EXPERIMENTAL SHIGELLA INFECTIONS

V. Studies in Germ-free Guinea Pigs

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ABSTRACT

FORMAL, SAMUEL B., (Walter Reed Army Institute of Research, Washington, D. C.), GUSTAVE DAMMIN, HELMUTH SPRINZ, DONALD KUNDEL, HERMAN SCHNEIDER, RICHARD E. HOROWITZ, AND MARTIN FORBES. Experimental shigella infections. V. Studies in germ-free guinea pigs. J. Bacteriol. **82**:284–287. 1961.—Germ-free guinea pigs succumb after oral infection with *Shigella flexneri* serotype 2a; they survive a similar challenge with either a strain of *Escherichia coli* or a culture of lactobacillus. Animals monocontaminated with *E. coli* survive, whereas those monocontaminated with lactobacilli succumb to subsequent challenge with dysentery bacilli. Prior subcutaneous inoculation of heat-killed *S. flexneri* 2a does not render germ-free guinea pigs resistant to the fatal infection with viable dysentery bacilli.

Guinea pigs modified either by starvation or by a subcutaneous injection of carbon tetrachloride succumb to a fatal enteric infection with a strain of *Shigella flexneri* serotype 2a (Formal et al., 1958, 1959). If, on the other hand, the animals are fed antibiotics 48 hr prior to challenge and maintained on these drugs, a chronic nonfatal infection results when an antibiotic resistant dysentery culture is fed (Freter, 1956). There are no doubt many factors which are responsible for this change in susceptibility of the conventional guinea pig, which usually resists shigella infection. Alteration of the normal intestinal flora has been logically implicated in providing a favorable environment for the dysentery bacilli to multiply.

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Increased susceptibility to the toxic products of the challenge organisms on the part of the starved or carbon tetrachloride treated animals has been suggested as a factor in bringing about the death of these animals (Formal, Noyes, and Schneider, 1960).

The germ-free guinea pig offers the advantage of a bacteriologically uncontaminated intestinal tract and a relatively immature defense system for the study of experimental intestinal disease. The purpose of this communication is to report the results of experiments on germ-free guinea pigs challenged with a strain of *S. flexneri* 2a.

MATERIALS AND METHODS

Animals. Hartley strain guinea pigs were obtained by Caesarean section of conventional full-term females in an operating unit of the Reyniers germ-free system. Only small numbers were available for use at any one time. Immediately after birth they were transferred into cages in a Reyniers holding tank, each cage holding no more than ten newborns. At age 4 weeks no more than six animals were allowed in any one cage. The animals were maintained on a modified Phillips (1959) diet supplemented with ascorbic acid and thiamine. The diet was liquid when given to the newborn animal but the water content was gradually decreased so that when the guinea pigs were 4 weeks of age the diet was quite dry. Temperature was maintained at 33 ± 1 C during the first week of life, 30 ± 1 C during the second, and 26 ± 1 C thereafter. Relative humidity was 55 ± 10% throughout life and an automatic light cycle of 12 hr on and 12 hr off was maintained. The microbiological procedures to determine absence of bacteria, fungi, protozoa, and pleuropneumonia-like organisms are described elsewhere (Levenson et al., 1959). The animals were used when they were 6 weeks old. At this time their average weight was approxi-

mately 250 g with a range of 180 to 350 g. Groups of similar average weights were employed.

Cultures. S. flexneri 2a strain 2457 was isolated by Oscar Felsenfeld in Tokyo in 1954. It has been maintained in the lyophilized state and used in our previous experiments. *Escherichia coli* strain HS was isolated from a healthy human being. A culture of lactobacillus, strain L3, was isolated from a conventional guinea pig.

RESULTS

In the first pilot experiment 1×10^7 *S. flexneri* 2a suspended in 10 ml of brain heart infusion broth (Difco) was administered by stomach tube to each of four germ-free guinea pigs. This dose was selected because it represents an LD_{50} for conventional animals modified either by starvation or by carbon tetrachloride. All animals succumbed within 48 hr with no evidence of diarrhea. At autopsy three of the animals exhibited no unusual gross changes with the exception of vascular congestion in the small intestine. Dysentery bacilli were isolated only from the gastrointestinal tract. The fourth animal on the other hand had a pneumonia. *S. flexneri* was isolated from the blood, liver, spleen, and lung in addition to the gastrointestinal tract of this animal.

