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Neutrophils Are Involved in Acute, Nonspecific Resistance to *Listeria monocytogenes* in Mice

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The importance of neutrophils in killing extracellular, pyogenic bacteria has long been established. However, there is only indirect evidence for a role for neutrophils in resistance against intracellular organisms. In this study, we directly demonstrate the involvement of neutrophils in defense against *Listeria monocytogenes* in normal C.B-17 immunocompetent and C.B-17 SCID mice. Because of the lack of sterilizing T-cell immunity, SCID mice are unable to completely eliminate listeriae systemically and become chronically infected. Both immunocompetent and SCID mice treated with a specific neutrophil-depleting monoclonal antibody during the early stages of *Listeria* infection were rendered remarkably sensitive to the organism, with a high level of mortality resulting from enhanced bacterial growth. At a late stage of infection in SCID mice, however, administration of neutrophil-depleting antibody did not affect mortality. In spite of the neutrophil depletion, other parameters of nonspecific immune function were normal. Macrophage infiltration to the site of infection and macrophage expression of major histocompatibility complex class II molecules were unaffected. Moreover, NK cell functions were normal as measured by infiltration to an infection site and gamma interferon production. These data demonstrate an important role for neutrophils in controlling the acute phase of *Listeria* infection, cooperating with, and yet independent of, macrophages and NK cells.

During the past several years, many laboratories have assessed the specific roles of various leukocytes in resistance to microbial pathogens. Our laboratory has used the well-characterized model of *Listeria monocytogenes* infection in mice to evaluate this subject. We have shown the importance of an early macrophage and natural killer (NK) cell interaction in generating a rapid, nonspecific response against listeriae, which is in part mediated by cytokines (8). Indeed, macrophage ingestion of listeriae induces the release of interleukin-1 (IL-1), tumor necrosis factor alpha, and IL-12 (30, 45, 48). IL-1 and tumor necrosis factor have been shown to be required for resistance to infection (9, 24, 39). Tumor necrosis factor and IL-12, at least in vitro, stimulate NK cells to produce gamma interferon (IFN-γ) (45). NK cell-derived IFN-γ in turn activates macrophages to express class II major histocompatibility complex molecules (7) and to curb infection (13) by producing antilisterial effector molecules like nitric oxide (10). This cellular system operates in the absence and presence of T cells (8). In fact, T- and B-cell-deficient SCID mice resist early *Listeria* infection as well as immunocompetent control mice do. However, in a SCID mouse, the absence of T-cell immunity permits listeriae residing in nonimmunological cell types to escape destruction, and therefore, the hosts become chronically infected. In a normal mouse, there is an obligate role for T cells in establishing sterilizing and long-lived immunity against *Listeria* infection (6, 11, 17, 22, 26, 31, 34, 35, 38, 41).

The involvement of neutrophils in protection against many pyogenic and blood-borne infections has been well demonstrated. However, there is limited information about the role of this cell type in resistance to intracellular pathogenic bacteria. Intraperitoneal (i.p.) infection of mice with intracellular pathogens like listeriae or mycobacteria induces rapid infiltration of high numbers of neutrophils, but the significance of this neutrophilia is not known (2–5, 44). In culture, the infiltrating neutrophils are capable of killing listeriae and release oxygen derivatives and enzymes with antilisterial activity (1, 12, 18; reviewed in reference 32). In our own studies, treatment of mice with monoclonal antibodies against IL-1 rendered them profoundly sensitive to listeriae (39). The interaction between neutrophils and IL-1 in antibacterial resistance has been suggested. For example, neutropenic mice were made more resistant to infections with *Pseudomonas aeruginosa* (46, 47) or *Candida albicans* (47) by treating them with IL-1. Administration of IL-1 enhanced *Listeria* resistance and also increased the number of neutrophils in tissues (16, 29). We have now shown that neutralization of IL-1 inhibited neutrophil influx into sites of infection (39a). We therefore decided to determine whether neutrophils were involved in *Listeria* resistance in vivo.

