flexneri* 2a strain 2457T induced diarrhea in 1 of 3 animals (33%) and did not cause significant disease in the remaining animals (data not shown). Therefore, three groups of *A. nancymaae* monkeys were orally challenged with *S. flexneri* 2a 2457T at either 5×10^9, 5×10^{10}, or 5×10^{11} CFU (Table 1)?> to determine a challenge dose that induced diarrhea in at least 75% of the animals. *Aotus* monkeys in the control group were mock challenged with PBS. One of the 10 animals (10%) in the PBS control group was positive for diarrhea for 9 days after inoculation with PBS. *Shigella* spp. were not recovered from the animal in fecal cultures, and the stools were *ipaH* negative by PCR at all time points.

In contrast, *S. flexneri* 2a strain 2457T induced diarrhea in 25, 56, and 100% of animals orally inoculated with 5×10^9, 5×10^{10}, and 5×10^{11} CFU, respectively. Disease was characterized as loose, low-volume stools with either gross or occult blood present. There was no significant difference in the colonization rate or duration between dose groups. Only the group administered 5×10^{11} CFU of *S. flexneri* 2a 2457T had a significantly higher number of days of diarrhea (Table 2) compared to PBS controls. On day 2 after challenge, one animal inoculated with 5×10^{11} CFU was euthanized due to severe disease. An additional animal that was

TABLE 2 Diarrhea and colonization after oral challenge of *A. nancymaae* with *S. flexneri* 2a strain 2457T

Treatment (CFU)	Diarrhea[a]				Colonization[b]		
	No. of animals	No. of cases	Mean no. of days to onset (range)	Mean no. of days to illness (range)	% Incidence	Median no. of days to onset	Median no. of days of duration (range)
S. flexneri 2a 2457T (5×10^9)	8[c]	2	4 (3–5)	5 (2–8)	100	1	10 (3–10)
S. flexneri 2a 2457T (5×10^{10})	9	5	2 (1–4)	7 (5–10)[d]	100	1	7.5 (2–10)
S. flexneri 2a 2457T (5×10^{11})	9	8[e]	3 (1–3)	5 (2–10)[f]	100	1	8.5 (5–10)
PBS	10	1	1	0.9 (0–9)	0		

[a] Diarrhea defined as at least one loose-watery stool on at least two consecutive days during the observation period (10 days) postchallenge.
[b] Colonization assessed by plating on HE agar with confirmatory slide agglutination or colony blotting. Negative samples confirmed with *ipaH*-specific PCR on frozen stool specimens.
[c] One animal excluded from data analysis due to diarrhea for 2 days prior to challenge.
[d] One animal removed from the study on day 6 postchallenge; full duration data not collected.
[e] One animal removed from the study at 2 days postchallenge; excluded from diarrhea incidence data analysis.
[f] $P = 0.002$ (Mann-Whitney; two-tailed, $\alpha = 0.05$) compared to PBS control group.

inoculated with 5×10^{10} CFU was euthanized 6 days postchallenge due to bloody vomitus and lethargy. Necropsy of the animals revealed hemorrhagic and necrotic small intestines and stomachs. Tissue samples collected from the colon, ileum, and stomach were macerated, cultured, and tested positive for *S. flexneri*. Tissue samples from the peritoneum tested negative for enteropathogens.

The incidence of clinical symptoms, which captures disease and death due to infection, was used to calculate a dose of 1×10^{11} CFU to result in a 75% attack rate. The dose of *S. flexneri* 2a strain 2457T expected to induce the targeted 75% attack rate in naive *Aotus nancymaae* was calculated by applying a linear fit to the line generated after plotting the $\log_{10}$-transformed doses (CFU) of *S. flexneri* 2a 2457T versus the attack rate achieved at each dose and interpolating the expected dose.

The animals were allowed to rest for 9 weeks, and then all groups were orally challenged with 1×10^{11} CFU of *S. flexneri* 2a strain 2457T. Veterans from the primary challenge were used to determine whether protection could be achieved after homologous rechallenge, whereas the veteran PBS control group was used to confirm that the calculated dose would result in a ≥75% attack rate.

Challenge of the veteran PBS groups with *S. flexneri* 2a strain 2457T resulted in an 80% attack rate confirming previous results (Table 1). Animals previously infected with *S. flexneri* 2a 2457T were not protected upon subsequent rechallenge with a homologous strain (Table 1) with the incidence of clinical symptoms ranging from 38% to 71%. There was no significant difference in the duration of diarrhea or colonization among the study groups. Three animals were euthanized due to complications from the second challenge, two animals from the group that previously received PBS during the primary challenge phase of the experiment and one animal that had received 5×10^{11} CFU. All euthanized animals had occult blood in the stool and bloody vomitus prior to death. Upon necropsy, blood and colitis were noted in the distal colon of two animals (*Shigella* veteran and PBS control veteran) with no pathology in the stomach or small intestine. Necropsy of the third animal (PBS control veteran) revealed loose stool without blood in the large intestine and necrosis in the stomach.

