INFECTION AND IMMUNITY, Dec. 1977, p. 636-645
Vol. 18, No. 3

Printed in U.S.A.

Anomalous High Native Resistance of Athymic Mice to Bacterial Pathogens

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Received for publication 23 June 1977

Congenitally athymic (nude) mice exhibited an anomalous high resistance against infections with the facultative intracellular parasite *Listeria monocytogenes* and other bacterial pathogens. Protection against lethal infection was demonstrated to result from the presence of naturally occurring activated macrophages in the reticuloendothelial organs of the nude mice. This was exemplified after intravenous challenge by enhanced bacterial clearance from the blood and augmented bacterial killing in the spleens and livers of nude mice as compared with immunologically competent control mice. Resident peritoneal macrophages of nude mice were not activated in terms of phagocytic, bactericidal, or tumoricidal potential. The development of activated fixed tissue macrophages appears to arise as a result of the T-lymphocyte deficiency since thymus implantation abrogated the enhanced resistance of nude mice. Antibiotic elimination of intestinal bacteria also modified resistance to bacterial infection, indicating a role of environmental factors on macrophage activation. Several possible mechanisms leading to macrophage activation and heightened resistance to infection in nude mice are offered.

The congenitally athymic (nude) mouse provides an excellent model for the study of immune responses in an immunologically compromised host. Nude (nu/nu) mice, due to embryological failure of thymus development (26), lack the mature T-lymphocyte component of the immune system (26). As a result, nude mice are capable of accepting foreign tissue grafts (26) as well as xenogenic tumors (27). Immunoglobulin responses to thymus-dependent antigens are reduced or absent (21), but antibody responses to thymus-independent antigens appear to be normal (14). Athymic mice also fail to develop delayed hypersensitivity to BCG (30).

Of interest is the effect of thymus deficiency on the course of bacterial infections. This is especially significant in infections caused by facultative intracellular parasites, which require competent specifically sensitized T-lymphocytes for induction of host macrophage activation and development of protective immunity (18). Whereas nude mice are highly susceptible to several naturally acquired bacterial and viral infections (9), recent evidence indicates that nude mice possess an unexpected heightened resistance to experimental infection with *Listeria monocytogenes* (5, 6, 33), *Brucella abortus* (5), *Salmonella typhimurium* (7), and *Candida albicans* (25); protective immunity against these agents requires development of cell-mediated immunity. Mice possessing the nu/nu mutation, however, do not exhibit greater than normal resistance against *Mycobacterium* species (28, 30) or *Streptococcus pneumoniae* (31).

To account for the observed increased resistance to bacterial infection, several investigators suggest that the macrophages of nude mice are activated and thus possess enhanced microbicidal activities (5, 7, 33). Supporting evidence indicates that peritoneal macrophages of nude mice phagocytize *L. monocytogenes* more efficiently, but there is some question as to whether this population of macrophages is more bactericidal than resident peritoneal macrophages from heterozygote (nu/+) littermates (5, 33). This study presents additional observations on the innate resistance of nude mice to several bacterial pathogens, and evidence is presented that fixed tissue macrophages are activated as a direct consequence of the thymic deficiency.

MATERIALS AND METHODS

Animals and husbandry. C3H/HeJ female mice 6 to 8 weeks of age were obtained from Jackson Laboratory, Bar Harbor, Maine.

The original breeders for the athymic (nu/nu) mouse colony were obtained from J. G. Michael, Department of Microbiology, University of Cincinnati Medical Center. These animals had been previously backcrossed for nine generations to a BALB/c genetic background and were thereafter randomly bred under modified, conventional conditions in animal facilities at the University of Cincinnati Medical Center. Male

homozygous nudes (nu/nu) were mated with female phenotypically normal heterozygotes (nu/+); nude females could not be used because of infertility (9). The room in which the colony was housed contained an Envirazone multipurpose module laminar air filtration unit (Envirco, Albuquerque, N.M.), which minimized the number of microbes in the immediate environment. Purina Lab Chow and drinking water containing Clorox at a concentration of 200 ppm were provided ad libitum. All nude mice showing evidence of the wasting syndrome, characteristic of conventionally raised nude mice (9), were discarded.

