INFECTION AND IMMUNITY, Nov. 1985, p. 425–430
0019-9567/85/110425-06$02.00/0

Vol. 50, No. 2

Phagocytes from Flora-Defined and Germfree Athymic Nude Mice Do Not Demonstrate Enhanced Antibacterial Activity

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Received 6 May 1985/Accepted 6 August 1985

In this study we directly compared the in vitro antibacterial activities of resident and inflammatory phagocytes obtained from athymic (*nu/nu*) and euthymic (*nu/+*) mice. Resident peritoneal macrophages obtained from flora-defined *nu/nu* and *nu/+* mice both demonstrated little ability to restrict the growth of *Listeria monocytogenes* in vitro. Inflammatory peritoneal neutrophils and macrophages obtained from flora-defined *nu/nu* and *nu/+* mice did not differ in their ability to kill *L. monocytogenes* in vitro. Likewise, inflammatory peritoneal neutrophils obtained from germfree *nu/nu* and *nu/+* mice killed equivalent numbers of listeria. In marked contrast, however, inflammatory macrophages obtained from germfree *nu/nu* and *nu/+* mice demonstrated very limited antilisteria activity in vitro. The reduced antilisteria activity of macrophages from germfree *nu/nu* and *nu/+* mice was associated neither with reduced phagocytosis of *L. monocytogenes* nor with an inability to generate an oxidative response. Unlike those of previous reports, these data suggest that resident and inflammatory phagocytes obtained from athymic mice that were maintained under conditions such that they were not subjected to underlying infections are not constitutively activated for enhanced antibacterial activity. Furthermore, these data suggest that stimulation of macrophages by products of the bacterial flora is required for the expression of macrophage antibacterial activity.

Despite their thymic dysgenesis, which makes them unable to express many biological responses that require mature T lymphocytes, athymic (*nu/nu*) mice are better able than are euthymic (*nu/+*) mice to restrict the growth of the facultative intracellular pathogen *Listeria monocytogenes* in the spleen and liver during the early phase of listerial infection (3, 6, 15). This anomalous enhanced antilisteria resistance has been proposed to be mediated by constitutive activation of macrophage antibacterial activity in *nu/nu* mice. Attempts to demonstrate directly enhanced listerial killing by *nu/nu* mouse macrophages, however, have yielded conflicting results (3, 15, 24). In the present study we reexamined this question and found that resident macrophages from flora-defined athymic (*nu/nu*) and euthymic littermate (*nu/+*) BALB/c mice both lacked the ability to kill *L. monocytogenes* in vitro. In contrast, inflammatory neutrophils obtained from flora-defined and germfree *nu/nu* and *nu/+* mice, as well as inflammatory macrophages obtained from flora-defined *nu/nu* and *nu/+* mice, all killed *L. monocytogenes* in vitro. Inflammatory macrophages obtained from germfree *nu/nu* and *nu/+* mice, however, had little antilisteria activity in vitro. These results indicate that neutrophils and macrophages obtained from nude mice are not activated constitutively for enhanced bacterial killing. Furthermore, it appears that the generation of antibacterial mononuclear phagocytes does not require the presence of a mature thymus but does require stimulation by bacterial products of the normal flora.

MATERIALS AND METHODS

Mice. Flora-defined and germfree athymic (*nu/nu*) and heterozygous littermate (*nu/+*) BABL/c mice were bred and maintained in flexible plastic isolators at the University of Wisconsin gnotobiotic laboratory. All mice were fed sterile water and laboratory chow. The flora-defined mice were associated with a previously described microbial flora consisting of nine species of gram-positive bacteria, and few, if any, gram-negative bacteria present (1). These mice were tested serologically by enzyme-linked immunosorbent assay and were found to be free of adventitious viral agents, including Sendai virus and mouse hepatitis virus.

