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Fluorescent-Antibody and Histological Study of Vaccinated and Control Monkeys Challenged with *Shigella flexneri*

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ABSTRACT

FORMAL, SAMUEL B., (Walter Reed Army Institute of Research, Washington, D.C.), T. H. KENT, S. AUSTIN, AND E. H. LABREC. Fluorescent-antibody and histological study of vaccinated and control monkeys challenged with *Shigella flexneri*. J. Bacteriol. **91**:2368–2376. 1966.—Groups of monkeys were fed four doses of a living *Escherichia coli-Shigella flexneri* 2a hybrid strain, and, together with control animals, were challenged with virulent *S. flexneri* 2a. Two experiments were carried out; in the first, the animals were challenged 10 days after and in the second, 1 month after the last vaccine dose was administered. At 48 hr after challenge, tissues were removed from the vaccinated and control animals, and examined by use of histological and fluorescent-antibody techniques. The results of this study demonstrate that the animals receiving the vaccine were protected from the tissue damage ordinarily observed after experimental challenge with virulent dysentery bacilli. The virulent challenge strain appeared to be unable to penetrate into the intestinal mucosa of immunized animals.

In a previous report (2), we presented evidence that monkeys fed living attenuated strains of *Shigella flexneri* 2a were resistant to subsequent oral challenge with virulent *S. flexneri* 2a. In that study, the capacity of the vaccine to protect was assessed by its ability to prevent diarrhea or dysentery. To test the effects of the vaccine in a more accurate and sensitive way, we have now studied the tissue response of vaccinated and control monkeys 48 hr after challenge with virulent *Shigella* organisms. We also hoped to gain insight into the mechanism of action of the vaccine.

MATERIALS AND METHODS

Male and female rhesus monkeys (*Macaca mulatta*), weighing 2 to 4 kg each, were housed in individual cages. The only animals used were those without diarrhea and with three stool cultures negative for *Salmonella* and *Shigella* during the week before the start of the experiment.

In the two experiments performed, the monkeys were divided into vaccinated and control groups. The vaccinated animals received four intragastric doses of *Escherichia coli-S. flexneri* 2a hybrid strain X16 at 3- to 4-day intervals. The characteristics of this hybrid have been described previously (1, 2). The organisms were grown on Brain Heart Infusion (BHI) agar (Difco) for 18 hr, and were harvested in BHI broth (Difco). Each intragastric dose consisted of 5×10^{10} viable cells in 20 ml of BHI broth. In previous studies, one or two doses of vaccine were shown to confer a significant degree of protection (2). In the first experiment, 24 vaccinated and 26 control animals were challenged intragastrically 10 days after the last dose of vaccine with 5×10^{10} virulent *S. flexneri* 2a strain M42-43 suspended in 20 ml of BHI broth. Prior to challenge, 12 animals from each group were arbitrarily chosen for sacrifice at 48 hr postchallenge, and the remaining animals were saved for observation. In the second experiment, 12 vaccinated and 12 control animals were fed 5×10^{10} virulent *S. flexneri* 2a 30 days after the last dose of vaccine, and all were killed at 48 hr postchallenge. We chose to kill the monkeys 48 hr after challenge, because it has been our experience that under the described conditions most of the animals which become ill do so within 48 hr.

Throughout both experiments, the spontaneously passed fresh stools were inspected daily for diarrhea, blood, and mucus, and cultures were taken daily to determine whether the vaccine strain or the virulent strain of *Shigella* was present. At autopsy, quantitative *Shigella* counts of approximately 1 g of cecal contents were obtained by plating 10-fold dilutions of the cecal content.

Blood was drawn from the monkeys before the start of the experiment, and 5 to 7 days after the vaccine group received its last dose. At the time of the bleed-

ings, fresh stool specimens were collected from some animals. A 50% suspension of feces in saline was centrifuged, and the supernatant fluid was frozen for later coproantibody determinations. The indirect hemagglutination test was used to detect serum and coproantibodies. The antigen used to coat the red blood cells was prepared by placing 10^{11} viable *S. flexneri* 2a cells suspended in 5 ml of 0.05 N NaOH in a boiling-water bath for 2 hr. The supernatant fluid was then neutralized with 0.1 N HCl, and dialyzed against 0.85% NaCl. A 0.1-ml amount of this extract was added to 1 ml of 10% washed sheep red blood cells, and the mixture was incubated for 1 hr at room temperature. The sensitized red blood cells were washed three times with saline, and resuspended in saline to make 1% suspension. Prior to testing for *S. flexneri* 2a agglutinins, the serum or stool extract was heated at 56 C for 30 min, and then adsorbed with normal, washed sheep red blood cells. Agglutination tests were performed by adding 0.1 ml of the sensitized sheep red blood cells to 0.2 ml of twofold serial dilutions of the serum or stool extract. The tests were incubated at room temperature for 2 hr and then read.