(ii) Immunological assessment. Individual animals were bled before challenge on day 0 and on days 7, 14, 21, and 49 after the primary challenge. Blood was also collected 1 and 2 weeks (days 70 and 77) after the second challenge. Serum IgG and IgA endpoint titers directed against *S. flexneri* 2a LPS and *S. flexneri* 2a Invaplex, IpaB, and IpaC were determined by ELISA (Fig. 1). *Shigella*-specific antibodies were not detected in serum of animals treated with PBS at any time point during the primary challenge phase of the study. Furthermore, baseline *Shigella*-specific antibodies were low across all groups prior to challenge. The serum IgG titers directed against LPS, Invaplex, IpaB, and IpaC followed a dose-dependent relationship with animals receiving the highest dose of *S. flexneri* 2a strain 2457T possessing the highest antigen-specific antibody titers (Fig. 1). Seroconversion to Invaplex (which includes LPS, IpaB, and IpaC antigens [25]) was challenge dose dependent with 25%, 63%, and 88% of *Aotus* monkeys challenged with 5×10^9, 5×10^{10}, and 5×10^{11} CFU, respectively, having at least a 4-fold increase in serum IgG titers. IpaC-specific titers were low to undetectable across all groups after the primary challenge. A slight decline was noted in the LPS- and Invaplex-specific IgG titers between samples collected on day 21 and day 49, whereas IpaB and IpaC titers remained constant or increased slightly during the same time period. *Shigella*-specific serum IgG titers increased ~0.5 to 2 log units after rechallenge with a homologous serotype. The kinetics of the serum IgG response directed against the *Shigella* antigens during the second challenge phase of the study in the PBS control group largely mirrored the kinetics of the response previously demonstrated after challenge with 5×10^{11} CFU in the primary challenge phase of the experiment.

Shigella-specific serum IgA responses after primary challenge with 5×10^9 and 5×10^{10} CFU were low, with less than 25% of animals seroconverting to any of the antigens (Fig. 1). Moderate levels of *Shigella*-specific IgA were elicited after oral inoculation with 5×10^{11} CFU of *S. flexneri* 2a strain 2457T with 38 to 50% of *Aotus* seroconverting after primary challenge. After rechallenge, there were significant increases in Invaplex- and IpaB-specific serum IgA titers, whereas IgA responses directed against IpaC and LPS were largely unchanged (Fig. 1).

Reactogenicity and colonization after oral immunization of *A. nancymaae* with strain SC602 or wild-type *S. flexneri* 2a strain 2457T. The reactogenicity, immunogenicity, and protective efficacy of a live-attenuated *Shigella flexneri* 2a vaccine strain, SC602, was assessed in the *Aotus nancymaae* model. SC602 has been previously shown to be immunogenic and protective against shigellosis in clinical trials (20) and in the rhesus macaque model (M. M. Venkatesan, unpublished data). In addition, a group of *Aotus* monkeys were immunized with *S. flexneri* 2a 2457T (1×10^9 CFU) to test the hypothesis that multiple immunizations with a subclinical dose could induce a protective immune response. As presented above, primary challenge with *S. flexneri* 2a 2457T at 5×10^9 CFU followed by rechallenge with 1×10^{11} CFU resulted in a strong immune response directed against multiple *Shigella* antigens (Fig. 1) conveying partial protection as evidenced by a low rate of diarrhea (Table 2), suggesting that low-level infections could confer protection against a larger bolus of *Shigella*.

Groups were orally immunized on days 0, 14, and 42 with strain SC602 (10^{10} or 10^{11} CFU/dose) or *S. flexneri* 2a strain 2457T (10^9 CFU/dose). Clinical symptoms and bacterial colonization were monitored for 10 days after each immunization (Table 3). As expected, animals mock immunized with PBS were not colonized with shigellae. All animals immunized with SC602 were colonized after each oral immunization for 1 to 8 days. The number of diarrhea cases was also low after each immunization with SC602. In contrast, oral immunization with *S. flexneri* 2a 2457T induced diarrhea in 75% (6/8) of animals after each immunization, which was significantly higher than the number of cases of diarrhea in PBS controls ($P = 0.007$ by Fisher's exact test), and colonization rates were also substantial (75 to 100%).