Serum samples obtained by cardiac puncture from apparently healthy heterozygote mice were analyzed by Microbiological Associates, Inc., Bethesda, Md., for the presence of antiviral antibodies. Positive titers were obtained for mouse hepatitis virus and minute virus of mice, indicating previous exposure to the viruses and perhaps latent viral infection as well.

Alternate breeding conditions were developed to produce nude mice devoid of intestinal bacterial flora. Mating was accomplished as described above, but mice were housed in a horizontal laminar flow hood and were handled aseptically. Purina Lab Chow Special Diet 5010C, San-I-Cell bedding (Paxton, Paxton, Ill.), water bottles, and cages were sterilized by autoclaving. Drinking water containing 0.5 g each of ampicillin, chloramphenicol, carbenicillin, and cephalothin per liter, designed to eliminate intestinal bacteria (personal communication, A. Roland, University of Alabama Medical Center), was provided ad libitum. Fecal samples were tested for bacterial content 1 week prior to use of mice in experiments. No bacterial growth was obtained on brain heart infusion (BHI) agar (BBL, Cockeysville, Md.) incubated aerobically or anaerobically. Bacteria were not evident in Gram-stained fecal smears, but yeast forms were present in small numbers.

Microorganisms. *L. monocytogenes* strain 19115 was obtained from the American Type Culture Collection (ATCC), Rockville, Md. *L. monocytogenes* CRC was obtained from A. C. Allison, Clinical Research Centre, Middlesex, England. No difference in the virulence of either of these strains for immunocompetent (nu/+) mice was observed, and therefore strain identification is not used in the text. *Staphylococcus aureus* 502-A (coagulase positive, nonencapsulated) was from departmental stock cultures, and *S. typhimurium* SR-11 was obtained from W. Johnson, Department of Microbiology, University of Iowa Medical Center, Iowa City. Overnight cultures of each organism prepared in BHI broth were dispensed in 2-ml portions, frozen, and stored at −70°C; this procedure assured genetic and physiological uniformity of organisms for the duration of the study.

Heat-killed *Corynebacterium parvum* (7 mg/ml) was obtained from Wellcome Research Laboratories, Beckenham, Kent, England. *C. parvum* was used to activate peritoneal macrophages (4) by intraperitoneal (i.p.) injection of 0.1 ml (0.7 mg, wet weight) of the commercial preparation.

Preparation of challenge inocula. Two milliliters of thawed stock culture was used to seed 50 ml of BHI broth. The appropriate bacterial culture was incubated at 37°C in a shaking water bath for 3 h. A 20-ml amount of the culture was centrifuged, and the pellet was washed once in 20 ml of phosphate-buffered saline (PBS) and adjusted to an optical density at 660 nm of 0.2; the suspension was then sonically treated for 1 min in an Ultrasonic cleaner (Cole-Parmer, Chicago, Ill.) to dissociate clumps of bacteria. Appropriate dilutions were made in PBS to obtain the desired number of bacteria required for mouse challenge. Actual viable counts were verified by subsequent plate count assays.

Calculation of LD_{50} values. Groups of five to six mice were injected intravenously (i.v.) with 0.2 ml of 10-fold dilutions prepared from a standard suspension of *L. monocytogenes* CRC. Deaths were recorded for a period of 14 days, and 50% lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (23).

Enumeration of viable bacteria in liver and spleen. Viable bacteria in spleen, liver, and blood were enumerated at various intervals after bacterial challenge in order to follow the course of infection or to provide a relative index of resistance. Challenge inocula and time intervals at which viable bacteria were determined varied for each experiment; these are described in the text.

Mice were killed by cervical dislocation. The spleen and a portion of liver were excised, weighed, and homogenized in 2.0 ml of sterile BHI broth. Appropriate 10-fold dilutions were plated in BHI agar, and colonies were counted 48 h after incubation at 37°C. The values presented are the geometric means of each group expressed as $\log_{10}$ viable bacteria per milligram.

Quantitation of bacteria in the blood was performed by collecting blood by cardiac puncture and plating appropriate dilutions in BHI agar as described above.

