Differing Contribution of Polymorphonuclear Cells and Macrophages to Protection of Mice against *Listeria monocytogenes* and *Pseudomonas aeruginosa*

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Bacterial growth and lethality of *Listeria monocytogenes* in mice were augmented by carrageenan-treatment and X-irradiation (8 J kg⁻¹), whereas growth and lethality of *Pseudomonas aeruginosa* were augmented by X-irradiation but not by carrageenan-treatment. Protection against *L. monocytogenes*, at least in the early phases, appears to depend mainly on macrophages, since carrageenan depletes macrophages but not polymorphonuclear cells (PMN), whereas protection against *P. aeruginosa* appears to depend mainly on PMN. Ineffectiveness of PMN in elimination of *L. monocytogenes* is supported by histological examination and observation of intracellular killing *in vitro*.

INTRODUCTION

Phagocytes, including polymorphonuclear cells (PMN) and monocytes or macrophages, are the final effector cells in the elimination from animals of a variety of bacteria and fungi. Many kinds of micro-organism are therefore able to produce severe infections in hosts whose phagocyte functions are depressed artificially by X-irradiation (Kaplan *et al.*, 1952; Gordee & Simpson, 1967), cyclophosphamide (Sharbaugh & Grogan, 1969; Tripathy & Mackaness, 1969; Buhles & Shifrine, 1977), vinblastine (Tripathy & Mackaness, 1969) or vincristine (Saslaw *et al.*, 1972). Such agents depress PMN, macrophages and lymphocytes, so that one cannot reach any conclusions as to which cells protect against individual micro-organisms. Even in systems in which immunological mechanisms can be excluded, the relative contributions of PMN and macrophages to protection remain to be analysed.

In the present study, *Listeria monocytogenes* and *Pseudomonas aeruginosa* were chosen as a facultative intracellular parasite and an extracellular parasite, respectively. Limitation of bacterial growth in early phases of infection with *L. monocytogenes* has been proved in our laboratory to be mainly the function of non-immune phagocytes (Mitsuyama *et al.*, 1978). Cellular immunity appears not to be important in protection against *P. aeruginosa* (Reynolds, 1974). To analyse the differing contributions of PMN and macrophages to protection, X-irradiation was used to deplete the total population of phagocytes and carrageenan was used to deplete selectively cells of the macrophage series (Catanzaro *et al.*, 1971).

METHODS

Animals. Female and male mice (8 to 12 weeks old) of an outbred ddN strain were obtained from the Breeding Unit of Experimental Animals, Kyushu University.

Micro-organisms. *Listeria monocytogenes* strain EGD was donated by Dr. Ishibashi, Research Institute for Diseases of the Chest, School of Medicine, Kyushu University. *Pseudomonas aeruginosa* was isolated

from the sputum of a patient suffering from acute pneumonia. The bacteria were maintained by serial passage in ddN mice before use in this study. Fresh isolates were obtained from spleens, subcultured once on Tryptic Soy Agar (Difco) and used for infection. The LD₅₀ by the intravenous route of *L. monocytogenes* was approximately 8×10^4 viable bacteria and that of *P. aeruginosa* approximately 1×10^7 .

Count of peripheral leukocytes. Blood specimens were collected by puncture of the retro-orbital venous plexus. Total numbers of leukocytes were counted after staining with Türk's solution and differential counts were carried out after staining of smears with Giemsa's solution.

Carbon clearance test. Colloidal carbon c11/1431a (Günther-Wagner, Pelikan-Werke, Hanover, FRG; 16 mg kg⁻¹) was injected intravenously and blood specimens were obtained at 3 min intervals for 15 min. The phagocytic index, K₁₆, was calculated from the pattern of disappearance of carbon from the blood by the Biozzi method (Stuart *et al.*, 1973).

Determination of bacterial growth. Mice were inoculated intravenously with 4×10^8 viable *L. monocytogenes* or 1×10^8 viable *P. aeruginosa* suspended in Hanks' balanced salt solution (HESS; Paul, 1973). 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; Paul, 1973) with Teflon homogenizers. The homogenized suspensions were serially diluted 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 at 37 °C for 20 h. 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.

X-irradiation. Mice were exposed to 8 J kg⁻¹ (800 rad) of whole body X-irradiation and infected 2 d later. The radiation was delivered from a Shimadzu 250 kV machine (Shimadzu, K.K., Tokyo, Japan) operating at 200 kV with 0.3 mm Cu and 1 mm Al filtration, 100 cm from the target focus.

Carrageenan. Carrageenan type II (Sigma) was dissolved in distilled water and injected intraperitoneally (200 mg kg⁻¹) 24 h before infection.

Preparation of PMN and macrophages. PMN were washed out of the peritoneal cavity with HBSS 3 h after injection of 2 ml 0.2% (w/v) sodium caseinate. The suspension contained about 80% PMN. Macrophages were harvested similarly 4 d after injection of 2 ml 2.4% (w/v) thioglycollate medium (Difco). Approximately 65% of the cells were macrophages. In the experiment assessing the function of carrageenan-treated PMN, peritoneal exudate cells were washed out from carrageenan-treated mice 16 h after injection of thioglycollate medium. To eliminate macrophages that had ingested carrageenan the cells were layered gently on Ficoll-Conray solution (Böyum, 1968) and the cell pellet was harvested by centrifugation at 400 g for 30 min.

