Bacterial Phagocytosis by Macrophages from Lipopolysaccharide Responder and Nonresponder Mouse Strains

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The phagocytic capacity of macrophages from C3H/HeJ mice was assessed against lipopolysaccharide-producing (*Escherichia coli*) and -nonproducing (*Staphylococcus aureus*) bacteria. Despite their gene-coded unresponsiveness to lipopolysaccharide endotoxin and lymphokines and their defective tumoricidal activity, proteose peptone-induced C3H/HeJ macrophages did not display a defective phagocytic capacity, but rather displayed an enhanced phagocytosis of both bacterial strains compared with macrophages from closely related C3H/HeN mice. Unstimulated peritoneal resident C3H/HeJ macrophages, on the other hand, displayed a normal phagocytic activity toward *E. coli* and enhanced phagocytosis toward *S. aureus*.

Phagocytosis and killing of bacteria by macrophages is one of the most important nonadaptive defence mechanisms against bacterial infections. Numerous extracellular and bacterial factors influencing phagocytosis have been recognized (8, 11, 12). Moreover, recent in vitro data from this laboratory have shown that phagocytosis is highly modulated by environmental factors, such as temperature and antibiotic concentrations (5; N. A. Carlone, A. M. Cuffini, and G. Cavallo, *In Symposium on the Significance of Subinhibitory Concentrations of Antibiotics*, in press; A. M. Cuffini, N. A. Carlone, and O. Cattaneo, *In Proceedings of the First Mediterranean Congress on Chemotherapy*, in press). It is also regulated by several genes located both outside and inside the major histocompatibility complex (2, 3).

In this paper, the phagocytic capacity of macrophages from C3H/HeJ mice is evaluated. These mice carry a single gene mutation that results in defective responses to all known in vivo and in vitro effects of *Escherichia coli* lipopolysaccharides (LPS) (7, 20). Moreover, their macrophages display defective tumoricidal capacity (14) and lack of responsiveness to lymphokines (14, 17). These defective biological responses are related to LPS (16) and are controlled by a gene closely linked or identical to the LPS response gene (16). In addition, several laboratories have shown that the C3H/HeJ mice exhibit a marked susceptibility to infection with *Salmonella* which also appears to be linked to the LPS gene defect (10, 13, 19). Finally, it has been demonstrated recently that these mice display defective Fc receptor-mediated phagocytosis

when cultured in vitro over a 24-h period (18).

To determine whether these defects also affect bacterial phagocytosis, a comparison was made with macrophages from closely related C3H/HeN mice, which are known to be LPS responsive.

Our findings showed that the phagocytic capacity of C3H/HeJ macrophages is not exactly defective, but enhanced toward both LPS-producing and -nonproducing bacterial strains compared with the phagocytic capacity of macrophages from the closely related C3H/HeN mice.

MATERIALS AND METHODS

Mice. C3H/HeJ mice (6 to 12 weeks old) were obtained from L. Ruco, Istituto di Anatomia Patologica, Roma, Italy. Male C3H/HeN mice of the same age were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md.

Peritoneal cells. Resident peritoneal cells from untreated mice or peritoneal exudate cells from mice treated 72 h previously with 2 ml of a solution of proteose peptone (Oxoid, London, England) in distilled water (pH 7.2) were harvested by repeated washings of the peritoneal cavity with a total of 15 ml of cold Earle balanced salt solution supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) and 100 U of heparin per ml. Fluids from five mice were pooled and washed three times by centrifugation at $100 \times g$ for 10 min at 4°C. The pellets were then suspended to a concentration of 10^6 cells per ml in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum screened by the *Limulus* test for bacterial endotoxin contamination (kindly donated by A. Mantovani, Istituto Mario Negri, Milano, Italy). Portions (2 ml) of the suspensions were plated onto plastic culture dishes (30 by 8 mm; Nunc, Copen-

hagen, Denmark) and incubated for 2 h at 37°C in 5% CO₂ in moist air. Then the medium was washed away and replaced with 2 ml of fresh RPMI 1640 medium, and the cultures were incubated for an additional 12 h. The nonadherent cells were vigorously washed away, and the adherent cell monolayers were used for the phagocytosis mixture. Total and differential cell counts were performed on control adherent cell monolayers, which were removed by scraping with a rubber policeman. Differential cell counts were made both by morphological criteria on Diff Quick (Harleco, Gibbstown, N.Y.)-stained smears and by counting latex bead-ingesting cells after 4 h of incubation. We used only preparations containing 90% macrophage-like cells, as judged by both criteria.