Macrophage listericidal assay. For the macrophage listericidal assay, a modification of the procedure used by Blanden et al. was employed (3). In this procedure, phagocytosis is allowed to proceed in the peritoneal cavity of mice before collecting peritoneal exudate cells. PBS (0.2 ml) containing approximately 10^7 *L. monocytogenes* organisms was injected i.p. After 15 min, mice were killed by cervical dislocation, and 5 ml of 75% NCTC-135 (GIBCO, Grand Island, N.Y.) plus 25% heat-inactivated fetal calf serum was injected i.p. The fluid was aspirated after 15 s of gentle abdominal massage, and 1.0 ml of the exudate was distributed to each of four tubes containing 1.0 ml of the tissue culture medium. Two tubes for the zero time period were immediately immersed in ice to inhibit further killing of phagocytized listeria. The remaining two tubes were incubated for 45 min at 37°C on a rocking platform adjusted to 16 cycles per min. One sample at each time period was centrifuged for 5 min at 200 × *g*, and serial dilutions of the supernatant were plated to quantitate extracellular bacteria. The remaining samples were sonically treated for 15 s at a power setting of 10 W on a Branson Sonifier (model W140). This disrupted all macrophages without any measurable effect on bacterial viability. Serial dilutions were plated to determine the numbers of total viable bacteria in each sample at the beginning and end of the 45-min incubation period. Subtraction of the extracellular from the total bacterial counts provided the values for macrophage-associated, viable listeria.

Irradiation and bone marrow reconstitution. Nude and heterozygote mice received 450 R of total body X-irradiation by placing the animals in a Westinghouse Quadrocondex therapy unit calibrated to deliver 95 R/min. Half of the mice received 1.0×10^7 bone marrow cells i.v. immediately after irradiation. Bone marrow cells were obtained aseptically by forcing PBS through the femurs of donor mice with a syringe and 27-gauge needle. Pooled cells were passed through an 80×-mesh stainless-steel screen, washed in PBS, and enumerated in a hemocytometer. Nude mice received bone marrow from nude mouse donors, and heterozygotes received cell transplants from heterozygote donors. Mice were challenged with 3.5×10^5 *L. monocytogenes* organisms 10 days after irradiation (i.e., 2 LD_{50} for unimmunized, immunocompetent mice).

Thymus grafting. Athymic (nu/nu) mice, 3 weeks of age, received thymus grafts from heterozygous (nu/+) littermates to minimize the possibility of graft versus host reactions. Thymuses were excised, sectioned into quarters, and implanted subcutaneously at the base of the neck on the dorsal surface. Mice receiving transplants were challenged i.v. with *L. monocytogenes* approximately 5 weeks after thymus implantation.

Cytotoxicity for tumor cells. Macrophage cytotoxicity was measured by [^{125}I]iododeoxyuridine release from prelabeled HEp-2 cells (ATCC CCL 23) at 48 h, as described by Norbury and Fidler (17). A macrophage/target cell ratio of 10:1 was used.

RESULTS

LD_{50} values of *L. monocytogenes* in nude and immunocompetent mice. A comparison of the LD_{50} values for *L. monocytogenes* in nude (nu/nu), heterozygous (nu/+), and C3H/HeJ mice is illustrated in Table 1. Mice were challenged i.v., and deaths were recorded over a 14-day period. In preliminary experiments with heterozygote mice, all deaths occurred prior to day 7. At day 7 the LD_{50} values for nude mice were 2.0 $\log_{10}$ units greater than those for the immunologically competent heterozygotes and C3H/HeJ mice. These considerable differences showed in a quantitative fashion that nude mice possess a surprisingly high native resistance to this facultative intracellular bacterial pathogen. The initial elevated resistance of nude mice diminished as evidenced by sporadic deaths of mice during week 2 of infection. As a consequence, the LD_{50} values at day 14 were approximately the same for all groups of mice. Deaths of the nude mice during week 2 could not be attributed in total to overwhelming listeria infection. In this experiment for which LD_{50} values are provided, no attempt was made to enumerate viable bacteria in the reticuloendothelial organs at the time of death. In subsequent experiments, however, the number of viable bacteria present in spleens and livers of infected nude mice that succumbed between 8 and 14 days after challenge was determined immediately after death. A small but significant proportion of nude mice apparently succumbed to listeria infection during week 2 after challenge, as evidenced by high bacterial counts, but in most instances, bacterial counts in livers and spleens were below lethal levels.