Other groups have postulated neutrophil involvement in mediating *Listeria* resistance on the basis of in vitro and nonspecific in vivo studies. In fact, Conlan and North showed that administration of a monoclonal antibody to the type 3 complement receptor compromised the ability of a mouse to resist *Listeria* infection (15). This study conjectured through histological work that neutrophils may actively lyse infected hepatocytes, exposing sequestered bacteria to inflammatory cell killing. This study is not definitive in showing a role for neutrophils since the antibody also inhibits macrophage and, possibly, NK cell migration (15). Both macrophages and NK cells are important mediators of T-cell-independent *Listeria* resistance, and both are highly reactive with the antibody. Actually, a parallel study from the same laboratory using the same monoclonal antibody claimed that monocyte infiltration into sites of infection was required for *Listeria* resistance (40). Moreover, another group showed that listericidal macrophage phagocytosis of complement-coated listeriae was inhibitable by antibody to the type 3 complement receptor (19). Therefore, although the conclusion that neutrophils are important determinants in acute *Listeria* resistance has been proposed, this study is the first to specifically evaluate it.

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In the current communication, we characterize a preexisting monoclonal antibody capable of specifically depleting neutrophils in vivo. By using this monoclonal antibody, we clearly demonstrate the requirements for neutrophils in the early stages of listerial infection. We also show that neutrophils are not required for the *Listeria*-induced recruitment and activation of macrophages or NK cells.

MATERIALS AND METHODS

Mice. C.B-17 and C.B-17 SCID mice, originally derived from a colony at Fox Chase Cancer Institute, Philadelphia, Pa., were bred at Washington University under pathogen-free conditions and used between the ages of 8 and 12 weeks.

Antibodies and reagents. RB6-8C5 is a rat immunoglobulin G2b (IgG2b) monoclonal antibody specific for murine neutrophils, a gift of Robert Coffman. RB6-8C5 is expressed at high levels on murine neutrophils and at much lower levels on the immature cells of the myeloid lineage (25). Most lymphocytes and macrophages do not express this antigen (25). Specificity of the antibody was examined by flow cytometry as well as by examination of bone marrow cells isolated by fluorescence-activated cell sorting (FACS) (25). F4/80 is a rat IgG2b antibody that reacts with murine macrophages. 25-9-17S is a mouse IgG2a antibody specific for the major histocompatibility complex class II molecule I-A^d. These latter two antibodies were obtained from the American Type Culture Collection (Rockville, Md.). 5E6 is a mouse IgG antibody that reacts with a subset of splenic NK cells, a gift of Michael Bennett (42). Monoclonal antibodies were purified by Protein G-Sepharose (Sigma Chemical Co., St. Louis, Mo.) and contained less than 50 endotoxin units per mg of protein as determined by using the QCL-100 endotoxin quantitation kit (Whittaker M.A. Bioproducts, Walkersville, Md.).

Cell analysis by FACS. A total of 10^6 cells was stained in 200 μl of Hanks balanced salt solution containing 10% fetal calf serum with 1 μg of primary antibody (RB6-8C5, F4/80, or biotinylated 25-9-17S) at 4°C for 60 min. Cells were washed three times in phosphate-buffered saline (PBS) and stained with 1 μl of streptavidin phycoerythrin (Molecular Probes, Eugene, Oreg.) to detect the biotinylated 25-9-17S or with goat anti-rat IgG conjugated to fluorescein isothiocyanate (Southern Biotech, Birmingham, Ala.) to detect the F4/80 or RB6-8C5 for 60 min at 4°C. Cells were washed three times with PBS and analyzed by FACStar.

Cytospin. Cells were suspended at 2×10^5 cells per ml in RPMI medium containing 10% fetal calf serum, and 200 μl was spun down onto gelatin-coated slides at 1,000 rpm for 5 min with a cytospin centrifuge (Shandon, Pittsburgh, Pa.). Slides were fixed in methanol for 1 min, stained with Giemsa for 20 min, and analyzed by light microscopy.

