EXPERIMENTAL SALMONELLOSIS

Intracellular Growth of *Salmonella enteritidis* Ingested in Mononuclear Phagocytes of Mice, and Cellular Basis of Immunity

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Received for publication October 7, 1960

Earlier reports from this and other laboratories have described the effects of living and killed vaccines on the course of experimental infections with *Salmonella enteritidis* in mice (Kobayashi and Ushiba, 1952; Ushiba et al., 1953; Mitsuhashi et al., 1958; Mitsuhashi, 1959; Mitsuhashi, Hashimoto, and Kawakami, 1960). As has been true for experimental infections with other group D salmonellae, e.g., *Salmonella dublin* (MacLeod, 1954), and with *Salmonella typhimurium* (e.g., Topley, 1929), killed vaccines have increased the survival time of mice after challenge but have been largely ineffective in preventing ultimate death from the infection. By contrast, suitable immunization with live vaccine of *S. enteritidis* was found to confer antilethal protection against challenge with a fully virulent culture of the same organism (Mitsuhashi et al., 1958; Misuhashi, 1959; Mitsuhashi et al., 1960). Resistance of the mouse to facultative cytotrophic bacteria such as these is first localized at the site of invasion. When the bacteria enter the blood stream, two kinds of defense mechanism of the host are mobilized: clearance of bacteria from the blood into cells of the reticuloendothelial system, and inhibition of intracellular growth of bacteria ingested by cells (Mitsuhashi et al., 1960).

In this paper results of studies of certain immune effects on the growth of bacteria in mononuclear phagocytes of mice are presented.

MATERIALS AND METHODS

Experimental animals. Six-week-old ddN strain mice (raised by the Central Animal Laboratory, Gunma University) weighing 17 to 20 g were used exclusively.

Immunization. For immunization with live vaccine, mice were inoculated intraperitoneally with 10^{-5} mg (dry weight) of attenuated strain SER and, 21 days later, with 10^{-7} mg of virulent strain 116-54 of *S. enteritidis.* These amounts corresponded to 3×10^4 and 3×10^2 viable cells, respectively. Immunized mice were obtained 21 days after the last injection. The virulence and antigenic structure of these strains were reported previously (Mitsuhashi et al., 1959*a*). The mouse LD_{50} of the virulent strain was $10^{-8.2}$ mg and that of the attenuated strain was 10^{-2} mg. Mice were immunized with dead vaccine by intraperitoneal injection of 2.8 mg of chrome-alum vaccine (Ando, Shimojo, and Tadokoro, 1952) prepared from virulent strain 116-54. They were considered immunized 14 days after vaccination.

Mononuclear phagocytes. Mononuclear phagocytes were obtained from the peritoneal cavity of normal and immune mice which had been given an intraperitoneal injection of 1 ml glycogen solution (0.01 mg/ml in Hanks' solution) 4 to 5 days prior to the collection (Suter, 1952). The mice were killed by heart puncture and given intraperitoneal injection of 3 ml of Hanks solution containing heparin (Hanks and Wallace, 1949). Cells were obtained by syringe from the abdominal cavity and washed twice by centrifugation at 1,500 rev/min for 3 min in the cold, then resuspended in cold Hanks' solution. Cells obtained from 5 mice were mixed and the final concentration of cells was adjusted to 500/mm^3.

Mouse serum. Mouse serum was obtained aseptically by heart puncture from normal mice, or mice immunized with live or dead vaccine. Serum obtained from 5 mice or more was pooled.

Phagocytic system and tissue culture. The bacterial suspension was prepared from a 18-hr culture on agar, and 0.1 mg/ml of bacterial suspension in Hanks' solution was made. One-tenth milliliter of bacterial suspension was added to a mixture of 5 ml of cell suspension and 0.1 ml of normal mouse serum. A ratio of approximately 10 bacilli to 1 mononuclear phagocyte was used.

