Importance of Antiserum and Phagocytic Cells in the Protection of Mice Against Infection by *Klebsiella pneumoniae*

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Rabbit antiserum to *Klebsiella pneumoniae* showed a powerful protective effect against intramuscular infection in normal mice. No protective effect was observed in mice whose monocytes and polymorphonuclear cells were depleted by X-irradiation. The antiserum had approximately the same protective effect in mice whose macrophages were blocked selectively by carrageenan as in normal mice. It is concluded that antiserum exerted its effect by opsonic function and that opsonized *K. pneumoniae* were eliminated mainly by polymorphonuclear cells rather than macrophages, at least in an early phase of the infection. These findings were supported by histological examination and observation of intracellular killing *in vitro*.

INTRODUCTION

Klebsiella species are opportunist pathogens (Dreizen *et al.*, 1974) causing, for example, infections of the respiratory and urinary tracts (Graybill *et al.*, 1973; Lorian & Topf, 1972). Specific antiserum plays an important role in protection against some encapsulated bacteria such as *Klebsiella pneumoniae* (Julianelle, 1926), but the involvement of cells in eliminating these bacteria remains undetermined.

Phagocytic cells contribute in different degrees and at different stages of infection to protection against many micro-organisms. For example, the final effector cells against *Listeria monocytogenes* are macrophages (Mitsuyama *et al.*, 1978; Tatsukawa *et al.*, 1979), whilst *Pseudomonas aeruginosa* is killed effectively by both polymorphonuclear cells (PMN) and macrophages (Van Furth *et al.*, 1978; Tatsukawa *et al.*, 1979).

In the present study, *K. pneumoniae* was chosen as a typical encapsulated *Klebsiella* species and the cells involved in the protection of mice were analysed in the presence of rabbit anti-*K. pneumoniae* serum.

X-irradiation was used to deplete the population of all phagocytes except tissue macrophages and carrageenan was used to deplete mononuclear phagocytes selectively (Catanzaro *et al.*, 1971). To facilitate the analysis, the susceptibility of *K. pneumoniae* to phagocytes was compared with that of *P. aeruginosa*.

METHODS

Animals. Outbred female CF1 mice were obtained from the Breeding Unit of Experimental Animals, Kyushu University, Japan. Athymic nude mice (nu/nu) of BALB/c background and their normal littermates (nu/+) were obtained from the Kyudo Laboratory for Experimental Animals, Kumamoto, Japan. Mice were used for experiments when 6 to 10 weeks old. Rabbits of both sexes, all weighing about 3 kg, were obtained from a local breeder.

Micro-organisms. *Pseudomonas aeruginosa* was isolated from the sputum of a patient suffering from acute pneumonia. Capsulated *Klebsiella pneumoniae* strain Fu-1 has been maintained in our laboratory. Micro-organisms were cultured overnight in Tryptic Soy Broth (Difco) at 37 °C for use in experiments. The LD₅₀ of *P. aeruginosa* for mice by the intravenous route was approximately 1×10^7 viable bacteria (Tatsukawa *et al.*, 1979). Out of 10 mice, only two survived 14 d after intramuscular inoculation of 10 viable *K. pneumoniae*.

Antiserum. In our preliminary experiments, mice immunized with *K. pneumoniae* produced antiserum with a low titre for agglutination which was inconvenient to use for experiments. A rabbit was therefore immunized subcutaneously with 6×10^9 formalin-killed *K. pneumoniae* in complete Freund's adjuvant. A week later, the rabbit was immunized intraperitoneally with 3.8×10^8 viable *K. pneumoniae*. Ten days after the second immunization, the rabbit was bled and about 50 ml of serum was removed from the clotted blood. Normal serum was obtained from three rabbits. The sera were heat-inactivated at 56 °C for 30 min and stored in 1 ml portions at -70 °C. A suspension of about 7×10^8 *K. pneumoniae* ml⁻¹ showed satisfactory agglutination with antiserum diluted to 1/64 with phosphate-buffered saline (PBS), but no agglutination occurred with normal serum.

Determination of bacterial growth in mice. Mice were inoculated intramuscularly into the middle of the right thigh with 1×10^5 viable *P. aeruginosa* or 1×10^4 to 1×10^5 viable *K. pneumoniae* suspended in Hanks' balanced salt solution (HBSS). At various times after inoculation, the whole muscle mass of an infected thigh was removed and homogenized in 10 ml PBS with a Teflon and glass homogenizer. Suspensions were diluted 10-fold serially with PBS and 0.1 or 1.0 ml of each dilution was spread on nutrient agar. Colonies were counted after incubation at 37 °C for 20 h (*P. aeruginosa*) or 15 h (*K. pneumoniae*).

