Toxigenesis and Infection of Mice by *Salmonella enteritidis* (34798)

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Prost and Riemann (12) reported that salmonellosis is a unique disease and that the mechanism of the pathogenic action of *Salmonella* is not yet fully explained. Roantree (13) pointed out that the reasons for the ability of certain species of *Salmonella* to infect and multiply in animals are still unknown. Miller and Bohnhoff (10) fed *S. enteritidis* by gavage to white mice and used the ability to recover the test organism from the spleen and stool as criteria for infection. Morello *et al.* (11) fed avirulent and virulent strain of *S. typhimurium* to mice and showed that the former strain gradually decreased in number to total elimination, while the latter strain either increased in number up until time of death of the test animal or produced a chronic carrier. Sato (14) infected wild rats with various doses of *S. pullorum, S. newington,* or *S. enteritidis* and found that *S. enteritidis* and *S. newington* caused longer fecal excretion and localization of the infecting organism in the submaxillary nodes. Sato also reported that *S. enteritidis* could be cultured from several organs and appeared to be transmitted from rat to rat more readily than *S. newington.* Berry and Mitchell (1, 2) found that intraperitoneal (ip) injection of *S. typhimurium* caused invasion of the blood within 2–3 hr postinfection of *S. typhimurium* and affected the blood count. Berry *et al.* (3) established that when *S. typhimurium* cells were injected (ip) in mice, the number of viable cells increased over 6 hr postinfection. Schewe (15) reported that mice, infected intravenously with 10^6 cells of *S. typhimurium,* had the test culture in the liver, kidneys, lungs, spleen, and in the remains of the carcass, and that these animals are supporting at death total populations of viable *Salmonella* of an approximate range of $10^9 - 10^{10}$ cells.

Salmonellosis is considered to be an animal disease, human food poisoning being an accidental consequence of the animal infection. It seems doubtful, however, that salmonellosis can be completely eradicated unless the animal reservoir is reduced and the cycle between animal and man is broken. The increase in the number of salmonellae patients in the past two decades made it necessary to examine the relationship between this organism and the host reservoir. Therefore, studies were conducted to examine the course of toxigenesis and infection caused by *S. enteritidis* in mice when fed, by gavage, a saline suspension containing a specific number of viable cells harvested from definite stages of growth (a negative acceleration and an early decline phase).

Materials and Methods. Test animals. Adult female white Swiss/1CR mice weighing 30–35 g (Blue Spruce Farms, Altamount, N. Y.) were examined upon arrival for the presence of *Salmonella* organisms and found to be clinically free. The mice were kept at constant room temperature (24°) in isolated cages and offered salmonella-free commercial laboratory pellets and water *ad libitum.*

Bacteria. S. enteritidis obtained from W. J. Payne (Dept. of Microbiology, University of Georgia) was kept by repeated transfer in tryptic soy broth (TSB-Difco) and incubated aerobically at 37°. Flasks of TSB were inoculated, using 1% inoculum, and incubated at 37°. The cells were harvested by centrifugation from two definite stages of growth; after 18-hr (negative acceleration phase) and 36-hr incubation (an early decline phase) and then washed three times with cold sterile

saline. The cells were then resuspended in sterile saline to two concentrations (10^3 and/or 10^8 cells/ml which were checked by plating on tryptic soy agar (TSA).

Infection technique. One milliliter of the saline suspension containing the desired number of salmonella, obtained after the 18- or 36-hr incubation, was fed to the mice by gavage. The mouse was anesthetized with diethyl ether and the suspension was then administered directly into the stomach using a 2-cc syringe with a 3-in. 17-gauge curved cannula covered with a polyethylene tubing to prevent injury to the animal (6). Efforts were made to reduce stress to the mice during infection and postfeeding (PF). Mice receiving sterile saline served as controls.

Criteria of toxigenesis and/or infection. The pathological condition caused by the endotoxins and/or the presence of the test organism in the host, were based on various differences between salmonella-fed and control mice. The criteria selected were: (a) amount and condition of the intestinal matter in the gastrointestinal tract; (b) activity of the mouse; (c) the condition of the mesenteries; and (d) the recovery of the test organism from liver, spleen, and blood.