Bacteria. *L. monocytogenes* EGD was passaged through mice to maintain its virulence, subcultured in tryptose phosphate broth, and stored as 0.5-ml portions at −70°C. Before each experiment a portion was quickly thawed and used to inoculate fresh tryptose phosphate broth. The listeria were incubated for 12 to 15 h at 37°C, recovered by centrifugation, and washed twice in cold Hanks balanced salt solution containing 0.25% bovine serum albumin (HBSA).

Recovery of peritoneal neutrophils and macrophages. Inflammatory peritoneal neutrophils and macrophages were elicited by injecting mice intraperitoneally (i.p.) with 1.0 ml of sterile 10% proteose peptone and harvesting the peritoneal exudate cells 4 and 48 h later, respectively. Resident and inflammatory peritoneal cells were harvested as described previously (5) by lavaging the peritoneal cavities twice with 8.0 ml of warm HBSA (Ca^{2+} and Mg^{2+} free, 0.01 M EDTA, 0.25% bovine serum albumin). The peritoneal exudate cells then were separated on Ficoll-Hypaque gradients into granulocyte-rich and mononuclear cell-rich populations. Viability was >95% as determined by exclusion of trypan blue. Microscopic examination of Diff-Quik-stained (American Scientific Products, McGraw Park, Ill.) cytospin smears revealed that the granulocyte and mononuclear cell fractions were >90% neutrophils and mononuclear cells, respectively.

Bactericidal assay. The bactericidal activity of resident and inflammatory phagocytes was determined as described pre-

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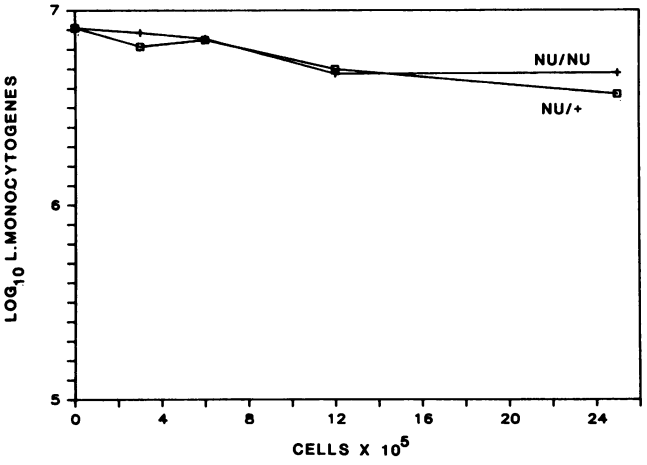


FIG. 1. Resident peritoneal macrophages from flora-defined *nu/nu* (+) and *nu/+* (□) BALB/c mice do not differ in their ability to kill *L. monocytogenes* in vitro. Results are expressed as the mean $\log_{10}$ number of viable listeria present after a 2-h incubation of 3×10^5 to 25×10^5 resident cells with 2.5×10^6 viable *L. monocytogenes* and 10% pooled normal BDF_1 mouse serum (two to three tubes for each data point). The $\log_{10}$ listeria indicated for zero cells represents the increased number of viable listeria present in cell-free control tubes at the end of the 2-h incubation period. Although not indicated, the standard error of the mean of all data points ranged from 0.01 to 0.03 on a $\log_{10}$ scale.

viously (5). Briefly, 3×10^5 to 50×10^5 neutrophils or macrophages were added to plastic tubes that contained 2.5×10^6 *L. monocytogenes* and 0.1 ml of pooled normal BDF_1 mouse serum in a total volume of 1.0 ml of HBSA. At the beginning of the incubation period and after rotation (8 rpm) at 37°C for 2 h, 0.1-ml samples were removed from each tube, diluted in sterile distilled water, and plated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) to determine the number of viable listeria. Results were expressed as the mean ± standard error of the mean $\log_{10}$ viable listeria.