By 4 h after intravenous inoculation of *K. pneumoniae* in mice, the bacteria had grown as well in the liver, spleen, lungs and kidneys as in the blood, but at 24 h bacterial growth was greater in the liver than in the other organs (unpublished results). Therefore, to observe the systemic spread of bacteria after intramuscular inoculation, bacteria were counted in the liver and blood. Mice were bled by cutting the femoral artery and the blood was diluted and plated as described above. Livers were removed, homogenized and counted as described above.

Treatment of mice with antiserum. The heat-inactivated antiserum or normal serum was diluted 20-fold with PBS and injected intraperitoneally in a volume of 1 ml 60 min before inoculation of *K. pneumoniae*.

X-irradiation. Mice were exposed to 8 J kg⁻¹ (800 rad) 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 at 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.

Phagocytosis and intracellular killing in vitro. PMN were washed out from 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 from the peritoneum 3 d after injection of 2 ml 10% (w/v) proteose-peptone (Difco); about 75% of the cells were macrophages.

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 the suspended cells, checked by dye exclusion of Trypan Blue, was over 98%. Cell suspensions (1.5 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. For *K. pneumoniae*, but not for *P. aeruginosa*, normal rabbit serum or antiserum was added to the cell suspension to obtain a final concentration of 5% (v/v) in the mixtures. After phagocytosis, the mixtures were washed by centrifuging three times to remove free bacteria. Cells were disrupted with 0.25% (w/v) sodium dodecyl sulphate (SDS) and viable bacteria in the suspension were counted. Such a concentration of SDS did not affect the growth of the bacteria (unpublished results). To observe the rate of intracellular killing, cell suspensions were incubated for 30 min after phagocytosis and washing. The numbers of bacteria remaining viable within cells were determined by culture on nutrient agar after disrupting the cells with SDS.

Histology. Right and left thigh muscles of antiserum-treated mice were each inoculated with 1×10^5 viable *K. pneumoniae*. Infected muscle of the left thigh was removed and the number of viable bacteria was counted 3 h, 6 h, 24 h and 72 h after inoculation. Infected muscle of the right thigh was removed for histological examination at the same times. Sections were stained with haematoxylin and eosin.

Fig. 1. Bacterial growth in the muscle of mice treated with antiserum (●), normal serum (■) or carrageenan and antiserum (○), and of X-irradiated mice treated with antiserum (▽), after intramuscular inoculation of 1×10^4 *K. pneumoniae*. Each point and bar indicate the mean result for five animals with the standard error. † indicates mice died.

RESULTS

Effect of antiserum on K. pneumoniae-inoculated mice and on bacterial growth in carrageenan-treated and X-irradiated mice

Mice pretreated with antiserum were protected completely from an intramuscular challenge with 1×10^7 *K. pneumoniae*, although the mortality of normal mice inoculated with 10^7 *K. pneumoniae* was 80%. Viable bacteria in infected muscle from groups of five mice were counted after intramuscular inoculation of 1×10^4 *K. pneumoniae*. In the antiserum-treated control mice, numbers of the bacteria decreased progressively over 120 h to reach an almost undetectable level (Fig. 1). No bacteria were detected in the liver and blood throughout the experiment. In mice treated with normal serum, numbers in the muscle increased rapidly by 72 h to reach over 1×10^8 . A few bacteria appeared at 6 h in the liver and blood and their numbers increased rapidly; none of these mice survived 5 d after infection. Bacterial numbers decreased by 72 h in antiserum- and carrageenan-treated mice, similar to the decrease in antiserum-treated control mice. In antiserum-treated X-irradiated mice, the numbers increased markedly by 24 h to reach more than 1×10^7 ; none of these mice survived 48 h after infection.

The degree of elimination of inoculated *K. pneumoniae* was compared with that of *P. aeruginosa*, which is killed by phagocytes effectively and rapidly (Tatsukawa *et al.*, 1979). When 1×10^5 *P. aeruginosa* were inoculated into untreated control mice, the $\log_{10}$ of the number of bacteria recovered from the muscle 15 min after inoculation was $4.96 \pm \text{s.e. } 0.09$, decreasing rapidly by 24 h to reach 2.79 ± 0.27 . The number decreased in carrageenan-treated mice in the same way as in control mice. The degree of elimination of *P. aeruginosa* in the absence of antiserum was apparently greater than that of *K. pneumoniae* in the presence of antiserum.

Growth of K. pneumoniae in the muscle of nude mice in the presence of antiserum

To observe the effect of the presence of the thymus on the elimination of inoculated *K. pneumoniae*, athymic nu/nu and control nu/+ mice were inoculated with 1×10^4 *K. pneumoniae*. Viable bacteria in the infected muscle from five antiserum-treated mice of each group were counted. The $\log_{10}$ of the numbers of bacteria in nu/nu and nu/+ mice 6 h after inoculation were 3.06 ± 0.10 and 3.10 ± 0.17 , respectively; no significant difference was observed between nu/nu and nu/+ mice.

