Antagonism of normal enteric flora has been considered for some time as a possible factor in resistance to enteric diseases.

Nissle (1) first reported that stool cultures from typhoid carriers became negative for Salmonella after oral administration of viable Escherichia coli to the patients. Gratia (2) subsequently described an in vitro antagonism between coliforms and there have been numerous later publications on colicines and other antibiotic substances produced by certain bacteria which inhibit the growth of other bacterial strains, species and genera. Halbert (3) attempted to correlate the number of antibiotic-producing bacteria in the intestine of human beings with resistance to bacterial dysentery, but he interpreted the evidence obtained as largely inconclusive.

More recently, another line of evidence for a possible protective effect of normal enteric flora was presented by Freter (4, 5) who found that fasting and oral administration of streptomycin rendered guinea pigs susceptible to enteric infection with a drug-resistant strain of Vibrio cholerae. At about the same time Bohnhoff, Drake, and Miller (6) reported that the susceptibility of mice to oral infection with Salmonella enteritidis was enhanced up to 100,000-fold after the animals had received a large oral dose of streptomycin. While these results suggest an antagonistic action on the part of normal flora, there are two alternative explanations: (a) streptomycin might directly affect the intestinal tract in such a way as to predispose it to the action of enteric pathogens, or (b) the drug might directly increase the virulence of the bacteria studied. If these interpretations were correct, the reduction of normal enteric flora by streptomycin would be merely incidental; i.e. not directly related to the observed increase in susceptibility.

The action of immune antibody has also been implicated as a protective mechanism against enteric infection, especially after the discovery by Davies (7) that antibody could be found in the intestinal lumen. Burrows and his coworkers (8, 9) studied this phenomenon in great detail and suggested the term coproantibody to...
denote fecal antibody. The study of the effect of immunization on resistance to enteric diseases has been hampered by the lack of experimental animals in which essential features of the human infections—such as an abundant growth of the pathogens in the intestine—could be easily reproduced. Studies on human volunteers and field studies have been carried out repeatedly (10, 11) but did not always yield very consistent and encouraging results. The question whether protective immunity to enteric diseases actually does exist is therefore far from being settled.

The present paper reports (a) a method, based on inhibition of normal flora, which permits long term, non-fatal enteric infections of mice and guinea pigs with *Shigella flexneri* and *Vibrio cholerae*, (b) direct evidence for the antibacterial action of antagonistic enteric flora, and (c) the lack of antibacterial effect of active and oral passive immunization in such infections. The accompanying publication (12) will describe the protective influence of these factors on the outcome of the short term, fatal cholera infection in guinea pigs.

**Materials and Methods**

All animals were infected with a streptomycin-resistant strain of *Shigella flexneri* type 2a or *Vibrio cholerae* by its introduction directly into the stomach. The resistant *Shigella* mutant was selected by the gradient plate technique from a strain isolated in 1954. The *Vibrio* strain was the same as used in previous studies (4, 5). After some preliminary experiments the following schedules were adopted.

**Infection of mice with *Shigella flexneri***.—The animals used were Swiss mice of 25 to 30 gm. weight. Two days before infection, 1 mg. erythromycin and 5 mg. streptomycin in 1 ml. water were given by stomach tube. No food was given on this day. Boiled tap water containing 0.1 mg. erythromycin, 4 mg. streptomycin, and 400 units nystatin (mystatin, Squibb) per ml. were supplied *ad libitum* throughout the entire experiment. Fox chow (Rockland) was given during the day before infection but withdrawn at night. On the day of infection, a known number of viable *Shigella* organisms from a 24 hour agar culture was fed by stomach tube. The bacteria were suspended in 1 ml. veal infusion broth (Difco) containing 5 mg. streptomycin and 50 mg. CaCO₃. In experiments requiring oral administration of antiserum this was given 2 hours before infection together with 25 mg. CaCO₃. The amount of CaCO₃ given with the bacteria was then reduced to 25 mg. Beginning with the 1st day after infection, fox chow (Rockland) was supplied *ad libitum* throughout the experiment. For assay of the infection, one stool pellet was collected from each mouse into 1 ml. sterile saline, emulsified, and streaked not later than 3 hours after collection on desoxycholate agar (Difco) containing 1 mg./ml. streptomycin. Preliminary experiments showed all bacteria growing out on the plates to be *Shigella*. However, confirmatory spot checks by slide agglutination were made in all experiments as a matter of routine. A rough quantitation of the number of *Shigella* organisms excreted was achieved in the following way. All plates in which the first streak with one loopful of fecal suspension resulted in solid bacterial growth were interpreted to indicate several million viable cells per milliliter of the original suspension. When the first streak of fecal suspension gave isolated colonies the result was reported as "few *Shigella* organisms per fecal pellet." Infected mice showed no gross symptoms of disease.

