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An In Vitro System to Study Listericidal Capacity of Macrophages from Separate Mice: Resident Macrophages Exhibit Different Activation Patterns

DORIS OTTENDORFER,* DIETER BITTER-SUERMANN, AND ULRICH HADDING

Institut für Medizinische Mikrobiologie, Johannes Gutenberg-Universität, D-6500 Mainz, Federal Republic of Germany

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An in vitro system with macrophages from individual mice was established to study their listericidal capacity. Because no antibiotics were used, bacterial killing was really due to macrophages in short-term culture. To restrict the extracellular growth of bacteria, cell culture medium was changed at 1-h intervals. We demonstrated that intracellular growth of listeria in macrophage pools from untreated animals varies considerably. Obviously, preactivated macrophages are constantly present, so that the common procedure of using macrophage pools from several animals is no longer acceptable. In addition, we demonstrated that in vitro mixtures of listeria-immune macrophages of one animal with cells from untreated animals at different ratios exhibit enhanced bacterial killing above a mere additive effect. Consequently, by using macrophages from individual untreated mice, we found that cells of different animals exhibited various activation stages, although unstimulated, inbred specific-pathogen-free mice of the same age, weight, and sex were used. When equal numbers of macrophages from untreated separate animals were mixed in vitro, intracellular growth of listeria was only moderate; that is, the number of preactivated macrophages of the individual animals determined listerial growth in the pooled preparation. Furthermore, we showed that identical doses of phorbol myristate acetate exerted different effects on the listericidal activities of macrophages as a function of their preactivation states. These experiments clearly demonstrate the advantage of using macrophages from individual mice for in vitro studies of macrophage activation.

Immunity to infection with facultative intracellular bacteria (listeria, brucella, mycobacteria) depends on the synergistic interaction of specific T cells and macrophages resulting in enhanced bactericidal activity of the latter (17). On stimulation with antigen, T lymphocytes produce a variety of lymphokines activating macrophages for increased antibacterial activity. The details of these mechanisms are not clear, although numerous attempts have been made to activate macrophages in vitro by using antigen-specific T cells or culture supernatants containing their secretion products (7, 22, 25).

In some of these studies, murine infection models with *Listeria monocytogenes* have been used to examine macrophage activation in vitro. For this purpose, many authors have developed in vitro systems to determine the bactericidal capacity of macrophages (5, 9). However, these various techniques revealed many technical difficulties resulting in conflicting data. In some of these studies it was not clear whether bactericidal activity was due to activated macrophages or it was the consequence of antibiotics used to restrict the extracellular growth of bacteria in the culture medium (8). In addition, it was suggested that secretory products of activated macrophages interfere with the correct determination of intracellular killing (24). Furthermore, some studies used Proteose Peptone-elicited macrophages as controls instead of untreated cells. A very important point is that all in vitro systems described in the literature used macrophage pools from several animals. However, by using this pooling procedure we found highly variable intracellular growth of listeria in unstimulated resident macrophages. These findings were obviously due to the presence of already activated macrophages in the cell pools.

Even when a specific-pathogen-free (SPF) inbred strain was used, the state of preactivation of their macrophages was unpredictable. To recognize the preactivation states of macrophages from individual animals, we were forced to develop an in vitro system with macrophages from separate animals. Furthermore, we investigated the influence of phorbol myristate acetate (PMA) on the intracellular multiplication of listeria in macrophages from individual animals.

MATERIALS AND METHODS

Mice. Female inbred C57BL/6 mice were used at 8 to 12 weeks of age. Specific pathogen-free animals were obtained from the Zentralinstitut für Versuchtstiere, Hannover, Federal Republic of Germany.

Tissue culture media and media for bacterial growth. Peritoneal exudate cells (PEC) were harvested with medium TC 199 (Boehringer GmbH, Mannheim, Federal Republic of Germany) supplemented with 1% glutamine (200 mM; Boehringer). Listeria were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). Viable intracellular bacteria were determined by plate counts of macrophage lysates appropriately diluted in phosphate-buffered saline (PBS).