Sampling. Both control and infected mice were examined at 1-hr intervals for 6 hr after feeding the sterile saline or the salmonella suspension and then after 8, 10, 12, 24, 48, and 72 hr PF. A ventral incision was made from the anus to the throat of the mouse and about 1 ml of blood was drawn by cardiac puncture (6). The spleen, liver, and cecum were aseptically excised, placed in sterile petri dishes, chilled immediately, and analyzed bacteriologically for the test organism. The blood was inoculated into 10 ml of mannitol purple broth (MPB) and incubated overnight at 37° (17). When positive reactions were obtained in MPB, an 0.2-ml culture was transferred to brilliant green tetrathionate (BGT) and incubated 24 hr. This culture was then streaked on brilliant green agar (BGA) and again incubated for 48 hr. Colonies were identified by reactions in triple sugar iron agar, lysine iron agar, and finally by reaction with salmonella polyvalent "H" antisera–G complex (Difco) as described by Hajna and Damon (7) and Kauffmann (9) to ensure identification of the isolate to that of the fed culture.

The organs were aseptically minced with a sterile scissors and swabbed thoroughly with a wet sterile swab which was then streaked on BGA plates and also used to inoculate TSB tubes. After overnight incubation, samples showing positive reactions were streaked on the BGA plates, and typical colonies were isolated, examined on other media, and then serotyped as previously outlined.

Ceca from infected or control mice were excised under aseptic conditions, minced with a sterile scissors, and thoroughly homogenized with cold saline for 2–3 min at 16,000 rpm in sterile, precooled Sorvall Omni-Mixer cups. The homogenates were then plated on BGA, and the number of salmonella recovered per gram of cecum was divided by the number of salmonella fed and the results were reported as cells recovered per cells fed (R/F value). This was performed to reduce the biological variability caused by slight differences (less than 1 log unit) in the number of cells fed and the speed of peristalsis.

Results. Toxigenesis in mice. Studies were conducted to determine the toxigenesis of *S. enteritidis* in mice fed *per os* various numbers of cells of this organism harvested from a definite growth phase. Five groups of mice, each consisting of 60 animals, were used. Each mouse of the first group was fed 10^3 salmonella from an 18-hr culture. Mice of the second group received the same number of cells, except for the salmonella fed which were harvested after 36-hr growth. The third group were fed 10^8 salmonella from an 18-hr incubation, the fourth group of mice were given 10^8 cells from a 36-hr culture, and the fifth received saline and thus served as controls. When toxigenesis and/or infection was initiated, the mouse would cease to eat and enter into a state of stupor. During this time the infected animal could be aroused but would not become excited by any stimuli including induced pain. This period of low response occurred throughout 3 hr PF and continued for various lengths of time depending on the number of cells and phase of growth of culture fed. When 10^3 cells of either

the 18- or the 36-hr culture were used, the duration of this low response was 3 hr, whereas when 10^8 cells were fed, the low response period lasted for 5 hr in the case of the 36-hr culture and for 72 hr for the 18-hr culture. The state of low response (mainly due to toxigenesis) was usually accompanied by a more rapid and labored breathing and an apparent increase in rate of heartbeat. During this period, the stomach and the small and the large intestine frequently lacked digested food material. They were filled with gas or fluid, and had some evidence of inflammation. Histopathological observations were performed on four control and eight infected mice, and the results revealed coagulation necrosis of the surface epithelium at the villi, which were shortened and blunted, especially in mice fed 10^8 cells of the 18-hr culture. This indicated that the mice suffered severe toxigenesis and enteritis within 3 hr, whereas, only mild toxigenesis and enteritis was noted in mice infected with 10^8 cells of the 36-hr culture. Control mice, fed saline, were normal and showed no inflammation. Since only small numbers of mice were examined histopathologically, the observations noted are, at most, only indicative of regression changes in the surface epithelium of the intestinal mucosa. In some instances diarrhea was evident, but in no case was vomiting ever observed. Before toxigenesis, the mesenteries were relatively translucent, but became opaque and mucus-like during toxigenesis and infection, and finally returned to normal toward the end of the infection period. This was evident within 3 hr in mice fed 10^8 cells of the 36-hr culture and was over by 5 hr, whereas in mice fed 10^8 cells of the 18-hr culture this condition persisted for 72 hr.

Survival of salmonella organisms in cecum. Time-course studies of the relationship between number of cells recovered from the cecum divided by number of cells fed (Fig. 1) indicated that there was very little change when 10^3 cells of either phase of growth were fed to mice, whereas when 10^8 viable cells of the 36-hr phase were fed, this ratio increased to a maximal value within 3 hr PF followed by a slight decrease to the fourth hour and very little changes thereafter. When 10^8 cells