Protective efficacy after oral immunization of *Aotus* with live-attenuated strain SC602 or wild-type *S. flexneri* 2a strain 2457T. Animals orally immunized on days 0, 14, and 42 were subsequently challenged with an oral dose of *S. flexneri* 2a strain 2457T (1×10^{11} CFU) on day 70 and monitored for 10 days (Table 4). Two animals immunized with strain SC602 were excluded from analysis due to the onset of diarrhea prior to challenge. The diarrhea attack rate in the placebo group was 70% (7/10 animals) and 14% (1/7 animals) in groups immunized with 1×10^{10} or 1×10^{11} CFU of strain SC602 (80% protective efficacy; $P = 0.05$). Comparison of the PBS control group to both groups immunized with SC602 (1×10^{10} and 10^{11} CFU) resulted in 80% protective

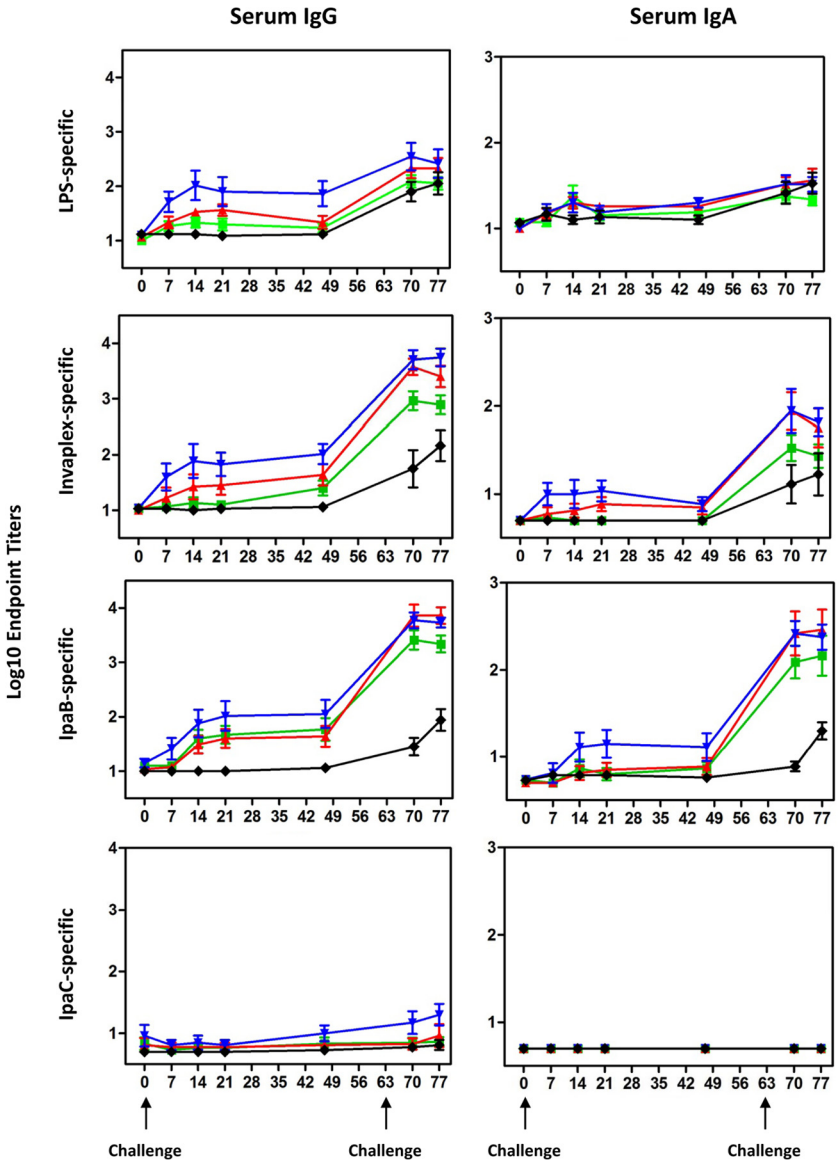

FIG 1 Kinetics of the *Shigella*-specific serum IgG and IgA responses in *A. nancymaae* after oral challenge with escalating doses of *S. flexneri* 2a strain 2457T and rechallenge with 1×10^{11} CFU of the homologous strain. Groups of *A. nancymaae* monkeys were orally challenged on study day 0 with 5×10^{9} (green square), 5×10^{10} (red triangle), or 5×10^{11} (blue inverted triangle) CFU of *S. flexneri* 2a 2457T. Another group was mock challenged with PBS (black diamond). On study day 63, all groups were orally challenged with 1×10^{11} CFU of *S. flexneri* 2a 2457T. Group mean titers ± 1 standard deviation (error bars) are plotted. The days the animals were challenged are indicated by the black arrows under the graphs at the bottom of the figure.