Bacteria and bacterial antigens. *L. monocytogenes* (strain EGD) was used in all experiments as the target organism. The strain was obtained from Stefan H. E. Kaufmann, Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany, and its virulence was maintained by continuous passage in mice (17). For monthly passage, C57BL/6 mice received 1×10^5 to 2×10^5 viable listeria (50% lethal dose, 9×10^5 listeria) by intraperitoneal injection.

* Corresponding author.

Three days later, bacteria recovered from the spleen of an infected mouse were cultivated overnight on blood agar plates followed by a 4-h culture in 4 ml of tryptic soy broth. Samples (200 μl) of this broth (10^7 bacteria per ml) were stored frozen at −70°C. For all experiments, 200 μl of this stock broth was freshly inoculated in 4 ml of tryptic soy broth, and 4 h later these listeria were used to infect macrophage cultures.

Viable bacteria were determined by plating 0.1 ml of serial 1:10 dilutions on soft agar plates. Colony forming units (CFU) were determined after incubation at 37°C for 18 to 24 h.

Heat-killed listeria which served as antigen were prepared by incubating 10^8 listeria per ml (suspended in PBS) at 62°C for 1.5 h (17). Heat-killed listeria were stored frozen in 1-ml samples at −70°C.

Collection and cultivation of macrophages from individual animals. To examine the intracellular multiplication of listeria in nonstimulated macrophages in vitro, we only used macrophages harvested from individual animals. Nonstimulated animals yielded between 1.5×10^6 and 4×10^6 cells, of which 39 to 44% were identified as macrophages by staining with nonspecific esterase as previously described (27). Cells of each animal were counted in a hemacytometer by microscopy. Thereafter, cell suspensions were centrifuged at 250 × *g* for 12 min and adjusted to 2×10^6/ml. A total of 10^6 cells were allowed to adhere to tissue culture plates (24 wells, 1.6 cm in diameter; Costar, Cambridge, Mass.) for 2 h in a 7% CO_2 atmosphere at 37°C. The resulting cell monolayers were washed three times with 500 μl of PBS, supplied with 500 μl of serum-free TC 199, and used immediately for bactericidal assay. Of the adherent cells, 85 to 90% were identified as macrophages by microscopic examination after differential staining (Hemacolor rapid blood smear; E. Merck AG, Darmstadt, Federal Republic of Germany). Viability of cells was determined by their ability to exclude 0.1% trypan blue (Merck). Viability was always >95%. Because PEC of each sample were adjusted to the same cell number (2×10^6 cells per ml), all cultures displayed confluent cell monolayers with equal amounts of macrophages with only small variations.

Infection of macrophage cultures with listeria and determination of bactericidal activity of macrophages. A 200-μl sample of the stock broth containing 10^7 listeria was inoculated in 4 ml of tryptic soy broth; 4 h later, listeria were washed twice with PBS (1 ml) and suspended in 1 ml of PBS. Because of the constant incubation time of 4 h, listeria concentrations always ranged from 3×10^8 to 6×10^8/ml. The washed listeria suspension (1 ml) was diluted to 1:100 with sterile PBS, and 50 μl of this dilution was added to 10^6 macrophages, resulting in an infectivity ratio of 1×10^5 to 3×10^5 listeria per 10^6 macrophages. Phagocytosis was allowed to proceed for 30 min. Cells were then washed three times with PBS, supplied with medium TC 199, and incubated for an additional 6 h. The starting point of this incubation represented $t = 0$ h (zero time). No antibiotics were added to macrophage cultures. Instead, the culture medium was changed at 1-h intervals as described by Harrington-Fowler and Henson (13). CFU in washing fluids at $t = 6$ h did not exceed 8 to 10% of the viable intracellular bacteria count. Viable intracellular bacteria at $t = 0$ and $t = 6$ h were determined by plate counts of macrophages lysed with sterile distilled water. The cell lysate was then appropriately diluted with sterile PBS and mixed with nutrient agar. In each figure, CFU values at $t = 6$ h represent the mean ± the standard deviation of triplicate determinations performed with one macrophage culture. Typically, two cultures could be obtained from one mouse. Sometimes there were sufficient macrophages for three cultures from one animal. Thus, base-line levels of intracellular listeria (CFU at $t = 0$) in each figure represent the means ± the standard deviations of two or three separate cultures from different animals. To ensure that inhibition of bacterial multiplication was due to macrophages and not to cell loss during the repeated changes of culture medium, macrophage protein was measured as described by Bradford (4). The method of protein determination is well accepted as a good control for measuring cell loss (26). Nevertheless, in some experiments we compared the cell number at $t = 0$ and $t = 6$ h by counting cells by microscopy under oil immersion after staining (Hemacolor rapid blood smear; Merck). We found that the percentage of cell loss determined by this technique was in good agreement with the protein determination. Protein content of monolayers was reduced by about 20% at the end of the experiments.