**Infection of Guinea Pigs with *Shigella flexneri* or *Vibrio cholerae***.—Animals of the "Wright strain" (Abrams Small Stock Breeders, Chicago), weighing 350 to 400 gm. were treated as follows. Two days before infection, 100 mg. streptomycin and 10 mg. erythromycin were
given by stomach tube in 10 ml. distilled water. No food was given on this day. Boiled tap water containing 4 mg. streptomycin, 0.1 mg. erythromycin, and 400 units nystatin was supplied \textit{ad libitum} throughout the entire experiment. Rockland guinea pig food was given on the day before infection but withdrawn at night. On the day of infection, 750 mg. CaCO$_3$ suspended in 10 ml. water was fed by stomach tube. Three hours later a known number of viable \textit{Shigella} or \textit{Vibrio} organisms from a 24 hour agar culture were given by the same route. The bacteria were suspended in 15 ml. veal infusion broth (Difco) containing 50 mg. streptomycin and 250 mg. CaCO$_3$. In experiments requiring oral administration of normal or immune serum this was given with the first dose of CaCO$_3$. Beginning with the 1st day after infection, Rockland guinea pig food was supplied \textit{ad libitum} throughout the experiment. With this method of infection, death due to enteric cholera occurred only rarely in guinea pigs of the size used. No death due to infection with \textit{Shigella} was observed. The infection was therefore assayed by performing bacterial counts on fecal specimen in the following way. The animals were placed in individual cages which had a wire mesh bottom suspended over a sterilized pan. After 2 hours, 10 fecal pellets were collected from the droppings and emulsified in 10 ml. sterile saline. The number of viable organisms in this suspension was determined by surface plate count on desoxycholate agar (Difco) containing 1 mg./ml. streptomycin. Only \textit{Shigella} or \textit{Vibrio} were recovered from the feces of infected animals. However, spot checks by slide agglutination were made as routine.

\textbf{Antisera and Active Immunization.}—All antisera employed for passive immunization were prepared in rabbits with the strain of \textit{Shigella} or \textit{Vibrio} that was used later for the challenge infections. For the preparation of antigens, the growth from 24 hour agar cultures was suspended in saline and heated for 10 minutes at 56°C. without previous washing. Six to eight intravenous injections of increasing amounts of antigen (0.2 mg. to 10 mg. dry weight) were given at 5 day intervals. The rabbits were bled on the 7th day after the last injection. All antisera were stored at $-20^\circ$C. without preservative.

Active immunization of mice was carried out by the intraperitoneal route with the strain of \textit{Shigella flexneri} that was used for the subsequent challenge infections. The streptomycin-resistant mutant of \textit{Escherichia coli} (25 rs) used in the present studies was selected by the gradient plate technique from a sensitive strain isolated in 1954 from an apparently healthy human being.

\section*{EXPERIMENTAL}

\textbf{Bacterial Antagonism.}—It was found in preliminary experiments that the enteric infections produced in mice and guinea pigs with the techniques described above were made possible by the action of streptomycin. No \textit{Shigella} or \textit{Vibrio} organisms could be recovered from the feces of orally infected animals which had not been treated with this drug. This was true even with inocula of several million viable organisms. Nystatin and erythromycin were given to prevent superinfections with enterococci and yeast-like fungi. The \textit{Shigella} or \textit{Vibrio} infections thus produced were found to persist for the entire duration of an experiment (about 2 weeks). Little or no decrease in the number of organisms excreted occurred during that period. In some experiments the 50 per cent infective dose was below 1000 organisms indicating a true infection; \textit{i.e.} rapid and apparently unlimited multiplication of the bacteria in the host.

As mentioned above, the observed increase in susceptibility of mice and guinea pigs to enteric infection after oral administration of streptomycin does
not in itself constitute conclusive proof for a protective action of normal enteric flora, because certain alternative explanations could also account for these results. This problem was investigated in the following way.

Six groups of 4 mice each were infected by stomach tube with mixtures of Sh. flexneri and the streptomycin resistant strain of E. coli (25 rs). All animals received the same number (10 x 10^6) of Shigella organisms and varying amounts of E. coli ranging from 10 x 10^8 to 1 x 10^9 organisms.

On the 1st day after infection stools from 19 of the 24 mice studied were positive for Shigella. On the 3rd day after infection Shigella could be recovered from only 4 out of 24 stool samples while 17 cultures showed only abundant growth of E. coli (3 stool samples were sterile). A control group of 8 mice which had received only Shigella organisms gave positive stool cultures on all 3 days. On later days, all 24 mice excreted only E. coli while the 8 control mice continued to give stool cultures with abundant growth of Shigella. Similar experiments in guinea pigs infected with Shigella flexneri or Vibrio cholerae showed identical results.