Analysis of RB6-8C5 monoclonal antibody effect in vivo. The basic experimental protocol consisted of injecting mice i.p. with 1 ml of pyrogen-free saline solution or 200 μg of purified monoclonal antibody diluted in 1 ml of pyrogen-free saline on day −1, 2, 5, or 7 of i.p. *Listeria* infection. *L. monocytogenes* (3×10^3 cells) was injected in a 0.5-ml volume of pyrogen-free saline. The number of viable bacteria in the spleens of infected animals was quantitated as described previously (7, 9). Peritoneal exudate cells (PEC) were isolated at days 1, 3, and 4 for analysis. Cells were examined by FACS and cytospin. Absolute numbers were determined by standard procedures with hemocytometers. Extensive controls with irrelevant rat or murine IgG antibodies failed to produce an effect on *Listeria* infection or in the inflammatory response (7–9, 39). Therefore, the control in most of these studies was the injection of saline solution.

Northern (RNA) blot. The spleens from mice of each treatment group were pooled, and poly(A)-selected RNA was prepared by the Fast track system (Invitrogen, San Diego, Calif.). Briefly, the tissue was homogenized in lysis buffer containing the recommended RNase protein degrader. Oligo(dT) cellulose was added to capture the poly(A)$^+$ RNA. Four micrograms of RNA per lane was electrophoresed on a 1% agarose formaldehyde gel. The RNA was transferred overnight in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto a Nylon-1 membrane (GIBCO-BRL, Gaithersburg, Md.). The membrane was baked for 2 h at 60°C and then prehybridized overnight at 42°C as described previously (14). The blot was then hybridized overnight at 42°C with radiolabelled probe, washed in 0.1×SSC–0.1% sodium dodecyl sulfate for 2 h, and exposed to X-ray film.

Radiolabelled probes. IFN-γ cDNA fragment was a gift of Kenneth Murphy. It was a *Pst*I-*Pst*I fragment originally derived from Gray and Goeddel (23). β-Actin cDNA fragment was a gift of Robert Schreiber (21). Both DNAs were labelled by random priming with hexamer mixtures and the Klenow fragment of DNA polymerase. Thus, 100 ng of DNA was labelled with 50 μCi of [^{32}P]dCTP (Amersham, Arlington Heights, Ill.) with an oligonucleotide labelling kit (Pharmacia, Piscataway, N.J.). The probe was boiled for 2 min and added straight to the Northern blot in prehybridization solution.

RESULTS

Monoclonal antibody RB6-8C5 stains neutrophils selectively. To define the specificity of the RB6-8C5 antibody, PEC from C.B-17 SCID mice infected i.p. with listeriae for 8 h were stained with RB6-8C5 and analyzed by FACS. The profile shown in Fig. 1A was gated by forward and side scatter on a cell population that is 85% neutrophils when analyzed by cytospin. The neutrophils stained very brightly. A small number of cells stained dully, and these were represented by infiltrating monocytes. Cells from spleens of C.B-17 SCID mice were analyzed for expression of 5E6, a marker of NK cells (42). The 5E6-positive cells were gated by forward and side scatter and analyzed for expression of RB6-8C5. Figure 1B and C demonstrates that NK cells and resident peritoneal cells from SCID mice (made up of greater than 99% macrophages) did not stain with RB6-8C5. B and T lymphocytes also did not stain with the monoclonal antibody (data not shown).