In vitro mixture of resident macrophages. To examine the intracellular growth of listeria in macrophages from pooled PEC, four separate macrophage pools, each with cells from the same five animals, were prepared. The peritoneal exudates were obtained from nonstimulated C57BL/6 mice. Each cell pool was divided into five cultures with a total of 2×10^6 cells each. Cells were allowed to adhere to plastic dishes (3.5-cm diameter; Nunc, Wiesbaden, Federal Republic of Germany) for 2 h, and then nonadherent cells were removed by vigorous washing with PBS. The resulting cell monolayers were then infected with listeria (10^5/100 μl), washed, and incubated for 8 h. Viable intracellular listeria expressed as CFU were determined at 0, 2, 4, 6, and 8 h.

In vitro mixture of resident macrophages and macrophages from listeria-immunized mice. Activated macrophages were produced as described by Harrington-Fowler and Henson (13). C57BL/6 mice were first immunized by intraperitoneal injection of a sublethal dose of 2×10^5 to 3×10^5 viable listeria, and 7 days later, cells were elicited by intraperitoneal injection of 10^7 heat-killed listeria as antigen. Macrophages were harvested 14 h after injection of the eliciting antigen. The mixtures of resident and activated macrophages (2×10^6 cells per culture) were allowed to adhere to plastic dishes (35-mm diameter; Nunc) for 2 h and were then washed to remove nonadherent cells. After proper incubation, the bactericidal activities of the different cell mixtures were examined as described above.

Activation of macrophages with PMA. PMA was obtained from Sigma Chemical Co., München, Federal Republic of Germany. A stock solution of 1 mg/ml in dimethyl sulfoxide was prepared, and 10-μl samples of this solution were stored frozen at −70°C. Macrophage cultures (10^6/500 μl) from individual animals were stimulated after phagocytosis of listeria (i.e., at $t = 0$) with 100 nM PMA (i.e., 62 ng) for 1 h. The cultures were then washed and incubated with TC 199 medium for 5 h. One macrophage culture from each animal was not treated with PMA and served as a control.

Cytotoxic antibody and complement. To exclude the modulating role of T cells in the in vitro mixing experiments with resident and activated macrophages, we used a monoclonal theta 1.2 antibody as a hybridoma supernatant of clone HO 13-4 (19). This antibody was kindly supplied by A. Reske, Institut für Immunologie, Mainz, Federal Republic of Germany. The final concentration of the cytotoxic antibody in the different cell mixtures was 1:30. The complement source used in this study was fresh guinea pig serum which had been absorbed before use with mouse spleen cells for 30 min at 0°C to neutralize cross-reacting antibodies. For killing T

cells in combination with cytotoxic antibody complement was used at a final concentration of 1:10.