These findings indicate a strong antagonistic action on the part of the coli strain, thus providing conclusive evidence for an in vivo protective effect of a common intestinal bacterium.

The coli strain used in the above experiments was tested for in vitro production of antibiotic substances which might be effective against the Shigella or Vibrio strains studied. The test was carried out using the method described by Fredericq (13):

A macrocolony of the coli strain, about 10 mm. in diameter was grown for 48 hours at 37°C. in the center of a veal infusion agar plate (veal infusion broth, Difco, with 1.5 per cent bacto agar and containing 1 mg./ml. streptomycin). The growth was then killed with chloroform vapor and the plate reseeded with the Shigella or Vibrio strain respectively.

No zone of inhibition was noticed in either case. The same negative result was obtained with a similar method described by Halbert (14), involving simultaneous growth of the suspected colicine producer with the test strain. Proteose No. 3 agar (Difco) with and without addition of streptomycin was used in these tests.

Active and Passive Immunization.—The following studies were carried out to detect any possible antibacterial effect of active and passive immunization in experimental enteric infections.

Table I gives the results of an experiment in which the duration and severity of Shigella infection in normal and actively immunized mice were compared.

The immunized mice had received 4 intraperitoneal injections of one-quarter LD_{60} dose of Shigella flexneri antigen, given at 10 day intervals. Infection was on the 7th day after

\footnote{All 4 samples were from mice in the group which had received the second smallest dose (100 x 10^8) of E. coli.}
the last antigen injection. The fractions in parenthesis in Table I refer only to those mice which excreted a large number (at least several million) of Shigella organisms per pellet while the other fractions refer to all mice with positive stool cultures, regardless of number of organisms recovered.

No consistent or significant difference was found in the excretion of Shigella flexneri by immunized mice as compared to the normal control group (Table I). At the end of this experiment (2 weeks) 5 mice each from the immunized and normal groups were bled and the agglutinin titers of their sera were deter-

<table>
<thead>
<tr>
<th>Time after infection</th>
<th>Group No.*</th>
<th>4</th>
<th>5</th>
<th>Total of groups 1 to 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>11,000</td>
<td>1,100</td>
<td></td>
</tr>
<tr>
<td>days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Normal</td>
<td>3/4</td>
<td>3/5</td>
<td>15/26 (15/26)</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>6/6</td>
<td>4/5</td>
<td>26/29 (26/29)</td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td>4/4</td>
<td>5/5</td>
<td>22/25 (9/25)</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>6/6</td>
<td>4/5</td>
<td>20/29 (16/29)</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>4/4</td>
<td>3/5</td>
<td>21/25 (9/25)</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>6/6</td>
<td>5/5</td>
<td>20/29 (7/29)</td>
</tr>
<tr>
<td>13</td>
<td>Normal</td>
<td>2/4</td>
<td>4/5</td>
<td>19/25 (14/25)</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>6/6</td>
<td>4/5</td>
<td>27/29 (19/29)</td>
</tr>
</tbody>
</table>

* Individual data on groups 1 to 3 (infected with 10,100,000 organisms respectively) are omitted from the table.

† 3/4, the stools from 3 out of 4 mice were positive for Shigella.

§ (3/4), 3 out of 4 mice gave stool specimens from which large numbers of Shigella were recovered.

Mined. Titers ranged between 1:250 and 1:1000 in the immunized group while the sera from normal mice were negative in a dilution of 1:4.

Similar experiments were carried out with Vibrio cholerae and Shigella flexneri in guinea pigs which had been passively immunized with a single dose of 2 ml. homologous antiserum given intragastrically 3 hours prior to infection.

Control animals received 2 ml. normal rabbit serum instead of antiserum. Table II shows the results obtained in 2 representative experiments of this type, in which the animals had been infected with 1.5 × 10⁶ viable cells of Shigella flexneri or 10 × 10⁶ viable cells of Vibrio cholerae respectively.

As can be seen, passive immunization had no demonstrable antibacterial effect. The slight reduction in numbers of vibrios recovered from immunized
animals (group D) on the first day after infection was statistically not significant. Other experiments with passively immunized mice and guinea pigs showed similar results. The mean numbers of shigella or vibrio recovered from the immunized animals on the first or subsequent days after infection were always insignificantly higher or lower than those of the controls. Complement given orally together with the antiserum also had no effect.