Monkeys used for morphological study were anesthetized with 2.5 mg/kg of phencydidrine hydrochloride (Sernylan, Parke, Davis and Co., Detroit, Mich.) given subcutaneously, and killed by opening the chest cavity after dissection of the intestines. The colon down to the sigmoid region was removed and opened. Segments from the cecum and descending colon were obtained for fluorescence microscopy study, and the remaining colon was pinned out flat and emersed in 10% formalin buffered with 2% sodium acetate. After fixation, at least two segments from each of the proximal and distal halves of the large intestine were examined by routine histological techniques. The ileocecal valve region from each animal was sectioned. Sections of distal ileum, jejunum, mesenteric lymph nodes, heart, lung, liver, pancreas, spleen, adrenal gland, and kidney were also examined. Fluorescent-antibody studies of frozen pieces of cecum, descending colon, and distal ileum were carried out as previously described (3, 4).

RESULTS

Effect of the vaccine. During the course of immunization, 6 of 24 animals in the first experiment had diarrheal stools (2 for 1 day and 4 for 2 days), and 1 of 12 of the animals in the second experiment had a single loose stool 48 hr after the last vaccine dose. None of the control animals had an abnormal stool prior to challenge with virulent *Shigella* organisms.

In the first experiment, all of the animals in the vaccine group shed the X16 strain in their feces intermittently. The pattern of shedding during the 10 days after the last dose of vaccine is summarized in Tables 1A and 1C. Although half of the animals shed the organisms for no more than 3 days, we isolated the vaccine strain from the remaining 12 animals sporadically. The hybrid strain was isolated from one animal on the 1st, 3rd, and 10th day after the last dose. In the second experiment, the vaccinated animals also shed the X16 strain in an unpredictable manner (Table 2A). After the last dose of vaccine, two of the animals failed to shed the organisms; four shed the vaccine strain for less than 3 days; and 6 shed the organisms intermittently. We isolated the organism from one animal on the day after the last dose, and not again for 22 days. Another animal shed the organisms intermittently for 21 days.

Five days after the last vaccine dose, 22 of 23 vaccinated animals from the first experiment had a fourfold or greater rise in the serum indirect hemagglutination titer to *S. flexneri* 2a, whereas none of 17 control monkeys had more

TABLE 1A. *Summary of studies carried out on monkeys challenged with Shigella flexneri 10 days after receiving four oral doses of a living attenuated vaccine, and sacrificed 48 hr postchallenge (experiment 1)*

Animal no.	Vaccine excretion	Serum antibody	Postchallenge stool	Cecal count	Gross lesions	Microscopic lesions	Bacterial penetration
M62-1	1*	<15; 120†	Formed	>10^7‡	Normal	Normal	None
M62-3	0	<15; 480	Formed	1 × 10^5	Normal	Slight, focal	None
M62-4	1, 3, 5, 7, 8	<15; 60	Formed	3 × 10^5	Normal	Normal	None
M62-5	1, 3, 4, 7	<15; 30	Pasty	3 × 10^4	Normal	Slight, focal	None
M62-7	1, 3, 5, 6	<15; 120	Pasty	2 × 10^5	Normal	Normal	None
M62-8	1	<15; 120	Formed	1 × 10^5	Mild, patchy	Moderate, patchy	Minimal
M62-9	1	<15; 120	Formed	2 × 10^3	Normal	Normal	None
M62-10	1, 3, 10	<15; 30	Formed	9 × 10^4	Normal	Normal	None
M62-11	1, 2, 3, 7	<15; 60	Formed	1 × 10^4	Normal	Normal	None
M62-12	5, 6	<15; 120	Formed	4 × 10^5	Normal	Normal	None
M62-13	1, 5	Not done	Formed	3 × 10^4	Normal	Normal	None
M62-26	1, 3, 5	15; 240	Formed	5 × 10^3	Normal	Normal	None

* Days after the last vaccine dose on which the vaccine strain was isolated from stool specimen.
† Reciprocal of pre- and postimmunization serum antibody titer to *S. flexneri* 2a.
‡ Number of *S. flexneri* 2a per gram of cecal contents.

TABLE 1B. *Summary of studies carried out on control monkeys challenged with Shigella flexneri 2a, and sacrificed 48 hr postchallenge (experiment 1)**

Animal no.	Serum antibody	Postchallenge stool	Cecal count	Gross lesions	Microscopic lesions	Bacterial penetration
M62-27	30; 15†	Formed	1×10^6‡	Mild, diffuse	Moderate, patchy	Minimal
M62-28	<15; <15	Dysentery	1×10^5	Severe, diffuse	Severe, diffuse	Extensive
M62-29	15; 15	Dysentery	1×10^7	Severe, diffuse	Severe, diffuse	Extensive
M62-30	<15; <15	Pasty	1×10^5	Mild, cecum	Mild, patchy	None
M62-31	30; 60	Diarrhea	1×10^5	Mild, patchy	Mild, focal	None
M62-32	<15; <15	Pasty	$>10^3 < 10^5$	Mild, cecum	Moderate, patchy	Minimal
M62-33	30; 15	Dysentery	1×10^7	Moderate, diffuse	Severe, diffuse	Severe
M62-34	30; 30	Dysentery	2×10^5	Severe, diffuse	Severe, diffuse	Moderate
M62-35	60; 60	Diarrhea	1×10^5	Mild, cecum	Severe, patchy	None
M62-36	30; 15	Pasty	$>10^3 < 10^5$	Mild, cecum	Severe, patchy	Moderate
M62-37	<15; <15	Diarrhea	1×10^4	Punctate	Moderate, focal	Minimal
M62-38	30; 30	Dysentery	1×10^5	Severe, diffuse	Severe, diffuse	Extensive

* For explanation of footnotes, see Table 1A.

