

EPITHELIAL CELL PENETRATION AS AN ESSENTIAL STEP IN THE PATHOGENESIS OF BACILLARY DYSENTERY

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Received for publication 17 July 1964

ABSTRACT

LABREC, EUGENE H., HERMAN SCHNEIDER, THOMAS J. MAGNANI, AND SAMUEL B. FORMAL. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. *J. Bacteriol.* **88**:1503-1518. 1964.—A parent strain of *Shigella flexneri* 2a and a colonial mutant derived from it were studied in three animal models. Both strains were equally virulent for mice when living cells suspended in hog gastric mucin were injected by the intraperitoneal route. Feeding the parent strain to starved guinea pigs, followed by the intraperitoneal injection of opium, resulted in the formation of ulcerative lesions in the intestinal tract and in the death of these animals. When the colonial variant was fed to similarly prepared animals, the animals survived and the intestinal tract remained normal. The parent produced diarrheal symptoms and intestinal lesions after its oral administration to rhesus monkeys; the variant caused neither symptoms nor pathology in this species. Studies were carried out to define the characteristics present in the parent strain and absent in the colonial mutant, which would enable the parent to produce ulcerative lesions of the bowel and death in the guinea pig model or intestinal lesions and diarrheal symptoms in the monkey. Neither serological studies nor growth studies conducted both in vitro and in vivo offered a clue to explain this difference. The virulent parent strain was shown to penetrate the bowel epithelium and enter the lamina propria; the avirulent mutant did not do this. Entrance to the lamina propria was by way of the epithelial cell of the mucosa. The avirulent mutant did not possess the capacity to penetrate this cell. This observation was extended to show that the virulent parent possesses the ability to infect and multiply within HeLa cells; furthermore, the organisms are able to penetrate epithelial cells of the guinea pig cornea, causing ulcerative lesions. The avirulent variant possesses neither of these capacities. It is suggested that epithelial cell penetration is a major factor in determining the pathogenicity of dysentery bacilli.

A major feature in the pathogenesis of bacillary dysentery involves the formation of ulcerative lesions of the intestinal tract. These lesions have long been considered to be the result of successive waves of absorption and excretion of toxic products of the dysentery bacillus through the bowel wall (Flexner and Sweet, 1906; Felsen, 1945). Although the parenteral injection of bacterial endotoxin may cause intestinal lesions in some animal species (Thomas, 1954; Gilbert, 1960), this is a situation quite different from absorption of this material from the bowel lumen.

Recently, Schneider and Formal (1963) in a preliminary communication described a smooth colonial variant of a strain of *Shigella flexneri* 2a which, when fed to starved guinea pigs, caused neither death nor pathology. Feeding the parent culture resulted in a fatal enteric infection with typical ulcerative lesions in the intestinal tract (Formal et al., 1958; Schneider and Formal, 1963). The present report summarizes experiments in which the parent and variant strains were studied in various in vivo and in vitro models. From the results of this work, a concept of the pathogenesis of bacillary dysentery is proposed which differs from that previously described, and, as a corollary, an insight into some properties which render a dysentery bacillus pathogenic is presented.

MATERIALS AND METHODS

Animals. White ICR mice, weighing 18 to 25 g, were used to study virulence of the *S. flexneri* 2a strains by intraperitoneal challenge. Hartley strain or Walter Reed strain guinea pigs, weighing 300 to 400 g, were used to study oral infections with the test *Shigella* strains. Monkeys (*Macaca mulatta*) weighing 6 to 8 lb (2.7 to 3.6 kg) were also used to study oral infections. All monkeys were held in quarantine for at least 8 weeks prior to delivery.

Cultures. *S. flexneri* 2a, strain 2457T, which is

virulent for guinea pigs by oral challenge was used in previous studies (Formal et al., 1958, 1963). This strain has a green-gold smooth translucent (T) colonial form when examined under the microscope by oblique transmitted light (Landy, 1950; Cooper, Keller, and Walters, 1957). It segregates a stable orange opaque (O) colonial variant (strain 24570) once in every 10^4 to 10^5 cell divisions (Schneider and Formal, 1963). Reversion from the variant to the parental form has not been observed. The colonial appearance of the parent and mutant is shown in Fig. 1. The parent and the variant strains were maintained in the lyophilized state, and a new ampoule was opened for each experiment. Before use in any one experiment, the strains were checked for colonial purity.

Media. Meat extract agar was used for routine cultivation of strains. Brain Heart Infusion (BHI) Broth (Difco) was used to suspend the challenge organisms. *Salmonella-Shigella* (SS) Agar (Difco) and MacConkey Agar (Difco) were used for isolation and enumeration of the test organisms. BHI broth, or liquid minimal glucose medium (Falkow, Rownd, and Baron, 1962) supplemented with 10 $\mu\text{g}/\text{ml}$ of both nicotinic and aspartic acids, was employed for in vitro growth studies of the *Shigella* strains.

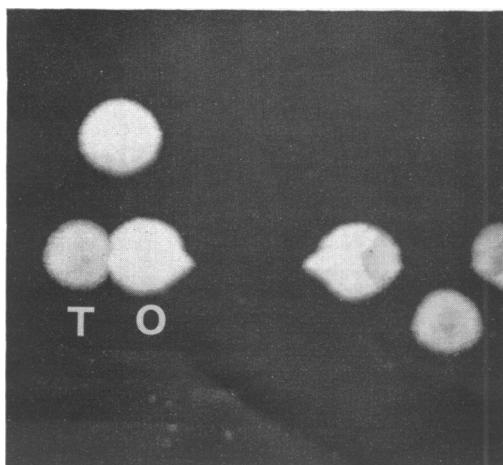


FIG. 1. Colonial forms of *Shigella flexneri* 2a. The translucent parent (T) is seen as a less-dense, smooth colony. The opaque variant (O) is seen as a smooth, more-dense colony. Photographed on an MEA agar plate with the use of oblique transmitted light. $\times 7.5$.

Oral infection of guinea pigs. The procedure as described by Formal et al. (1958) was employed, except that calcium carbonate, previously used to neutralize gastric acidity, was not administered. Animals were starved for 4 days; the challenge dose containing approximately 5×10^7 organisms suspended in 10 ml of BHI broth was fed by stomach tube; 1 ml of tincture of opium was injected intraperitoneally after challenge.

Oral infection of monkeys. Upon arrival from the quarantine section, the monkeys were placed in individual cages or in restraining chairs. They were observed daily for at least 1 week for the occurrence of diarrhea, and stool specimens were cultured for enteric pathogens on at least three occasions. Approximately 18% of the animals which were found to carry *S. flexneri* 4 were not used in the study.

After the period of observation, the monkeys were challenged by stomach tube with approximately 5×10^{10} of either the parent-T or the variant-O organisms suspended in 20 ml of BHI broth. The animals were observed daily for 7 days for diarrheal symptoms. The feces were cultured daily on SS agar to determine the presence of the challenge organisms.

Mouse virulence tests. Groups of 20 animals containing equal numbers of males and females were injected intraperitoneally with 0.5 ml of serial tenfold dilutions of the test culture suspended in 5% hog gastric mucin (pH 7.2). Deaths were recorded for 72 hr after challenge, and the LD₅₀ and standard error (SE) were estimated by the method of Miller and Tainter (1944).