Phagocytosis assay. Phagocytosis of *L. monocytogenes* was determined as described previously (5). Briefly, 2.5×10^6 macrophages were added to plastic tubes that contained 2.5×10^7 *L. monocytogenes* and 0.1 ml of pooled normal BDF_1 mouse serum in a total volume of 1.0 ml of HBSA. The tubes were rotated at 37°C for 30 min and then removed to an ice bath. The phagocytes were washed free of extracellular bacteria by three low-speed centrifugations ($100 \times g$ for 5 min at 4°C) and were resuspended in 1.0 ml of HBSA. The cell suspensions were used to prepare cytospin smears that were air dried and stained with Diff-Quik. At least 200 cells on each smear were examined by light microscopy (oil immersion objective) and scored for the percentage of phagocytes that contained listeria and for the average number of listeria per ingesting phagocyte. Results were expressed as the phagocytic index (percentage of phagocytes with ingested listeria × average number of listeria per ingesting phagocyte × 100).

Phagocytosis of *L. monocytogenes* was confirmed by electron microscopy. Reaction mixtures were treated as described above and then were fixed with modified Karnovsky fixative, stained with osmium tetroxide and uranyl acetate, and embedded in epoxy. The prepared thin-section grids were examined in a Philips 410 electron microscope, and representative areas were photographed. Phagocytosis was quantitated by examining at least 50 cells on each grid; results were expressed as the phagocytic index.

Release of superoxide anion. Macrophage oxidative activity was assessed by spectrophotometric determination (550 nm) of the reduction of cytochrome *c* by superoxide anion as described previously by Johnston (10). Suspensions of macrophages in phenol-red-free Hanks balanced salt solution (10^6/ml) were rotated in plastic tubes at 37°C for 90 min. Release of O_2^- was stimulated by phorbol myristic acetate (PMA; Sigma Chemical Co., St. Louis, Mo.; 0.5 μg/ml) or by opsonized *L. monocytogenes* (100 bacteria per phagocyte). Controls included unstimulated macrophages and PMA-stimulated macrophages to which was added superoxide dismutase (Sigma; 40 μg/ml). Results were expressed as nanomoles of cytochrome *c* reduced per 10^6 macrophages by using the molar extinction coefficient $\Delta E_{550} = 21.0 \times 10^3\ M^{-1}\ cm^{-1}$.

Statistical analysis. The data were analyzed for statistical significance by a one-way analysis of variance followed by Fisher's least significant difference test (22). The level of significance was set at $P < 0.05$.

RESULTS

Bactericidal activity of resident and inflammatory phagocytes from flora-defined nude mice. Because it has been suggested by others that macrophages from nude mice exhibit enhanced antibacterial activity against *L. monocytogenes*, we first compared the antilisteria activity of resident peritoneal macrophages from flora-defined *nu/nu* and *nu/+* mice. Neither cell population was able to significantly kill *L. monocytogenes* in vitro and exerted at best a slight inhibition of listerial growth (Fig. 1). We next asked whether inflammatory neutrophils and macrophages obtained from flora-defined *nu/nu* and *nu/+* mice would differ in their ability to kill listeria in vitro. Inflammatory neutrophils obtained from *nu/nu* and *nu/+* flora-defined mice demonstrated an equivalent ability, on a per cell basis, to kill *L. monocytogenes* in vitro (Fig. 2). Likewise, inflammatory macrophages obtained from *nu/nu* and *nu/+* flora-defined mice demonstrated simi-