Three-tenths milliliter of the mixture was placed in a culture-chamber provided with a cover glass and incubated at 37 C. The mononuclear phagocytes adhered to the cover glass. After 60 min incubation at 37 C, the supernatant fluid was removed and exchanged with culture medium containing normal mouse serum and antibiotics. This consisted of 7 ml of Hanks' solution, 3 ml of normal mouse serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Culture medium was exchanged every other day. As described previously (Sato, Tanaka, and Mitsuhashi, 1958), tissue culture of mononuclear phagocytes obtained from mice according to the roller-tube or Porter-flask method was unsuccessful, whereas Suter (1952) obtained good results with tissue culture of mononuclear phagocytes of the guinea pig. By the glass-ring method described by Lyang (1960), mouse mononuclear phagocytes were maintained in good condition without loss of cell numbers for about 3 weeks. The cover glass, to which mononuclear phagocytes adhered, was taken from the culture-chamber every day,

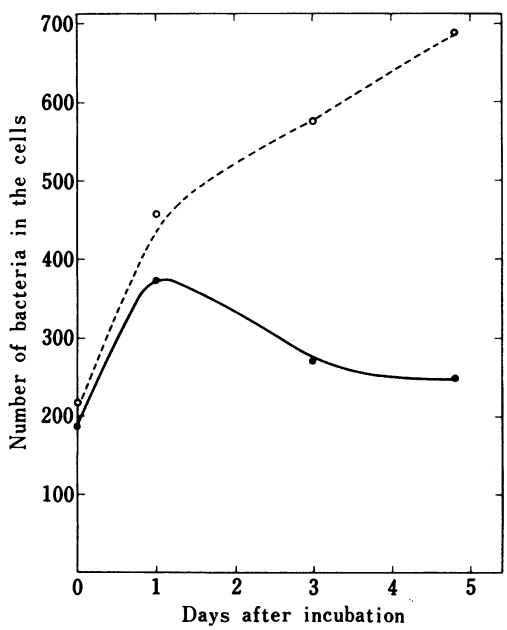

Fig. 1. Intracellular multiplication of virulent or attenuated strain of *Salmonella enteritidis* in monocytes derived from normal mice. The number of bacteria indicates the total number of intracellular bacteria in 100 monocytes which ingested bacteria. O-----O, Virulent strain; ●——●, attenuated strain.

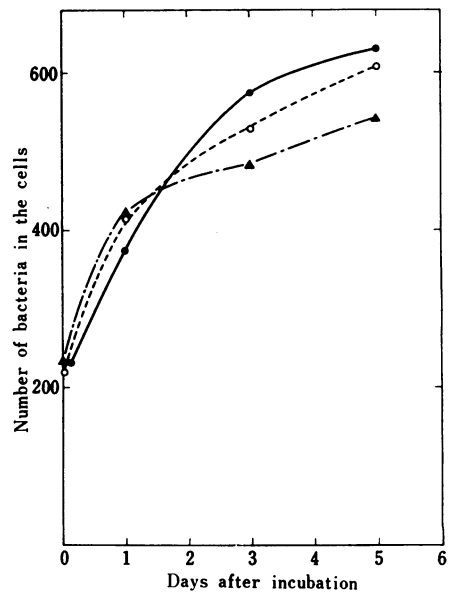

Fig. 2. Bacteriocidal effect of serum derived from normal or immunized mice on the intracellular multiplication of the virulent strain 116-54 of *Salmonella enteritidis*. The number of bacteria indicates the total number of intracellular bacteria in 100 monocytes which ingested bacteria. O-----O, Serum of normal mouse; ▲- - -▲, serum of mouse immunized with dead vaccine; ●——●, serum of mouse immunized with live vaccine.

dried in air, fixed in methanol, and stained in Giemsa solution. The phagocytic index was calculated by the following ratio: (number of phagocytes which ingested bacteria)/(total number of phagocytes examined).

RESULTS

Intracellular behavior of attenuated SER or virulent 116-54 strains of S. enteritidis. When mononuclear phagocytes derived from the peritoneal cavity of normal mice and bacterial suspensions of attenuated SER or virulent 116–54 strains were incubated in a culture-chamber, under the conditions described above, about 80% of the cells which ingested organisms phagocytized 1 to 2 bacteria. The phagocytic index was approximately 30%. When attenuated strain SER was ingested into the cell, the bacteria became small in size and coccoid in shape within 1 day of incubation. Sometimes a halo was