Histological procedures. The method for freezing and sectioning specimens of intestine for fluorescent antibody studies, and procedures to determine the specificity of the reaction between the *Shigella* organisms and the homologous fluorescein-labeled rabbit antibody, were previously described (LaBrec and Formal, 1961). However, several modifications of the method for preparing the fluorescein-labeled antiserum were used. (i) Rabbit antisera were fractionated with dry sodium sulfate by the method of Schur and Becker (1963). The γ -globulin obtained after final precipitation with 12% (w/v) Na₂SO₄ usually gave a strong γ -globulin band when tested against sheep antirabbit serum by immunoelectrophoresis. (ii) A 1% globulin solution in saline adjusted to pH 9.0 with 0.5 M sodium carbonate-bicarbonate buffer was con-

jugated with crystalline fluorescein isothiocyanate (BBL; lot #302699) at a dye-protein ratio of 1:50. The dye, dissolved in acetone, was added to the chilled globulin solution with constant stirring over a period of 2 hr; stirring was continued overnight. The labeled globulin was then passed through a column of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Rochester, Minn.) to remove the unconjugated dye. The fluorescein-labeled globulin was then dialyzed against 0.01 M phosphate-buffered saline to restore the proper pH (7.2) for the antigen-labeled antibody reactions. Before use, suitably diluted labeled globulin was adsorbed one time with 50 mg of guinea pig or rabbit liver powder. Rhodamine-labeled bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio), used as a counterstain, was added to the adsorbed conjugate before sterilization by filtration.

Tissues for routine histological examination were fixed in 10% neutral formalin with 1% sodium acetate, embedded in paraffin, and sections were cut and stained with hematoxylin and eosin.

Cell-culture methods. HeLa S-3 cell monolayers, obtained from Microbiological Associates, Inc., Bethesda, Md., were used to study the infectivity characteristics of the parent-T and variant-O strains in an in vitro biological system. The cell cultures were maintained in prescription bottles as monolayers grown in Minimal Essential Medium (Eagle) supplemented with 10% fetal calf serum, 20 µmoles/ml of glutamine, 50 units per ml of penicillin G, and 50 µg/ml of streptomycin (complete medium; CM). The cultures were maintained in a humidified incubator in an atmosphere of 5% CO₂ and 95% air. For passage, the medium (CM) was removed and the cells were washed once with 2 ml of 0.25% trypsin (Difco, 1:300) in saline D-1 (trypsin D-1), (Ham and Puck, 1962). The wash was removed and was replaced with 2 ml of trypsin D-1. The monolayers were incubated at 37°C with occasional shaking until the monolayers loosened from the surface. After further dispersion by gentle agitation, the cells were dispensed into new prescription bottles.

The cover-slip culture technique of DeMars (*personal communication*) was used to prepare monolayers for infection experiments. Five clean sterile no. 1 cover slips, 18 mm in diameter, were immobilized onto the bottom surface of a plastic

tissue culture dish (60 mm; Falcon Plastics, Los Angeles, Calif.) by drawing a flap of plastic over the edge of the cover slip with a hot spatula. Each dish was then seeded with approximately 10⁵ cells, and the CM was changed at 18 hr and every 48 hr thereafter; 24 hr prior to infection, the CM was removed and replaced with CM lacking antibiotics. A second change of CM without antibiotics was made just prior to infection. Infection of the HeLa cell monolayers was accomplished by simple addition of washed bacteria to yield a final concentration of 3 × 10⁷ bacteria per ml, and the monolayers were incubated. After 3 hr, the CM lacking antibiotics was removed, the monolayers were washed three times with Hank's Balanced Salt Solution (BSS), and fresh CM without antibiotics was added. The washing procedure was repeated every 2 hr to minimize extracellular multiplication of the infecting *Shigella* strains. Usually, cover-slip samples were taken at 3, 5, and 7 hr after inoculation of the *Shigella* strains. The cover-slip preparations were washed in three changes of saline, fixed in a mixture of 3:1 methanol and acetic acid, and then stained with Giemsa stain at pH 6.5 (0.01 M phosphate buffer).

Agglutinin and agglutinin-adsorption tests. Antisera against the parent-T and the variant-O strains were prepared in rabbits by intravenous inoculation of suspensions of living cells. To determine agglutinin titers, 0.5 ml of saline suspension of living cells was added to 0.5 ml of serial twofold dilutions of antiserum. The tubes were incubated at 52°C for 18 hr, and the agglutinin titers were recorded.

For reciprocal agglutinin-adsorption tests, 4 ml of a 1:20 dilution of each antiserum was adsorbed two times with 10¹¹ of the appropriate living cells for periods of 1 hr at room temperature. After final adsorption, the serum was separated from the bacteria by centrifugation, and was tested for agglutinins as described above.

Immunodiffusion studies. Antisera prepared as above were tested by the Ouchterlony method to determine the qualitative antigenic structure of the two strains. Heavy suspensions of living cells or cell juice from sonically disrupted bacteria were used as antigens. The precipitin bands were allowed to develop for 1 week at room temperature in a humid atmosphere.

In vitro growth studies. A total of 10⁵ cells of either the parent or the mutant strains were

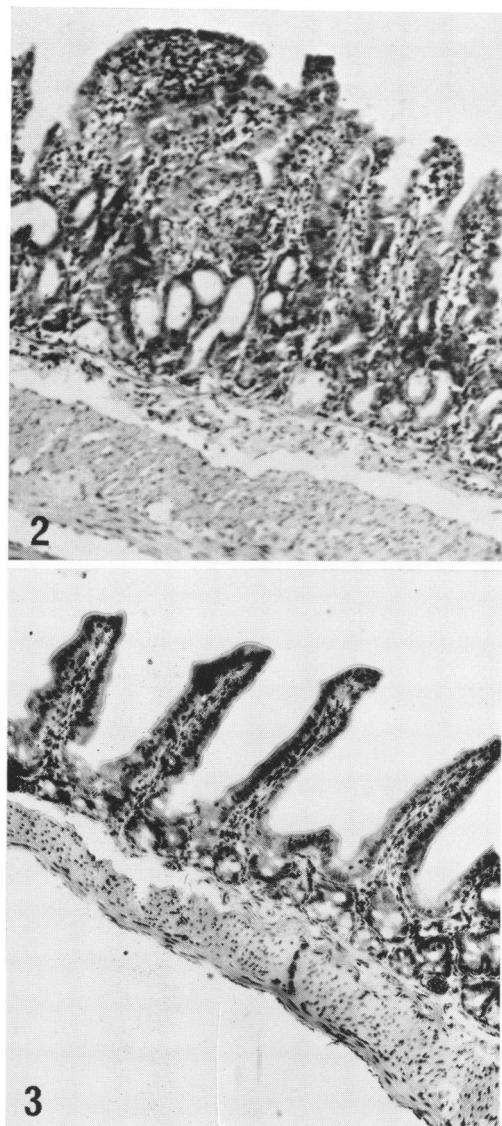


FIG. 2. Ileum of a guinea pig starved 4 days, fed the virulent parent strain of *Shigella flexneri* 2a, and sacrificed 24 hr after challenge. Thinning of the epithelium can be seen, with some loss of epithelial cells. There is fusion of the villi. The lamina propria contains large numbers of inflammatory cells. The villus-crypt ratio is altered, indicating a high turnover of epithelial cells. Goblet cells are empty. $\times 95$.

FIG. 3. Ileum of a guinea pig starved 4 days, fed the avirulent variant strain of *Shigella flexneri* 2a, and sacrificed 24 hr postchallenge. The normal mucosal architecture is maintained. The villus-crypt ratio is normal. The epithelial cells are normal in polarity and height, with a well-defined brush border. Goblet cells are full. $\times 95$.

inoculated into 15 ml of BHI broth or liquid minimal glucose medium. At various intervals, samples were removed and viable cell counts were determined.

RESULTS

Oral infection of guinea pigs with the parent and variant strains. Formal et al. (1958) reported that starved guinea pigs are susceptible to oral infection with *S. flexneri* 2a, strain 2457T, providing a drug such as opium is injected after challenge. In this experimental model, oral challenge with 10^7 dysentery bacilli usually results in the death of approximately 80 to 90% of the animals, and ulcerative lesions of the intestines similar to those seen in human infections are observed. On the other hand, similarly treated guinea pigs survive oral challenge with *Escherichia coli*, and the intestinal mucosa of these animals remains normal.

