Three Phases of Phagocyte Contribution to Resistance against *Listeria monocytogenes*

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The contribution of phagocytes to protection against *Listeria monocytogenes* was analysed in outbred ddN mice. Most of the bacteria injected intravenously at a dose of 3×10^3 to 4×10^3 were trapped in the liver within 10 min. There was a transient 10-fold decrease in the number of bacteria by 6 h. Anti-listeria activity in the initial phase was resistant to X-irradiation but was inhibited by carrageenan, and was not influenced by immunization. The protection in this very early stage of infection seemed to be attributable to the function of fixed macrophages. Viable bacteria in the organs increased progressively but slowly from 6 h to 72 h to reach maximum numbers. Bacterial growth during this period was markedly enhanced by X-irradiation or treatment with carrageenan. Accumulation of free phagocytes seemed to suppress the bacterial growth in this phase. The number of bacteria began to decrease from day 4 and became undetectable by day 9. The suppressive effect on bacterial growth in this last phase may be dependent on immunologically activated macrophages and was reversed by X-irradiation and carrageenan. The course of local infection was similar to that of systemic infection except for the lack of initial decrease. We conclude that the course of infection with *L. monocytogenes* can be divided into three phases with regard to the roles of phagocytes in resistance.

INTRODUCTION

It is well known that after intravenous inoculation of *Listeria monocytogenes* into normal mice, the number of live bacteria per organ increases during the first 3 d of infection but begins to decrease from day 4 (Mackaness, 1962). Such protection has been presumed to depend primarily upon macrophages activated by sensitized lymphocytes. Therefore, a fulminating course of infection would be expected to occur in athymic nude mice. However, nude mice showed a persistent form of infection (Emmerling, Finger & Bockemühl, 1975; Takeya *et al.*, 1977). During the first 3 d of infection, the number of bacteria in the liver or spleen was smaller in nude mice than in normal littermates. Takeya *et al.* (1977) showed that the number of bacteria after 72 h of infection was strikingly increased by whole body irradiation with a lethal dose (900 R), or by administration of blocking agents of the reticuloendothelial system. Free phagocytes appear to suppress a fulminating bacterial growth, especially at an early stage of infection. Activated macrophages appear to be required for complete elimination of bacteria at a later stage of infection.

The present study was undertaken to analyse further the contribution of phagocytes to resistance at different stages of listeria infection. The course of infection appears to be separated into three phases with regard to the contribution of phagocytes to the resistance.

METHODS

Animals. Female mice of an outbred ddN strain were obtained from the Breeding Unit of Experimental Animals, Kyushu University. Mice were used for experiments at 8 to 12 weeks of age.

Micro-organism. *Listeria monocytogenes* (EGD) was kindly donated by Dr Ishibashi, Research Institute for Diseases of the Chest, School of Medicine, Kyushu University. The bacteria were maintained by serial passage in outbred CF₁ mice and passed through ddN mice for a few generations before use in this study. Fresh isolates were obtained from spleens, grown once on Tryptic Soy Agar medium (Difco) and used for infection. The LD₅₀ by the intravenous route of infection was approximately 8×10^4 viable bacteria.

Determination of bacterial growth. Mice were inoculated intravenously with 3×10^3 to 4×10^3 or 1×10^6 viable bacteria in Hanks' balanced salt solution. At various times after inoculation, they were bled by cutting the femoral artery, and their livers and spleens were removed. Individual organs were homogenized separately in 10 ml phosphate-buffered saline (PBS) with Teflon homogenizers. The homogenized suspensions were diluted serially 10-fold with PBS and 0.1 ml of each dilution was spread on nutrient agar containing 0.3 % (w/v) glucose. Three plates were prepared for each dilution of each specimen. Colonies were counted after incubation for 20 h at 37 °C. To observe bacterial growth in a local infection, bacterial suspensions were inoculated intramuscularly into the middle of the right thigh. The whole mass of the muscle of an infected thigh was removed and treated in the same way as described for the estimation of bacterial growth in the liver and spleen.