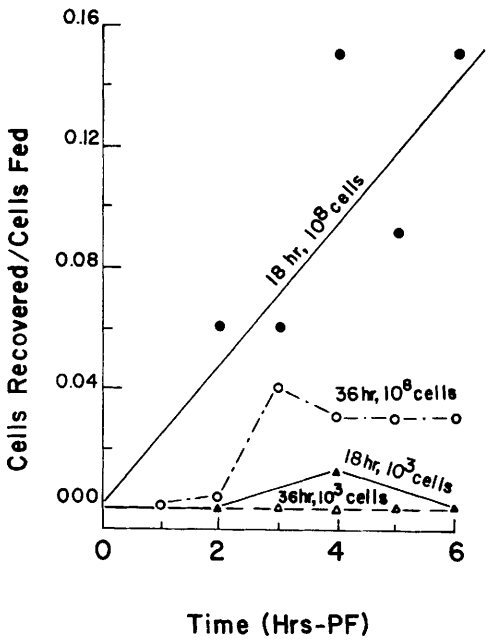

FIG. 1. Number of salmonella recovered per gram of cecum/number of salmonella fed. Different age and numbers of *S. enteritidis* were fed to mice.

of the 18-hr culture were fed, a dramatic increase in the ratio resulted within 1–2 hr and continued thereafter to 6 hr PF, indicating definite survival and possibly growth of the organisms in the cecum. Analysis of the cecum after 24, 48, and 72 hr PF revealed the presence of the test culture at all intervals.

Recovery of salmonella from organ tissue. The data (Fig. 2) showed that when 10^8 cells of the 18-hr culture were fed to mice, the percentage of incidence of positive culture recovery of salmonella from spleen (the presence of 30 or more colonies/plate) increased to 47% on the third hour with slight change thereafter. When 10^8 cells of the 36-hr culture were fed, the incidence increased to 20% on the second hour followed by a gradual decline. Positive recovery from blood was small in mice fed 10^8 cells of the 36-hr culture with a slight increase on the fifth and sixth hours PF. On the other hand, viable salmonella (in large numbers) persisted in blood, and a higher incidence was noted at all times, reaching 56% within 3 hr (indicative of bacteremia) in mice fed 10^8 of

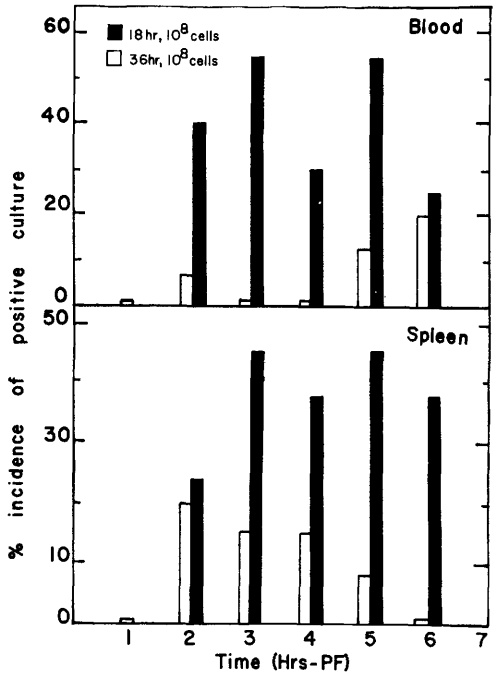

FIG. 2. Presence of *S. enteritidis* in spleen and blood obtained from mice fed (*per os*) different age *Salmonella* cells.

the 18-hr cultures. Incidence of recovery of test culture from liver of mice fed 10^8 cells of the 36-hr culture was nil and only to the extent of 7.0% on the third hour. On the other hand, when 10^8 cell of the 18-hr culture were used, a higher incidence of positive recovery from liver was noted, reaching 13% on the second hour and 37% on both the third and fifth hours and 13% on the sixth hour PF. Salmonella was not recovered from mice fed 10^3 cells of either the 18 or the 36-hr cultures during the entire experiment from blood or organs (spleen, liver) even when enrichment technique was made on all samples examined.

Discussion. The data presented indicated that salmonellosis in mice depends on number and phase of growth of viable cells fed *per os* and that 10^8 of the 18-hr-grown cells of *S. enteritidis* were necessary to induce toxigenesis and infection as evident both physiologically and bacteriologically. These results confirm those reported by Miller and Bohnhoff (10) and Morello *et al.* (11). However, the former authors stated that infected mice, even those that were bacteremic, rarely showed signs of illness. While in the present study, definite symptoms ranging from stupor to diarrhea and inflammation of the small intestine were noted. The rapid appearance of the symptoms may be due to the presence of salmonella endotoxin and high concentration of O-antigen in the cell wall. Shands (16) reported that the amount of endotoxin produced in a host and the response of the latter to the endotoxin are important factors in the establishment of gram-negative infections and the production of disease syndrome. Collins (4) reported that the cell-wall composition of *S. enteritidis* could be altered by changes in the growth rate. On the other hand, Shands (16) established that differing conditions of growth failed to alter the toxicity of *S. typhimurium* and that small, slowly grown organisms appear to be as toxic as large, rapidly grown cells. However, the fact that it required a large number of viable cells (10^8) of *S. enteritidis* to induce toxigenesis and infection indicates that mice have a high degree of resistance to this organism.