Administration of antibody RB6-8C5 results in selective depletion of neutrophils. We have shown in the context of a different study that i.p. infection with listeriae results in a rapid, transient influx of neutrophils in immunocompetent mice (44). SCID mice infected with listeriae have a similar rapid influx of neutrophils which, unlike in immunocompetent mice, persists throughout the course of infection (39a). In the present experiment, SCID mice were pretreated with either saline or RB6-8C5 antibody and infected i.p. with listeriae. PEC were isolated and analyzed by cytospin 24 and 72 h later. The numbers of peritoneal neutrophils were reduced in antibody-treated mice by 97 and 96% at the two time points examined (Fig. 2A). Antibody administration, however, did not affect the number of infiltrating monocytes (Fig. 2B) or lymphoid cells (mainly NK cells in SCID mice; Fig. 2C) at either time point. Thus, neutrophils migration is not required for monocytes or NK cells to migrate to the peritoneum in response to *Listeria* infection. RB6-8C5 like-

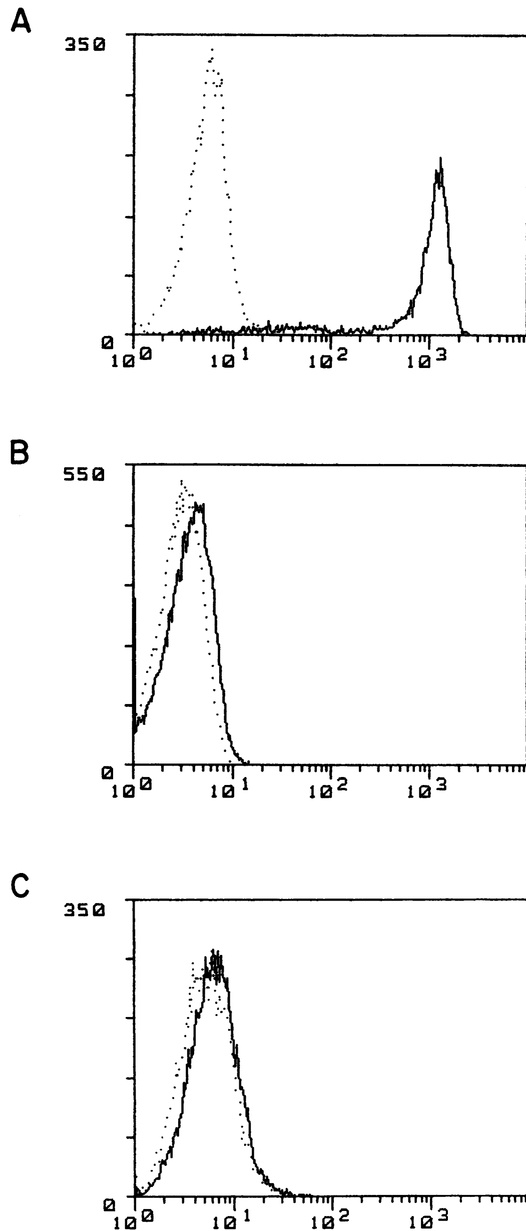

FIG. 1. Cytofluorimetric analyses of murine leukocyte populations from SCID mice. (A) PEC, 8 h after *Listeria* infection, composed of neutrophils (85%) and monocytes (15%); (B) splenic NK cells; (C) resident peritoneal macrophages. All cells were stained with RB6-8C5 monoclonal antibody and then with goat anti-rat IgG conjugated to fluorescein isothiocyanate (described in Materials and Methods).