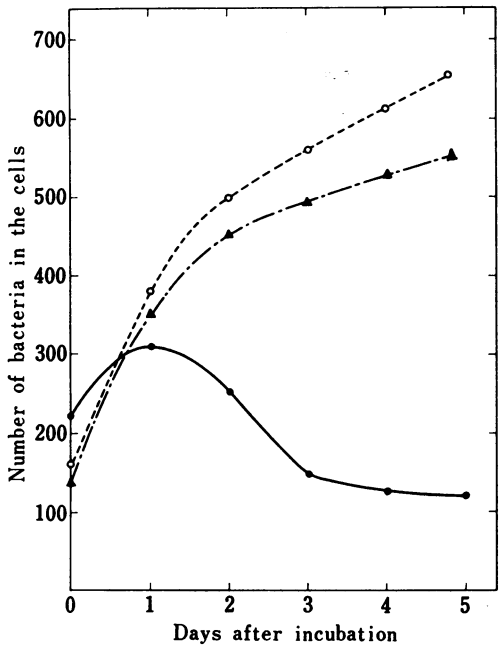

Fig. 3. Intracellular multiplication of the virulent strain 116-54 of *Salmonella enteritidis* in monocytes derived from normal or immunized mice. The number of bacteria indicates the total number of intracellular bacteria in 100 monocytes which ingested bacteria. ●——●, Cells derived from mice immunized with live vaccine; ▲- - -▲, cells derived from mice immunized with dead vaccine; O-----O, cells derived from normal mice.

observed around the ingested bacteria, as previously reported (Mitsuhashi et al., 1959*b*). Intracellular increase of bacteria was observed within 1 day of incubation, but thereafter the intracellular numbers decreased slowly and became constant after 7 days of incubation. As Ushiba (1959) stated, intracellular growth of the virulent strain was rapid and most of the cells were destroyed within 3 days of incubation (Fig. 5*a*, *b*, *c*).

The virulent strain 116-54 also became small in size within the cells at 1 day of incubation; this change in size was slight in comparison with that of the attenuated strain SER. The results are shown in Fig. 1.

Effect of serum obtained from mice immunized with live or dead vaccine on intracellular growth of virulent strain 116-54. The virulent strain 116-54 of *S. enteritidis* was ingested by mononuclear phagocytes of normal mice in culture media consisting of Hanks' solution containing antibiotics and normal mouse serum, or serum obtained from mice immunized with dead or live vaccine. The pooled sera obtained from mice immunized with dead vaccine showed agglutinin titers of 1:160 against O antigen and 1:1,280 against OH antigen of virulent strain 116-54. Pooled sera obtained from mice which received live vaccine showed an O agglutinin titer of 1:80 against the virulent strain 116-54. Culture media

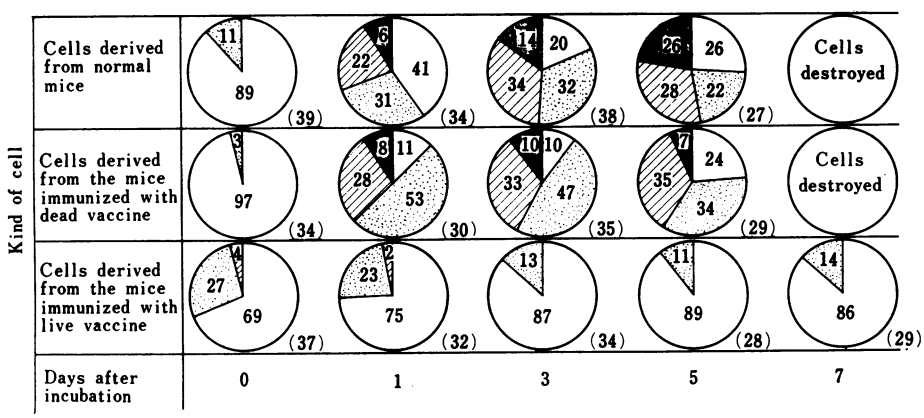

Fig. 4. Intracellular multiplication of the virulent strain 116-54 of *Salmonella enteritidis* in monocytes derived from normal or immunized mice. Monocytes are classified into 4 groups by the number of intracellular bacteria: 1–2, 3–5, 6–10, and more than 10.

□ 1–2, ▨ 3–5, ▨ 6–10, ■ more than 10.

Figures in parentheses in each column indicate the phagocytic index.