TABLE 1. *LD_{50} of L. monocytogenes in mice challenged i.v.*[a]

Mouse strain	LD_{50}	
	Day 7	Day 14
nu/nu[b]	8.9×10^6	1.6×10^5
nu/+[c]	1.2×10^5	1.2×10^5
C3H	1.1×10^5	8.0×10^4

[a] Calculated by Reed and Muench method (23).
[b] Athymic nude mice.
[c] Heterozygote littermates.

Growth kinetics of *L. monocytogenes* in reticuloendothelial organs. Viable listeria in the livers and spleens of infected mice were determined to characterize the time course and disposition of the infection in nude and heterozygote mice. Figure 1 illustrates growth kinetics in the spleen and liver of mice challenged i.v. with 9.2×10^5 listeria (>5 LD_{50} for nu/+ mice). The kinetics of bacterial growth in livers and spleens were found to be similar; listeria multiplied rapidly in the reticuloendothelial organs of the heterozygote mice, all of which succumbed to infection after 72 h. Nude mice, in contrast, responded by suppressing the infection effectively. During day 1 of infection, organ counts rose slightly, after which a reduction in bacterial numbers occurred during days 2 and 3. The nude mice then developed a persistent, low-level infection as also noted by Emmerling et al. (6). In view of the failure of nude mice to completely

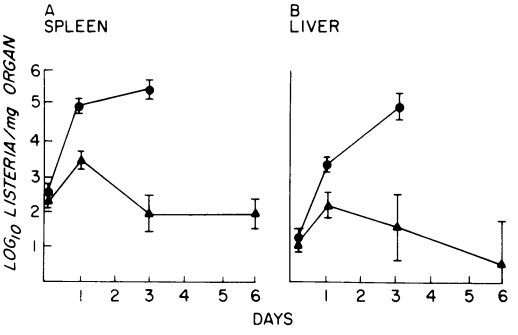

FIG. 1. *Growth of L. monocytogenes in spleens (A) and livers (B) of nude and heterozygous mice. Nude (▲) and heterozygous (●) mice received 9.2×10^5 L. monocytogenes organisms i.v. on day 0. Values represent the geometric means of three to four mice ± standard error of the mean.*

eliminate listeria from tissues, it was of interest to ascertain whether total elimination of bacteria could be accomplished when small inocula were used for challenge (i.e., <1 LD_{50}; Fig. 2). After an initial increase in the level of infection, heterozygote mice mounted effective antibacterial immunity, which resulted in the clearance of listeria from tissues by day 10. Nude mice were found to eliminate almost all of the listeria in the liver by day 10, but a stabilized infection in the spleen persisted until the termination of the experiment. Failure to eliminate the infection completely can be attributed to the absence of T-cell function required for the development of specific cell-mediated immunity (19, 20).

Resistance of athymic mice to *S. aureus* infection. To further evaluate the paradoxical resistance of nude mice to bacterial infection, an experiment was performed to compare staphylococcal infection in the nude and immunocompetent mice. Groups of nude mice and inbred C3H mice were challenged i.v. with 8.0×10^7 *S. aureus* organisms. Numbers of viable staphylococci recovered from the livers and spleens at 3 and 48 h after challenge are shown in Fig. 3. It is readily apparent that fewer *S. aureus* organisms were recovered from nude mice than from C3H mice. The differences are significant after 3 h, but reach dramatic proportions by 48 h when almost 3.0 $\log_{10}$ units more of staphylococci are found in the reticuloendothelial organs of the immunocompetent C3H mice. The naturally high resistance of the nude mice, therefore, is not restricted to *L. monocytogenes*. C3H and heterozygote mice behave in the same manner when challenged i.v. with the bacterial pathogens used. In all instances, LD_{50} values are significantly greater for the immunocompetent than for the nude mice.

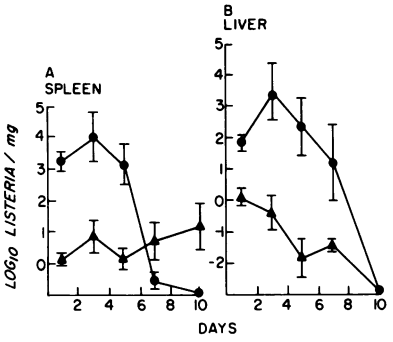

FIG. 2. *Course of infection in spleens (A) and livers (B) of nude (▲) and heterozygous (●) mice challenged with a sublethal dose of 2.7×10^4 L. monocytogenes organisms i.v. on day 0. Values represent the geometric means of three to four mice ± standard error of the mean.*