The virulence of the parent-T strain was compared with that of the variant-O strain by feeding the cultures to starved guinea pigs. In a series of experiments, 54 of 60 animals fed the parent-T strain succumbed, whereas only 1 of 44 of those fed the variant-O died. It is readily apparent that this mutation, expressed phenotypically by colonial variation from translucent to opaque, is also accompanied by a loss of virulence for the starved guinea pig.

The ability of these strains to produce histological changes in the guinea pig intestine was compared in similar feeding experiments. Animals fed either of the strains were sacrificed 24 hr after challenge. Figure 2 shows the typical ulcerative lesion of the intestine seen in animals fed the virulent parent culture. This lesion is characterized by a heavy infiltration of inflammatory cells, congestion and extravasation of erythrocytes, and a general loss of villus architecture. The histological picture in animals infected with the avirulent variant is strikingly different, as can be seen in Fig. 3 where the intestinal tract is essentially normal. The O-variant, therefore, not only has lost its ability to kill starved guinea pigs but also is incapable of causing significant histological alteration of the intestinal mucosa.

Peritoneal infection of mice with the parent and variant strains. Others (Cooper et al., 1954; Watkins, 1960) reported that colonial variants of *Shigella* strains differed in their ability to kill

TABLE 1. Virulence for mice of the parent and colonial variant strains of *Shigella flexneri* 2a*

Strain	Challenge dose†			LD ₅₀	SE
	10 ⁴	10 ³	10 ²		
Parent.....	33/40‡	26/40	12/40	2.4 × 10 ²	4.2 × 10 ²
Variant.....	35/40	25/40	18/40	3.5 × 10 ²	5.5 × 10 ²

* Data were pooled from two experiments.

† Challenge was suspended in 0.5% hog gastric mucin, and was injected intraperitoneally in 0.5-ml volumes.

‡ Death/total.

mice when injected by the intraperitoneal route. Similar mouse virulence assays were performed in our laboratory. The parent-T and variant-O strains suspended in hog gastric mucin were inoculated into separate groups of mice to see whether the differences in virulence noted for orally challenged guinea pigs could be extended to this laboratory model. The results (Table 1) show that the T and O strains are equally virulent for mice challenged in this manner. The toxicity of the parent and variant cultures was also compared. Weighed amounts of acetone-killed and dried cells suspended in saline were injected intraperitoneally, and deaths which occurred within 3 days were recorded. The LD₅₀ for cells of both the parent and the variant strains was 8 mg.

Oral infection of monkeys with the parent and variant cultures. The parent culture of *S. flexneri* 2a and its variant were fed to Rhesus monkeys to determine whether either or both would cause symptoms associated with bacillary dysentery. A suspension of approximately 5 × 10¹⁰ parent organisms was fed by stomach tube to each of ten monkeys. Observations for signs of illness and stool cultures were made daily. Within 48 hr of challenge, three of the animals had frank dysentery with blood and mucus and a fourth exhibited simple diarrhea. The translucent form of *S. flexneri* 2a was isolated from the diarrheal stools. Of the six monkeys which appeared normal over the period of the experiment, three shed the parent-T organisms on only the day after oral challenge. The stools of the other three monkeys were negative throughout the course of the experiment.

The variant, on the other hand, produced no apparent symptoms when five doses of 5 × 10¹⁰ cells were fed to 25 monkeys at intervals of 3

days. The stools remained normal, and variant cells were recovered from the feces of all animals at one time or another over the course of the experiment, but never on more than 2 consecutive days.

Histological examination of tissues from animals infected with the parent strain, and exhibiting symptoms of classical bacillary dysentery, showed typical lesions of the colon. Histological alterations of the ileum were observed, but ulcerative lesions were rare (Fig. 4 and 5). In contrast, the intestinal tracts of monkeys fed 18 consecutive doses of the variant culture were normal (Fig. 6 and 7).

Thus, the parent-T strain fatally infects mice when injected intraperitoneally, causes death and bowel pathology when fed to starved guinea pigs, and causes clinical symptoms of dysentery and intestinal lesions when administered orally to monkeys. The O-variant, though equally virulent for mice when inoculated intraperitoneally, causes neither death nor pathology in the guinea pig model, and is ineffective in producing either symptoms or pathology in monkeys.

A series of studies were then carried out in an attempt to define the characteristics possessed by the parent form, and absent or masked in the variant culture, which are responsible for the pathogenicity of the parent for starved guinea pigs and monkeys.

Serological studies. Experiments were conducted to determine whether mutation from the translucent to the opaque colonial form in *S. flexneri* 2a, strain 2457T, was accompanied by any discernible change in the antigenic mosaic of the two strains. First, antisera prepared in rabbits against the living T and O cells were tested in reciprocal adsorption tests. Qualitative differences in the surface antigens were not apparent

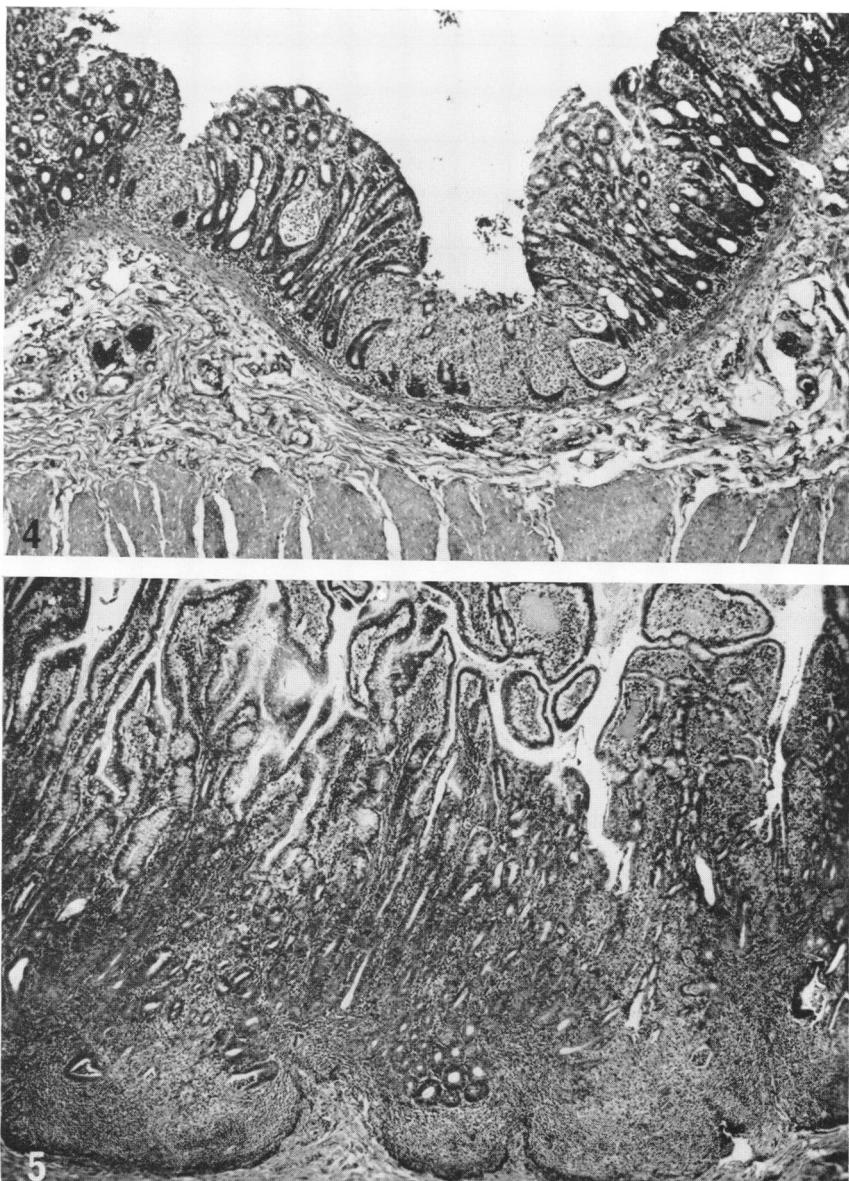


FIG. 4. Colon of a monkey killed 3 days after oral challenge with the virulent parent strain of *Shigella flexneri* 2a. Mucosal ulcerations and focal crypt abscesses containing large numbers of inflammatory cells are numerous. The tubular glands are elongated. Goblet cells are empty. $\times 110$.