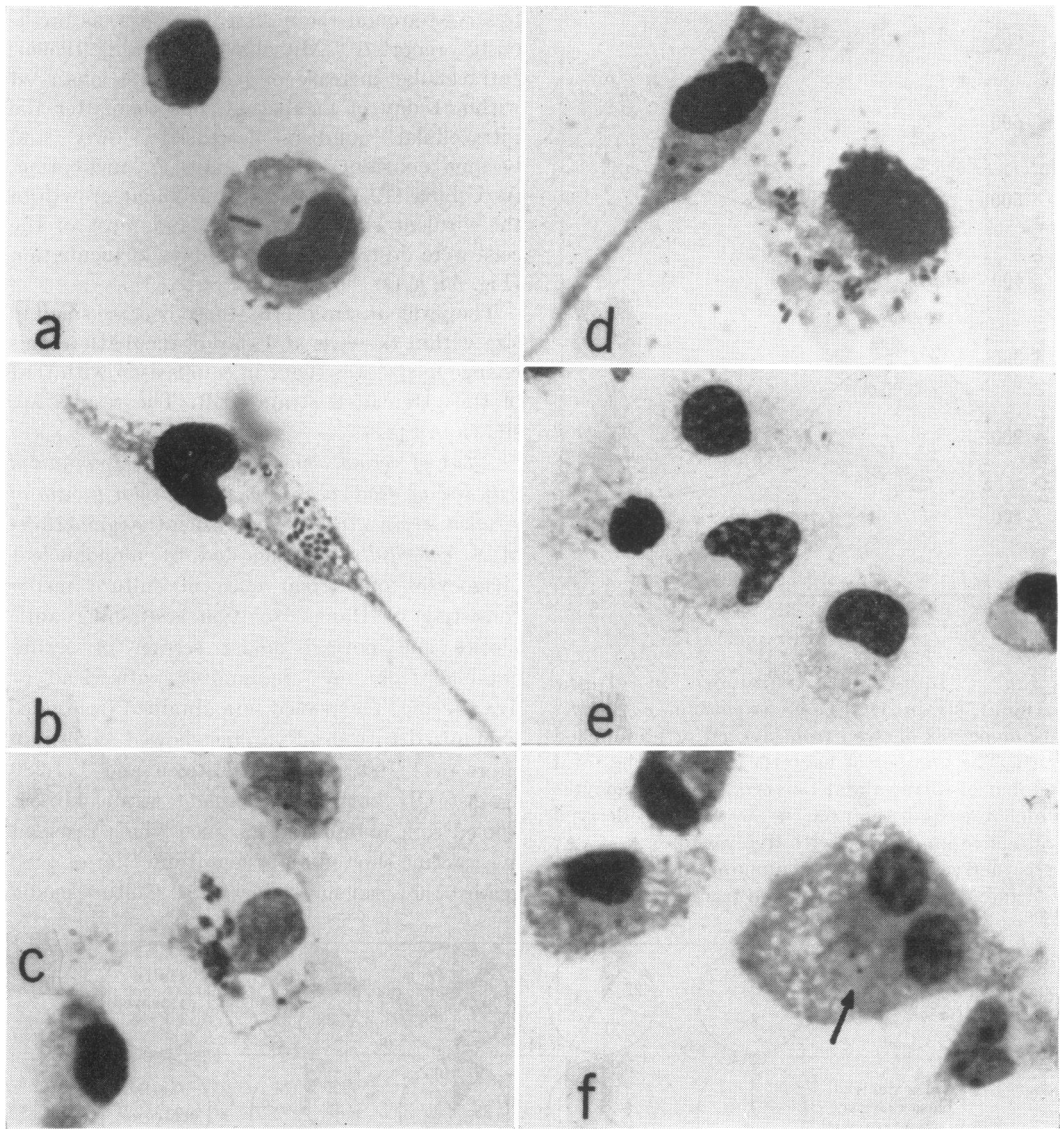

Fig. 5. a) Virulent strain 116-54 of *Salmonella enteritidis* ingested into the mononuclear phagocyte of normal mouse. Immediately after ingestion (magnification, 1,250×).

b) Intracellular multiplication of virulent strain 116-54 of *S. enteritidis* in the mononuclear phagocyte of normal mouse. Three days after incubation (magnification, 1,250×).

c) Destruction of mononuclear phagocyte of normal mouse by the intracellular multiplication of virulent strain 116-54 of *S. enteritidis*. Five days after incubation (magnification, 1,250×).

d) Destruction of mononuclear phagocyte of mouse immunized with dead vaccine by the intracellular multiplication of virulent strain 116-54 of *S. enteritidis*. Five days after incubation (magnification, 1,250×).

e) Inhibition of intracellular multiplication of virulent strain 116-54 of *S. enteritidis* by the mononuclear phagocyte of mouse immunized with live vaccine. Three days after incubation (magnification, 1,250×).

f) Same as *e*). Bacteria show coccoid form. Five days after incubation (magnification, 1,250×).

contained these sera in a concentration of 30%. The results are shown in Fig. 2.