TABLE 1C. *Summary of studies on vaccinated and control animals challenged with Shigella flexneri 2a and held for observation (experiment 1)**

Vaccine group					Control group			
Animal no.	Vaccine excretion	Serum antibody	Postchallenge stool	Fate	Animal no.	Serum antibody	Postchallenge stool	Fate
M62-2	0*	15; 480†	Diarrhea	Survived	M62-39	<15; <15†	Dysentery	Died; 72 hr
M62-14	3, 5	30; 480	Formed	Survived	M62-40	15; <15	Dysentery	Survived
M62-15	1, 3, 5	<15; 60	Pasty	Survived	M62-41	120; 60	Dysentery	Survived
M62-16	1	15; 120	Formed	Survived	M62-42	<15; <15	Dysentery	Died; 72 hr
M62-17	1, 6	15; 60	Pasty	Survived	M62-43	<15; <15	Dysentery	Died; 96 hr
M62-18	1	<15; 120	Pasty	Survived	M62-44	<15; <15	Pasty	Survived
M62-19	1, 2	120; 480	Formed	Survived	M62-45	Not done	Dysentery	Survived
M62-20	1	30; 120	Pasty	Survived	M62-46	<15; <15	Dysentery	Died; 96 hr
M62-21	2, 3, 4	<15; 120	Formed	Survived	M62-47	60; 60	Dysentery	Died; 96 hr
M62-22	1	<15; 120	Formed	Survived	M62-48	<15; <15	Diarrhea	Died; 96 hr
M62-23	1, 3	<15; 120	Formed	Survived	M62-49	30; 15	Dysentery	Died; 96 hr
M62-25	1, 3	60; 120	Formed	Survived	M62-50	30; 15	Dysentery	Survived
					M62-51	<15; 15	Dysentery	Survived
					M62-52	30; 30	Dysentery	Survived

* For explanation of footnotes, see Table 1A.

than a twofold rise (Tables 1A, 1B, and 1C). In the second experiment, 11 of 12 vaccinated animals had a fourfold or greater rise in *S. flexneri* 2a antibodies, whereas none of the controls exhibited a significant rise in titer (Tables 2A and 2B). We attempted to detect copro-antibody in the 12 vaccinated animals in the second experiment. Two animals had a twofold or greater rise in titer, two had a onefold rise, and eight had no rise in titer to *S. flexneri* 2a.

Protection tests. In the first experiment, vaccinated and control monkeys were fed virulent *S. flexneri* 2a 10 days after the last vaccine dose. After 48 hr, 1 of 24 vaccinated monkeys had diarrhea without blood or mucus; 15 of 26 control animals had classical dysentery (bloody mucoid diarrhea), and 4 had diarrhea without blood and mucus (Tables 1A, 1B, 1C). Of the 14 control animals which were kept for observation, 7 died between 72 and 96 hr post-challenge. The one monkey with diarrhea in the vaccine group became normal after 3 days. In the second experiment, in which vaccinated and control animals were fed virulent *S. flexneri* 2a 30 days after the last vaccine dose, 1 of 12 vaccinated animals had dysentery 48 hr post-challenge. At this time, 1 of 12 control animals had dysentery, and 1 had diarrhea without blood and mucus (Tables 2A and 2B).

The gross and microscopic findings in vacci-

TABLE 2A. *Summary of studies carried out on monkeys challenged with Shigella flexneri 2a 1 month after receiving four oral doses of a living attenuated vaccine, and sacrificed 48 hr postchallenge (experiment 2)**

Animal no.	Vaccine excretion	Serum antibody	Postchallenge stool	Cecal count	Gross lesions	Microscopic lesions	Bacterial penetration
M66-6	1, 2, 3, 4, 5, 6, 8, 10, 12, 13, 17, 21*	15; 60†	Formed	3×10^5‡	Normal	Normal	None
M66-9	1, 2, 3, 4, 9	<15; 60	Formed	5×10^3	Normal	Slight, focal	Minimal
M66-11	0	15; 60	Formed	1×10^5	Normal	Normal	None
M66-12	1, 8, 9	15; 120	Formed	7×10^4	Normal	Slight, focal	None
M66-14	1	<15; 120	Formed	6×10^5	Normal	Normal	None
M66-15	1	60; 240	Formed	3×10^3	Normal	Normal	None
M66-16	2, 5, 7	<15; 120	Formed	1×10^5	Normal	Normal	None
M66-20	1, 5	<15; 480	Formed	7×10^3	Normal	Normal	None
M66-22	1, 22	<15; 60	Dysentery	1×10^8	Severe, diffuse	Severe, diffuse	Extensive
M66-24	0	60; 120	Formed	$<10^3$	Normal	Slight, focal	Minimal
M66-25	2	30; 240	Formed	1×10^5	Normal	Slight, focal	None
M66-27	1	60; 480	Formed	5×10^4	Normal	Normal	None

* For explanation of footnotes, see Table 1A.