efficacy ($P = 0.01$ by Fisher's exact test). Immunization with *S. flexneri* 2a 2457T did not result in significant protection (46%; $P = 0.34$), a delay in the mean day of onset or a decrease in the duration of illness (Table 5). There was a significant reduction in the duration of diarrhea after challenge in groups orally immunized with 1×10^{10} CFU of SC602 ($P = 0.05$ by Mann-Whitney test) and 1×10^{11} CFU of SC602 ($P = 0.025$) compared to the PBS control group. There was no difference in the colonization rate or duration after oral challenge with *S. flexneri* 2a 2457T in any of the immunized groups and the placebo controls.

Immune responses after oral immunization of *Aotus* with live-attenuated strain SC602 or wild-type *S. flexneri* 2a strain 2457T and subsequent oral challenge. Blood samples collected on study days 0, 21, 49, 70, 77, and 84 were assayed by ELISA for IgG and IgA endpoint titers directed against *S. flexneri* 2a LPS, Invaplex, IpaB, and IpaC (Fig. 2). Animals mock immunized with PBS did not mount an antigen-specific serum IgG or IgA response during the immunization phase of the study. Furthermore, *Shigella* antigen-specific serum IgG and IgA titers on study day 0 were low in all experimental groups. In contrast, robust levels of serum IgG directed against *S. flexneri* 2a LPS, Invaplex, and IpaB were induced after three oral immunizations with strain SC602 or *S. flexneri* 2a strain 2457T, and the responses were of significantly higher magnitude ($P < 0.01$) than the responses in the PBS control group. Seroconversion after immunization with strain SC602 was dose dependent and most evident in antigen-specific serum IgA. For example, 43% (3/7) of *Aotus* monkeys receiving SC602 (1×10^{10} CFU) seroconverted to LPS compared to 100% (7/7) of

TABLE 3 Clinical symptoms after oral immunization with either live-attenuated vaccine strain SC602 or wild-type *S. flexneri* 2a strain 2457T

Time and clinical parameter	Value for treatment group[a]			
	SC602 ($n = 8$) (1×10^{10} CFU)	SC602 ($n = 8$) (1×10^{11} CFU)	*S. flexneri* 2a 2457T ($n = 8$) (1×10^{9} CFU)	PBS ($n = 10$)
After vaccination 1				
No. of cases of diarrhea[b]	1	1	6	2
Mean day of onset (range)	7 (NA)	1 (NA)	3.8 (1–8)	7 (1–9)
Mean no. of days of illness (range)	2 (NA)	10 (NA)	3.5 (2–9)	3.5 (2–5)
% Colonization[c]	100	100	75	0
Median duration (range)	1 (1–3)	4 (1–8)	7.5 (2–10)	
After vaccination 2				
No. of cases of diarrhea	2	1	6	0
Mean day of onset (range)	2.5 (2–3)	1 (NA)	2 (1–5)	0
Mean no. of days of illness (range)	3 (2–4)	5 (NA)	6 (2–10)	0
% Colonization	100	100	100	0
Median duration (range)	2 (1–3)	2 (2–4)	5 (1–8)	
After vaccination 3				
No. of cases of diarrhea	0	2	6	0
Mean day of onset (range)	0 (NA)	1 (NA)	2.8 (1–6)	0
Mean no. of days of illness (range)	0 (NA)	8 (6–10)	4 (2–8)	0
% Colonization	100	100	100	0
Median duration (range)	2 (1–2)	2 (1–3)	6 (1–8)	

[a] NA, not applicable.
[b] Diarrhea defined as at least one loose-watery stool on at least two consecutive days during the observation period (10 days).
[c] Colonization assessed by plating of HE agar with confirmatory slide agglutination or colony blotting. Negative samples confirmed with *ipaH*-specific PCR on frozen stool specimens.

animals receiving SC602 (1×10^{11} CFU). Similarly, none of the animals immunized with SC602 (1×10^{10} CFU) had IpaB-specific serum IgA, whereas 57% (4/7) of *Aotus* immunized with SC602 (1×10^{11} CFU) seroconverted to IpaB. Interestingly, only animals immunized with *S. flexneri* 2a 2457T had detectable anti-IpaC serum IgG (38% [3/8]) and IgA (13% [1/8]) responses after immunization, albeit at low levels. However, there was no significant difference in seroconversion rates between animals immunized with SC602 (1×10^{10} or 1×10^{11} CFU) and animals immunized with *S. flexneri* 2a 2457T across all antigens assayed. An increase in serum IgG and IgA directed against LPS, Invaplex, and IpaB was demonstrated after challenge, indicating that vaccination effectively primed the ensuing immune response.