Bacterial strains. *E. coli* TEM⁺ and *Staphylococcus aureus* ATCC 6538 were maintained on solid agar medium. Colonies were transferred to 12 ml of brain heart infusion broth (Oxoid) containing 50 μ Ci of [³H]uracil (specific activity, 26 Ci/mmol; Sorin, Saluggia, Italy). After 18 h of growth at 37°C, the bacteria were washed five times with 10 ml of cold brain heart infusion broth. A final concentration of 10⁸ colony-forming units per ml of brain heart infusion broth was obtained by a spectrophotometric method and was confirmed by colony counts in quadruplicate.

Phagocytosis mixture. A 1-ml amount of bacterial suspension was added to each macrophage monolayer, which was then incubated on a rocking platform at 37°C in 5% CO₂ in moist air. After progressive incubation periods, the supernatant was removed, and the plates were washed vigorously five times with 3 ml of cold Earle buffer. The adherent cell monolayers were dissolved in 1 ml of 0.1% sodium lauryl sulfate in distilled water; 100- μ l samples were placed in 3 ml of a scintillation liquid (Picofluor 15; Packard, Downers Grove, Ill.) and counted by liquid scintillation spectrometry. We made a parallel determination of the number of colonies and the radioactivities of control suspensions incubated under the same conditions without adherent cell monolayers, and the numbers of control bacterial colonies per count per minute of control bacterial suspensions were determined. The number of bacteria associated with adherent cells at a given time was calculated as described by Adam et al. (1), using the following formula: number of adherent cell-associated bacteria = (number of control bacterial colonies $\times$ counts per minute of cell-associated bacteria)/counts per minute of control bacterial suspensions. Each experiment was performed at least four times. A representative experiment is reported.

RESULTS

Phagocytic activity of resident peritoneal macrophages. First, the phagocytic capacity of resident macrophages from LPS-deficient C3H/HeJ mice obtained without deliberate stimulation was tested toward both an LPS-nonproducing strain (*S. aureus* ATCC 6538) and an LPS-producing strain (*E. coli* TEM⁺). On *E. coli* (Fig. 1) the phagocytic activities of macrophages from C3H/HeJ and C3H/HeN mice were substantially the same. In contrast, phagocytosis

FIG. 1. Phagocytosis of *E. coli* by resident peritoneal macrophages obtained without deliberate stimulation from LPS-deficient C3H/HeJ mice (dashed line) and normal C3H/HeN mice (solid line). Each point represents the mean $\pm$ 1 standard deviation of triplicate determinations.

FIG. 2. Phagocytosis of *S. aureus* by resident peritoneal macrophages obtained without deliberate stimulation from LPS-deficient C3H/HeJ mice (dashed line) and normal C3H/HeN mice (solid line). Each point represents the mean $\pm$ 1 standard deviation of triplicate determinations.

of *S. aureus* was the same for both macrophage substrains for the first 120 min only (Fig. 2). Thereafter, the number of *S. aureus* cells phagocytized by C3H/HeJ macrophages were nearly twice as high. During the first 120 min, essentially equal numbers of *E. coli* and *S. aureus* cells were phagocytized. However, at 180 min substantially more *S. aureus* cells were phagocytized (Fig. 1 and 2).

Phagocytic activity of proteose peptone-induced macrophages. C3H/HeJ and C3H/

HeN macrophage-like cells from proteose peptone-induced exudates displayed enhanced phagocytic capacity compared with unstimulated resident peritoneal cells. Phagocytosis of both bacteria increased about 20 to 30 times after the first 60 min, with no significant differences between the two C3H substrains (Fig. 3 and 4). After this time, however, C3H/HeJ macrophages showed a much higher bacterial uptake. At 120 and 150 min, this uptake was nearly twice as high against both *E. coli* (Fig. 3) and *S. aureus* (Fig. 4). At 180 min, a significant difference in the numbers of macrophage-associated bacteria was still evident, even though the total

amount of radioactivity recovered in the macrophages decreased (Fig. 3 and 4). This decrease was probably due to a slowing down of cell phagocytic activity and a release into the medium of fragments of digested bacteria.

