The Synergistic Contribution of Macrophages and Antibody to Protection against *Salmonella typhimurium* during the Early Phase of Infection

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The contribution of phagocytes and antibody to protection against *Salmonella typhimurium* during the early phase of infection in mice was analysed. Following intravenous injection, most of the bacteria were trapped in the liver and spleen within 10 to 60 min and killed within 6 h; surviving organisms began to multiply in these tissues after 24 h and reached a maximum at 5 to 7 d. The transient killing phase was abrogated by treatment with carrageenan, a macrophage blocker, but not by whole-body X-irradiation. These observations suggest that carrageenan-sensitive, but radio-resistant macrophages play an important role in the early phase of the infection. Actively immunized mice showed accelerated trapping and killing; the protection observed at the early stage of infection in immunized mice could be passively transferred to normal mice, whereas carrageenan-treated mice did not kill the bacteria even after receiving immune serum. It seems that the synergistic action of macrophages and antibody provides the main initial primary defence in immune animals.

INTRODUCTION

In the case of infection caused by facultative intracellular bacteria, effective protection seems to be exerted only by cell-mediated immunity. The degree of the contribution of antibody to protection appears to be negligible from observations of experimental listeriosis and tuberculosis (Miki & Mackaness, 1962; Lefford *et al.*, 1973). It also appears that radio-resistant, non-immune macrophages play a protective role at the early stage of listeria infection (Mitsuyama *et al.*, 1978; Newborg & North, 1980).

Salmonella species have been included in the category of facultative intracellular bacteria (Suter, 1956), but there are many reports showing that antibody contributes in high degree to the protection (Rowlay *et al.*, 1964; Hochadel & Keller, 1977; O'Brien *et al.*, 1979*a*). It is controversial whether or not antibody alone accounts for the greater part of protection in salmonella infection.

In the present paper, *Salmonella typhimurium* was used to analyse the protective mechanisms in mouse salmonellosis. The contribution of macrophages to protection was examined by studying the effects of X-irradiation and treatment with carrageenan. The role of antibody was also investigated in serum transfer experiments.

METHODS

Animals. Female mice of a colony-bred ddY strain (5 to 8 weeks old) were purchased from a local breeder (Kyudo, K. K., Kumamoto, Japan).

Micro-organism. *Salmonella typhimurium* strain LT2 was used in all experiments. The bacteria were maintained by serial passage in ddY mice. Fresh isolates were obtained from the spleen, subcultured once in Tryptic Soy Broth (Difco) and used for challenge. The LD₅₀ of this strain was about 6×10^4 viable bacteria by the

intravenous route and more than 10^7 by the intramuscular route. The viable count of the challenge dose was assessed by plating appropriately diluted bacterial suspensions on nutrient agar plates.

Determination of bacterial growth. Mice were inoculated intravenously with 10^4 or 10^6 viable bacteria suspended in phosphate-buffered saline (PBS). At various times after inoculation, blood samples were taken by cutting the femoral artery and livers and spleens were removed. The organs were homogenized in PBS and dilutions of blood or homogenized organs were plated for colony counts as described previously (Tatsukawa *et al.*, 1979), except that nutrient agar without glucose was used. The number of viable bacteria in the whole organ was expressed as $\log_{10}$ (bacterial count).

Active and passive immunization. Mice were immunized by the intravenous inoculation of 6.3×10^3 bacteria or by the intramuscular inoculation of 10^5 bacteria. They were used for experiments 3 to 4 weeks after immunization. Antiserum raised against *S. typhimurium* was obtained from mice immunized intramuscularly 3 weeks previously. Immune serum from 40 mice was pooled and sterilized by membrane filtration just before use. Heat inactivation of the serum was not carried out. Mice of the same age were employed as a source of normal serum. Immune serum was tested for whole-cell agglutinin titre using microtitre plates. Serum (25 μ l) was added to the first series of wells, containing an equal volume of PBS, and serial dilutions were made into further wells; 25 μ l volumes of a suspension in PBS of formalin-killed, methylene blue-stained bacteria (2×10^9 cells ml^{-1}) were then added. The microtitre plates were incubated at 37 °C for 1 h and then held overnight at 4 °C before scoring the agglutinin titre.

X-irradiation and carrageenan treatment. These were done as described previously (Tatsukawa *et al.*, 1979): the X-ray dose was 8 J kg^{-1} , 2 d before infection; the carrageenan dose was 200 mg kg^{-1} , 24 h before infection.

RESULTS

Time course of bacterial growth after intravenous inoculation in normal mice

The time course of bacterial growth in normal mice is shown in Fig. 1. Approximately 20% of the inoculum was recovered from both the liver and the spleen 30 min after inoculation. The number of bacteria in each organ had decreased roughly 10-fold by 6 h; it then increased logarithmically to reach a maximum at 5 to 7 d. Gradual elimination of bacteria occurred after 9 d. Some of the mice showed abscess formation (predominantly in the liver, occasionally in the spleen) later in the infection. These abscesses caused the unexpectedly greater bacterial recovery observed in the liver 11 and 13 d after inoculation.