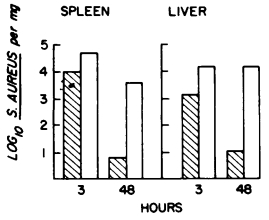

FIG. 3. *Viable S. aureus in the reticuloendothelial organs of nude and C3H mice at 3 and 48 h after infection. Nude (nu/nu; ▨) and C3H/HeJ (□) mice received 8.0×10^7 S. aureus organisms i.v. at zero time. Values represent the geometric means of four mice.*

Blood clearance of bacterial pathogens by athymic mice. To further generalize the phenomenon, nude and C3H mice were challenged i.v. with either *S. aureus, L. monocytogenes,* or *S. typhimurium.* Blood clearance of the three bacterial pathogens was ascertained; in addition, viable bacteria in the livers and spleens were also enumerated (Table 2). In the three cases, fewer viable bacteria were present in the blood of nude mice at 3 h after challenge, indicating greater than normal phagocytosis by reticuloendothelial elements. In addition, in the case of listeria and staphylococcal challenge, fewer bacteria were recovered from the major reticuloendothelial organs, suggesting a greater than normal antibacterial activity of tissue macrophages in nude mice.

The demonstration of antibacterial resistance of nude mice as early as 3 h after challenge would suggest that the extraordinary resistance is innate and not elicited by bacterial challenge

TABLE 2. *Blood clearance by reticuloendothelial organs of mice challenged i.v. with bacterial pathogens*

Tissue	Log_{10} viable units per mg or ml		
	nu/nu	C3H	*P* value[a]
S. aureus[b]			
Blood	3.1 ± 0.06[c]	3.7 ± 0.08	0.0005
Spleen	3.7 ± 0.06	4.7 ± 0.11	0.0005
	3.2 ± 0.08	4.4 ± 0.04	0.0005
L. monocytogenes[d]			
Blood	3.2 ± 0.15	4.6 ± 0.06	0.0005
Spleen	4.2 ± 0.05	4.7 ± 0.05	0.0005
Liver	3.5 ± 0.12	4.2 ± 0.05	0.005
S. typhimurium[e]			
Blood	5.2 ± 0.53	6.1 ± 0.06	0.1
Spleen	4.3 ± 0.08	4.6 ± 0.05	0.05
Liver	3.1 ± 0.06	3.1 ± 0.03	0.3

[a] Computed from Student's *t* test.
[b] 7.8×10^7 *S. aureus.*
[c] Geometric mean of four mice ± standard error of the mean; values from tissues at 3 h after challenge i.v.
[d] 1.7×10^8 *L. monocytogenes.*
[e] 1.3×10^7 *S. typhimurium.*

per se. To account for this phenomenon, we hypothesized that one or more populations of macrophages in untreated (control) nude mice are activated by mechanisms not immediately apparent. This would account for the rapid bacterial clearance from blood and the significant killing of phagocytized microbes during the initial 3-h period of infection. An alternative also considered as a possible mechanism was that nude mice possessed antibacterial substances in serum not present in conventional mice. This possibility was dismissed when it was determined that *L. monocytogenes* multiplied equally well in blood or serum of either nude or heterozygous mice (data not presented).

Bactericidal activity of peritoneal macrophages. Since the previous experiments showed that spleen and liver macrophages of nude mice were activated, it was of interest to determine whether resident peritoneal macrophages of nude mice were also activated. The results are presented in Table 3. The data show no significant differences in phagocytosis or killing of bacteria by resident macrophages from normal nude and heterozygous mice during the 45-min in vitro incubation. Peritoneal macrophages of heterozygous mice immunized 7 days previously with *L. monocytogenes* were shown to be activated, as judged by three criteria: (i) recovery of fewer bacteria from the peritoneal cavity, indicating rapid phagocytosis and killing during the 15-min in vivo phagocytosis period; (ii) greater killing of cell-associated bacteria during the 45-min in vitro incubation period; and (iii) more efficient in vitro phagocytosis of listeria, as evidenced by restricted multiplication of extracellular bacteria in the tissue culture medium, which supports bacterial multiplication. In contrast, peritoneal macrophages of nude mice immunized with *L. monocytogenes* did not increase in bactericidal activity above the normal level. These data are consistent with the mechanism of T-cell-mediated macrophage activation for development of immunity against *L. monocytogenes* (18).