Phagocytosis and intracellular killing of bacteria in vitro

In the presence of antiserum, 1.63×10^6 *K. pneumoniae* were phagocytized by 1×10^7 macrophages. The degree of phagocytosis by PMN was about eightfold lower than that by macrophages. Unphagocytized bacteria could not be removed completely by washing three times, and small numbers of these were counted as phagocytized bacteria. When allowance was made for this source of error, macrophages or PMN seemed to be almost unable to phagocytize *K. pneumoniae* in the absence of antiserum. Approximately one-half of the phagocytized *K. pneumoniae* were killed by PMN or macrophages during 30 min incubation in the presence of antiserum.

In the absence of antiserum, 8.7×10^5 and 1.7×10^5 *P. aeruginosa* were phagocytized by 1×10^7 macrophages and 1×10^7 PMN, respectively. The degree of intracellular killing of *P. aeruginosa* by macrophages or PMN in the absence of antiserum was about fourfold greater than that of *K. pneumoniae* in the presence of antiserum.

Bacterial growth in the muscle and histological examination of cellular infiltration into infected sites in antiserum-treated mice

At 15 min after inoculation with 1×10^5 *K. pneumoniae*, the $\log_{10}$ of the number of bacteria recovered from the muscle from five mice was 4.73 ± 0.06 . Slight cellular infiltration by PMN was observed at 3 h, when the $\log_{10}$ of the number of bacteria had reached 5.71 ± 0.10 . The $\log_{10}$ of the number then decreased to reach 4.78 ± 0.48 at 6 h, at which time marked cellular infiltration was observed. Approximately 95% of the infiltrating cells observed were PMN. Bacterial numbers decreased by 72 h, but the degree of cellular infiltration remained approximately constant. The proportion of macrophages and lymphocytes increased progressively from 24 to 72 h and these cells comprised about 85% of the infiltrating cells observed at 72 h.

DISCUSSION

No significant phagocytosis of *K. pneumoniae* by PMN or macrophages was observed *in vitro* in the absence of antiserum. *In vivo*, inoculated *K. pneumoniae* was virulent. *Klebsiella pneumoniae* inoculated intramuscularly without antiserum grew progressively until the mice died. Systemic spread of bacteria occurred soon after inoculation. On the other hand, *K. pneumoniae* inoculated into antiserum-treated mice remained localized within the inoculated muscle and were finally eliminated.

Carrageenan may be used to block selectively the functioning mononuclear phagocytes (Tatsukawa *et al.*, 1979). On the other hand, the numbers of both PMN and monocytes decreased markedly in X-irradiated mice (unpublished results). Therefore, carrageenan-treated mice may be regarded as macrophage-depleted and X-irradiated mice as PMN- and monocyte-depleted. Although *K. pneumoniae* was eliminated efficiently in controls given antiserum, progressive growth of bacteria was observed in X-irradiated mice even in the presence of antiserum. Thus, antiserum had no significant direct bactericidal effect against *K. pneumoniae*; its protective effect depended on radiosensitive cells, including both phagocytes and lymphocytes. However, T lymphocytes appeared not to affect protection against *K. pneumoniae* in the presence of antiserum at an early phase of infection, because *K. pneumoniae* was eliminated in athymic nude mice as efficiently as in normal littermates. We conclude that the main effect of antiserum was opsonization for phagocytosis.

To obtain more information, protection against *K. pneumoniae* was compared with that against *P. aeruginosa*. *Pseudomonas aeruginosa* inoculated into muscle is known to be eliminated efficiently by PMN even in the absence of macrophages (Tatsukawa *et al.*, 1979). Growth patterns of *K. pneumoniae* in the presence of antiserum in carrageenan-treated mice

and normal controls are fundamentally similar to those of *P. aeruginosa* in the absence of antiserum. It was concluded tentatively that *K. pneumoniae* inoculated in the presence of antiserum was killed effectively by PMN even in the absence of normally functioning macrophages.

In antiserum-treated mice, the number of bacteria started to decrease at 6 h, when marked infiltration of PMN was observed, but only very small numbers of macrophages were observed. The rate of phagocytosis of *K. pneumoniae* by macrophages in the presence of antiserum *in vitro* was greater than that by PMN, and macrophages killed *K. pneumoniae* as efficiently as did PMN. These results suggest that PMN protect efficiently against *K. pneumoniae* by their rapid accumulation at infected sites even in the absence of macrophages, although the latter may also contribute.

Dependence of protection on antiserum and relative resistance to intracellular killing by phagocytes may account for some of the pathogenicity of opportunistic infection by members of encapsulated *Klebsiella* species for immunologically impaired or otherwise susceptible patients.

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