RESULTS

Intracellular growth of listeria in four macrophage pools from five animals. Different intracellular listeria growth in resident macrophages was demonstrated by using PEC of four separate macrophage pools, each with cells from the same mice. All cells were harvested from nonstimulated SPF C57BL/6 mice. The peritoneal exudates of groups of five animals each were pooled and then adjusted to 10^6 cells per ml. Then, from a given cell pool five cultures were performed with a total of 2×10^6 cells. After infection of monolayers with listeria and washing, macrophage cultures were incubated for 8 h. Viable intracellular listeria were determined at 0, 2, 4, 6, and 8 h expressed as CFU (Fig. 1). Macrophages of pool 3 inhibited the intracellular growth of listeria, although cells were obtained from nonstimulated SPF mice. Bacterial numbers expressed as CFU at $t = 0$ were 9,400 ± 140 CFU; 6 h later, viable intracellular listeria reached only 9,600 ± 1,440 CFU. In contrast, macrophages from pools 1, 2, and 4 were not able to restrict the intracellular growth of listeria. Because of the bacteriostatic activity of macrophages from pool 3, we assumed the presence of preactivated macrophages in the peritoneal exudate of one or more animals. These obviously preactivated macrophages could influence the other resident cells in a still unknown way to restrict intracellular growth of listeria. Because no reproducible intracellular listerial growth could be obtained by using macrophage pools, we were forced to examine only macrophages from individual animals. This indeed should exclude the risk of not recognizing preactivated macrophages leading to a useless control in experiments designed to determine the effect of an external stimulus.

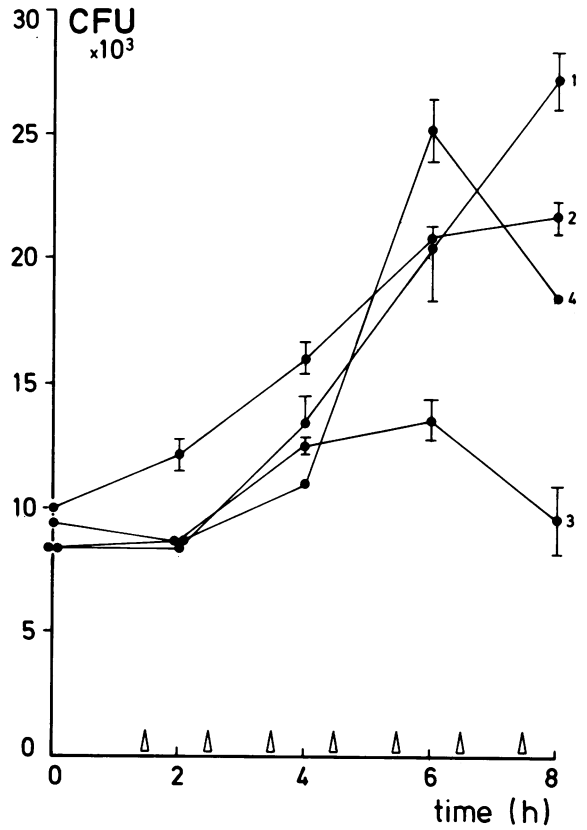

FIG. 1. Intracellular growth of *L. monocytogenes* in four separate macrophage pools. PEC of five animals were pooled, and intracellular growth of listeria was determined. CFU were measured at 0, 2, 4, 6, and 8 h. Arrows indicate the changes of cell culture medium to restrict the growth of extracellular listeria.