TABLE 2B. *Summary of studies carried out on control monkeys challenged with Shigella flexneri 2a, and sacrificed 48 hr postchallenge (experiment 2)**

Animal no.	Serum antibody	Postchallenge stool	Cecal count	Gross lesions	Microscopic lesions	Bacterial penetration
M66-28	15; 15†	Pasty	10^3‡	Moderate, cecum	Severe, patchy	Moderate
M66-29	15; 15	Dysentery	Not done	Moderate, patchy	Severe, patchy	Extensive
M66-31	30; 30	Formed	10^3	Normal	Moderate, patchy	Moderate
M66-35	15; 15	No stools	10^3	Severe, patchy	Severe, patchy	Extensive
M66-36	15; 15	Formed	1×10^4	Normal	Moderate, patchy	Minimal
M66-37	15; 15	Dysentery	2×10^8	Severe, diffuse	Severe, diffuse	Extensive
M66-38	15; 15	Formed	1×10^5	Normal	Moderate, focal	Minimal
M66-40	15; 15	Formed	3×10^5	Normal	Slight, focal	Moderate
M66-41	15; 15	Formed	Not done	Normal	Slight, focal	Minimal
M66-42	30; 30	Formed	Not done	Normal	Normal	None
M66-43	15; 15	Diarrhea	6×10^7	Moderate, patchy	Severe, patchy	Moderate
M66-44	30; 15	Formed	1×10^8	Moderate, patchy	Severe, patchy	Moderate

* For explanation to footnotes, see Table 1A.

nated and control animals are summarized in Tables 1A, 1B, 2A, and 2B. In the first experiment, 1 of 12 vaccinated animals had a moderate patchy colitis; 5 of 12 control animals had severe diffuse colitis, 2 had severe patchy colitis, and 4 had moderate patchy or focal colitis. In the second experiment, 1 of 12 vaccinated animals had severe diffuse colitis, while 1 of 12 control animals had severe diffuse colitis, 5 had severe patchy colitis, and 3 had moderate patchy or focal colitis. In both experiments, a total of eight animals—5 vaccinated and 3 controls—exhibited a very mild focal colitis.

Gross lesions were confined to the large intestine. In the most severely affected animals, the colonic contents were scanty and consisted of a bloody mucoid or mucopurulent exudate. The mucosa was thickened and diffusely red (Fig. 1). The cecal region was usually more severely involved than the distal colon. Moderately involved animals had patchy areas of red granular mucosa which were most often in the cecal region. In other areas, punctate red lesions less than 1 mm in diameter were often seen. The ileocecal valve was often severely congested; however, congestion of the valve was seen in some animals in which there was no evidence of shigellosis. The gross lesions in vaccinated and control