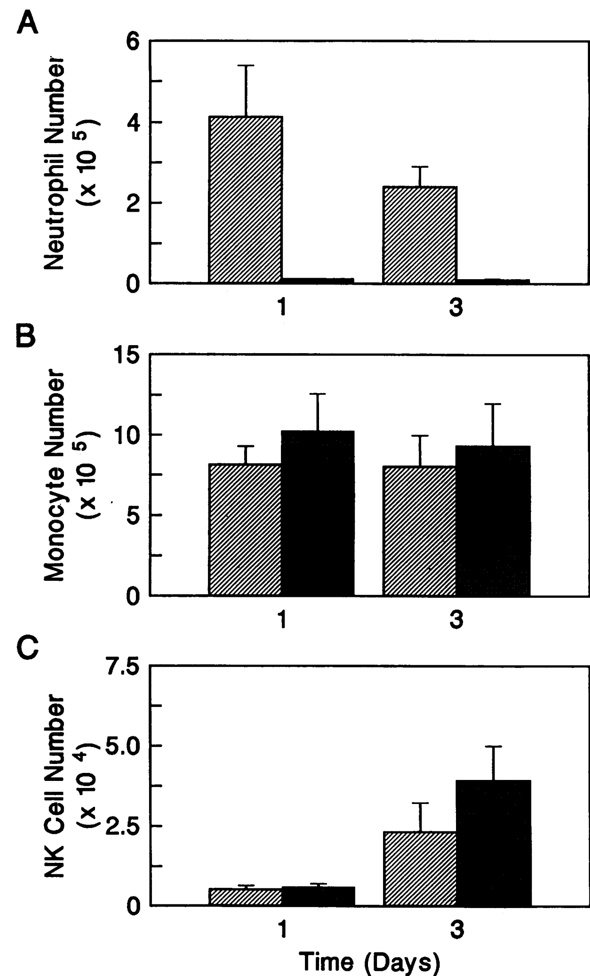

FIG. 2. Effect of RB6-8C5 administration on inflammatory cell influx in response to *Listeria* infection. SCID mice were injected on day −1 with saline (▨) or 200 μg of RB6-8C5 (■) and then infected with 3,000 listeriae on day 0. The peritoneal neutrophils (A), monocytes (B), and lymphoid cells (C), mainly represented by NK cells, were quantitated on days 1 and 3 of infection. Each point represents the mean ± standard error of the mean for five mice per group.

wise deleted the number of circulating neutrophils by close to 100% without an effect on monocytes. For example, in one representative experiment, SCID mice infected with listeriae for 24 h exhibited the following leukocyte counts (in cells per cubic millimeter): neutrophils, 3,103 ± 970; monocytes, 371 ± 162; lymphoid cells, 35 ± 12. Those mice administered RB6-8C5 and infected with listeriae had the following leukocyte counts (in cells per cubic millimeter) neutrophils, 70 ± 63; monocytes, 398 ± 55; lymphoid cells, 43 ± 12.

Administration of RB6-8C5 antibody has no effect on monocyte or NK cell activation. Neutrophil depletion had no effect on macrophage activation in response to listeriae as assayed by induction of high levels of surface major histocompatibility complex class II molecules. Class II induction was measured by FACS analysis of peritoneal cells for surface expression of I-A^d compared with that of F4/80, a macrophage-specific marker expressed by bright staining on unactivated macrophages and dull staining on activated macrophages (20). Figure 3A demonstrates the typical staining pattern of resident macrophages with about 90% unactivated cells (I-A^d, dull; F4/80, bright) and 10% activated cells (I-A^d, bright; F4/80, dull). Three days after infection with 3×10^3 listeriae, almost all macrophages were activated (I-A^d, bright; F4/80, dull) in the saline-pretreated mice (Fig. 3B). Administration of RB6-8C5 antibody prior to *Listeria* infection did not change the day-3 staining pattern, with almost all of the peritoneal macrophages activated (Fig. 3C). In a different experiment, mice infected with 10^3 listeriae with