Burrows and Havens (15) reported that antibody to *Vibrio cholerae* administered orally to guinea pigs was not inactivated to any large degree in the intestine. These authors could recover 25 per cent of the original oral dose in the feces, collected over a period of 1 week, and demonstrated that additional amounts of antibody had diffused into the blood stream. As will be described later (12), the method of oral passive immunization employed in the present experiments protected guinea pigs against death from the fatal enteric cholera infection. Consequently, excessive destruction of antibody in the intestinal tract cannot account for the lack of antibacterial protection observed in the present studies.

Another possible objection to the above experiments may be that the rapid growth of the enteric pathogens in the intestine, which was possible because of the lack of inhibitory normal flora, may have masked any slight antibacterial effect of the antibody. The following experiment was designed to test this hypothesis.

Three groups of 7 mice each (A, B, and C) were infected in the usual way with $1 \times 10^6$ cells of *Shigella flexneri*. Two of these groups (B and C) received an additional $1 \times 10^6$ cells of *E. coli* 25 rs. One of the two latter groups (C) received 0.5 ml. antiserum to the *Shigella* strain (agglutinin titer 1:12500) orally, while the other mice (groups A and B) were given

### TABLE II

*Mean No. of Sh. flexneri or V. cholerae per Fecal Pellet* Recovered from Normal and Passively Immunized Guinea Pigs

<table>
<thead>
<tr>
<th>Time after infection</th>
<th>Infected with <em>Sh. flexneri</em></th>
<th>Infected with <em>V. cholerae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
<td>Group B</td>
</tr>
<tr>
<td></td>
<td>Normal serum</td>
<td>Antiserum titer 1:2500</td>
</tr>
<tr>
<td>days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mean No.</td>
<td>$5 \times 10^6$</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Mean No.</td>
<td>$63 \times 10^6$</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>Mean No.</td>
<td>$7 \times 10^6$</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>11</td>
</tr>
</tbody>
</table>

n—no. of animals tested.

* Determined from a sample of 10 fecal pellets.
normal rabbit serum instead. Stool specimens were cultured on subsequent days. Table III illustrates the results of this experiment.

There was no observable effect of oral passive immunization on the 1st day after infection (Table III). It seems reasonable to expect that any antibacterial effect of the immunization on *Shigella* growing under such competitive conditions would have become apparent in the stool cultures.

**DISCUSSION**

The experiments described in the first part of this paper demonstrate that bacterial antagonism is a definite factor in resistance to enteric infection. Only one strain of *E. coli* and one strain each of *Shigella flexneri* and *Vibrio cholerae* were used in the present studies and no definite conclusions can therefore be drawn as to how common this phenomenon may be. The fact that the *coli* strain used was originally isolated from a healthy human being suggests however, that at least some individuals might harbor similar strains. The *in vivo* antagonistic effect of the *coli* strain could be demonstrated equally well in two different species, guinea pigs and mice. This observation adds some weight to the assumption that a similar protective mechanism might also be operative in human beings which carry a suitable normal enteric flora.

It is interesting to note that no diffusible antibiotic substance could be demonstrated with either Halbert’s (14) or Fredericq’s (13) technique in plate cultures of the *coli* strain studied. This might possibly explain the lack of correlation between resistance to *Shigella* infection and the presence of colicine-producing bacteria reported by Halbert (3).

Several possible explanations can be advanced to account for the *in vivo* inhibitory action of the *coli* strain used in the present studies. This strain
might overgrow the *Shigella* or *Vibrio* in the intestine because of the production of an extremely unstable antibiotic substance, such as that described by Wynne and Norman (16), or it might successfully compete for essential nutrients. A mere difference in growth rate might also account for the effects observed.

The lack of antibacterial effect of passive immunization was observed for two different microorganisms and in two different kinds of animals. The same negative result was obtained in *Shigella* infections of actively immunized mice. The evidence presented does not preclude that different techniques might reveal some degree of antibacterial effect of immunization such as that found by Burrows and his coworkers (8, 9, 17). It can however be concluded with some certainty that any antibacterial effect of immunization in experimental enteric infections would be at least far inferior to that of antagonistic normal flora.

**SUMMARY**

A method has been devised for inhibiting the normal enteric flora, permitting long term asymptomatic enteric infections of mice and guinea pigs with streptomycin-resistant strains of *Shigella flexneri* or *Vibrio cholerae*.

Introduction of a streptomycin-resistant strain of *E. coli* into the intestinal tract of experimental animals resulted in a rapid elimination of the enteric pathogens studied. No *in vitro* production of antibiotic substances by this *coli* strain could be demonstrated.

Active and oral passive immunization did not noticeably influence the number of *Shigella* or *Vibrio* organisms recoverable from the feces of infected animals.

**BIBLIOGRAPHY**