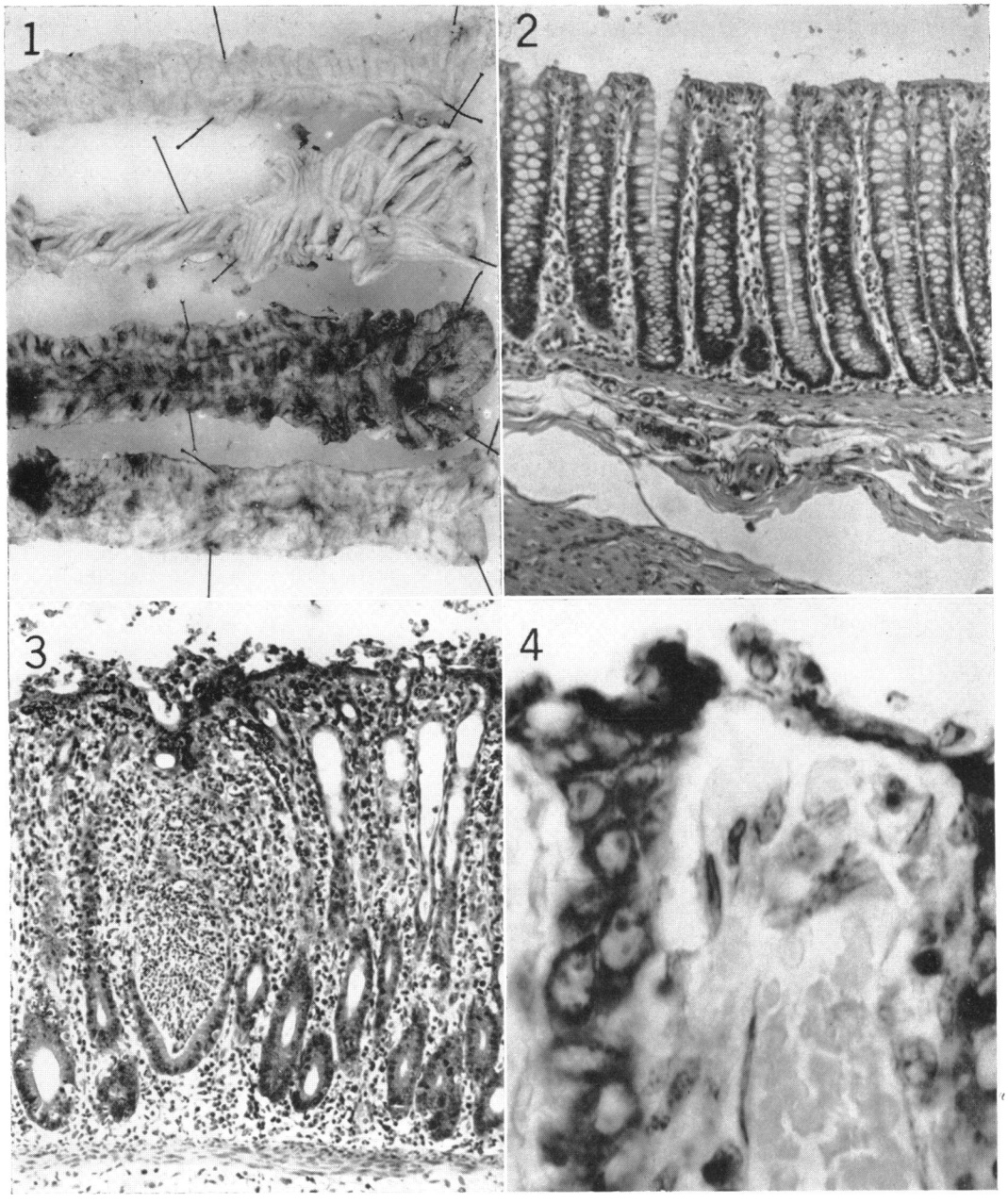

FIG. 1. *Upper pieces are left and right colon from a monkey vaccinated with Escherichia coli-Shigella flexneri 2a hybrid strain and killed 48 hr after challenge with virulent S. flexneri 2a. The lower pieces are right and left colon from an unvaccinated monkey killed 48 hr after challenge with S. flexneri 2a. The black areas represent severe congestion and bloody exudate. Note that the left colon is less severely involved, and punctate lesions are present away from the more diffusely involved areas.*

FIG. 2. *Colonic mucosa from a vaccinated monkey 48 hr after challenge with virulent Shigella flexneri. This is normal. Hematoxylin and eosin stain. × 110.*

FIG. 3. *Colonic mucosa from unvaccinated monkey 48 hr after challenge with virulent Shigella flexneri. This severe diffuse lesion is characterized by surface exudate, degeneration of surface and crypt epithelial cells with depletion of mucus content, crypt abscesses, and infiltration of the lamina propria by leukocytes. Hematoxylin and and eosin stain. × 110.*

FIG. 4. *Surface of colonic mucosa from monkey with severe diffuse shigellosis. Bacilli are present in the attentuated surface epithelial cells. Capillaries in the lamina propria are severely congested. Giemsa stain. × 800.*

animals are summarized in Tables 1A, 1B, 2A and 2B.

Microscopically, the large intestinal mucosa of the most severely affected animals was diffusely inflamed. The characteristic findings included mucopurulent exudate in the lumen, degeneration of surface and crypt epithelial cells with decreased mucus content, crypt abscesses, infiltration of the lamina propria by polymorphonuclear leukocytes, and severe congestion of mucosal capillaries (Fig. 3 and 4, compare with normal in Fig. 2). Ulceration was very infrequent at this stage of the disease. The abundant mucosal-submucosal lymphoid patches were usually the site of microabscesses (Fig. 5 and 6). Moderately involved animals had patchy or focal mucosal involvement (Fig. 7) and lymphoid abscesses. In some animals, lymphoid abscesses were the predominant manifestation of shigellosis. In Giemsa-stained sections, bacilli were seen in surface and crypt epithelial cells, and in the glands which extend into the lymphoid patches (Fig. 4 and 6). Bacilli were present but less conspicuous in the lamina propria and in the inflammatory exudate of crypts and lymphoid patches. In many of the most severely affected animals, there was mucoid exudate in the lumen of the ileum, and the lamina propria of the ileum was slightly more cellular than usual with occasional polymorphonuclear leukocytes. However, a well-developed acute inflammatory response was not present in the ileum, not even in the intracolonic portion of the distal ileum. In severely affected animals, an early acute lymphadenitis was present in lymph nodes along the colonic serosa, but not in more distal nodes. No significant lesions were observed in other organs. The microscopic lesions of the colon in vaccinated and control animals are summarized in Tables 1A, 1B, 2A, and 2B.

Fluorescent-antibody studies on tissue taken from the 12 control animals in the first experiment indicated that extensive tissue invasion occurred in four monkeys, moderate invasion in two, and minimal invasion in three; in the remaining three control animals, we saw no evidence of invasion. In the immunized group of the first experiment, 1 animal presented evidence of minimal invasion, and no invasion was observed in the remaining 11 monkeys. Extensive tissue invasion was observed in 3 of the 12 control animals in the second experiment; moderate invasion was seen in 5, minimal invasion in 3, and no invasion occurred in the 1 remaining animal. Of the 12 vaccinated animals in the second experiment, 1 exhibited evidence of extensive tissue invasion, 2 monkeys had minimal invasion, and in the remaining 9 animals no invasion was detected (Tables 1A, 1B, 2A, and 2B).