DISCUSSION

The three most common bacterial pathogens responsible for traveler's diarrhea include ETEC, *Campylobacter*, and *Shigella* (6). In addition, significant morbidity and mortality are associated with these enteric pathogens in impoverished areas where the disease is endemic (26). Substantial efforts over the past decade have resulted in the generation of several vaccine candidates to prevent the causes of diarrhea by these enteric bacterial pathogens. Ideally, a combination vaccine capable of protecting against ETEC, *Shigella*, and *Campylobacter* will be developed and deployed. A single animal model to evaluate immunogenicity and efficacy of a combination enteric vaccine may greatly facilitate development and evaluation.

The *A. nancymaae* model has been used to evaluate the immunogenicity and efficacy of several ETEC (15) and *Campylobacter* (14, 27) vaccines. The attack rates in naive *Aotus* monkeys orally inoculated with 5×10^{11} to 7×10^{11} CFU of *C. jejuni* are typically ≥70%. Similar attack rates are achieved after oral inoculation of naive *Aotus* with 1×10^{11} to 5×10^{11} CFU of ETEC. Disease in the

TABLE 4 Diarrhea incidence, clinical symptoms, and protective efficacy in *Aotus nancymaae* orally immunized with live-attenuated SC602 vaccine strain or *S. flexneri* 2a strain 2457T and then challenged with *S. flexneri* 2a 2457T

Treatment (CFU dose)	No. of animals	Diarrhea incidence (%)[a]	Clinical symptoms (%)[b]	Protective efficacy (%)[c]	*P* value[d]
SC602 (1×10^{10})	7[e]	14	14	80	0.05
SC602 (1×10^{11})	7[e]	14	14	80	0.05
S. flexneri 2a 2457T (1×10^{9})	8	38	38	46	0.34
PBS	10	70	70		

[a] Diarrhea defined as at least one loose-watery stool on at least two consecutive days during the observation period (10 days).
[b] Clinical symptom defined as diarrhea, bloody stools for 2 days, or death.
[c] Protective efficacy = [(percentage of clinical symptoms of the control group − percentage of clinical symptoms in the vaccinated group)/(percentage of clinical symptoms of the control group)].
[d] Fisher's exact test compared to the PBS control group.
[e] One animal excluded from data analysis due to diarrhea for 2 days prior to challenge.

TABLE 5 Diarrhea duration, clinical symptoms, and colonization after oral immunization of *A. nancymaae* with live-attenuated vaccine strain SC602 or *S. flexneri* 2a strain 2457T and oral challenge with *S. flexneri* 2a 2457T[a]

Treatment	No. of animals	Diarrhea[b]			Colonization[c]		
		No. of cases	Mean no. of days to onset (range)	Mean no. of days illness (range)	% Incidence	Median no. of days to onset	Median no. of days duration (range)
SC602 (1×10^{10} CFU)	7[d]	1	3	4	100	1	7.9 (3–10)
SC602 (1×10^{11} CFU)	7[d]	1	2	2	100	1	8.4 (4–10)
S. flexneri 2a 2457T (1×10^{9} CFU)	8	3	5 (3–6)	3 (2–5)	100	1	6.6 (2–10)
PBS	10	7	3.3 (1–7)	4.6 (2–8)	100	1	8.0 (4–10)

[a] Groups of monkeys were orally immunized on days 0, 14, and 42 with either live-attenuated *Shigella* vaccine strain SC602 or with wild-type *S. flexneri* 2a 2457T at a subclinical dose. The animals were then orally challenged with 1×10^{11} CFU of *S. flexneri* 2a 2457T on day 70.
[b] Diarrhea defined as at least one loose-watery stool on at least two consecutive days during the observation period (10 days).
[c] Colonization assessed by plating of HE agar with confirmatory slide agglutination or colony blotting. Negative samples confirmed with IpaH-specific PCR on frozen stool specimens.
[d] One animal excluded from data analysis due to diarrhea prior to the challenge.

ETEC and *Campylobacter* challenge models is typically characterized by diarrhea and bacterial colonization evidenced by positive stool culture. Although there are similarities between the three challenge models in terms of infectious dose and inducing diarrhea, there are also several key differences between the ETEC- and *Campylobacter-Aotus* models compared to the *Shigella* model. In the *Shigella* model, melena or black tarry stool with gross blood is a typical outcome, whereas gross blood is rarely seen in the ETEC and *Campylobacter* models.