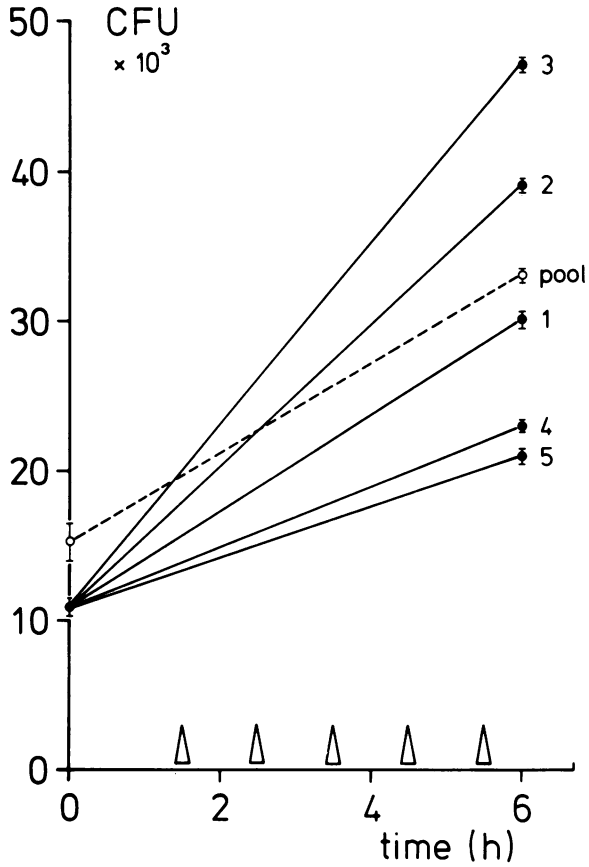

FIG. 2. Intracellular growth of listeria in macrophages from five individual C57BL/6 mice (numbers 1 to 5) compared with that in a pool of macrophages from the same animals. PEC of each mouse were prepared, washed, and adjusted to 2×10^6 cells per ml. An additional experiment was performed with pooled macrophages from all five animals. With PEC of each animal and with pooled macrophages, two cultures were set up with 10^6 cells each. Viable intracellular listeria were determined at $t = 0$ and $t = 6$ h.

Intracellular growth of listeria in macrophages from different mice compared with listerial growth in pooled PEC. The next series of experiments was conducted with peritoneal exudate macrophages harvested from individual nonstimulated C57BL/6 mice to investigate the fate of intracellular listeria. With peritoneal macrophages of one animal, two cell cultures were set up with 10^6 cells each. One culture served for determination of viable intracellular listeria at $t = 0$; the other served for determination of CFU 6 h later. In addition, PEC of the same animals were also pooled. Figure 2 shows the intracellular growth of listeria in macrophages from five individual animals compared with intracellular growth in the same pooled macrophage preparation. It could be seen that macrophages from different inbred mice varied greatly in their abilities to restrict the intracellular growth of listeria. Although prepared from nonstimulated SPF mice of

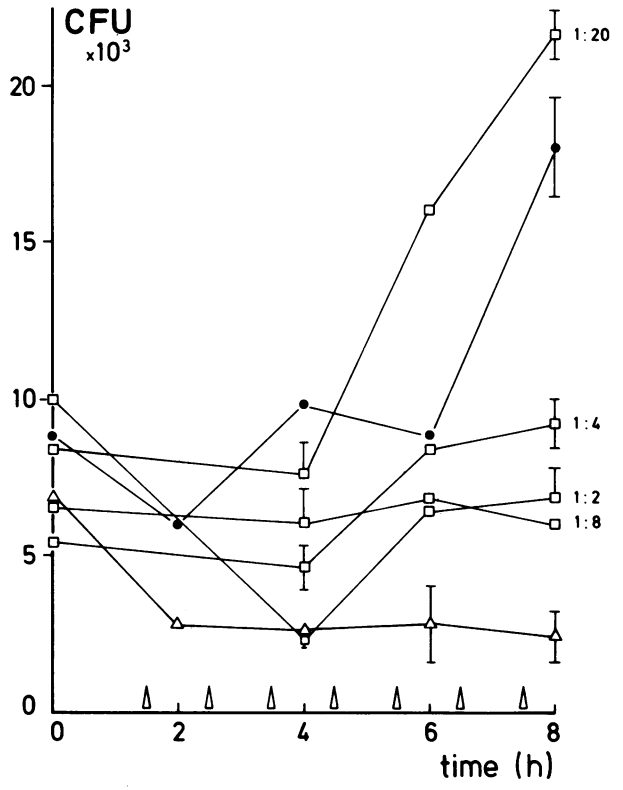

FIG. 3. Influence of activated macrophages on resident macrophages with regard to the intracellular growth of listeria. Listeria-immune (△) and resident (●) macrophages were mixed in vitro in concentrations ranging from 1:2 to 1:20. CFU were determined at 0, 2, 4, 6, and 8 h. □, Mixtures of immune and resident macrophages.

the same age and sex as the other mice, macrophages from animals 4 and 5 were able to inhibit the intracellular growth of listeria. In contrast, listeria multiplied well in macrophages from animal 2 and 3. It became apparent from these results that there are critical functional differences in so-called resident macrophages with regard to the intracellular growth of listeria. Listerial growth in pooled macrophages was only moderate compared with macrophages from, e.g., animal 3. Thus, the advantage of using macrophages from individual animals was clearly demonstrated. Concerning intracellular listerial growth in pooled macrophages, we speculated that, in particular, preactivated macrophages from animals 4 and 5 would have influenced the final CFU value seen with pooled cells. Next we examined the possibility that listeria-activated macrophages would have an influence on the intracellular growth of listeria in resident macrophages.