FIG. 5. Terminal ileum of a monkey killed 3 days after oral challenge with the virulent parent strain of *Shigella flexneri* 2a. The villus tips are swollen and edematous. There is thinning of the epithelium, and the brush border is indistinct. Goblet cells are diminished in number. High epithelial cell turnover is indicated by the altered villus-crypt ratio. Large numbers of inflammatory cells can be seen in the lamina propria. $\times 180$.

(Table 2). Preliminary experiments with use of the Ouchterlony immunodiffusion technique were also carried out with both live and sonically disrupted cells of the T and O strains as antigens

and sera from rabbits immunized with live cells of either strain. Each antigen preparation was tested against antisera from four rabbits (two antisera for each strain). Analysis by this method

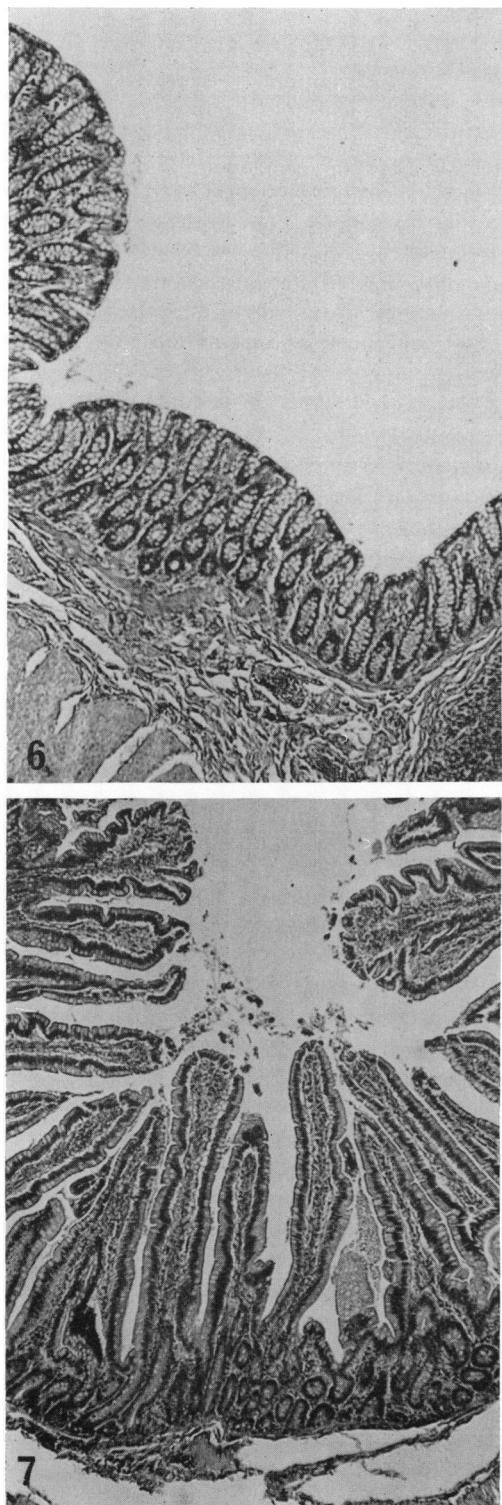


TABLE 2. Reciprocal agglutinin adsorption tests with antisera against *Shigella flexneri* 2a strain 2457 and its colonial variant

Test antigen	Antiserum prepared against					
	Parent		Variant			
	Unadsorbed	Adsorbed by	Unad-	Adsorbed by	Parent	Variant
Parent	10,240*	40	40	5,120	40	40
Variant	10,240	40	40	10,240	40	40

* Values equal reciprocal of highest dilution in which agglutination occurred.

revealed no qualitative antigenic differences between the T and O strains.

Growth studies. Earlier studies (Formal et al., 1963) indicated that multiplication in the small intestine is an important factor in initiating the disease in starved guinea pigs. To determine whether the loss of pathogenicity of the O-variant is a function of generation time, in vitro and in vivo growth studies were conducted. The in vitro growth rate of the two strains was compared in a complex medium, BHI broth, and in a synthetic medium, minimal glucose broth. The results (Fig. 8) demonstrate that the rates of growth of the parent and variant strains do not differ in these media.

The ability of the two strains to grow in vivo was assessed in the experimental guinea pig model. In the first experiment, starved guinea pigs were orally infected with 5×10^7 cells of either strain, and groups were killed 8 and 24 hr postchallenge. The entire small intestine was removed and ground in a mortar with sterile sand and saline. The total volume was noted, ten-

FIG. 6. Colon of a monkey fed 18 consecutive daily doses of the avirulent variant strain of *Shigella flexneri* 2a and sacrificed 24 hr after the last dose. The mucosal architecture is normal. $\times 180$.

FIG. 7. Ileum of a monkey fed 18 daily doses of the avirulent variant strain of *Shigella flexneri* 2a and sacrificed 24 hr after the last dose. The mucosal architecture is essentially normal, with only a slight hypercellularity of the lamina propria. Normal polarity of the epithelial cells is seen, and the brush border is well defined. The normal villus-crypt ratio is preserved and goblet cells are filled. $\times 180$.

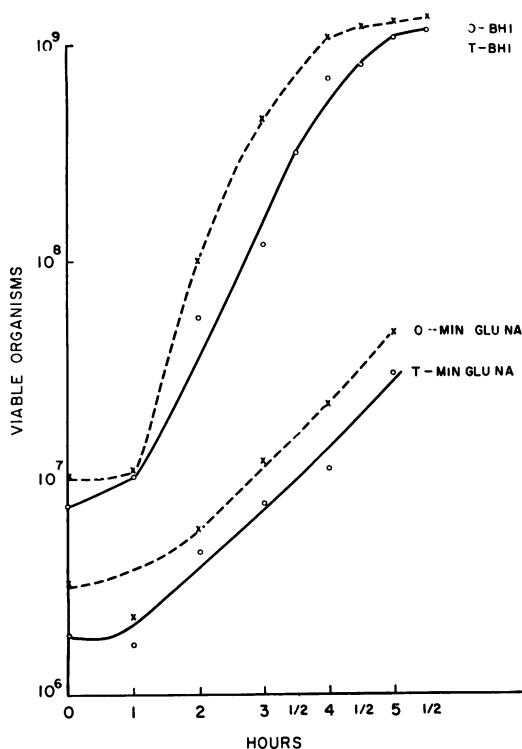


FIG. 8. *In vitro* growth rate of the virulent parent and the avirulent variant strains of *Shigella flexneri* 2a in a complex medium (BHI) and a synthetic medium (Min Glu). The points on the curves represent the number of viable cells per milliliter.

fold serial dilutions were made, and the appropriate dilutions were spread on the surface of SS agar plates. *S. flexneri* colonies, confirmed by serological typing, were then enumerated (Fig. 9). At 8 hr postchallenge, the avirulent O-variant appeared to maintain itself in the small bowel as well as the virulent parent. However, at 24 hr after challenge, the O-variant was present in much smaller numbers than was the virulent parent.

Because the avirulent mutant was unable to maintain itself in the small bowel as well as the parent culture, it was considered possible that it is more sensitive than the parent strain to some factors present in the small intestine. This concept was evaluated by comparing the growth of the two cultures in the small intestines of starved guinea pigs subjected to ligation of the terminal ileum. This, in effect, produced a closed tube for bacterial growth in the presence of any possible inhibitory substance. Laparotomy and ligation

were accomplished by use of aseptic technique, with no significant interference with the intestinal blood supply. Immediately thereafter, 10⁶ cells of the proper culture were injected intraduodenally, and the incision was closed. At various intervals after the completion of the operation, the animals were killed, and the total numbers of dysentery bacilli in the small intestine were determined. The growth curves constructed from these data (Fig. 10) demonstrate that, under the conditions of this experiment, the avirulent mutant multiplied as well as did the virulent parent.