Extensively invaded colonic tissue was characterized by specifically fluorescing bacilli in the following areas: lumen, surface and crypt epithelium, crypt abscesses, lamina propria, and lymphoid abscesses (Fig. 8b). Small numbers of fluorescing bacilli were seen in the epithelium and lamina propria of the ileum only in animals with severe diffuse lesions of the colon. In moderately affected animals, fluorescing bacilli were localized mainly in the surface epithelium and in lamina propria beneath these areas (Fig. 8c and d). Few organisms were observed in the crypt areas. However, focal collections of fluorescing shigellae were present in mucosal and submucosal lymphoid patches. Minimal involvement consisted of a few foci containing small numbers of fluorescing bacilli in the surface epithelium, lamina propria, or in lymphoid patches. In general, the gross and microscopic lesions corresponded well with the degree of bacterial invasion as demonstrated by the fluorescent-antibody technique. Histological lesions were seen in a few animals in which bacterial invasion was not demonstrated. The failure to demonstrate bacterial invasion in these animals can be explained by inadequate sampling of involved areas, for in some of these monkeys organisms were observed in Giemsa-stained sections of the lesions.

DISCUSSION

In the present study, oral administration of the *E. coli-S. flexneri* 2a hybrid strain effectively protected monkeys for at least 1 month from the development of colonic lesions of shigellosis. Although the virulent *Shigella* organisms produced moderate to severe colitis in 19 of 24 control monkeys, the same challenge suspensions elicited a moderate or severe reaction in only 2 of 24 animals which had previously received the oral vaccine.

This study demonstrates that the bloody, mucoid diarrheal stool of experimental dysentery is associated with a diffuse inflammation of the colonic mucosa. Severe congestion of subsurface mucosal capillaries is a conspicuous feature of the lesion at the time the bloody diarrhea begins. In these experiments, many monkeys which had diarrheal stools without blood and mucus or normal stools also had morphological evidence of shigellosis. The lesions in these animals were patchy or focal or predominantly involved the lymphoid patches of the large intestine. Diffuse colonic lesions were seen only in animals with bloody, mucoid diarrhea. In these experiments, morphological evaluation of the colon was a much more sensitive method of detecting shigellosis