Effect of X-irradiation and carrageenan treatment on the growth of bacteria

To analyse the very early phase of infection more precisely, a large challenge dose ($>10 \times \text{LD}_{50}$) was used. Doses of 10^6 viable bacteria were inoculated intravenously into X-irradiated mice, carrageenan-treated mice and controls. X-irradiation impairs polymorphonuclear cells (PMNs) and free monocytes but not fixed macrophages, and carrageenan treatment impairs fixed macrophages and free monocytes but not PMNs: the treatment conditions were chosen on the basis of our previous results (Tatsukawa *et al.*, 1979). There was no difference between the three groups of mice in the number of bacteria recovered in the liver after 30 min (Fig. 2*a*). In both irradiated mice and controls, the bacterial count decreased progressively from 30 min to 6 h in a similar pattern and multiplication did not begin until after 12 h. In carrageenan-treated mice, multiplication began without a transient decrease in bacterial numbers.

The course of infection was also followed for 7 d after the intravenous inoculation of 10^4 bacteria (Fig. 2*b*). In this case, bacterial growth was enhanced by X-irradiation and carrageenan treatment, the degree of enhancement being more pronounced in the X-irradiated mice. The growth rate of bacteria in the carrageenan-treated mice was similar to that in the controls, but the recovery of bacteria was always greater in the former group.

Enhanced elimination of bacteria in immunized mice

To examine the pattern of bacterial elimination in immunized mice, control and immunized animals (inoculated intravenously with 6.3×10^3 bacteria 4 weeks previously) were challenged intravenously with 10^4 bacteria (Fig. 3). Some of the immunized mice showed macroscopic

Fig. 1. Time course of bacterial growth in the liver (○) and spleen (●) of normal mice after intravenous challenge with 10^4 *S. typhimurium*. Each point and bar indicates the mean result for four animals $\pm$ S.E.M.

Fig. 2. Effect of X-irradiation and carrageenan treatment on bacterial counts in the liver at (a) the very early stage of infection after intravenous challenge with 10^6 *S. typhimurium*, and (b) the early stage of infection with 10^4 *S. typhimurium*: ○, control mice; ●, X-irradiated mice; □, carrageenan-treated mice. Each point and bar indicates the mean result for four animals $\pm$ S.E.M.

Fig. 3. Continuous decrease in the number of bacteria in the organs of immunized mice after intravenous challenge with 10^4 *S. typhimurium*: ○, liver of control mice; ●, spleen of control mice; □, liver of immunized mice; ■, spleen of immunized mice. Each point and bar indicates the mean result for four animals $\pm$ S.E.M. The shaded area indicates an undetectable level of bacterial count by colony assay.

Fig. 4

Fig. 5

Fig. 4. Elimination pattern of *S. typhimurium* in the very early stage of infection in control and immunized mice after intravenous challenge with 10^6 bacteria: ○, liver of control mice; ●, spleen of control mice; □, liver of immunized mice; ■, spleen of immunized mice. The histograms represent bacterial counts ml^{-1} in the blood at 10 min, 1 h and 6 h: □, control mice; ■, immunized mice. Each point or column and bar indicates the mean result for four animals $\pm$ S.E.M.

Fig. 5. Effect of immune serum transfer to normal mice and carrageenan-treated mice on the elimination of *S. typhimurium* from the liver and blood after intravenous challenge with 10^6 bacteria; the histograms represent bacterial counts ml^{-1} in the blood: ○, □, normal mice given normal serum; ●, ■, actively immunized mice; □, □, normal mice given immune serum; ■, ■, carrageenan-treated mice given immune serum. Each point or column and bar indicates the mean result for four animals $\pm$ S.E.M.

abscess formation, mainly in the liver. Such abscess formation was never observed in the organs until at least 7 d after infection. On each day of determination of the bacterial count in organs, animals that exhibited abscess formation were excluded from the experiment. In the immunized animals, bacteria in both organs became undetectable within 3 d and no bacterial growth was observed thereafter.

Elimination pattern of bacteria in the very early phase of infection in immunized mice

The course of infection in the very early phase, following challenge with 10^6 bacteria, was investigated in controls and in animals immunized 3 weeks previously by intramuscular inoculation with 10^5 bacteria (Fig. 4). In the immunized animals, bacteria were eliminated from the liver, spleen and muscle within 3 weeks (results not shown). The immunized mice had trapped 85% of the inoculum in the liver and 6% in the spleen by 10 min after challenge. The clearance of bacteria from the blood was also accelerated in the immunized animals. The trapping of bacteria in the livers of control mice was smaller than that in immunized mice and the delayed trapping resulted in the prolongation of bacteraemia. Accelerated killing of bacteria was observed in the immunized mice, especially in the liver.