Fluorescent antibody studies. This series of

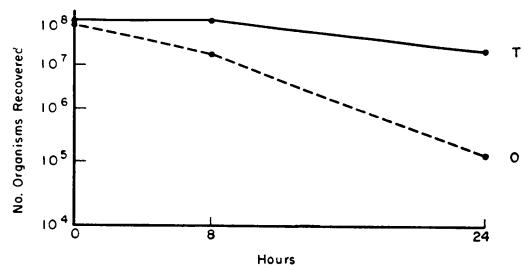


FIG. 9. *In vivo* growth of the virulent parent and the avirulent variant strains of *Shigella flexneri* 2a in the small intestine of guinea pigs starved 4 days and then fed 5 × 10⁷ cells of either the parent or variant strains. The points on the curves represent the geometric mean of the number of organisms recovered from the entire small intestine of four animals.

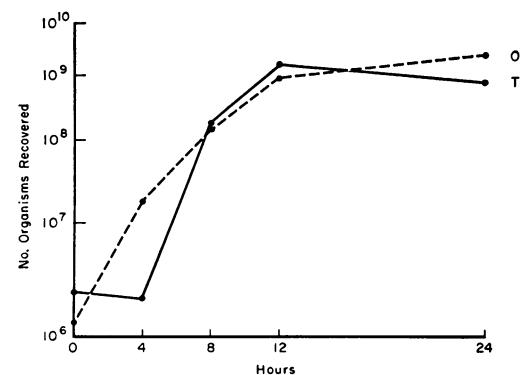


FIG. 10. *In vivo* growth of the virulent parent and the avirulent variant of *Shigella flexneri* 2a in the ligated small intestine. The terminal ileum was ligated, and the organisms were inoculated into the duodenum. The points on the curves represent the geometric mean of the organisms recovered from two animals.

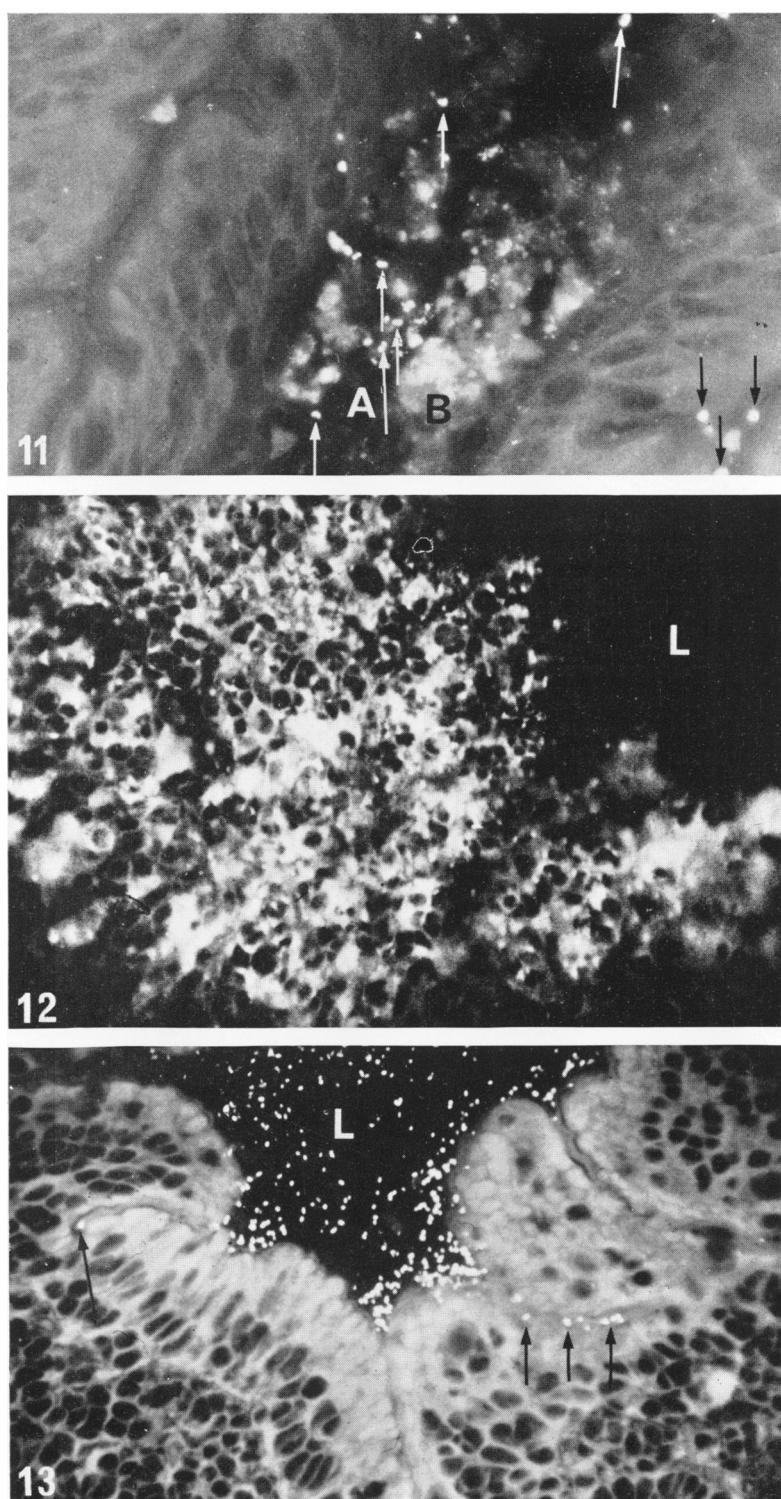
growth experiments indicates that the avirulent culture multiplies as well as the parent under static conditions both in vitro and in vivo where the terminal ileum is ligated. Under conditions of the experimental infection where the organisms were fed and intestinal motility was inhibited by opium, the O-variant appeared to do as well as the parent for only 8 hr. By 24 hr, when the effects of the opium had worn off, the virulent parent was present in numbers 100-fold larger than the avirulent mutant. It is likely that the variant strain was cleared from the small intestine by virtue of that organ's motility (Dixon, 1960; Formal et al., 1963). On the other hand, the recovery of the virulent parent in relatively undiminished numbers from the small intestine can be explained by the fact that it possesses the ability to penetrate the mucosal epithelium early in the infection and to multiply in the lamina propria, thus rendering it resistant to the cleansing effects of bowel motility. This phenomenon, mucosal penetration, might represent a step in the formation of intestinal ulcers, the characteristic lesion of classical bacillary dysentery (LaBrec and Formal, 1961). In view of the inability of the variant strain to cause intestinal pathology in either guinea pigs or monkeys, a study with the use of fluorescent antibody methods was undertaken to determine whether the avirulence associated with the O-variant is a result of a loss in ability to penetrate the epithelial barrier of the intestinal mucosa.

Guinea pigs experimentally infected with either the parent or the mutant strain were killed at various intervals after challenge, and frozen sections of intestinal specimens treated with specific fluorescein-labeled antibody to *S. flexneri* 2a were examined. In confirmation of our previous work, fluorescing dysentery bacilli were observed in the lamina propria of the intestine within 8 hr after the animals were fed the virulent parent strain (Fig. 11). By 24 hr postchallenge, the full range of intestinal involvement described above was observed (Fig. 12). In contrast, penetration of the intestinal mucosa was not observed in specimens from guinea pigs fed the avirulent mutant and sacrificed 6 and 8 hr postchallenge, although moderate to large numbers of specifically fluorescing bacilli were seen in the lumen (Fig. 13). Bacteria were not observed in the intestinal mucosa, very few fluorescing cells were seen in the intestinal lumen,

and the villus architecture was normal 24 hr after challenge with the avirulent strain.