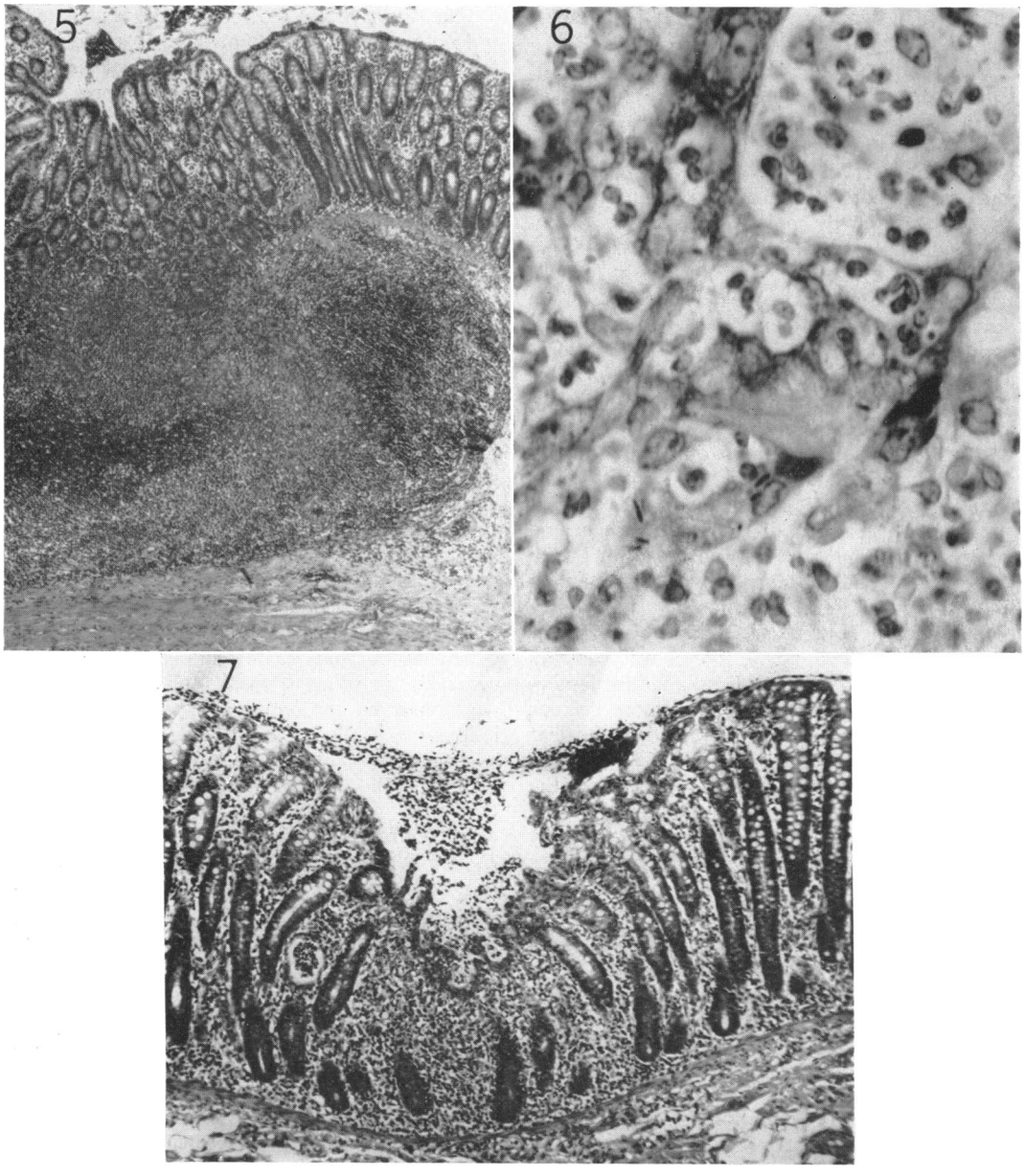

FIG. 5. *Abscess in colonic lymphoid patch. Although the cell detail cannot be seen at this magnification, the lighter staining areas of the lymphoid patch contain masses of polymorphonuclear leukocytes while the two darker staining areas represent the remaining lymphocytes. The overlying mucosa is not appreciably involved. Hematoxylin and eosin stain.* × *45.*

FIG. 6. *Mucosal crypt overlying a lymphoid patch such as that in Fig. 5. Bacilli are present in degenerating crypt epithelial cells. Polymorphonuclear leukocytes are present in the crypt lumen (upper right), in the epithelium, and in the tissue surrounding the crypt. Giemsa stain.* × *800.*

FIG. 7. *Focal colonic mucosal lesion of shigellosis. Leukocytic exudate is present in the lumen and in one crypt (left), and there is focal involvement of the surface epithelium in the depressed area. The mucosa at the right is not appreciably involved. Hematoxylin and eosin stain.* × *80.*