Another characteristic of the *Shigella-Aotus* challenge model that differs from the ETEC- and *Campylobacter-Aotus* challenge models is death in a subset of animals. Necropsies of *Aotus* monkeys challenged with *Shigella* revealed hemorrhagic and necrotic small intestines and stomachs in a subset of animals, similar to reports using rhesus macaques (10). Death after oral challenge of nonhuman primates with shigellae has been documented in several studies (28, 29). Oral challenge of *M. fascicularis* monkeys with 1×10^{10} or 1×10^{11} CFU of *S. flexneri* 2a strain 2457T resulted either in death 3 and 4 days postinoculation or a moribund state requiring humane euthanasia (28, 29). After oral challenge of 40 rhesus monkeys with *S. flexneri* 2a 2457T (3.2×10^{10} CFU), five animals died, reportedly due to necrotizing enteritis characteristic of acute shigellosis (29). In the *Aotus* model, several animals were humanely euthanized or died within a week of oral challenge with $\geq 5 \times 10^{10}$ CFU of *S. flexneri* 2a 2457T. In the subsequent study in which *Aotus* monkeys were challenged with 1×10^{11} CFU of *S. flexneri* 2a 2457T, none of the animals died or required humane euthanasia. It is difficult to speculate on the disparate results between the two *Aotus* studies due in part to the small number of animals in each study. Future work using more animals should help to address the inconsistency.

The majority of results achieved in the current study are consistent with reports describing oral infection of rhesus monkeys with virulent shigellae. The similarities between the two animal models include the dose ($\sim 1 \times 10^{11}$ CFU) required for reproducible infection (28–30), time course and severity of the disease (28, 30, 31), and protection against infection after immunization of rhesus monkeys with live-attenuated SC602 vaccine strain (unpublished results). One difference in the results achieved in the *Aotus* model compared to the rhesus monkey model is protection afforded after homologous rechallenge. A seminal study conducted by Formal and colleagues clearly demonstrated that prior infection with *S. flexneri* 2a protected rhesus monkeys against subsequent challenge with homologous *S. flexneri* 2a but not against heterologous *Shigella sonnei*, despite serum IgG responses directed against the highly conserved Ipa proteins (32). In the rhesus monkey model, serotype-specific LPS responses were suggested as the protective antigen. In a similar fashion, *Aotus* monkeys were challenged with increasing doses of *S. flexneri* 2a, rested for 9 weeks, and then rechallenged with *S. flexneri* 2a. In contrast to rhesus monkeys, no protection was afforded after a homologous back challenge of the *Aotus* monkeys. The discordant results between the two studies may reflect differences in genetic background and susceptibility to infection but may also be a product of significant differences in the experimental procedures. In the *Aotus* studies, all monkeys were screened for serum IgG responses to *S. flexneri* 2a LPS to ensure that there was no preexisting immunity, whereas Formal et al. focused on stool cultures to ensure that no carrier state or active infection was identified. The inoculum dose used by Formal et al. was 2×10^{10} CFU delivered orally in brain heart infusion (BHI) to animals weighing 2.3 to 3.2 kg. The *Aotus* monkeys in the present study weighed ~850 g and were inoculated with $\sim 1 \times 10^{11}$ CFU delivered in a rice-based buffer. The challenge inoculum/weight ratio in the rhesus monkey study (2×10^{10} CFU/2 to 3 kg) may have resulted in a less robust challenge (54% attack rate). Finally, the BHI nutrient medium used in the challenge bolus given in the Formal et al. study may have also impacted gene expression and perhaps invasiveness of the shigellae.

In the challenge/rechallenge study, serum IgG responses directed against *S. flexneri* 2a LPS were low after the primary infection with 5×10^{9} or 5×10^{10} CFU and of moderate magnitude after challenge with 5×10^{11} CFU. However, upon rechallenge, all groups demonstrated a significant boost in the anti-LPS serum IgG titers. These results suggested that perhaps a single infection of *Aotus* monkeys with *S. flexneri* 2a did not sufficiently prime the immune system to provide protective efficacy, but perhaps repeated infections may be required to produce the necessary protective immune response.