Immunization. Mice were inoculated intravenously with 3×10^3 to 4×10^3 viable bacteria and used as immunized hosts 14 d later. Preliminary experiments had shown that the degree of acquired immunity was higher at 14 d than at 7 or 21 d after infection.

X-irradiation. Mice were exposed to 900 or 1000 R of whole body X-irradiation and infected 3 d later. The radiation was delivered from a Shimadzu 250 kV machine operating at 200 kV with 0.3 mm Cu and 1 mm Al filtration at 100 cm from the target focus. In order to protect the liver from damage by irradiation, the lower chest and upper abdomen were covered with two layers of a lead plate 0.9 mm thick and 12 mm wide. Approximately 95 % of the X-ray dose was estimated to be blocked by this shielding method.

Carrageenan. Carrageenan type II (Sigma) was dissolved in distilled water (to give 5 mg ml⁻¹) and injected intraperitoneally at a dose of 200 mg kg⁻¹ 24 h before infection.

RESULTS

Time course of bacterial growth after intravenous inoculation in normal mice

The time course of *L. monocytogenes* infection in normal mice is shown in Fig. 1. Approximately 90 % of the inoculum was recovered from the liver 10 min after inoculation. The number of bacteria decreased 10-fold by 6 h and increased again to reach a maximum at 2 to 3 d. The number decreased gradually from day 4 to become undetectable by day 9. In the spleen, the number of live bacteria taken up in 10 min was smaller than in the liver. The number increased rapidly without the early transient reduction observed in the liver. The decrease began from day 4 in the spleen as well as in the liver. Preliminary experiments showed that the blood (approximately 1 ml) contained less than 1 % of the inoculum after 10 min, so the bacterial counts in the organ were regarded as trapped bacteria.

The course of infection in the liver was divided tentatively into three phases: the first phase up to 6 h, the second phase up to day 3 and the third phase from day 4 to day 9.

Effect of X-irradiation on the course of infection

To analyse more precisely the first phase in the liver, a large dose (over 10 LD₅₀) of inoculum was used. Viable *L. monocytogenes* (10⁶) were injected intravenously into 1000 R-irradiated mice and non-irradiated controls. The trapping of bacteria by 10 min and the progressive decrease from 10 min to 6 h showed similar patterns in both groups (Fig. 2a).

The course of infection was plotted daily after inoculation of 2×10^3 bacteria into irradiated mice. In the liver, irradiation enhanced bacterial growth slightly on day 1, and markedly on days 2 and 3. Shielding of the liver with lead plates did not prevent progressive bacterial growth by day 3 (Fig. 2b). In the spleen, bacterial growth was enhanced appreciably by irradiation.

Fig. 1. Time course of bacterial growth in the liver (○) and spleen (●) after intravenous inoculation of 3.2×10^3 *L. monocytogenes* into normal mice. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

Fig. 2. Effect of 1000 R-irradiation on liver counts at (a) a very early stage of infection after intravenous inoculation of 10^6 *L. monocytogenes* and (b) an early stage of infection after inoculation of 2×10^3 bacteria: ○, control mice; ●, irradiated mice; □, mice irradiated with the liver shielded. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

Fig. 3. Effect of carrageenan on liver counts at (a) a very early stage of infection after intravenous inoculation of 10^6 *L. monocytogenes* and (b) an early stage of infection after inoculation of 4.1×10^3 bacteria: ○, control mice; ●, mice treated with carrageenan. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

Fig. 4. Enhancement by treatment with carrageenan of bacterial growth in (a) the liver and (b) the spleen after intravenous inoculation of 3.5×10^3 *L. monocytogenes*: ○, control mice; ●, mice treated with carrageenan 60 h after challenge. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