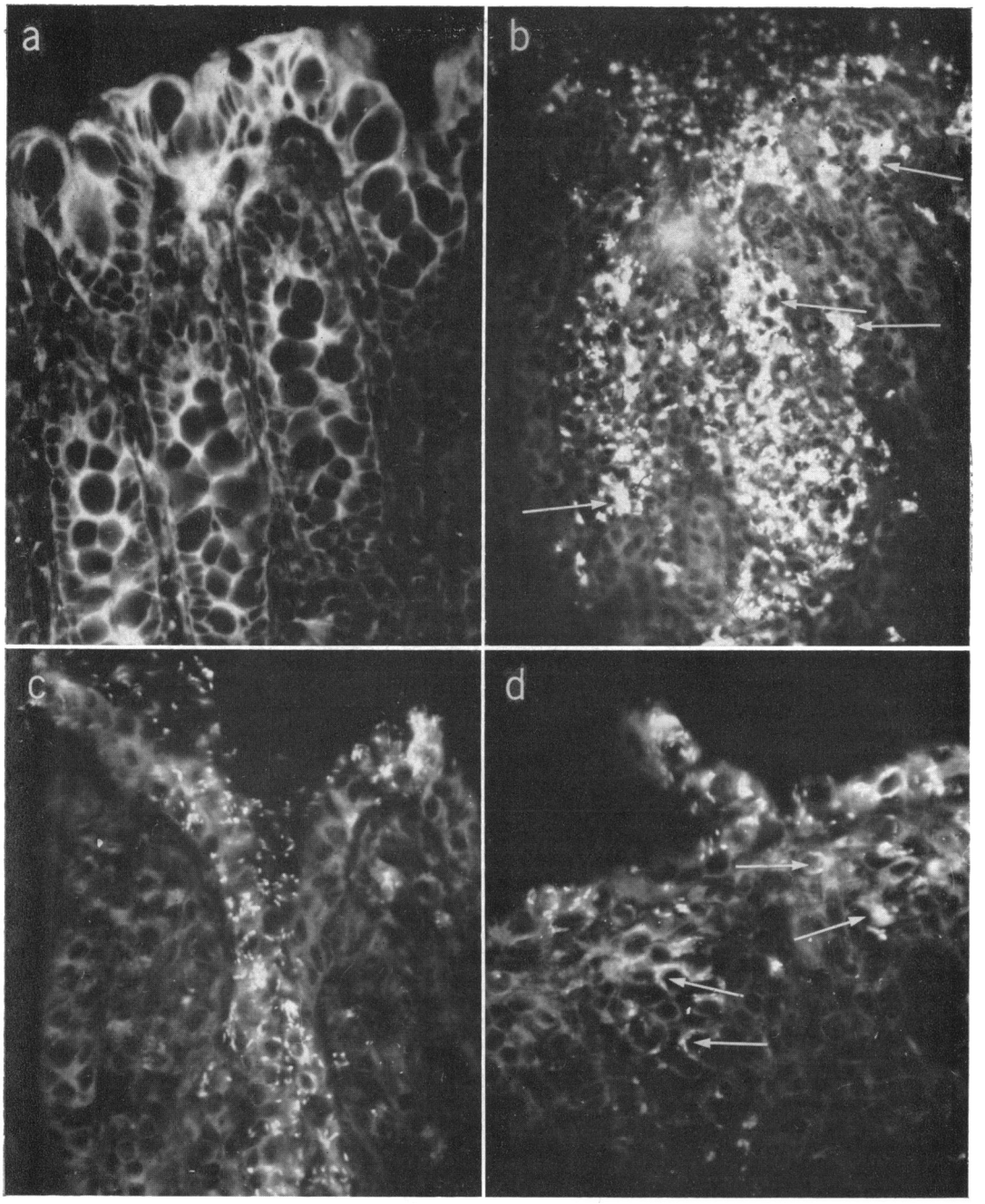

FIG. 8. *Frozen sections of cecum from monkeys challenged orally with the virulent strain of Shigella flexneri 2a. All sections were fixed in acetone, and treated with fluorescein-labeled rabbit anti-S. flexneri 2a globulin. Rhodamine-labeled bovine serum albumin was used as an orange fluorescent counterstain. (a) Cecum from an immunized monkey. No fluorescing bacilli are seen in the tissue. The normal architecture is demonstrated by the counterstain. × 860. (b) Cecum from control unimmunized monkey. The mucosa is extensively invaded. Specifically fluorescing shigellae are seen in the surface and crypt epithelium, in the lamina propria, and within inflammatory cells (arrows). A crypt abscess is at the center of the photograph, and the lumen is at the top. Compare with Fig. 3. × 540. (c) Cecum from control unimmunized monkey. Fluorescing shigellae are present in the surface and crypt epithelial cells. Few bacilli are seen in the lamina propria. The section is representative of moderate invasion. × 840. (d) Cecum from control unimmunized monkey. Moderate invasion, with fluorescing bacilli seen only in the surface epithelium and in the lamina propria immediately adjacent. Inflammatory cells containing phagocytosed shigellae can be seen in the lamina propria (arrows). × 840.*

than evaluation based on the clinical signs of illness. In the control groups, 8 of 13 monkeys with formed or pasty stools had a moderate to severe colitis, whereas only 1 of 23 monkeys in the vaccine groups with formed or pasty stools had a moderate colitis.

Our previous work has indicated that at least two steps are involved when dysentery bacilli cause severe intestinal mucosal damage (1, 4). First the organisms must penetrate the intestinal epithelium and enter the lamina propria, and then they must multiply to some extent within the lamina propria. The effect of the vaccine in rendering the animals resistant to experimental challenge could be due to blocking or limiting of either of these two processes. The fluorescent-antibody studies on our animals indicate that the virulent challenge strain was not present in the colonic mucosa of most vaccinated animals 48 hr after challenge in spite of the presence of relatively large numbers of organisms in the lumen of the large intestine. Since we have examined the tissue from animals at only one time interval (48 hr) after challenge, we cannot rule out the possibility that the virulent organisms penetrated the mucosa and were inhibited in their ability to multiply. However, if appreciable tissue invasion did take place and multiplication was inhibited, one might expect some of the vaccinated animals to have a definite but mild inflammatory response. We have observed such a mild inflammatory response 48 hr after feeding the vaccine strain to monkeys (2; *unpublished data*), and we know from our studies in guinea pigs (1) that the vaccine strain enters the tissue and does not multiply to a great extent.

Our observations presented here differ somewhat from those made previously (2). In our former study, we failed to isolate the vaccine strain for more than 4 days after the last dose in a small group of animals given five doses, and in a larger group fed either one or two doses. This pattern of shedding was adhered to by half of the animals in the present report, but the remaining half shed the strain X16 unpredictably; one animal passed the strain in its feces intermittently for 22 days.

In our previous experiments, we could not detect rises in serum antibody titer in animals fed two doses of vaccine. Of 23 animals tested in the present study, 22 responded with increased serum antibody levels of fourfold or greater after 4 doses of the vaccine. We do not know whether this difference in response of the latter group is due to the increased number of vaccine doses or to the increased interval of time between the administration of the first and the last dose of vaccine. In any event, we do not imply that circulating antibody is responsible for protection. However, by detecting this rise in serum antibody levels, we do have assurance that antigenic material is reaching antibody-forming cells.

We are still having difficulty in detecting coproantibody in our animals (2). Of 12 animals tested, 4 exhibited detectable coproantibody. Whether the remaining animals had levels too low to detect is a matter for conjecture, and it will remain so until more sensitive techniques are devised to detect antibody in the intestinal lumen. We consider that it will be difficult to explain the precise mechanism of the protection afforded by this oral vaccine unless we can establish, with some degree of certainty, the role of corproantibody in resistance to infection.

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