To test this hypothesis, *Aotus* monkeys were immunized with a subclinical dose of *S. flexneri* 2a (5×10^{9} CFU) on days 0, 14, and 42. Anti-LPS, anti-IpaB, and anti-IpaC serum IgG and IgA titers

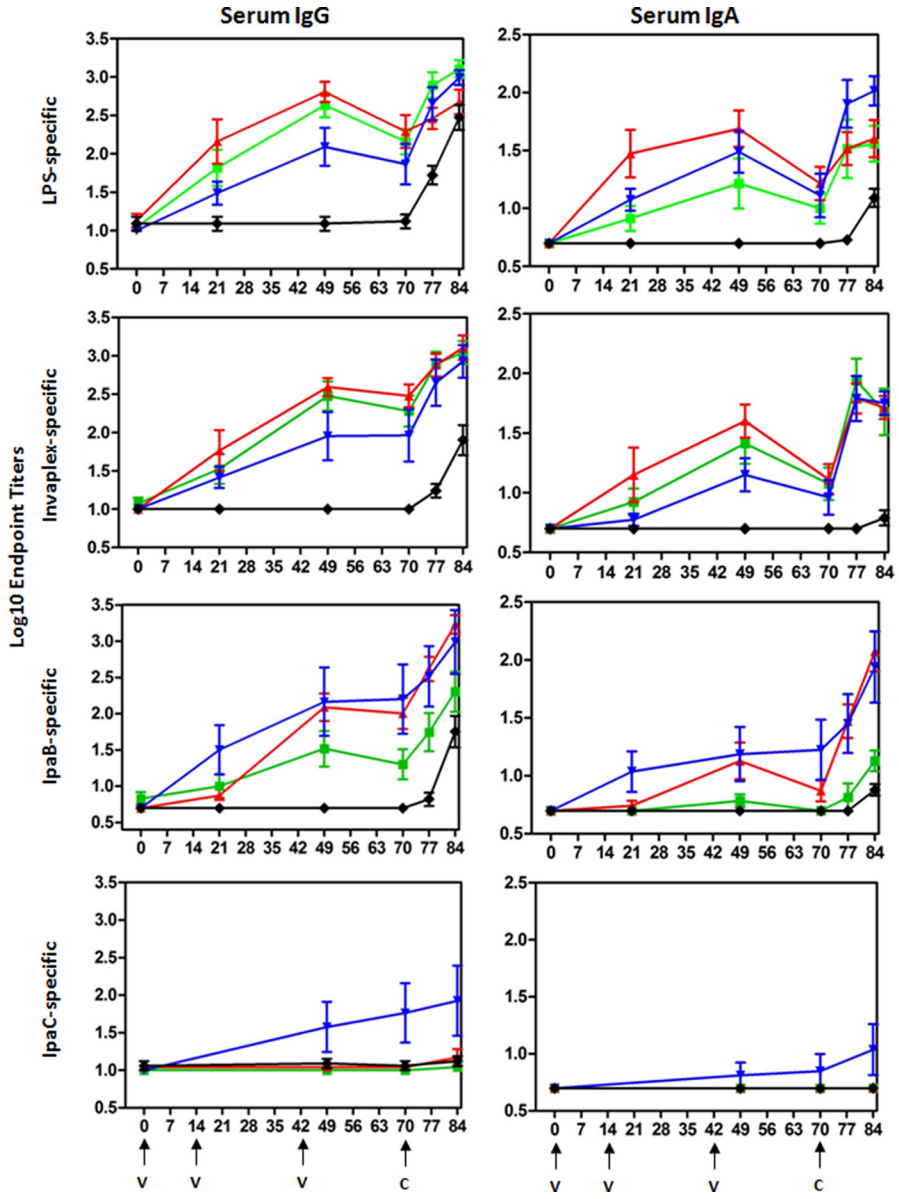

FIG 2 Kinetics of the *Shigella*-specific serum IgG and IgA responses in *A. nancymaae* after oral immunization with live-attenuated vaccine strain SC602 or wild-type *S. flexneri* 2a strain 2457T and oral challenge with *S. flexneri* 2a 2457T. Groups of *A. nancymaae* monkeys were orally immunized on study days 0, 14, and 42 with 1×10^{10} CFU of SC602 (green square), 1×10^{11} CFU of SC602 (red triangle), or 1×10^{9} (blue inverted triangle) CFU of *S. flexneri* 2a 2457T (black diamond). The control group was mock immunized with PBS (black). On study day 70, all groups were orally challenged with 1×10^{11} CFU of *S. flexneri* 2a 2457T. Group mean titers ± 1 standard deviation are plotted. The days of immunization (v) and the days of challenge (c) are indicated by the black arrows under the graphs at the bottom of the figure.