Fig. 5. Continuous decrease in the number of bacteria in the liver of immunized mice after intravenous re-challenge with (a) 10^6 or (b) 3×10^3 *L. monocytogenes*: ○, control mice; ●, immunized mice. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

Effect of carrageenan on the course of infection at various stages of infection

Mice were inoculated with 10^6 *L. monocytogenes* 24 h after treatment with carrageenan. The number of live bacteria trapped in the liver by 10 min was reduced 10-fold compared with the number in non-treated controls. Moreover, the number of bacteria increased gradually without the reduction observed in controls (Fig. 3a). Figure 3(b) shows a growth curve of *L. monocytogenes* in carrageenan-treated mice after challenge with 4.1×10^3 bacteria. Administration of carrageenan enhanced bacterial growth slightly on days 1 and 2, and markedly on day 3.

When carrageenan was injected intraperitoneally into normal mice 60 h after challenge with 3.5×10^3 bacteria, the number of live bacteria in the liver began to increase (Fig. 4a). In the spleen, bacterial counts increased slightly from day 3 to 6 (Fig. 4b).

Effect of host treatments on bacterial growth in immunized mice

To examine the pattern of bacterial elimination in immunized mice, 10^6 *L. monocytogenes* were inoculated into immunized mice and non-immunized controls. The same numbers of bacteria were trapped in the liver of both groups by 10 min and the numbers decreased from 10 min to 6 h in both groups in a similar fashion. Thereafter, the number of bacteria in

Fig. 6. Effects of (a) 900 R-irradiation and (b) carrageenan on bacterial growth in the livers of immunized mice. In (a), symbols indicate: $\circ$, non-irradiated non-immunized controls; $\bullet$, irradiated non-immunized mice; $\square$, non-irradiated immunized mice; $\blacksquare$, irradiated immunized mice. In (b): $\circ$, non-immunized controls; $\bullet$, immunized mice; $\square$, immunized mice treated with carrageenan. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

Fig. 7

Fig. 8

Fig. 7. Time course of bacterial growth in infected thigh muscles after intramuscular inoculation of 1.1×10^4 *L. monocytogenes*. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

Fig. 8. Effects of 900 R-irradiation, carrageenan and immunization on bacterial growth in infected thighs after intramuscular inoculation of 10^6 *L. monocytogenes*: $\circ$, control mice; $\bullet$, immunized mice; $\square$, irradiated mice; $\blacksquare$, mice treated with carrageenan. Each point and bar indicates the mean result for five animals $\pm$ S.E.M.

controls increased progressively until 24 h, while the number in immunized mice continued to decrease (Fig. 5a).

When 3×10^3 viable bacteria were inoculated into immunized mice, bacteria in the liver decreased to reach an undetectable level within 3 d without proliferation (Fig. 5b).

The effects of X-irradiation and carrageenan treatment on bacterial growth in the liver of immunized mice were investigated. Immunized mice were exposed to 900 R-irradiation 3 d before challenge with 10^6 *L. monocytogenes*. Bacterial growth in immunized mice was modified by whole body irradiation to a pattern similar to that in irradiated, non-immunized mice (Fig. 6a). Injection of carrageenan into immunized mice 24 h before the challenge reduced the trapping of bacteria by 10 min and abolished the protective mechanism for continuous elimination in immunized mice (Fig. 6b).

Bacterial growth after intramuscular (local) infection

The growth pattern of *L. monocytogenes* in the thigh muscle was examined after inoculation of 1.1×10^4 bacteria. The number increased progressively by 24 h without any transient reduction at a very early stage of infection as observed in the liver (Fig. 7). A constant level of approximately 10^6 bacteria was sustained from day 1 to day 5 and the number decreased thereafter.

The effects of several treatments on the local growth of bacteria at an early stage of infection were studied. A suspension containing 10^6 *L. monocytogenes* was inoculated into normal mice, irradiated mice, mice treated with carrageenan and immunized mice. In immunized mice, the number of live bacteria decreased from 6 h to 48 h with a transient increase by 6 h. Bacterial growth in normal mice was enhanced substantially by X-irradiation. The effect of treatment with carrageenan was not so prominent on local bacterial growth (Fig. 8).