Cytotoxicity of PEC for HEp-2 cells. Activated macrophages have been shown to possess cytotoxic activity for tumor cells in vitro (17). The cytotoxic activity of PEC from normal heterozygous and nude mice was compared, as well as PEC from nude and heterozygote mice stimulated i.p. with *C. parvum* 7 days previously. The results are presented in Table 4. Cytotoxicity as measured by percent release of [^{125}I] iododeoxyuridine from HEp-2 cell monolayers was identical for nude and heterozygote mice. Stimulation of PEC by *C. parvum* greatly increased the release of radioactive label by PEC from both nude and heterozygote mice. These results confirm those of the bactericidal studies indicating that the normal resident peritoneal macrophages of nude mice are not activated.

Bone marrow as source of effector cells. Hahn (11) demonstrated that monocyte precur-

TABLE 4. *Cytoxicity of normal and C. parvum-activated nude (nu/nu) and heterozygote (nu/+) PEC for [^{125}I]iododeoxyuridine-prelabeled HEp-2 cells at 48 h*

Treatment	Mouse strain	cpm released[a]	Cytotoxicity (%)[b]
Control	nu/+	1,730 ± 22	18
	nu/nu	1,669 ± 161	16
C. parvum	nu/+	2,975 ± 90	51
	nu/nu	3,250 ± 97	68

[a] Each value based on calculations from six replicate samples.

[b] Percentage of HEp-2 cells in monolayers killed after exposure to macrophages.

TABLE 3. *Phagocytosis and killing of L. monocytogenes by PEC from normal and listeria-treated athymic nu/nu mice and heterozygous nu/+ mice*

Mouse strain	Pretreatment	Time (min)	No. of listeria per 5 ml					
			Total	Decrease (%)	Cell associated	Decrease (%)	Extracellular	Increase (%)
nu/nu	None	0	4.5×10^6		4.1×10^6		4.4×10^5	
		45	1.5×10^6	67	7.6×10^5	81	7.1×10^5	61
nu/+	None	0	5.1×10^6		4.8×10^6		3.6×10^5	
		45	1.5×10^6	71	8.8×10^5	82	5.9×10^5	64
nu/nu	Listeria[a]	0	4.8×10^6		4.6×10^6		1.4×10^5	
		45	1.2×10^6	75	9.1×10^5	80	2.7×10^5	93
nu/+	Listeria[a]	0	7.1×10^5		6.2×10^5		9.8×10^4	
		45	1.6×10^5	98	3.0×10^4	95	1.3×10^5	33

[a] Injected i.v. 7 days previously with 4.0×10^4 *L. monocytogenes*.

sors of macrophages responsible for effective protection against listeria infection are sensitive to X-irradiation and are derived from bone marrow. The following experiment was designed to determine whether the cells of nude mice responsible for increased resistance to infection are present in bone marrow as irradiation-sensitive precursors. Groups of nude and heterozygous mice received 450 R of total body X-irradiation. Animals were reconstituted by an i.v. injection of 10^7 bone marrow cells from normal mice on the day of irradiation. Reconstituted and control mice were challenged 10 days after irradiation with 3×10^5 *L. monocytogenes* organisms. Since many mice exhibited signs of radiation sickness, it was necessary to enumerate viable bacteria in certain groups at 24 h after challenge (Fig. 4). Viable bacteria in the remainder of the animals in this experiment were enumerated at 48 h after challenge (Fig. 5). For most experiments, 48-h bacterial organ counts were chosen, as these provide a valid measure of immunity to listeria infection (11) and are prior to any observable specific cell-mediated response by the animals to the challenge per se (34). The data calculated at 48 h (Fig. 5) indicate that irradiation resulted in elimination of the natural resistance of nude mice to bacterial infection. Irradiated bone marrow-reconstituted nudes, assayed at 24 h (Fig. 4), in contrast were restored to their anomalous high resistance. Heterozygous animals, on the other hand, demonstrated comparable low resistance to listeria infection whether or not they were irradiated and reconstituted with bone marrow before challenge. These observations provide evidence that the cells responsible for increased bactericidal activity in the reticuloendothelial organs of nude mice are of bone marrow origin.