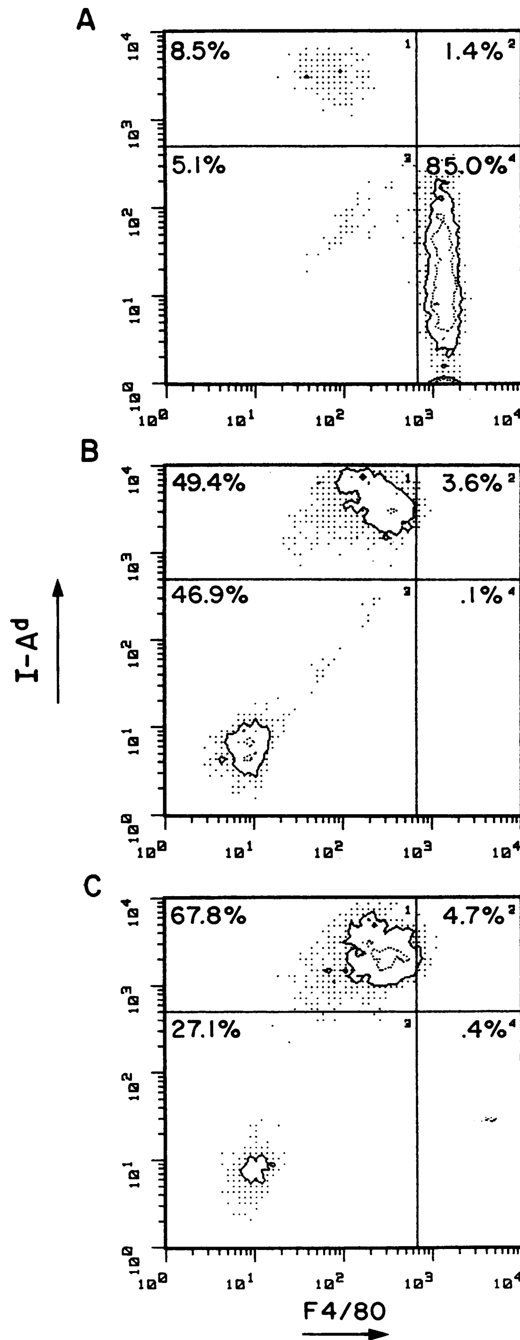

FIG. 3. Representation of two-color cytofluorometric analyses of SCID PEC. (A) Resident PECs; (B) PEC from *Listeria*-infected SCID mice; (C) PEC from *Listeria*-infected SCID mice pretreated with RB6-8C5. Cells were stained with F4/80 and 25-9-17S conjugated to biotin and were detected with goat anti-rat IgG conjugated to fluorescein isothiocyanate and streptavidin conjugated to phycoerythrin. F4/80 staining is measured on the horizontal axis; I-A^d with the monoclonal antibody 25-9-17S is measured on the vertical axis.

and without RB6-8C5 administration were also examined on day 4. Again, both groups had almost 100% I-A^d-positive macrophages. Neutrophil depletion also had no effect on macrophage activation in immunocompetent mice (not shown). Thus, peritoneal macrophages were almost all

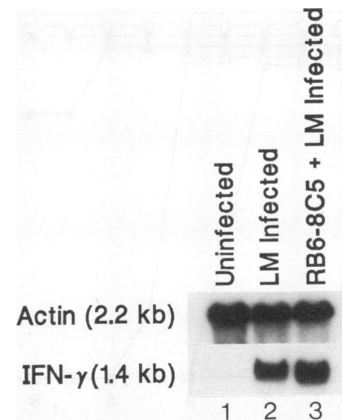

FIG. 4. Effect of RB6-8C5 administration on IFN-γ production by *Listeria*-infected spleen. Northern blots were performed with RNA isolated from an uninfected SCID spleen (lane 1), a day-3 *Listeria*-infected SCID spleen (lane 2), and an RB6-8C5-pretreated, day-3 *Listeria*-infected SCID spleen (lane 3) (five mice per group). IFN-γ and β-actin were detected by using specific probes.

stained brightly for I-A^d and dully for F4/80 regardless of prior RB6-8C5 administration.

Splenic IFN-γ production in SCID mice was assessed as a measure of NK cell activation. IFN-γ mRNA was readily detected by Northern blot of splenic RNA from SCID mice infected with listeriae for 3 days (Fig. 4). The spleens of *Listeria*-infected mice pretreated with RB6-8C5 antibody had roughly equivalent levels of IFN-γ message. Uninfected mice had no detectable IFN-γ mRNA. (We have not consistently detected IFN-γ mRNA by Northern blot from PEC, probably because of the limited number of NK cells.)