were similar in groups administered *S. flexneri* 2a twice (challenge/rechallenge study) and groups administered *S. flexneri* 2a three times. In agreement with previous results, *Aotus* monkeys administered *S. flexneri* 2a strain 2457T were not significantly protected against back challenge with a homologous *Shigella* serotype. However, in the same study, *Aotus* monkeys immunized with strain SC602 (10^{10} or 10^{11} CFU) were protected against challenge with *S. flexneri* 2a strain 2457T. There were no significant differences in serum IgG or IgA responses specific for Invaplex, LPS, and IpaB in groups immunized with SC602 (10^{10} or 10^{11}CFU) and groups immunized with *S. flexneri* 2a 2457T. Mucosal immune responses were not assessed in the current study and may be responsible for the differences in protective efficacy obtained between 2457T and SC602. After inoculation with 2457T, 75% of *Aotus* monkeys had episodes of diarrhea, whereas only 11 to 22% of *Aotus* monkeys immunized with SC602 experienced similar loose stools. Episodes of diarrhea did not result in a significant reduction in colonization but may have impacted the generation of robust anti-LPS serum antibodies or affected the induction of gut-homing mucosal IgA in the large intestine if an adequate microenvironment was not maintained.

Several other nonhuman primate models (other than *Aotus nancymaae*) have been utilized to investigate *Shigella* pathogenesis and the immunogenicity and efficacy of *Shigella* vaccines, includ-

ing both rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*). *Shigella flexneri* (1×10^{11} CFU) fed to rhesus monkeys resulted in lesions in the colonic epithelium (33). Rhesus monkeys fed 10^8 to 10^{10} CFU of *S. flexneri* 2a had clinical signs of acute shigellosis within 48 h of challenge (34), including lethargy, prostration, and diarrhea with liquid or semisolid mucohemorrhagic stools (12). In addition to rhesus monkeys, cynomolgus monkeys have been infected with *Shigella* spp. (35). Interestingly, intragastric (10^{11} CFU) but not intraduodenal (10^9 CFU) injection of *Shigella dysenteriae* 1 was able to induce shigellosis despite colonization and serum antibody responses after intraduodenal administration.

Shigella flexneri 2a vaccine strain SC602 carries deletions in *virG* (*icsA*) and *iuc* (encoding aerobactin) genes (21). SC602 is Congo red positive, indicating that it has retained the virulence plasmid and is unable to cause keratoconjunctivitis in the guinea pig eye (Sereny test negative) (21). In the rhesus model, the monkeys were orally immunized on days 0, 10, and 20 with 8×10^{10} CFU of strain SC602. The immunized rhesus monkeys (≥44%) secreted liquid stools with mucus within 72 h after each vaccination with SC602 (unpublished results). After the first vaccination, all monkeys shed *S. flexneri* 2a for 3 days. All immunized animals also shed *S. flexneri* 2a after the second and third vaccinations, but the carriage rate was diminished with each successive immunization. On study day 48, control animals ($n = 8$) and SC602-immunized animals ($n = 16$) were orogastrically challenged with 1×10^{11} CFU of *S. flexneri* 2a strain 2457T. Vaccination of rhesus monkeys with strain SC602 was associated with 75% protection against overt dysentery ($P = 0.002$). Similar to the rhesus model, 80% protection was achieved in the current study after oral immunization of *Aotus nancymaae* with SC602 on days 0, 14, and 42.

Oral immunization of humans with $\geq 10^6$ CFU of strain SC602 causes shigellosis in the majority of volunteers (20). In contrast, immunization with 10^4 CFU results in transient fever or mild diarrhea in a small percentage of volunteers. Moreover, volunteers immunized with strain SC602 (10^4 CFU) and subsequently challenged with wild-type *S. flexneri* 2a strain 2457T were completely protected against fever and severe shigellosis (4), while six of seven controls experienced shigellosis. Expanded safety evaluation of strain SC602 (10^4 CFU) resulted in fever and diarrhea in 15% of volunteers, as well as headaches (35%) and abdominal cramps (24%) warranting further attenuation for clinical development (36). Similar levels of loose stools were induced after administration of SC602 to rhesus macaques and *Aotus nancymaae*, albeit at higher dose levels, suggesting that the monkey models may mimic, in part, the immunogenicity and reactogenicity in humans.

The described *Aotus nancymaae* model provides another means to study pathogenesis and *Shigella* vaccine immunogenicity and efficacy. Moreover, the model opens the possibility for future testing of combination vaccines to combat infection with the three most prevalent enteric bacterial pathogens encountered by travelers, military, and most importantly, children living in areas where these pathogens are endemic. Significant research needs to focus on building upon immunological evaluation in the *Aotus* model to include assessing antigen-specific fecal IgA, memory B cells, and IgA-secreting plasma cells in search of immune correlates of protection. Future efforts will also focus on expanding the challenge model to include additional *Shigella* serotypes to facilitate the efficacy testing of different *Shigella* vaccine formulations and constructs and exploring the potential of a broad-based immune response capable of cross-protecting against multiple, relevant *Shigella* serotypes.

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