Influence of macrophages from listeria-immune mice on resident macrophages in vitro. In the next set of experiments, we examined the quantitative aspects of the fact that resident macrophages could be stimulated to restrict intracellular growth of listeria by the presence of listeria-activated macrophages. As described above, activated macrophages were obtained from listeria-immune mice 14 h after challenge with antigen. Washed monolayers contained 95 to 97% macrophages as judged by morphology and their capacity to phagocytose heat-killed listeria. A small number of polymorphonuclear leukocytes were present in the macrophage cultures. It is conceivable that soluble factors released from polymorphonuclear leukocytes influenced our results. However, polymorphonuclear leukocyte contamination was estimated to be less than 3%, so this possibility seems unlikely. Listeria-immune macrophages were mixed in various proportions with cells from nonstimulated mice. Thereafter, the bactericidal capacity of these mixtures was examined (Fig. 3). The addition of listeria-immune macrophages to resident macrophages in vitro in concentrations varying from 1:8 to 1:2 clearly reduced intracellular growth of listeria compared with resident macrophages alone. This result demonstrates why it is very difficult to obtain reproducible controls by using pooled PEC. To exclude a role for T cells or T cell-secreted lymphokines on the observed effects, we used a cytotoxic T cell-specific antibody and complement to eliminate T cells which could have escaped the washing procedures. Before using the cytotoxic antibody for the mixing experiments, we investigated its killing capacity with pooled PEC from normal animals. Compared with non-antibody-treated control cells, complement-mediated lysis with monoclonal anti-theta antibody was 49%, which represents an almost complete depletion of T cells, which constitute about 50 to 52% of the PEC as previously described (6; 28). Furthermore, the antibody killed > 98% thymocytes, which express a high amount of theta antigen. The different cell mixtures of resident and listeria-immune macrophages were treated with antibody after adherence and washing, when most of the nonadherent cells were already washed off. After treatment, a sample was stained (Hemacolor rapid blood smear; Merck) and examined microscopically. No cells with lymphocyte morphology could be seen in the adherent monolayers. After the cell cultures had been treated in this way, they were washed again and then infected with listeria as usual. This precaution to exclude T cells had no effect. The data were identical with those shown in Fig. 3.

Macrophages from nonstimulated inbred SPF mice exhibited variable activation states with regard to the intracellular multiplication of listeria (Fig. 2). We therefore investigated the influence of PMA, a well-known macrophage activator. It could be possible that PMA would exert different effects on macrophages, depending on their preactivation states.

Influence of PMA on the intracellular growth of listeria in resident macrophages harvested from individual animals. The influence of PMA on the listericidal activity of macrophages is shown in Fig. 4. Macrophages were harvested from six individual C57BL/6 mice. PEC from each mouse yielded two cultures, which were treated as follows. Both macrophage cultures served for determination of viable intracellular listeria at $t = 6$ h. One of the 6-h cultures was stimulated after phagocytosis (i.e., at $t = 0$) with 100 nm PMA; the other one remained untreated as a control. The CFU value at zero time shown in Fig. 4 is the mean ± the standard deviation determined with four separate cultures, two from animal 6 and two from animal 2. In Fig. 4, cells of individual animals are indicated by corresponding numbers. As suspected, macrophages from the different animals exhibited quite distinct reactions in response to PMA, depending on their preactivation states. Macrophages from animal no. 2 exhibited a 50% reduction of listerial growth on stimulation with PMA compared with the untreated control (100%). In contrast, cells from animal 6 were really resident, but the CFU value in the PMA-treated culture was only slightly reduced. Of 45 animals used for PMA experiments, the macrophages of 6 did not respond to PMA, although the controls were really resident. Macrophages from animal 4 were not stimulated by PMA because its untreated control showed

listericidal activity. Macrophage populations from animals number 1, 3, and 5, which allowed only moderate listerial growth, also exhibited different reactions on stimulation with PMA. Only macrophages from animals 3 and 5 responded with a reduction of bacterial growth. In contrast, PMA-stimulated macrophages of animal 1 did support intracellular listerial growth, even above the value of the untreated control. Thus, depending on their activation states, macrophages do respond quite differently to PMA.