Administration of RB6-8C5 antibody decreases resistance to *Listeria* infection in SCID and immunocompetent mice. SCID mice (five mice per group) were injected i.p. with RB6-8C5 antibody at various time points before and after i.p. infection with listeriae (Fig. 5A). The chosen dose of listeriae was about one-third the 50% lethal dose. Mice treated with antibody on either day −1 or day 2 quickly succumbed to infection, while saline-treated control mice did not. Only one mouse in the group injected with antibody 5 days after infection died, and none in the group injected after 7 days died. By day 7 of infection, the numbers of listeriae in SCID mice are near maximum levels and remain at this plateau for several weeks (7, 8). These data indicate that neutrophils are involved mostly during early infection when the organism is rapidly growing and disseminating. Increased mortality to *Listeria* infection due to RB6-8C5 administration correlated with increased numbers of listeriae in the spleens of infected mice; on days 1 and 3 of *Listeria* infection, saline-pretreated mice had splenic *Listeria* loads of 342 (range, 67 to 933) and 48,417 (range, 12,000 to 92,300), respectively (mean of four mice in each group). However, mice pretreated with RB6-8C5 and then infected with listeriae for 1 and 3 days had splenic counts of listeriae of 9,411 (range, 4,566 to 16,300) and 1,031,500 (range, 149,333 to 2,726,000). The dramatic effects of RB6-8C5 on survival from infection were not limited to SCID mice in that treatment with RB6-8C5 antibody the day before infection with listeriae also dramatically increased mortality in immunocompetent C.B-17 mice (Fig. 5B).

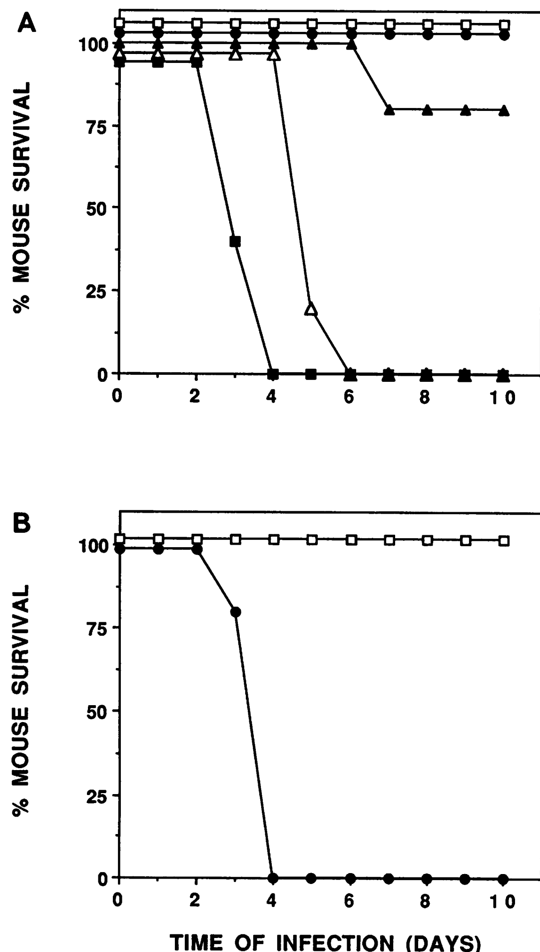

FIG. 5. Effect of administration of RB6-8C5 on survival from *Listeria* infection in SCID and immunocompetent mice. (A) SCID mice were injected i.p. with saline on day −1 (□) or with 200 μg of RB6-8C5 on day −1 (■), 2 (△), 5 (▲), or 7 (●) and infected i.p. with 3,000 listeriae on day 0. (B) Immunocompetent C.B-17 mice were injected i.p. with saline (□) or 200 μg of RB6-8C5 on day −1 (●) and infected i.p. with 3,000 listeriae on day 0.

DISCUSSION

These data indicate that neutrophils are required to prevent uncontrolled *Listeria* growth early in infection but not at later time points. Moreover, neutrophil depletion does not affect macrophage and NK cell migration to the peritoneum or activation in response to listeriae. These data, therefore, establish the important role of neutrophils in *Listeria* resistance, which to date has not been directly proven for intracellular pathogenic microorganisms. Thus, treatment of SCID and immunocompetent mice with monoclonal antibody RB6-8C5 depleted neutrophils in vivo and profoundly decreased survival from *Listeria* infection. Counts of splenic listeriae emphasize the importance of neutrophils since the depletion of neutrophils increased the *Listeria* burden by over 20-fold. However, by days 5 to 7 of infection, administration of the antibody no longer affected survival. The lack of any effect of neutrophil depletion late in infection may be explained by the presence of the listericidal activated macrophages in the exudates.