DISCUSSION

Our data showed that macrophage phagocytosis of bacteria is not impaired but enhanced in LPS-defective C3H/HeJ mice, compared with phagocytosis by macrophages from the closely related and histocompatible strain C3H/HeN. Substantial activity was displayed against both an LPS-nonproducing strain (*S. aureus*) and an LPS-producing strain (*E. coli*). Moreover, in several cases both resident and proteose peptone-induced C3H/HeJ macrophages displayed greater phagocytosis after prolonged incubation periods. C3H/HeJ phagocytosis of these bacteria, therefore, does not appear to be directly linked to other defective macrophage functions.

C3H/HeJ mice are refractory to most biological effects induced by the lipid A moiety LPS on B lymphocytes and macrophages (9). Their macrophages are not killed by LPS in vitro (6), and their phagocytic activity is not increased by in vivo LPS injections (14). They also fail to respond to the macrophage-activating factor to kill tumor cells or to the macrophage-inhibitory factor (15, 17) and exhibit defective Fc receptor-mediated phagocytosis when cultured in vitro over a 24-h period (18). Finally, the C3H/HeJ LPS gene defect also appears to affect in vivo natural resistance to *Salmonella* infections (10, 13, 19). By contrast, C3H/HeJ mice display a markedly enhanced bacterial phagocytosis, which does not seem to be due to different background levels of macrophage activation depending on environmental factors (4), since the two mouse strains were kept in the same room for 5 weeks before the experiments, and the experiments were always performed in parallel. Moreover, the initial phagocytic activities of C3H/HeJ and C3H/HeN mice were identical in our experiments.

The rate of phagocytosis of *S. aureus* by resident macrophages was the same for both substrains during the first 120 min. After this, C3H/HeJ macrophages ingested twice as many bacteria. A similar increase was shown by C3H/HeJ proteose peptone-induced macrophages against both *S. aureus* and *E. coli* after 60 min.

The only situation in which C3H/HeJ macrophages did not display an enhanced phagocytic activity was when unstimulated peritoneal resident macrophages were tested against *E. coli*. Here both strains displayed identical phagocytosis patterns throughout the test.

FIG. 3. Phagocytosis of *E. coli* by proteose peptone-induced macrophages from LPS-deficient C3H/HeJ mice (dashed line) and normal C3H/HeN mice (solid line). Each point represents the mean $\pm$ 1 standard deviation of triplicate determinations.

FIG. 4. Phagocytosis of *S. aureus* by proteose peptone-induced macrophages from LPS-deficient C3H/HeJ mice (dashed line) and normal C3H/HeN mice (solid line). Each point represents the mean $\pm$ 1 standard deviation of triplicate determinations.

There is no obvious reason for this. It may depend on some bacterium-macrophage factors unrelated to LPS and LPS gene-coded unresponsiveness. Alternatively, the enhanced activity which began during the test period may require a multistage activation sequence, as has been shown for other C3H/HeJ macrophage functions (15). *S. aureus* alone can turn on this greater activity in unstimulated macrophages after a 2-h incubation.

Protease peptone-induced macrophages, on the other hand, have already received all of the preparatory signals, and their enhanced activity appears earlier. These signals, however, cannot be provided to unstimulated macrophages by *E. coli*, since they are LPS defective. Even so, the induction of suppressive mechanisms by LPS-producing *E. coli* capable of blocking unstimulated macrophages only cannot be ruled out.

In these studies, it was assumed that the radioactivity associated with plastic adherent macrophage-like cells depends mainly on phagocytized bacteria. This assumption and the primary role of macrophage-like cells are clearly confirmed by our previous data obtained in parallel with electron microscopy studies (5; Carlone et al., in press; Cuffini et al., in press).

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