The serum obtained from mice immunized with dead vaccine did not inhibit the intracellular growth of virulent strain 116-54, as Gelzer and Suter (1959) stated in their work on intracellular infections with *S. typhimurium* in mononuclear phagocytes of rabbits. The serum obtained from mice immunized with live vaccine also had no inhibitory effect on intracellular growth of virulent strain 116-54, whereas these mice showed high antilethal resistance to intravenous infection with the virulent strain. In the conditions of tissue culture described above, there was no difference between normal mouse serum and serum of immunized mice in effect on intracellular growth of virulent strain 116-54.

Intracellular growth of virulent strain 116-54 in mononuclear phagocytes obtained from immunized mice. Mononuclear phagocytes were obtained from the abdominal cavity of mice immunized with dead or live vaccine. Culture medium consisted of Hanks' solution containing antibiotics and normal mouse serum. The conditions of tissue culture were the same as in previous experiments. Phagocytes derived from mice immunized with dead vaccine showed a small degree of inhibition on the intracellular growth of bacteria but these, as well as the cells of normal mice (Fig. 5*d*), were destroyed after 3 days of incubation. The cells derived from mice receiving live vaccine did not allow the intracellular growth of virulent strain 116-54 during incubation (Fig. 5*e*, *f*). The results are shown in Fig. 3. More detailed aspects of intracellular multiplication of virulent strain 116-54 in mononuclear phagocytes are shown in Fig. 4. The cells derived from mice immunized with live vaccine inhibited the intracellular multiplication of bacteria regardless of the presence of antibody in the culture media. But in the case of the cells of normal mice or mice immunized with dead vaccine, bacteria increased intracellularly, and the cells were destroyed during the course of incubation.

DISCUSSION

Vaccination with dead organisms is largely ineffective in preventing the death of mice experimentally infected with *S. enteritidis* (Kobayashi and Ushiba, 1952; Mitsuhashi et al., 1958; Mitsuhashi, 1959). A primary sublethal infection with some viral and bacterial agents of disease, however, provokes a powerful resistance to further infection by the same pathogen, and this has been found to hold for infections with *S. enteritidis*. According to a recent detailed study of antilethal resistance of mice to infection with *S. enteritidis*, mice immunized with live vaccine resisted intravenous injection with 1,000 MLD of a virulent strain. The survivors of this challenge then completely resisted intravenous challenge with 10,000 MLD of the same organism (Mitsuhashi et al., 1960).

In the course of infection with facultative cytotrophic bacteria (e.g., *S. enteritidis* in mice), bacteria are ingested by the reticuloendothelial cells of the blood, and certain definable relationships between numbers of bacterial cells in the liver and blood are characteristic of each stage of infection (Mitsuhashi et al., 1960). The antilethal resistance of mice immunized with live vaccine is mainly attributable to clearance of bacteria from the blood shortly after challenge and to inhibition of intracellular growth of the bacteria.

The histogenesis of mononuclear phagocytes obtained from the abdominal cavity of mice is not clearly understood; but the virulence of *S. enteritidis* for this host is recognized at the cellular level in vitro (Saito, Ono, and Akiyama, 1957; Sato et al., 1958; Sato, Tanaka, and Mitsuhashi, 1960). Mononuclear phagocytes from mice which received live vaccine inhibited intracellular growth of the virulent strain 116-54, regardless of the presence of antibody, whereas cells from mice immunized with dead vaccine did not.

Up to 18 hr after inoculation of attenuated strain SER into mice, antibiotics are effective in sterilizing the infection. After 24 hr, however, a "carrier state" has developed in which the salmonellae, presumably because of intracellular habitat, are resistant to antibiotics (Mitsuhashi et al., 1959; Smadel, 1960).

The study of bacteria grown in vivo has recently contributed importantly to problems of pathogenesis and immunity in some infectious diseases. It may be expected that this method, if properly exploited, will help to explain the greater effectiveness of live vaccines. Investigations in this direction are in progress.

SUMMARY

Infection in vitro with *Salmonella enteritidis* of mononuclear phagocytes derived from the

abdominal cavity of mice showed that the intracellular multiplication of virulent strain 116-54 was rapid, and phagocytes were destroyed within 3 days of incubation. However, the intracellular growth of attenuated strain SER was inhibited after a slight increase in number of bacteria, and reached a "carrier state."

Serum obtained from normal mice or from mice immunized with live or dead vaccine had no inhibitory effect on intracellular growth of virulent strain 116-54. Cells obtained from mice immunized with live vaccine inhibited intracellular multiplication of virulent strain 116-54, regardless of the presence of antibody in the medium, whereas the cells of mice immunized with dead vaccine did not.

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