A second pilot experiment was conducted to determine the effect of a challenge with *E. coli* or high dose heat-killed *S. flexneri*. Three germ-free guinea pigs were fed 6.6×10^6 *E. coli* by stomach tube. In a similar manner two additional animals were given 9.9×10^{10} autoclaved *S. flexneri*. One of the animals fed viable *E. coli* died within 48 hr of challenge; bacteria were isolated from blood, liver, spleen, lung, and gastrointestinal tract. Both animals fed heat-killed Shigellae survived. Three weeks later the two surviving animals contaminated with *E. coli* and the animals previously fed killed Shigella were challenged with 5×10^6 viable *S. flexneri* 2a administered by stomach tube. The latter animals succumbed after 3 to 4 days; dysentery bacilli were isolated from the gastrointestinal tract of these animals. The *E. coli*-contaminated animals survived and when they were killed 2 weeks later only *E. coli* was isolated from the intestines. *S. flexneri* 2a cells were not found.

Two additional experiments were conducted to confirm the impression that guinea pigs monocontaminated with *E. coli* are resistant to *S. flexneri*. A total of 11 germ-free animals were contamined with *E. coli* by adding this organism to the food and water. One of these animals died 48 hr after exposure to *E. coli*. At autopsy this animal grossly exhibited no unusual features. The surviving animals were challenged with 5×10^6 *S. flexneri* 2a administered by stomach tube. Half were fed the dysentery bacilli 1 week after contamination with *E. coli*. The remaining five received the challenge 3 weeks after exposure to *E. coli*. All these animals survived following administration of the Shigella inoculum, and when killed only *E. coli* was isolated from the bowel. All of eight germ-free animals serving as virulence controls succumbed to the dysentery challenge. The results of these two experiments are summarized in Table 1.

Another experiment was performed to determine if guinea pigs monocontaminated with a culture of lactobacillus isolated from a conventional guinea pig would survive challenge with *S. flexneri* 2a. All of five germ-free animals survived exposure to food and water contaminated with the lactobacillus culture. One week after this exposure, these animals and five germ-free guinea pigs were fed 5×10^6 *S. flexneri*. All animals succumbed within 48 hr.

An experiment involving five germ-free guinea pigs was next carried out to determine if animals

TABLE 1. *Deaths in germ-free and monocontaminated Hartley strain guinea pigs fed Shigella flexneri 2a*

Expt*	No. animals at start of expt	Animals contaminated with	Animals surviving after contamination	Animals surviving Shigella challenge
1	6	*Escherichia coli*	5	5
	4	Nothing (germ-free)	—	0
2	5	*E. coli*	5	5
	5	Nothing (germ-free)	—	0
3	5	Lactobacillus	5	0
	5	Nothing (germ-free)	—	0

* Animals in experiment 1 were challenged with *S. flexneri* 2a 3 weeks after exposure to *E. coli*; the interval between contamination and challenge with *S. flexneri* was one week in experiments 2 and 3.

would suffer a fatal infection if exposed only to food and water contaminated with *S. flexneri* 2a. One animal was fed the pathogen by stomach tube, the others were infected from food and water heavily contaminated with the organisms. All animals were dead or moribund 24 hr after challenge. Dysentery bacilli were isolated from the gastrointestinal tract of all animals and from the blood of the animal fed by stomach tube and from three of the four animals which ingested contaminated food.

The purpose of the final experiment was to determine the effect of prior parental administration of heat-killed *S. flexneri* 2a on the germ-free guinea pigs' ability to resist infection with viable dysentery bacilli. A saline suspension of 1 $\times$ 10^9 ml viable *S. flexneri* 2a was placed in a flask and sterilized in the autoclave of the Reyniers germ-free tank. Over a period of 1 week, five 0.5-ml amounts were inoculated subcutaneously into nine germ-free guinea pigs. All the animals survived this treatment. However, one of ten control germ-free animals inoculated with saline died after the fourth injection. One week after the final inoculation, the tank was heavily contaminated with *S. flexneri* 2a. All the animals previously inoculated either with saline or heat-killed *S. flexneri* 2a were dead or moribund within the next 31 hr. Immunized guinea pigs succumbed as rapidly as unvaccinated controls. Two moribund animals which had received the injections of the *S. flexneri* vaccine were bled. Their sera possessed agglutinin titers against *S. flexneri* 2a of 1:80 and 1:640.