From the above observations, it was considered that transmigration of dysentery bacilli across the epithelial barrier may be the primary mechanism involved in the pathogenesis of the disease. It was of major importance, then, to determine whether this phenomenon occurred by passage through individual epithelial cells or between them. Figure 14 clearly demonstrates the presence of dysentery bacilli in an intestinal epithelial cell of a guinea pig fed the virulent parent strain. This was not an isolated observation, but was a consistent feature of the experimental infection. It is likely that penetration into the epithelial cell and passage into the lamina propria are active rather than passive processes. Although the avirulent variant is present in relatively large numbers in the intestinal lumen, and is in intimate contact with the epithelial surface, it was never observed within epithelial cells or in the lamina propria (Fig. 13). Thus, one might tentatively consider the chain of events in the infection to consist of penetration of the mucosal epithelial cell (Fig. 14), entrance into the lamina propria by passage through this cell (Fig. 11), multiplication within the mucosa, and subsequent ulcer formation (Fig. 12). The avirulent variant has lost the ability to initiate this chain of events.

A similar study was carried out in monkeys with the use of tissues from animals killed after infection with either the parent or mutant strain. Animals fed the virulent parent culture and exhibiting symptoms of clinical bacillary dysentery were killed. Frozen sections of intestinal specimens were examined by the fluorescent antibody technique. Specifically fluorescing dysentery bacilli were seen in the lamina propria of the cecum and colon in the presence of an apparently intact mucosal epithelium. Most of the specimens exhibited severe ulcerations of the mucosa and focal crypt abscesses containing large numbers of fluorescing *Shigella* (Fig. 15). Although these changes obscured the mucosal architecture, the phenomenon of epithelial cell penetration by virulent *Shigella* was seen sufficiently often to be also a consistent feature of the disease process in the monkey (Fig. 16). Again, we failed to observe fluorescing dysentery bacilli either in the lamina propria or in the luminal contents of monkeys fed 18 consecutive daily doses of the O-variant



FIGS. 11-13

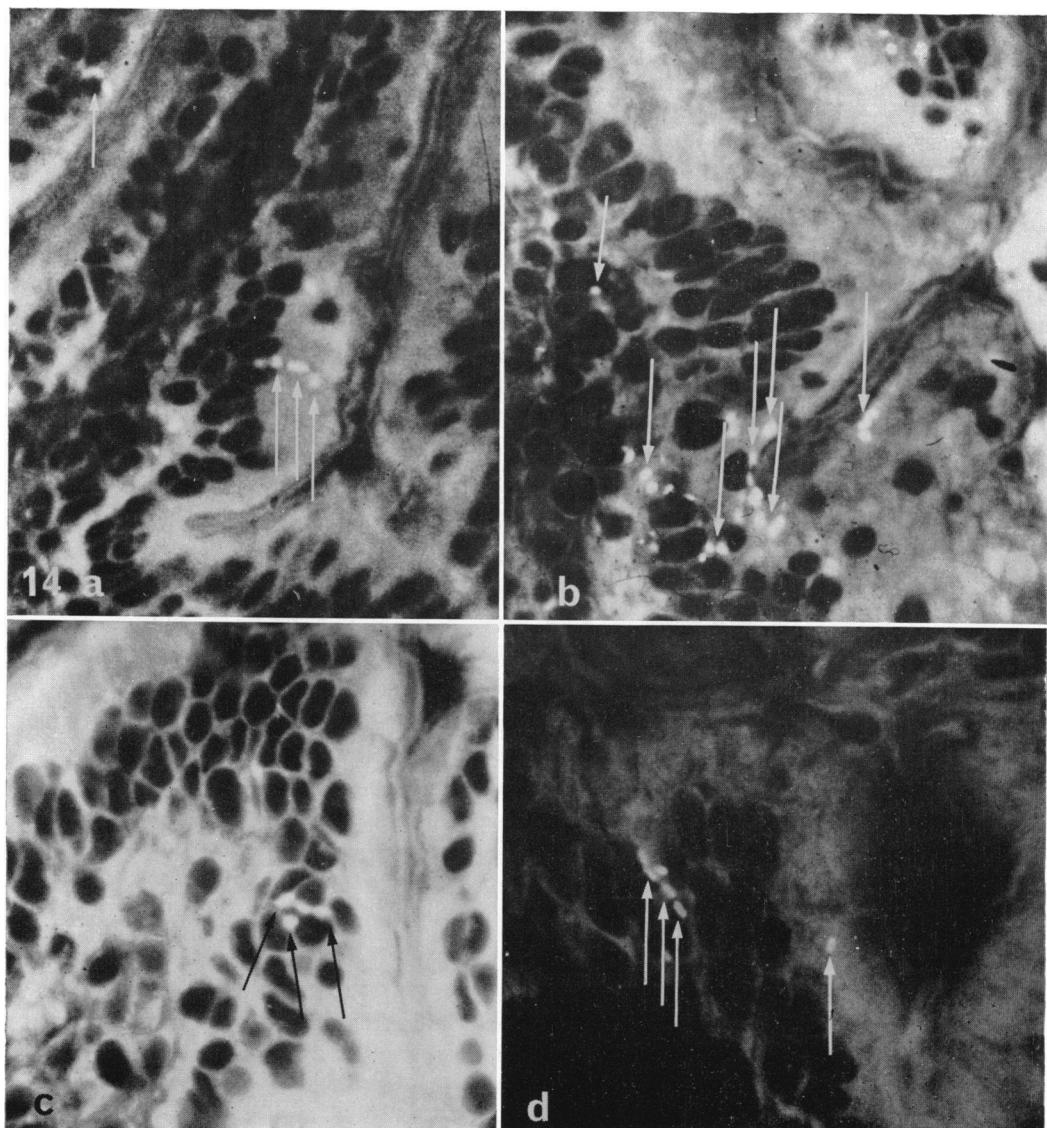


FIG. 14. Frozen sections of ileum from four guinea pigs killed 8 hr after feeding the virulent parent strain of *Shigella flexneri* 2a. All sections were treated with fluorescein-labeled rabbit anti-*S. flexneri* 2a globulin. Several examples of penetration of the mucosal epithelium by virulent dysentery bacilli can be seen (arrows). Fluorescing *Shigella* can be seen in the epithelial cells (a, b, d), although the brush border appears intact. Original magnification, $\times 240$ (a and b); original magnification, $\times 540$ (c and d).

FIG. 11. Frozen section of ileum from a starved guinea pig fed the virulent parent strain of *Shigella flexneri* 2a and killed 8 hr after challenge. The section was treated with fluorescein-labeled rabbit anti-*S. flexneri* 2a globulin. Note the specifically fluorescing bacilli in the lamina propria of a single villus (A; see white arrows). B denotes the primary fluorescence of inclusions in macrophages normally present in the intestine. The black arrows point to other shigellae between two villi. Original magnification, $\times 540$.

FIG. 12. Frozen section of ileum, from a starved guinea pig fed the virulent parent *Shigella flexneri* 2a and killed 24 hr after challenge. The section was treated with fluorescein-labeled rabbit anti-*S. flexneri* 2a globulin. A typical ulcerative lesion with complete loss of the normal architecture is seen. The lesion contains fluorescing *Shigella*, inflammatory cells, and necrotic debris. L denotes the intestinal lumen. Original magnification, $\times 240$.

FIG. 13. Frozen section of ileum from a starved guinea pig fed the avirulent variant strain of *Shigella flexneri* 2a and killed 8 hr after challenge. The section was treated with fluorescein-labeled rabbit and anti-*S. flexneri* 2a globulin. Specifically fluorescent variant-O *Shigella* are seen in the intestinal lumen (L), between the villi, and in the infoldings of the villus epithelium (arrows). Original magnification, $\times 240$.