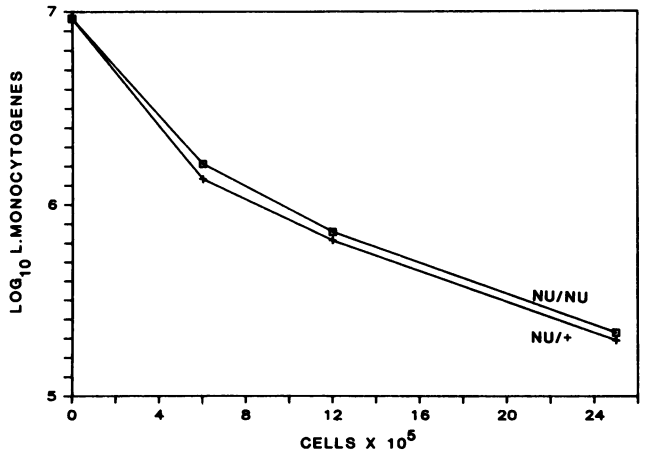


FIG. 2. Equivalent killing of *L. monocytogenes* by inflammatory neutrophils obtained from flora-defined *nu/nu* (□) and *nu/+* (+) mice 4 h after intraperitoneal injection of 1.0 ml of 10% sterile proteose peptone. Results are expressed as described in the legend to Fig. 1. Although not indicated, the standard error of the mean of all data points ranged from 0.02 to 0.10 on a $\log_{10}$ scale.

lar abilities to kill *L. monocytogenes* in vitro (Fig. 3), although at the 12×10^5 and 25×10^5 cell concentrations, killing of *L. monocytogenes* by inflammatory macrophages from *nu/+* mice was statistically better than was killing of *L. monocytogenes* by macrophages from *nu/nu* mice ($P < 0.05$).

Bactericidal activity of inflammatory neutrophils and macrophages obtained from germfree nude mice. It has been suggested previously that macrophages in nude mice are persistently activated because the animals are subjected to a chronic state of subclinical infection by various microorganisms present in their normal flora (3, 15, 24). We decided to examine the possible effects of microbial stimulation on nude mouse phagocyte function by determining the antilisteria activity of inflammatory neutrophils and macrophages obtained from germfree *nu/nu* and *nu/+* mice. Inflammatory neutrophils obtained from germfree *nu/nu* and *nu/+* mice were not significantly different in their listericidal activity (Fig. 4) and demonstrated an ability to kill listeria that was similar to that shown above for neutrophils obtained from flora-defined mice (Fig. 3). In marked contrast to the unimpaired killing of listeria by inflammatory neutrophils, inflammatory macrophages obtained from germfree *nu/nu* and *nu/+* mice only slightly inhibited the growth of *L. monocytogenes* in vitro.

Having observed impaired antilisteria activity by germfree *nu/nu* and *nu/+* macrophages, it was necessary to determine whether the observed killing deficiency was actually the result of impaired phagocytosis of *L. monocytogenes* by macrophages from germfree mice. Table 1 indicates that germfree *nu/nu*, germfree *nu/+*, and flora-defined *nu/+* mice phagocytosed equivalent numbers of listeria as determined by light and electron microscopy. The lower estimate of phagocytosis obtained by electron microscopy probably reflects the fewer cells that were available for counting and the difficulty in positively identifying intracellular bacteria that may be largely out of the plane in which the section was prepared. Phagocytosing macrophages from germfree *nu/nu*, germfree *nu/+*, and flora-defined *nu/+* mice exhibited similar morphologies (Fig. 5). These data therefore suggest that the

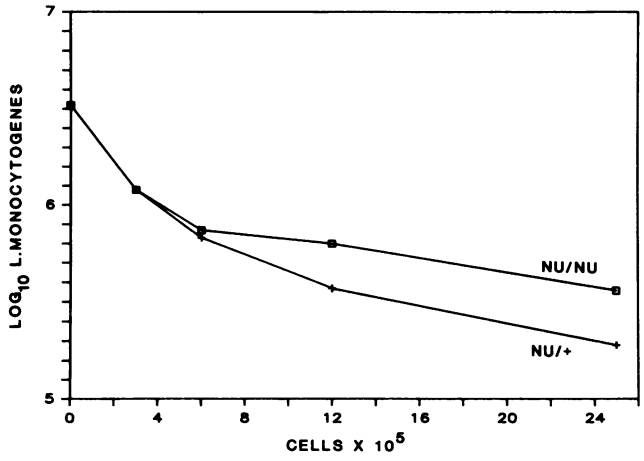


FIG. 3. Comparable killing of *L. monocytogenes* by inflammatory macrophages obtained from flora-defined *nu/nu* (□) and *nu/+* (+) mice 48 h after intraperitoneal injection of 1.0 ml of 10% sterile proteose peptone. Results are expressed as described in the legend to Fig. 1. Although not indicated, the standard error of the mean of all data points ranged from 0.01 to 0.09 on a $\log_{10}$ scale.