Contribution of antibody in immunized mice to the rapid elimination of bacteria

To analyse the contribution of antibody to the rapid elimination of bacteria in immunized mice, the effect of immune serum transfer was investigated (Fig. 5). Transfer of immune serum was done by intraperitoneal injection of pooled immune serum (0.5 ml volumes) into each normal mouse. At 24 h after serum transfer, 10^6 bacteria were inoculated intravenously into each group of mice. Accelerated trapping of bacteria like that observed in immunized mice was found in normal mice after immune serum transfer, and a rapid and progressive

elimination of bacteria was also observed, in a pattern comparable to that in immunized mice. The transfer of immune serum did not, however, confer any protective effect to mice pre-treated with carrageenan. The bacterial agglutinin titres of immune serum and of normal serum were 16 and less than 4, respectively.

DISCUSSION

Protection against bacterial infections depends on many factors. Phagocytes seem to be the most important because of their rapid accumulation into the infected focus. The phagocytes consist of PMNs and macrophages, and these two different series of cells provide differing degrees of protection depending on the infecting bacterial species. In a previous paper, we showed that the protection against *Pseudomonas aeruginosa* was exerted mainly by PMNs, whereas that against *Listeria monocytogenes* was by cells of macrophage series (Tatsukawa *et al.*, 1979).

In this study, we first examined the relative contribution of PMNs and macrophages to protection against *Salmonella typhimurium*. The course of infection was assessed by bacterial growth in the liver and spleen. Most of the injected bacteria were trapped in both organs and subsequently killed. This initial trapping and transient killing of bacteria is similar to that observed in experimental listeriosis in mice as reported previously (Mitsuyama *et al.*, 1978). The killing process was abrogated by treatment with carrageenan but not by X-irradiation. The cells contributing to the protection in this very early phase are probably fixed macrophages, since the differentiated macrophage population is resistant to X-irradiation (Kornfeld & Greenman, 1966; Gillette & Lance, 1973) but not to carrageenan (Catanzaro *et al.*, 1971; Tatsukawa *et al.*, 1979). Carrageenan is a macrophage-toxic agent whose mode of action is very similar to that of silica (Allison *et al.*, 1966), and silica is reported to decrease the resistance of mice to *S. typhimurium* (O'Brien *et al.*, 1979*b*).

The killing of trapped bacteria continued for 6 to 10 h, but complete elimination was not achieved. The bacteria proliferated in the liver after 12 h in normal mice; X-irradiation markedly enhanced this proliferation after 12 h. The recovery of bacteria was always greater in carrageenan-treated mice than in controls. This result showed the inhibitory effect of macrophages on bacterial growth, and suggested that the accumulation of macrophages might play an inhibitory role in this phase.

In actively immunized mice, bacteria were eliminated almost completely within 1 or 2 d after administration of a sublethal challenge dose. The rate of trapping and killing of bacteria was very high especially in the liver. The trapping of bacteria in the spleens of immunized mice was lower than in controls. This may be due to the accelerated trapping in the liver. Such a prominent resistance in the very early phase of infection was not observed in mice treated with zymosan or BCG, both activators of macrophages (results not shown). The pattern of trapping and killing found in actively immunized mice could be completely reproduced in normal mice after the transfer of serum obtained from immunized mice. This effect of passive transfer was observed only in normal mice, carrageenan-treated mice failing to exhibit the enhanced trapping and killing of bacteria even after passive immunization. These results suggest that intact fixed macrophages are necessary for the expression of resistance in passively immunized mice. The bactericidal activity of serum was assessed by observing bacterial growth *in vitro* in the presence of immune and control serum. The growth of bacteria in the presence of immune serum showed no significant difference from that in the presence of control serum (results not shown). From the results reported here, it can be argued that the synergistic action of fixed macrophages and antibody is responsible for the rapid trapping and accelerated killing of bacteria in immunized mice.

We must also take into consideration how cell-mediated immunity contributes to protection in salmonellosis. Mice infected with listeriae have been shown to exhibit cross-resistance to antigenically unrelated *S. typhimurium*. The macrophages from salmonella-infected mice have

also been shown to have enhanced bactericidal activity *in vitro*, and this acquired resistance was attributed to immunologically activated macrophages (Blanden *et al.*, 1966). However, the expression of resistance in adoptively immunized mice did not require the presence of activated macrophages in the very early phase of infection. To us, it seems that activated macrophages are necessary for the elimination of salmonellae during the course of a primary infection, but that the synergistic action of fixed macrophages and antibody provides the initial primary defence in immune animals.

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