DISCUSSION

The present report describes an in vitro system for investigating the listericidal activity for macrophages from individual mice. For demonstration of macrophage-mediated resistance to facultative intracellular bacteria in vitro, several methods have been established (2, 13, 14). A central problem concerning these systems is how to control extracellular bacterial multiplication for evaluation of true intracellular bacteria. For this purpose, antibiotics have usually been used to inhibit extracellular multiplication of bacteria. In this case, careful examination is necessary to ensure that antibiotics are not internalized by macrophages, leading to antibiotic intracellular killing as previously described (3, 8). In addition, macrophage functions could be modulated by antibiotics (10, 12). Similarily, our studies have shown that penicillin in concentrations ranging from 0.3 to 2.4 μg/ml kills listeria intracellularly (data not shown); therefore, we avoid the use of antibiotics. Owing to repeated changes of tissue culture medium, as described by Harrington-Fowler (13), during the test period, we were able to restrict extracellular bacterial growth without using antibiotics. Concerning our test system, the next important problem was the fact that macrophage pools from several animals led to extremely variable results with regard to the intracellular growth of listeria (Fig. 1). In quite a number of experiments, we found macrophage preparations able to kill listeria intracellularly although unstimulated inbred mice were used which were usually thought to yield resident macrophages. A possible explanation for these findings might be that the peritoneal exudates contained a small portion of preactivated cells which might have stimulated the other resident macrophages for enhanced listericidal activity. Perhaps these preactivated cells resulted from an inapparent infection with bacteria or viruses in some animals. Consequently, we used only macrophages from individual animals (Fig. 2). Surprisingly, intracellular growth of listeria in these macrophages was again quite different. Although it has become increasingly evident that macrophages may exist in a variety of activation stages (15, 20), the observed differences did not agree with (i) the identical genetic background of the animals; (ii) the same age, weight, and sex of the animals; and (iii) the identical treatment of the animals before cell harvesting (SPF breeding, food, no eliciting agent). A report by Harrington-Fowler and Wilder (14), in which it is documented that pooled peritoneal macrophages of nonstimulated animals contain subpopulations with different antilisterial activities, points in the same direction. They separated mouse peritoneal macrophages on density gradients and obtained macrophage populations with different capacities for controlling intracellular growth of listeria.