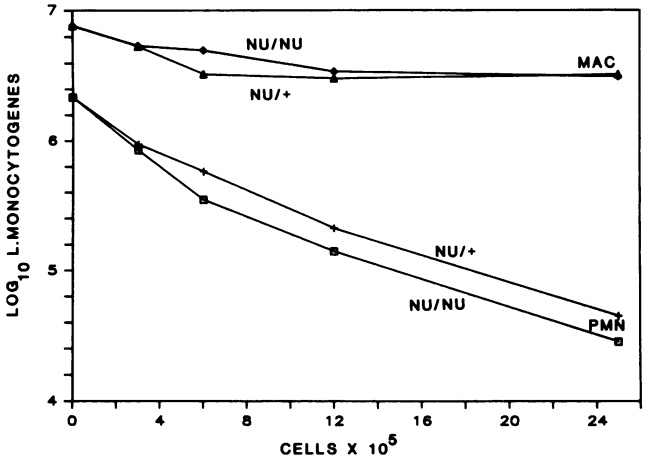


FIG. 4. Killing of *L. monocytogenes* by inflammatory neutrophils (PMN), but not by macrophages (MAC), obtained from germfree *nu/nu* (□, ◇) and *nu/+* (+, △) mice 4 and 48 h, respectively, after intraperitoneal injection of 1.0 ml of 10% sterile proteose peptone. Results are expressed as described in the legend to Fig. 1. Although not indicated, the standard error of the mean of all data points ranged from 0.01 to 0.10 and from 0.01 to 0.05 for the neutrophil and macrophage data, respectively.

weak antibacterial activity of germfree mouse macrophages is the result of decreased intracellular killing rather than deficient phagocytosis.