Two issues need a brief analysis. The first is the exact role of neutrophils in acute nonspecific *Listeria* resistance. The simplest explanation is that the neutrophil has direct listericidal activity and thereby limits the extent of growth and dissemination of extracellular listeriae during the early stages of infection. Other groups have shown that neutrophils and neutrophil products have the capacity to kill listeriae in vitro (1, 12, 18; reviewed in reference 32). In a recent study, we showed that nitric oxide made by activated macrophages had listericidal properties (10). Even though neutrophils produce nitric oxide (33) under some conditions, the 8-h *Listeria*-induced neutrophil peritoneal exudates did not release detectable amounts (our unpublished results). This suggests that other listericidal molecules may be employed in the early neutrophil exudates. As discussed previously, it has been postulated that neutrophils may also lyse infected hepatocytes, thereby exposing the intracellular listeriae to killing by inflammatory cells (15). There is also evidence that neutrophil infiltration is required for subsequent IL-8-dependent $CD4^+$ T-cell recruitment into subcutaneous tissues (28). Therefore, a possible role of neutrophils may be in mediating the influx and activation of other leukocytes, like monocytes and NK cells. However, in our model, this activity of neutrophils can be ruled out (Fig. 2B and C). Another possibility that should be considered is whether neutrophils, which produce vasoactive compounds (32), play a role in vascular changes that limit the spread of *Listeria* infection. Indeed, it has been demonstrated that both prostaglandin and thromboxane production are important in early *Listeria* defense (43).

A second issue relates to the cellular and molecular processes that initiate and terminate the neutrophil influx. We have shown that administration of antibodies to IL-1, which enhances susceptibility to *Listeria* infection (39), also decreases neutrophil infiltration (39a). IL-1 may be involved by way of induction of neutrophil-specific chemotactic cytokines. This conclusion is supported by the findings that neutrophil chemotactic cytokines of the IL-8 family are rapidly induced by IL-1 in many cell types (reviewed in reference 37).

Although the mechanisms of induction of acute inflammation are slowly being elucidated, little is known about how inflammatory processes are terminated. When a mouse is infected i.p. with listeriae, there is a rapid influx of neutrophils, peaking at 8 to 12 h postinfection (44). Neutrophil counts then gradually decrease over the next several hours. This infiltration by neutrophils may be dependent on a persistently large local load of listeriae that decreases with the onset of the acute inflammatory response. An alternative explanation is that subsequent cellular infiltrates inhibit the neutrophil attraction into sites of infection. For example, NK cells and macrophages may inactivate acute neutrophil-mediated inflammation through the production of IFN-γ and nitric oxide. It has been shown that IFN-γ inhibits IL-8 production by human fibroblasts and that nitric oxide inhibits neutrophil adhesion and migration across the endothelium (27, 36). In contrast to the above model, SCID mice infected i.p. with listeriae and immunocompetent mice infected i.p. with mycobacteria both have persistently high levels of peritoneal neutrophil infiltrates (2, 5). Further evaluation of these experimental infections and how they differ from *Listeria* infection in normal mice may give important insights into the regulation of neutrophil recruitment.

In conclusion, this study indicates that neutrophils are crucial in the control of acute listerial infection. This finding argues against the strict separation of effector cell responses, one represented by neutrophils (responsible for resistance to

extracellular, pyogenic infections) and the other represented by macrophages mediating resistance to intracellular pathogens. Clearly, in the *Listeria* model of intracellular infection, the participation of both cell types is critical for mounting an effective inflammatory response and eventual control of growth and dissemination of the bacteria.

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