Phagocytosis and intracellular killing in vitro. Cell suspensions were washed three times with HBSS and resuspended at 2×10^7 cells ml⁻¹ in HBSS containing 0.1% (w/v) gelatin and 10% (v/v) fresh autologous serum. The viability of suspended cells was over 95%. Cell suspensions (2.5 ml) were mixed with equal volumes of bacterial suspensions containing 2×10^7 bacteria ml⁻¹ and incubated at 37 °C for 15 min to allow phagocytosis to occur. The mixtures were then centrifuged repeatedly to remove free bacteria. Cells were disrupted with sodium dodecyl sulphate (SDS; 0.1% final concn) for 1 min at room temperature and bacteria in the suspensions were counted. In order to observe the rate of intracellular killing, washed cell suspensions were incubated further after phagocytosis as described above. The numbers of bacteria remaining viable within cells were determined by culture on nutrient agar after disrupting the cells with SDS at appropriate times.

Histology. *Listeria monocytogenes* (2×10^6) or *P. aeruginosa* (1×10^6) were inoculated intramuscularly into the right thigh and the infected muscle was removed 6, 24 or 72 h later. Sections were stained with Giemsa's solution and infiltrating cells were observed.

RESULTS

Effects of carrageenan

Numbers of PMN and macrophages (monocytes) in the peripheral blood were counted at intervals after mice had been treated with carrageenan (Fig. 1). The number of PMN increased approximately fourfold by 1 or 2 d after treatment compared with untreated controls and then decreased slowly to reach the control level by day 15. The number of macrophages decreased eightfold by day 2 and then increased slowly to reach the control level by day 15.

To observe the effect of carrageenan on the function of fixed macrophages, the rate of

Fig. 1. Effect of carrageenan on the number of PMN (○) and macrophages (●) in the peripheral blood. Carrageenan was injected intraperitoneally (200 mg kg⁻¹) on day 0. Each point and bar indicates the mean number for four mice $\pm$ s.d.

carbon clearance was estimated after treatment. The phagocytic index (K_{18}) was $0.035 \pm$ s.d. 0.006 before treatment but decreased to 0.013 ± 0.003 at 1 d and increased slowly thereafter. The inhibition of carbon clearance lasted for 7 d. From these results, it seemed likely that carrageenan decreased or impaired cells of the macrophage series.

In order to evaluate the function of PMN in carrageenan-treated mice, the capacity to kill *P. aeruginosa* was estimated *in vitro*. Killing by untreated PMN was 1.31 ± 0.12 expressed as $\log_{10}$ (proportional decrease in number of viable bacteria) in 30 min, while that by carrageenan-treated PMN was 1.30 ± 0.38 . Thus, the capacity of PMN in treated mice was comparable to that in normal controls. These results suggested that carrageenan mainly affected macrophages and not PMN.

Mortality rate after bacterial infection in carrageenan-treated mice and X-irradiated mice

Mice showed large decreases in the numbers of PMN and macrophages at 2 d after exposure to whole body irradiation (results not shown). Lethal effects after intravenous inoculation of 8×10^3 *L. monocytogenes* or 2×10^4 *P. aeruginosa* were compared among carrageenan-treated, X-irradiated and untreated control groups. Such doses were not lethal to untreated mice (Fig. 2). After inoculation with *L. monocytogenes*, all carrageenan-treated and X-irradiated mice died within 8 d. All X-irradiated mice died within 4 d after inoculation with *P. aeruginosa*, while 67% of carrageenan-treated mice survived beyond 2 weeks.

Bacterial growth in organs after intravenous inoculation

Numbers of viable bacteria in the liver and spleen were determined 1, 2 and 3 d after intravenous inoculation with 4×10^3 *L. monocytogenes*. Recoveries of bacteria from the livers on day 1 ranged from 2×10^4 to 9×10^4 showing no significant differences among controls, carrageenan-treated mice and X-irradiated mice. At day 3, the number was approximately 10^5 in untreated controls, but reached 5×10^7 or more in the carrageenan-treated and X-irradiated groups. Similar patterns of bacterial growth were observed in the spleen.

Numbers of bacteria in the liver, spleen and blood were determined 4, 50 and 80 h after intravenous inoculation of 1×10^4 *P. aeruginosa*. Control mice and carrageenan-treated mice cleared their blood of bacteria by 4 h and their livers and spleens showed no bacterial growth at 50 or 80 h after inoculation. Large numbers of bacteria (over 10^5) were recovered from some X-irradiated mice.

Fig. 2. Mortality after intravenous challenge of (a) 8×10^8 *L. monocytogenes* and (b) 2×10^4 *P. aeruginosa*. ○, Control mice; ●, carrageenan-treated mice; □, X-irradiated mice.