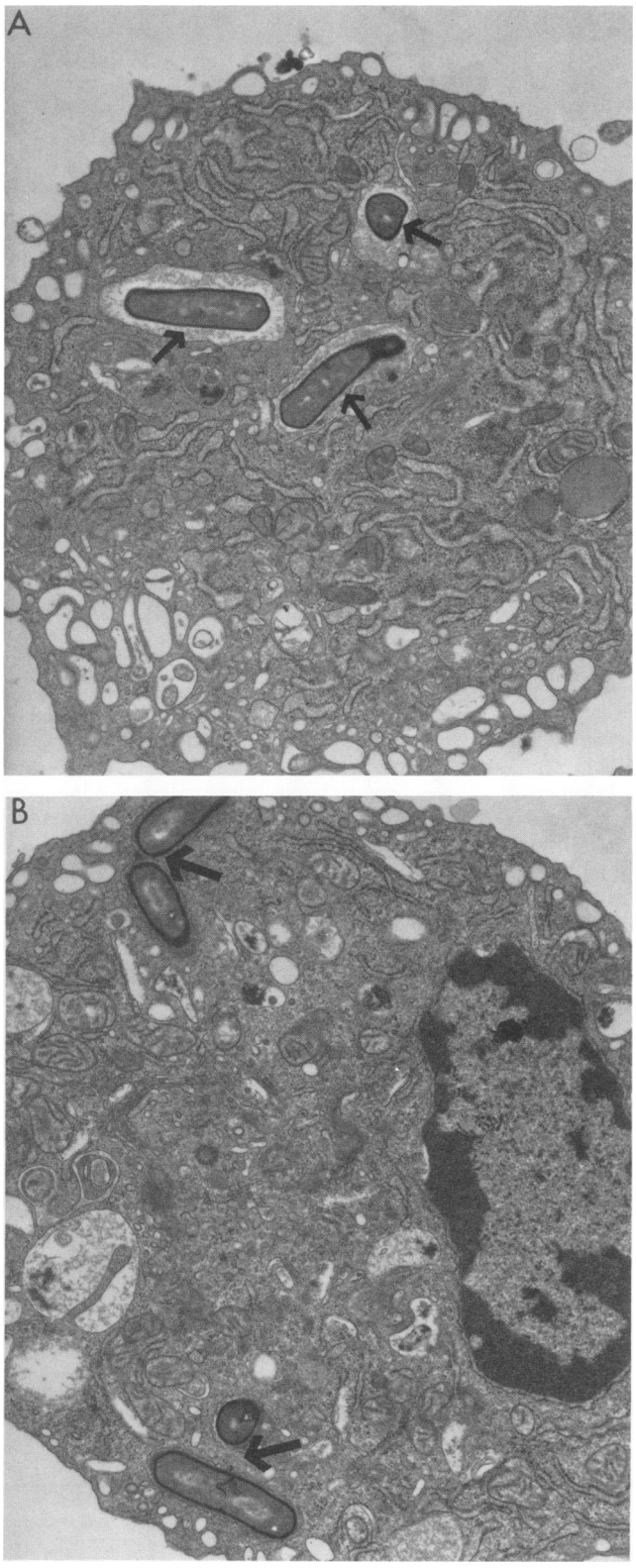
Production of reactive oxygen intermediates has been proposed as an important mechanism by which macrophages can kill tumor cells and microbes (12, 13). Because production of these oxygen products by mononuclear phagocytes in vitro has been shown to be exquisitely sensitive to the presence of microbial products (16), we next considered the possibility that the poor antibacterial activity of macrophages from germfree mice might be the result of inadequate stimulation of macrophages because of the absence of a microbial flora. We therefore compared the production of the reactive oxygen intermediate superoxide anion (O_2^-) by germfree *nu/nu*, germfree *nu/+*, and flora-defined *nu/+* macrophages. All three groups gave a significant O_2^- response to PMA ($P < 0.05$) but not to opsonized *L. monocytogens* ($P > 0.05$) as compared with unstimulated cells. The results indicated that there was no substantial difference ($P > 0.05$) in the abilities of these three groups of macrophages to release O_2^- in response to PMA stimulation (Table 2), thus

TABLE 1. Comparable phagocytosis of *L. monocytogenes* by inflammatory peritoneal macrophages obtained from germfree *nu/nu*, germfree *nu/+*, and flora-defined *nu/+* mice

Group	Phagocytic index	
	Light microscopy[a]	Electron microscopy[b]
Germfree *nu/nu*	1.187	404
Germfree *nu/+*	1.169	308
Flora-defined *nu/+*	1.131	340

[a] Estimated by light microscopic examination (oil immersion objective) of at least 200 cells on Diff-Quick-stained cytospin smears.

[b] Estimated by electron microscopic examination (magnification, ×9,100) of at least 50 cells on each grid.