DISCUSSION

In our previous work with experimental dysentery infections in conventional animals it was necessary to modify the host either by starvation or by injection with carbon tetrachloride to render the host susceptible to a fatal injection. In addition, it was necessary to neutralize the stomach acid with calcium carbonate before and to inhibit intestinal motility with opium after challenge for the most consistent results. These manipulations were not required to achieve the same end result with germ-free guinea pigs.

Another difference which was observed between the modified conventional and the germ-free guinea pig was the distribution of the dysentery bacilli within moribund animals. In the conventional guinea pig, these organisms were limited to the gastrointestinal tract, whereas in somewhat less than 50% of the moribund germ-free animals, *S. flexneri* was isolated from organs outside of the intestinal tract. Hematogenous spread occurred in the untreated germ-free animals as well as in germ-free animals fed or inoculated with dead *S. flexneri*. It also was independent of the manner by which the animals became infected (challenge by stomach tube or contaminated food). Thus it is apparent that the defense mechanism of the conventional animal which contains the infection within the bowel is lacking in some germ-free guinea pigs.

Cohendy and Wollman (1922) observed that 10- to 15-day-old, germ-free guinea pigs succumbed 6 to 9 days following oral administration of *Vibrio comma*. At autopsy organisms were isolated from the blood as well as the intestinal tract. Previous contamination with either a strain of staphylococcus or *Agarbacterium mesentericus* did not influence the course or outcome of the infection. Thus the response of germ-free guinea pigs to challenge with either *V. comma* or *S. flexneri* is in many respects similar, although one must keep in mind that our animals were about 6 weeks old, whereas those of Cohendy and Wollman were 1 to 2 weeks of age. On the other hand, germ-free chickens, mice, and rats do not succumb after exposure to *S. flexneri* (Wagner, 1959). The organisms multiplied well in the intestinal tract, rapidly reaching a density of 10^9 cells per gram of cecal contents; yet the animals exhibited no signs of illness.

S. flexneri 2a caused a fatal enteric infection of germ-free guinea pigs, but *E. coli* usually did not. Nineteen of 23 animals survived infection with this latter organism for at least 6 days. Animals monocontaminated with *E. coli* strain HS did not succumb to subsequent challenge with *S. flexneri*. Indeed, 1 week after the administration of the dysentery bacilli it was not possible to isolate these organisms from the intestine. Only *E. coli* was present. This observation is similar in some respects to that made by Freter (1956) who found that a strain of *E. coli* could inhibit multiplication of Shigellae or *V. comma* in the intestines of antibiotic-treated mice or guinea pigs if the *E. coli* cultures were fed at the same time as the pathogen. The mechanism of this growth inhibition is not understood. The *E. coli* strain which was used in this study exhibited no

colicine activity against the dysentery culture, nor did it carry a phage that would lyse the pathogen. Ransom, Ceder, and Formal (1960, *unpublished data*) worked in vitro with the same *E. coli* and *S. flexneri* cultures used in this investigation. Employing a continuous culture procedure they found that a growing culture of *E. coli* in the steady state inhibited multiplication of the *S. flexneri* 2a strain which was subsequently introduced if the environment was anaerobic. Under aerobic conditions inhibition was not observed. These data confirm similar observations made by Freter (1959).

As far as could be determined with a small number of animals, a single previous feeding of heat-killed *S. flexneri* did not render germ-free animals resistant to subsequent oral challenge with viable dysentery bacilli. Multiple subcutaneous injections of autoclaved dysentery organisms also were ineffective in conferring resistance, and animals monocontaminated with a strain of lactobacillus succumbed to subsequent infection with *S. flexneri*. There is a possibility that the presence of *E. coli* in the bowel of the guinea pig alters the animal's ability to respond to the challenge with Shigellae. Experiments are planned in which large numbers of dead *E. coli* or *S. flexneri* 2a are fed over a period of several days prior to the administration of dysentery bacilli to determine if this procedure will increase the resistance of the test animal.

Although the presence of *E. coli* in the bowel protects the germ-free guinea pig from subsequent dysentery challenge, this organism probably does not play a significant part in rendering the conventional animal resistant to this infection. *E. coli* is not a consistent member of the normal enteric flora of this animal species (Crecelius and Rettger, 1943; Roine and Elvehjem, 1950). Ransom and Formal (1959 *unpublished data*) have studied the normal flora of guinea pigs at Walter Reed Army Institute of Research over a period of 1 year and found that *E. coli* was absent during the summer and present during the winter months. During the summer months the animals are resistant and during the winter sensitive to experimental shigellosis when modified by procedures already published (Formal et al., 1958; 1959).

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