Virulence, by definition, is the relative capacity of an organism to produce a disease, not necessarily the ability to kill the host. Hsu and Radcliffe (8) showed that avirulent *S. typhimurium* were destroyed faster than the virulent strain of the same organism within the host macrophages. These authors also reported that the virulent strain appeared to be much more resistant to the intracellular digestion and could overcome it after 3 hr of intracellular residence, whereas, the avirulent strain was less capable of adapting to the intracellular environment and required 4 hr to overcome this intracellular digestion. Yamamoto (18) stated that virulent *S. enteritidis* was capable of surviving and even multiplying in the polymorphonuclear phagocytic cells. It is possible that the higher incidence of recovery of the test culture from the organs and blood of mice fed 10^8 cells of the 18-hr culture may be due to the ability of these organisms to survive phagocytosis. It is also possible that these organisms were not completely destroyed in the stomach and living bacteria were able to pass to more caudal sections of the alimentary tract and other

organs, and their action in these areas may add to their toxigenesis. It seems evident that the important factor of the salmonella infection is the ability of the organism to multiply from small to very large numbers in the tissues of mice fed 10^8 viable cells of the 18-hr culture, but not the 36-hr culture. Furness and Ferreira (5) reported that a minimum lethal dose (MDL) of 10^7 cells or more was rquired of an avirulent strain of *S. typhimurium* for the albino male mice. Using this one parameter for the establishment of virulence can in some cases be very misleading. The results obtained in this investigation were excellent examples of such usage. The $LD_{50/48hr}$ of *S. enteritidis* (the test culture obtained after 18-hr growth) was found to be 7.1×10^8 cells, and 4.5×10^9 cells for the 36-hr culture and, accordingly, this strain should be labeled avirulent. However, this organism has the capability of producing severe toxigenesis as well as symptoms of salmonella infection and could qualify, by definition, as virulent though not lethal when administered *per os*.

Age of the culture (*i.e.*, the time after which cells were harvested during growth) was found to be an important factor for the establishment of salmonellosis. Cells harvested after 18 hr of growth were able to adapt themselves more readily to the changes in environment and probably survive phagocytosis and thus could induce infection.

The fast heartbeat and breathing rate observed would facilitate the efficiency of the reticuloendothelial system. It is believed that as this rate decreased, viable cells could be more readily detected in the blood as shown toward the end of the test period for the 10^8 cells of the 36-hr culture. The 10^8 cells of the 18-hr culture showed only a continual increase in these apparent rates due to the length of the infection period as evident in the steady increase in the percentage of positive incidence in the blood of the infected mice. When 10^3 cells of salmonellae from either the 18- or 36-hr culture were fed to test animals, the incidence of recovery of the test cultures from the various organs was negative. Organisms were detected in the cecum but were below significant countable levels except at the 4 hr PF. Miller and Bohnhoff (10) showed that infection *per os* of small inocula of *S. enteritidis* failed to multiply during transit through the gastrointestinal tract of the mice. It is also possible that the small inocula (10^3 cells) encountered an environment quite unsatisfactory for their growth and multiplication. The inability of the test organisms, of this concentration, to cause infection may also be due to competition with normal flora.

Summary. Toxigenesis and infection studies caused by *S. enteritidis* ($LD_{50/48hr} = 7.1 \times 10^8$ cell) were evaluated. Mice were fed *per os* 10^3 or 10^8 viable cells harvested from an 18- or 36-hr culture. Time of toxigenic onset, duration, and persistence of test organisms in organ tissues were confirmed bacteriologically, physiologically and by pathological differences between infected and control mice. No toxigenesis and/or infection was noted in mice fed 10^3 cells of either culture, while severe syndrome was observed within 3 hr in mice fed 10^8 cells of both cultures. Feeding 10^8 cells harvested after 36 hr of growth produced short-lived toxigenic manifestation (less than 5 hr PF), but mice receiving 10^8 cells obtained after 18 hr of growth had a longer toxigenic syndrome, and organisms persisted in the GI tract for 72 hr PF. High incidence of recovery of the test organism from the liver, spleen, and blood of mice was noted in mice fed 10^8 cells of the 18-hr culture but not from the 36-hr culture. It appeared that toxigenesis and infection of *S. enteritidis* required a minimum of 3 hr PF and 10^8 cells of the 18-hr culture to induce salmonellosis in mice.

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