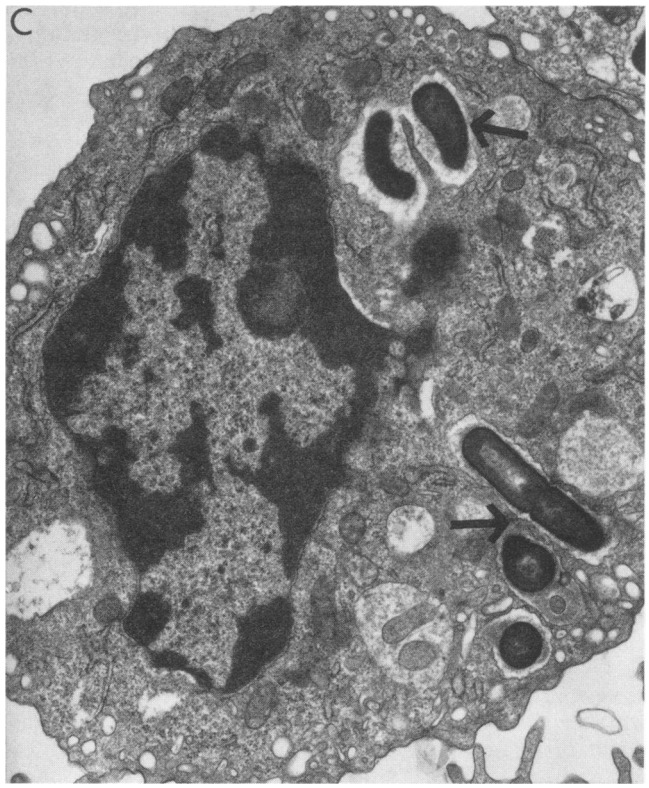


FIG. 5. Ingestion of *L. monocytogenes* (arrows) in vitro by inflammatory peritoneal macrophages obtained from germfree *nu/nu* mice (A), germfree *nu/+* mice (B), and flora-defined *nu/+* mice (C). Magnification, ×11,500.

suggesting that the poor antibacterial activity of germfree mouse macrophages that we observed was not related to an innate defect in their ability to generate an oxidative response.

DISCUSSION

The results of this study indicate that when nude mice are maintained in a flora-defined environment where they remain free of underlying infection, their macrophages do not exhibit increased bactericidal activity compared with macrophages from *nu/+* mice. This finding is in contrast to a previous report that suggested that macrophages in nude mice are constitutively activated to an enhanced microbicidal state (3). The short healthy life-span reported for the nude mice in that study, however, suggests that they may have been suffering from the wasting syndrome that plagues nude mice when they are maintained in conventional animal housing where they are exposed to various bacterial pathogens. In support of our observations, Nickol and Bonventre (15) also did not observe enhanced bactericidal activity by resident macrophages obtained from nude mice that had been maintained under conditions that minimized bacterial infections.

Because we observed similar killing of listeria by flora-defined and germfree *nu/nu* and *nu/+* neutrophils, the present study also suggests that inflammatory neutrophil bactericidal activity is not influenced markedly by the absence of either a mature thymus or an intestinal microflora. Likewise, the presence of a mature thymus is not required for mice to develop inflammatory macrophages capable of killing listeria in vitro; however, stimulation by products of the bacterial flora does appear to be necessary for the in vitro expression of antibacterial activity by inflammatory macrophages. This finding suggests that macrophage antibacterial activity may

TABLE 2. Comparable release of O_2^- by inflammatory peritoneal macrophages obtained from germfree *nu/nu*, germfree *nu/+*, and flora-defined *nu/+* mice

Stimulant	nmol of cytochrome *c* reduced/10^6 macrophages[a]		
	Germfree *nu/nu*	Germfree *nu/+*	Flora-defined *nu/+*
PMA (0.5 μg)	13.52 ± 3.42	13.83 ± 1.57	12.20 ± 1.61
PMA (0.5 μg) + superoxide dismutase (40 μg)	4.52 ± 1.59	2.72 ± 0.04	0.40 ± 0.10
Listeria[b]	4.26 ± 0.01	1.70 ± 0.78	1.44 ± 0.25

[a] Mean ± standard error of the mean for 10^6 cells in suspension at 37°C for 90 min.
[b] Opsonized *L. monocytogenes* at a 100:1 bacteria-to-phagocyte ratio.

be more dependent on stimulation by bacterial products than it is by T-cell factors. Alternatively, it may be that the low levels of T cells and their functions previously reported for nude mice both in vivo and in vitro (7, 8, 18) may be sufficient, in the presence of additional stimulation by products of the bacterial flora, to activate macrophage bactericidal activity. A stepwise activation sequence has been clearly demonstrated for macrophage killing of tumor cells. In that system, activation of macrophage tumoricidal activity requires an initial priming event, by mediators such as gamma interferon, that must be followed by a second signal that is usually bacterial lipopolysaccharide or some other bacterial product (17). In light of the data presented here, it seems likely that a similar sequence of events may regulate macrophage killing of bacteria. Because the flora-defined animals in this study possess a bacterial flora that contains few, if any, gram-negative bacteria, it is possible that microbial products other than lipopolysaccharide may be able to stimulate macrophage bactericidal activity. Precedent for this possibility has been set by other studies that showed that gram-positive bacteria, including *L. monocytogenes*, can serve as the second stimulus for activation of macrophage tumor cell killing (2).