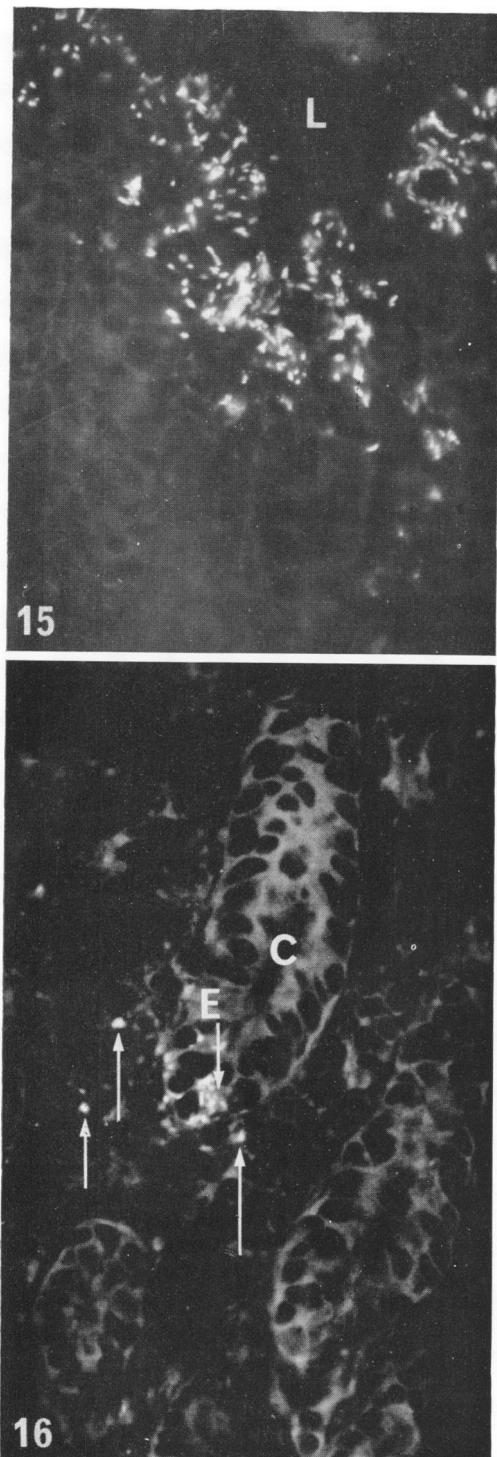


FIG. 15. Frozen section of the colon from a monkey fed the virulent parent strain and killed 3 days later. The section was treated with fluorescein-labeled

and killed 24 hr after the last dose was administered.

Studies with the corneal epithelium. For a number of years, workers have differentiated strains of *Shigella* on the basis of their ability to cause a keratoconjunctivitis after inoculation of the conjunctival sac (Zoeller and Manoussaki, 1924; Sereney, 1955; Kerekes, 1962). An essential feature of this infection is the invasion of the epithelial cell of the cornea (Piéchaud, Szturm-Rubinsten, and Piéchaud, 1958), which results in an ulcerative lesion. When this test was carried out in guinea pigs, it was found that a positive reaction was obtained with the parent strain when approximately 5×10^7 cells were used as an infecting dose.

The virulent parent invaded the epithelial cell of the cornea (Fig. 17), and an ulcerative lesion resulted (Fig. 18). On the other hand, the cornea of eyes inoculated with as many as 5×10^9 cells of the avirulent mutant strain remained normal, and organisms were never observed within the epithelial cells.

Tissue culture studies. If a major difference between the parent and the mutant strain is reflected by the ability of the parent to penetrate epithelial cells, it seemed possible that this difference might be shown in a less complex system. Accordingly, a study of the behavior of the parent and mutant strains in HeLa cell culture was undertaken. HeLa cell cultures were infected with either the parent or the mutant strain as described above. Both strains multiplied equally well in the cell culture medium. However, 5 hr after inoculation of the cell cultures with the parent strain, bacteria were observed within the HeLa cells (Fig. 19), and, by 7 hr, degeneration of the cell culture was evident. On the other hand, the avirulent mutant was not observed

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rabbit anti-*Shigella flexneri* 2a globulin. The photograph shows a microulcer with many fluorescing *Shigella* in the lamina propria, in epithelial cells, and phagocytosed inflammatory cells. L denotes the intestinal lumen. Original magnification, $\times 240$.

FIG. 16. Frozen section of colon from a monkey fed the virulent parent strain and sacrificed 48 hr later. The section was treated with fluorescein-labeled rabbit anti-*Shigella flexneri* 2a globulin. C denotes the lumen of a tubular gland. The arrow at E points to specifically fluorescing *Shigella* in epithelial cells. The other arrows show individual shigellae in the lamina propria. Original magnification, $\times 240$.

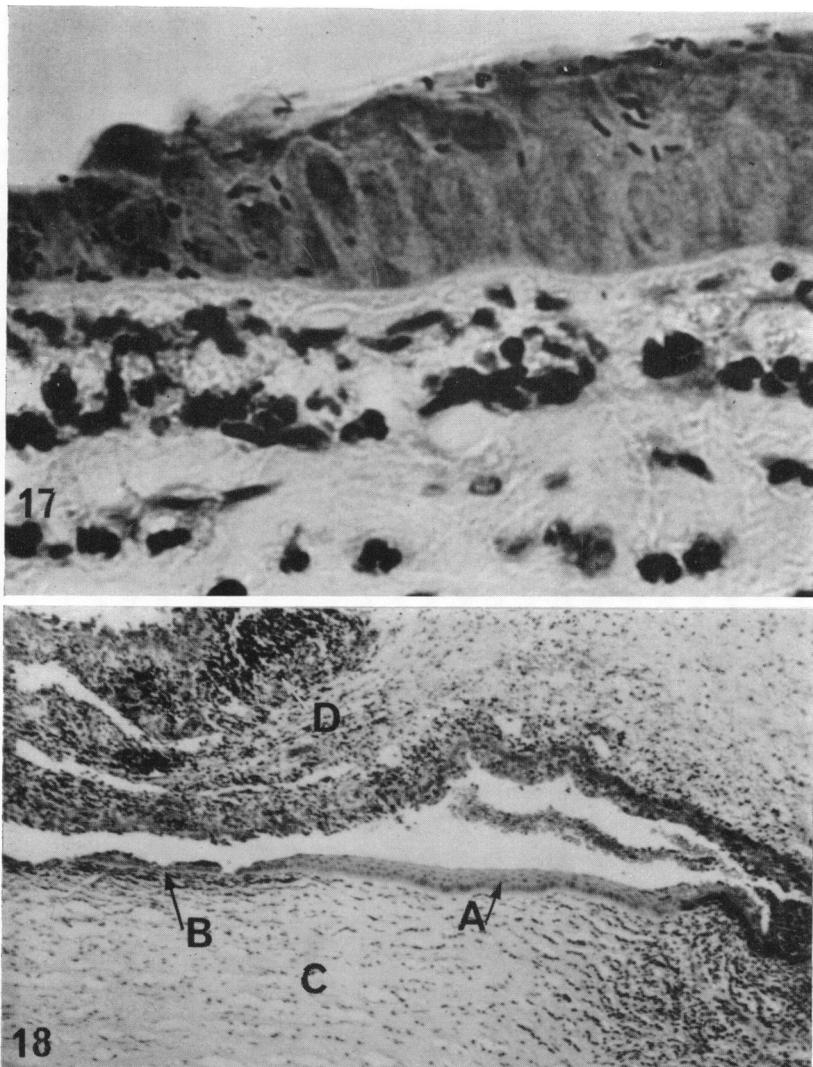


FIG. 17. Section of eye from a guinea pig killed 24 hr after inoculation of the conjunctiva with the virulent parent strain of *Shigella flexneri* 2a. Numerous bacilli can be seen in the corneal epithelium. $\times 896$.

FIG. 18. Section of eye from a guinea pig killed 24 hr after inoculation of the conjunctiva with the virulent parent strain of *Shigella flexneri* 2a. Note the intact corneal epithelium at A and an ulcerated portion at B. Edema and leukocytic infiltration of the corneal stroma are prominent (C). Ulcerative lesions of the conjunctiva can also be seen (D). $\times 53$.

within HeLa cells, and 7 hr after inoculation the monolayer appeared no different from uninfected controls (Fig. 20).