DISCUSSION

The growth pattern of *L. monocytogenes* in the liver or spleen has been widely used for the assessment of protective immunity against this facultative intracellular parasite. Tripathy & Mackaness (1969) observed an initial fall in the bacterial count in the liver but not in the spleen, but gave no detailed analysis. From the results of the present studies, the trapping of bacteria and a decrease in number at a very early stage of infection appear to be attributable to radio-resistant phagocytes, whose functions were depleted by carrageenan. The cells responsible for protection in this first phase may be the fixed macrophages of the liver, Kupffer cells, since differentiated macrophage populations are resistant to irradiation (Kornfeld & Greenman, 1966; Nelson, 1969; Gillette & Lance, 1973) but sensitive to carrageenan (Catanzaro, Schwartz & Graham, 1971). Furthermore, the efficiency of such protection in the liver was not augmented by immunization. The early protection was undetectable in the spleen after systemic infection or in the muscle after local infection. The lack of the early protection in local infection is presumably a consequence of the small number of fixed macrophages in the muscle, and its absence in the spleen may be due to some differences in the number or activity of fixed macrophages from those in the liver.

In mice exposed to irradiation or treated with carrageenan, the number of bacteria increased nearly exponentially from 6 h. The suppression of such a fulminating bacterial growth by 72 h which occurs in normal mice may be ascribed to normal phagocytes, since the suppression is similar in athymic nude mice and normal littermates (Takeya *et al.*, 1977). The cells responsible for such protection may be some population of circulating phagocytes but not the fixed macrophages in the liver, since shielding of the liver in whole body irradiation did not prevent fulminating bacterial growth by 72 h. Such an explanation may be supported by the results of North (1970) who showed that local irradiation of the liver, with the rest of body shielded, did not enhance bacterial growth by 72 h. It is reported that neutrophils begin to accumulate in the lesion a few hours after infection and macrophages become detectable at 48 h and comprise the majority of infiltrating cells at 96 h (North, 1970). A fulminating bacterial growth from 48 to 72 h was evoked by treatment with carrageenan which was reported to be cytotoxic to macrophages but not to lymphocytes (Allison, Harington & Birbeck, 1966; Catanzaro *et al.*, 1971). Carrageenan appears to affect macrophages preferentially, since our experiments (unpublished data) showed that carrageenan decreased the number of circulating monocytes but increased polymorphs. Blood monocytes or their precursors are radio-sensitive unlike fixed macrophages (Benacerraf *et al.*, 1959; Volkman & Collins, 1971). Therefore, prevention of a fulminating growth at the second phase, i.e. by 72 h, appears to depend upon the accumulation of immature macrophages, although the contribution of polymorphs cannot be ruled out completely.

Complete elimination of *L. monocytogenes* from organs depends upon the accumulation

of activated macrophages mediated by cellular immunity (Mackaness, 1962, 1969). Accumulation of a large number of macrophages in the lesions has been ascribed to local proliferation of fixed macrophages (North, 1969) or assembling of monocyte-derived macrophages in inflammatory sites (McGregor & Koster, 1971).

In immunized mice, no bacterial multiplication occurred in the organs. The first phase of killing, attributable to fixed macrophages, appears to be followed by the third phase, attributable to immunologically activated macrophages. The establishment of immunity did not influence the uptake and killing of bacteria in the liver after re-challenge.

In the course of local infection, protection appears to initiate from the second phase depending upon the accumulation of free circulating phagocytes.

Thus, we divided the course of infection with *L. monocytogenes* tentatively into the first phase depending on fixed macrophages, the second phase depending on the accumulation of free macrophages and the third phase depending on the accumulation of immunologically activated macrophages.

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