Although this study does not support the concept of constitutive activation of macrophage bactericidal activity in nude mice, it does provide evidence for the positive influence of microbial stimulation on the expression of macrophage antibacterial activity. Because comparable numbers of listeria were phagocytosed by germfree and flora-defined mice (Table 1), it seems likely that the poor antibacterial activity of germfree macrophages reflects a reduction in intracellular killing rather than a defect in phagocytosis. Additional evidence has been presented indicating that the marked reduction of microbial products that occurs in the germfree state can compromise macrophage function and resistance to infection. Germfree euthymic mice are reported to have less resistance to listeria infection than do mice that contain a microbial flora (9). Other investigators, however, have reported that listeria infection in germfree and specific-pathogen-free athymic mice follows a similar time course and elicits a comparable histopathological response (23). This may indicate that the colonization of the gastrointestinal tract by *L. monocytogenes* that will occur after parenteral injection of listeria into germfree mice is a sufficient stimulus for induction of macrophage microbicidal activity. The previously reported nonspecific enhanced resistance of listeria-immunized athymic mice also may indicate stimulation of macrophage microbicidal activity by listerial products that were present during the initial listeria infection (14). In addition to the poor antibacterial activity of germfree mouse macrophages that we observed in this study, other investigators have reported that macrophages obtained from germfree mice have markedly reduced antitumor (11) and antiviral (19) activities in vitro.

We considered the possibility that a common mechanism to explain the reduced effector functions of macrophages from germfree mice could be decreased production and release of the reactive oxygen intermediates that have been suggested to be important for macrophage killing of both tumors and microbes (12, 13). We reasoned that because the production of reactive oxygen products by mononuclear phagocytes in vitro requires the presence of microbial products (16), the poor antibacterial activity of macrophages from germfree *nu/nu* and *nu/+* mice might reflect their reduced ability to form these microbicidal reactive oxygen intermediates. When we compared the abilities of PMA-stimulated inflammatory macrophages from germfree *nu/nu*, germfree *nu/+*, and flora-defined *nu/+* mice to release the microbicidal oxygen product superoxide anion (O_2^-), however, we found a comparable release of O_2^- from all three cell types. Thus, it appears that germfree mice, whose diet is not totally devoid of microbial products, receive sufficient microbial stimulation for their macrophages to release levels of O_2^- that are comparable to those of flora-defined mice and are consistent with previously reported values (10). Our observation that macrophages from athymic mice were able to release normal levels of O_2^- after PMA stimulation in vitro suggests that mature functional T cells may not be an essential prerequisite for the in vivo development of mononuclear phagocytes that are capable of responding with a vigorous oxidative burst to stimulating agents in vitro. Finally, these data suggest that the impaired killing of *L. monocytogenes* in vitro by macrophages from germfree *nu/nu* and *nu/+* mice may not be the result of an innate defect in the oxidative activity of germfree mouse macrophages. *L. monocytogenes* has been reported previously to be very susceptible to nonoxidative killing mechanisms such as cationic proteins (4, 20, 21). Perhaps differences in macrophage nonoxidative antibacterial activity account in part for the reduced antilisteria activity of inflammatory peritoneal macrophages obtained from germfree mice.

ACKNOWLEDGMENTS

We thank Joann Croft and her staff at the University of Wisconsin gnotobiotic laboratory for their assistance in maintaining the gnotobiotic mice. We also thank Renate Bromberg for doing the electron microscopy, and the School of Veterinary Medicine word processing personnel for the preparation of this manuscript.

This work was supported by Public Health Service grant AI-21343 from the National Institutes of Health and by funds from the University of Wisconsin Graduate School (135-6702).

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