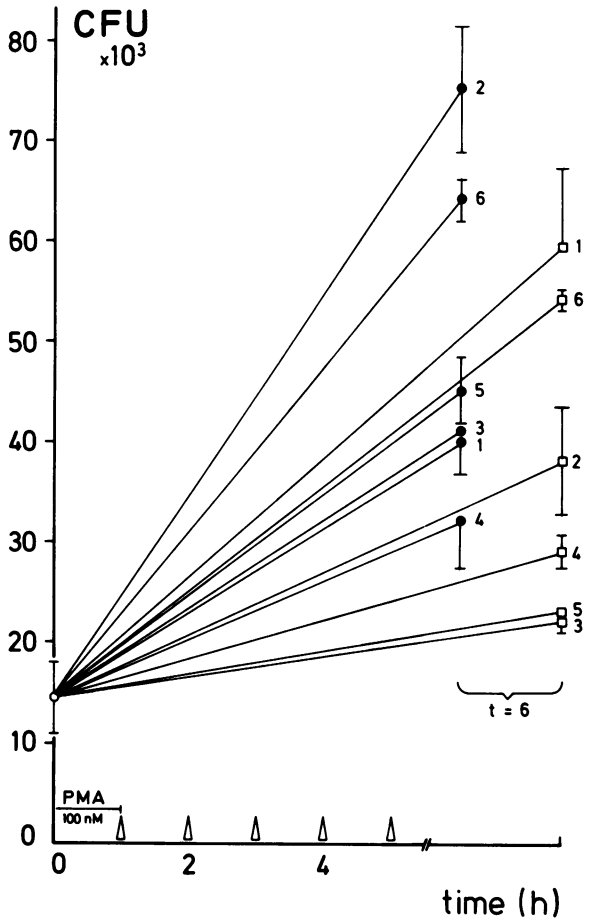

FIG. 4. Influence of PMA on intracellular multiplication of listeria in resident macrophages harvested from six separate C57BL/6 mice (numbers 1 to 6). Peritoneal exudate macrophages from individual inbred mice were prepared. Cells of each mouse yielded two macrophage cultures (10^6 cells per 500 μl each). Both cultures served for determination of viable intracellular listeria at t = 6 h. One of these two cultures was stimulated after phagocytosis of listeria (at t = 0) with 100 nM PMA; the other culture served as a control. CFU at t = 0 were determined with four separate cultures of two different animals (mean ± the standard deviation). Cells from individual animals are indicated by corresponding numbers. ●, Controls; □, PMA-stimulated cells.

We confirm their results by our experiment shown in Fig. 2. If pooled peritoneal exudate macrophages had been used, only moderate listerial growth would have been measured, so that the functional state of macrophages from, e.g., animal 3 could not be recognized.

By mixing in vitro macrophages from listeria-immune mice with cells from untreated mice, we assessed the quantitative aspect of having preactivated cells in a pooled cell suspension. We could demonstrate that activated macrophages from listeria-immune mice were able to stimulate resident macrophages for enhanced listericidal activity. This could occur via release of monokines by activated macrophages. On the other hand, lymphokines released by the small percentage of listeria-specific T cells in our macrophage cultures could also activate resident macrophages. This possibility was excluded by killing the few T cells remaining in culture after washing. In addition, stimulation of resident macrophages by lymphokines requires a 6- to 8-h period (11). This time span, however, is too long to play a critical role in our test system. By stimulating macrophages from individual animals with PMA, we again observed functional differences in the intracellular growth of listeria

(Fig. 4). PMA was able to activate macrophages for enhanced listericidal activity only when the cells were really in the nonactivated state. In contrast, PMA treatment of preactivated macrophages led to no reduction in the intracellular growth of listeria, and sometimes intracellular growth was even enhanced, confirming previous data on the intracellular killing of *Mycobacterium microti* by lymphokine-activated and PMA-stimulated murine macrophages (29). Our observations that macrophage populations showed functional heterogeneity is confirmed by many recent reports in the literature. Density gradient separation leads to populations of macrophages heterogeneous in a variety of activities and membrane markers, including the synthesis of complement components (21, 31), expression of complement receptors (30), phagocytic activity (23), and induction of Ia molecules (1). Recent reports that resident and elicited macrophages differ in their capacities to respond to lymphokines are equally relevant (18). Moreover, intracellular killing by elicited murine macrophages of *Leishmania tropica* amastigotes was markedly reduced on stimulation with lymphokines compared with resident cells (16). The failure of inflammatory macrophages to respond to lymphokines parallels our observations with preactivated cells and PMA as a stimulus. Very recently we have shown (U. Mauer-Gross, D. von Steldern, U. Aadding, D. Bitter-Suermann, and R. Burger, Immunology, in press) that distinct activation states of guinea pig macrophages could be recognized by monoclonal antibodies. Some of these antibodies do not bind to normal or elicited (mineral oil) macrophages. However, in vitro stimulation of normal guinea pig macrophages with PMA results in macrophage populations recognized by monoclonal antibodies. We suggested that PMA initiated the maturation of macrophages in vitro, leading to the expression of membrane-bound antigens only expressed by activated macrophages. The common property of all of these observations is, however, that elicited macrophages or other cells behave quite differently compared with resident cells, as we could confirm with PMA as a stimulus.

In conclusion, we were able to define distinct activation states of resident macrophages by using only macrophages from separate animals to determine their listericidal capacity. According to our mixing experiments with macrophages from individual animals and the nonreproducible growth of listeria in pooled macrophage preparations, the use of pooled cultures is not recommended. By using macrophages from individual animals, we confirmed other reports of distinct activation states of resident macrophages and were able to show that a potent macrophage stimulator, e.g., PMA, exerts different effects depending on the preactivation states of the macrophages under investigation.

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