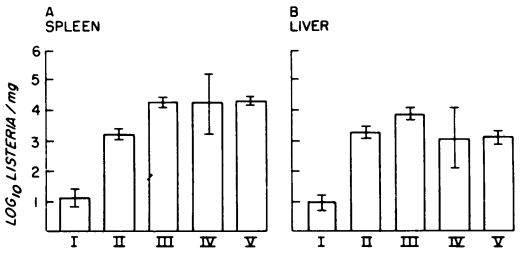

FIG. 4. *Viable L. monocytogenes in the spleens (A) and livers (B) of irradiated and bone marrow-reconstituted mice at 24 h after challenge. The number of viable L. monocytogenes organisms was determined in the spleens and livers of irradiated, bone marrow-reconstituted nude (I), normal nude (II), irradiated heterozygous (III), irradiated, bone marrow-reconstituted heterozygous (IV), and normal heterozygous (V) mice at 24 h after i.v. challenge with 3.5×10^5 L. monocytogenes organisms. Irradiated mice received 450 R of X-irradiation 10 days before challenge.*

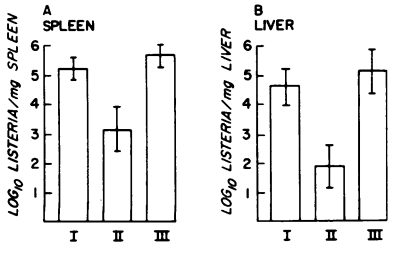

FIG. 5. *Viable L. monocytogenes in the spleens (A) and livers (B) of normal and irradiated mice at 48 h after challenge. The number of viable L. monocytogenes organisms was determined in the spleens and livers of irradiated nude (I), normal nude (II), and normal heterozygous (III) mice at 48 h after i.v. challenge with 3.5×10^5 L. monocytogenes organisms. Irradiated mice received 450 R of X-irradiation 10 days before challenge.*

Restoration by thymic grafts. Attempts were made to alter the native high resistance of the nude mice by restoration of T-cell function by thymus grafting. Nude mice received thymus transplants from heterozygous littermates. After 5 weeks these mice and appropriate controls were challenged i.v. with 4.6×10^5 *L. monocytogenes* organisms (about 5 LD_{50} for heterozygous mice). A 5-week interval was used to assure the disappearance of activated macrophages that may have been present before thymus implant. Spleens and livers were then assayed for viable bacteria at 48 h after challenge (Fig. 6). It appeared that thymus grafting removed to a considerable extent the anomalous resistance of nude mice to *L. monocytogenes* infection. Thus, the high native resistance of nude mice to bacterial infection is associated with the absence of thymus-derived cells and results, at least in part, from that deficit.

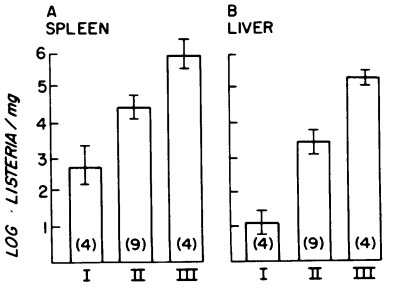

FIG. 6. *Viable L. monocytogenes in spleens (A) and livers (B) of nude (I), thymus-grafted nude (II), and heterozygous (III) mice. Nude mice received subcutaneous thymus grafts at 5 weeks before i.v. challenge with 4.6×10^5 L. monocytogenes organisms. Values represent the geometric means of organ-associated viable bacteria at 48 h after infection ± standard error of the mean. Numbers in parentheses are numbers of mice assayed in each group.*