Fig. 3. Bacterial growth in the muscle after intramuscular inoculation of (a) 1.0×10^6 *L. monocytogenes* and (b) 8.5×10^6 *P. aeruginosa*. ○, Control mice; ●, carrageenan-treated mice; □, X-irradiated mice.

Bacterial growth after intramuscular inoculation

Local proliferation of bacteria was observed after intramuscular inoculation into the thighs of carrageenan-treated mice, X-irradiated mice and untreated controls. When 1×10^6 *L. monocytogenes* were inoculated into controls, the number of bacteria increased slightly by 24 h and then decreased to about 10^6 by 72 h (Fig. 3). The numbers of bacteria increased progressively from 6 h to 72 h in carrageenan-treated mice and X-irradiated mice, although the degree of increase was higher in the latter than in the former.

When 8.5×10^6 *P. aeruginosa* were inoculated into untreated controls, the number decreased progressively from 3 h to 48 h to reach around 10^4 . In carrageenan-treated mice, the number decreased similarly. On the other hand, the number increased progressively and extensively from 3 h to 24 h in X-irradiated mice; none of these mice survived 48 h after infection.

Fig. 4. Phagocytosis and intracellular killing of (a) *L. monocytogenes* and (b) *P. aeruginosa* by macrophages and PMN *in vitro*. The number of viable bacteria at 0 min represents phagocytized bacteria after 15 min preincubation (see Methods). ○, Macrophages incubated at 37 °C; ●, macrophages at 0 °C; □ PMN at 37 °C; ■, PMN at 0 °C.

Histological examination of cellular infiltration into infected sites

Considerable infiltration of PMN was detected 6 h after inoculation with *L. monocytogenes* or *P. aeruginosa* and PMN comprised approximately 80% of infiltrating cells at 24 h. The proportion of macrophages or lymphocytes increased slightly thereafter. At 72 h after inoculation with *P. aeruginosa*, the absolute number of infiltrating cells decreased compared with earlier phases and macrophages and lymphocytes increased relatively. At 72 h after inoculation with *L. monocytogenes*, on the other hand, the absolute number of infiltrating cells increased compared with earlier phases. Considerable numbers of macrophages were detected in such lesions.

Phagocytosis and intracellular killing of bacteria in vitro

Listeria monocytogenes was phagocytized efficiently by macrophages, but the degree of phagocytosis by PMN was about 10-fold lower than by macrophages. Intracellular killing was scarcely detected in macrophages or in PMN (Fig. 4). *Pseudomonas aeruginosa* was phagocytized and killed efficiently by PMN and macrophages, although the efficiency of intracellular killing was slightly higher in PMN than in macrophages.

DISCUSSION

The number of PMN increased considerably after carrageenan injection and their capacity to kill *P. aeruginosa* was retained normally. The number of macrophages and rate of carbon clearance decreased in such carrageenan-treated mice and so these animals may be tentatively regarded as macrophage-depleted. The numbers of PMN and macrophages decreased strikingly in X-irradiated mice, which may therefore be regarded as PMN- and macrophage-depleted animals.

Mortality after inoculation of a sublethal dose of either *L. monocytogenes* or *P. aeruginosa* was markedly augmented by X-irradiation. Carrageenan-treatment increased the lethal effect of *L. monocytogenes* but only slightly augmented that of *P. aeruginosa*. Bacterial growth after systemic or local infection with *L. monocytogenes* was enhanced in both carrageenan-treated mice and X-irradiated mice. Bacterial growth of *P. aeruginosa* was enhanced in X-irradiated mice, but not in carrageenan-treated mice. These results suggest that

in vivo protection against *P. aeruginosa* depends mainly on PMN whereas that against *L. monocytogenes* depends mainly on macrophages.

Polymorphonuclear cell infiltration became detectable within a few hours after inoculation with *L. monocytogenes* and *P. aeruginosa*. The latter started to decrease in number at this stage, but *L. monocytogenes* continued to increase until 24 h in the presence of PMN accumulation. This supports the tentative conclusion that PMN are effective in elimination of *P. aeruginosa* but not of *L. monocytogenes*. One reason for ineffectiveness of PMN in eliminating *L. monocytogenes* may be the low degree of phagocytosis observed *in vitro*.

Macrophages may also contribute to elimination of *P. aeruginosa*, but PMN can protect efficiently against *P. aeruginosa* by their rapid accumulation at infected sites even in the absence of macrophages. It is possible that macrophages eliminate *P. aeruginosa* in the absence of normally functioning PMN. This is supported by the fact that prior stimulation with complete Freund's adjuvant or BCG increased the lethal dose of *P. aeruginosa* for mice treated with cyclophosphamide (Buhles & Shifrine, 1977). Complete Freund's adjuvant or BCG has been reported to stimulate macrophage function (Blanden, 1968; Gentry & Remington, 1971).

The rates of phagocytosis and intracellular killing of *L. monocytogenes* by macrophages *in vitro* were shown to be slower than those by PMN in experiments with human cells (Steigbigel *et al.*, 1974; Peterson *et al.*, 1977). The difference in effectiveness of PMN in resistance against *L. monocytogenes* as observed in their and in our experiments may be due to different functions of cells in different host species.

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