DISCUSSION

These studies emphasize several factors concerned with the pathogenesis of bacillary dysentery. First, they demonstrate quite clearly that ability of a dysentery strain to infect fatally

mice after intraperitoneal injection does not necessarily correlate with its capacity to provoke symptoms when administered by the oral route. The parent and the variant used in this study were equally virulent for mice, but only the parent was pathogenic when orally administered to monkeys or to starved guinea pigs. Indeed, the latter two species appeared to react no differently to the avirulent variant than had the ani-

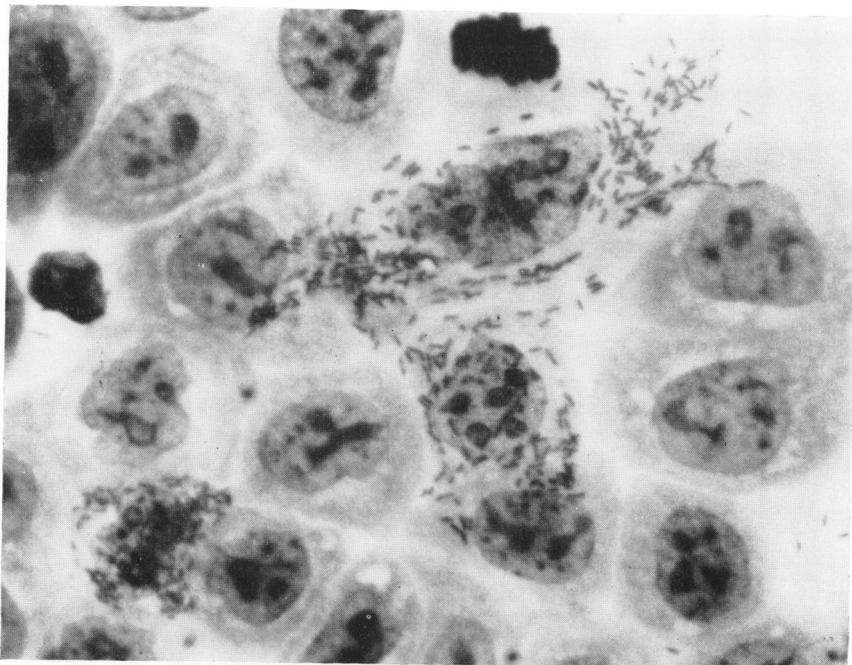


FIG. 19. Cover-slip monolayer of HeLa S-3 cells 5 hr after inoculation with the virulent parent strain of *Shigella flexneri* 2a. Several HeLa cells containing numerous bacilli can be seen. Giemsa stain. $\times 640$.

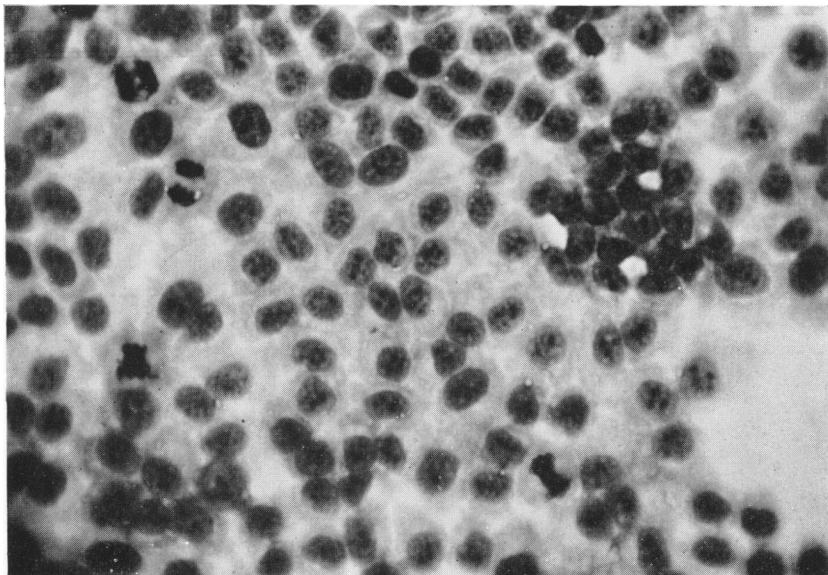


FIG. 20. Cover-slip monolayer of HeLa S-3 cells 7 hr after inoculation with the avirulent variant strain of *Shigella flexneri* 2a. The morphology of the HeLa cells is preserved, and no bacilli can be seen in the illustration. A lower magnification is used to show a larger field. Giemsa stain. Original magnification, $\times 240$.

mals been fed *E. coli* cultures isolated from normal individuals. Thus, the standard mouse virulence test, as used over the years, is of limited value for study of the pathogenesis of bacillary dysentery.

On the positive side, our results give further insight into the mechanism of ulcer formation and, from this, a beginning to an explanation concerning the characteristics which make a dysentery bacillus pathogenic. Earlier workers (Flexner and Sweet, 1906; Felsen, 1945) suggested that, in dysentery infections, lesions of the intestines gradually developed after several waves of absorption and excretion of stable toxic bacterial products through the bowel wall. However, we fed monkeys large numbers of acetone-killed and dried dysentery bacilli daily for 30 days, with no detectable unfavorable effects (Formal, *unpublished data*); on the basis of our present findings, it seems that an alternative mechanism for the formation of ulcerative lesions is justified.

LaBrec and Formal (1961), using the fluorescent antibody technique, previously observed dysentery bacilli in the lamina propria of the ileum, cecum, and colon of experimentally infected guinea pigs; the epithelium of the mucosa overlying these regions appeared to be intact. This finding was confirmed in the present study, and was extended to show that a similar situation also occurs in a natural host, the monkey, when it is fed the virulent parent, i.e., dysentery bacilli in the lamina propria in the presence of an intact mucosal epithelium. It seemed most likely that the dysentery bacilli passed either between the epithelial cells of the mucosa or through them. Our observations with the fluorescent antibody technique, and those of Takeuchi (*unpublished data*) with electron microscopic techniques, demonstrate clearly that virulent dysentery bacilli have a capacity to enter the epithelial cell of the bowel mucosa. One may now construct a tentative chain of events in the evolution of an ulcerative lesion. First, the dysentery bacillus penetrates the epithelial cell (at present we do not know the mechanism). The bacteria reach the lamina propria via this cell. At this stage, the bacteria are present in the lamina propria, while the epithelial barrier remains intact. Here the organisms multiply further, and may elaborate toxic products, resulting in the death of the epithelium and ulcer formation.

Although the ability to penetrate the intestinal

epithelial cell and reach the lamina propria is a characteristic of our virulent parent strain, it is not an attribute of the nonpathogenic variant. This difference also was seen in other experimental models. The parent strain penetrated and multiplied in cultured HeLa cells; the O-variant was not observed to enter these cells. The parent entered the epithelial cells of the cornea of the guinea pig, multiplied, and ulcerative lesions resulted. When exposed to the variant strain, the cornea remained normal, and bacteria were not observed in the epithelial cells.

The only difference in the parent and the variant strains which we were able to detect is the ability of the parent to invade epithelial cells. It is possible that this characteristic is a major factor in endowing the parent culture with the capacity to cause disease. Once the organism reaches the lamina propria, its effect may be relatively nonspecific if the subsequent changes which occur are due largely to the effect of bacterial endotoxin (Thomas, 1954; Gilbert, 1960), a component of bacteria belonging to a variety of genera. If this is the case, then it is conceivable that epithelial cell penetration and at least limited survival in the lamina propria are the necessary attributes for pathogenicity of dysentery bacilli, and are characteristics which set them apart from nonpathogenic *E. coli* strains and avirulent *Shigella*. Because, to date, there has been no reasonable means to explain the pathogenicity of dysentery bacilli, these possibilities should be considered.

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