Relation of intestinal bacterial flora on resistance. Parenteral administration of bacterial cell wall components such as phospholipids (7) and lipopolysaccharide (12) are known to increase the phagocytic and bacterial killing by mononuclear phagocytes of mice. Latent bacterial infections or cell wall components derived from the intestinal tract of the nude mice may have been responsible, at least in part, for the activation of macrophages since these animals lack T-cell-regulated homeostatic functions. Mice raised under sterile conditions and fed water containing an antibiotic cocktail (see above) to eliminate bacterial flora from the gastrointestinal tract were compared with conventionally raised animals to test this possibility. Viable bacterial counts were performed on spleens and livers of the nude mice raised under the diverse regimens, 48 h after challenge with 5.5×10^5 *L. monocytogenes* organisms (Fig. 7). These data show that the absence of an intestinal bacterial flora modifies the resistance of the immunocompetent as well as the immunodeficient mice. It appears, therefore, that the normal bacterial content of the gut may stimulate macrophages of the reticuloendothelial system and accounts in part for heightened resistance to infection. However, this phenomenon apparently holds for both the nude and the immunologically competent heterozygous mice since the dichotomy of innate resistance to infection of nu/nu and nu/+ mice persists after bacterial sterilization of the gastrointestinal tract. This suggests that still other factors accounting for activated macrophages in nude mice are yet to be defined. The regimen of antibiotic sterilization used in these experiments does not eliminate yeasts and viruses. Yeast forms, although present in small numbers, were observed in stained fecal smears of antibiotic-treated mice, and measurable amounts of antibody against mouse hepatitis virus and minute virus of mice were detected in the sera of heterozygote mice in the breeding colony.

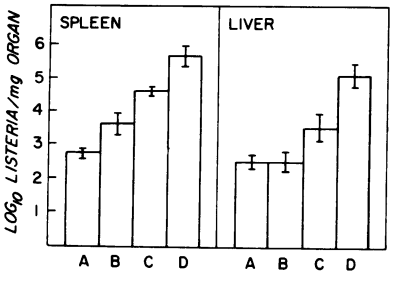

FIG. 7. *Viable L. monocytogenes in the organs of normal and antibiotic-sterilized nude and heterozygote mice. Bacterial counts were performed at 48 h after i.v. challenge with 5.5×10^5 L. monocytogenes organisms in normal nude (A), antibiotic-sterilized nude (B), normal heterozygous (C), and antibiotic-sterilized heterozygous (D) mice.*

DISCUSSION

These experiments show convincingly that congenitally athymic (nude) mice raised under conventional conditions possess an anomalous, high resistance to several bacterial pathogens. Two of the bacterial species tested are recognized as facultative intracellular parasites. The data suggest that nude mice possessing a natural microbial flora develop populations of macrophages in the major reticuloendothelial organs that are in the activated state prior to experimental infection. Resistance of nude mice was demonstrable immediately after i.v. challenge with the bacterial pathogens and was apparent by a rapid bacterial clearance from the circulation and bacterial destruction in the spleen and liver exceeding that observed in immunocompetent heterozygote littermates or other inbred mice used as experimental controls. As documented previously (6), resistance was not absolute, as shown by a persistence of chronic low-level listeria infection in the reticuloendothelial organs of nude mice. As has been shown by other investigators using inbred mice (20), we observed that immunologically competent heterozygotes eliminated a sublethal infection of *L. monocytogenes* after the development of specific cell-mediated immunity. Nude mice survived infection with a challenge dose of listeria lethal for immunocompetent mice. Similar observations have been published recently (5, 6).

The differences in phagocytic clearance and killing of *S. typhimurium* in nude and C3H mice were not as great as those observed for the other bacterial pathogens (Table 2). Effective immunity to *S. typhimurium* is dependent upon not only activated macrophages but also opsonic antibody (12). Inefficient phagocytosis of *S. typhimurium* by macrophages would preclude a demonstration of increased bactericidal capacity. Phagocytosis of nonencapsulated *S. aureus* and *L. monocytogenes,* in contrast, is not dependent upon specific opsonins. This may account for the way in which nude mice responded to challenge with *S. typhimurium* as compared with *L. monocytogenes* or *S. aureus* (Table 2).

In contrast to reports by others (5, 15, 22, 33), peritoneal macrophages of the conventionally raised nude mice from our colony were not activated when the criterion of greater than normal phagocytic activity, enhanced bacterial killing, or tumor cell cytotoxicity was applied. These discrepancies may be due to diverse genetic backgrounds of the nude mice used or to differ-

ences in environmental conditions utilized for maintaining the respective animal colonies.

At 10 days after X-irradiation, it was observed that nude mice became less resistant to listeria infection. Bone marrow reconstitution of irradiated nude mice, however, restored the natural high resistance of nude mice. Macrophage precursors are derived from bone marrow, and their destruction by X-irradiation is known to inhibit the development of resistance against *L. monocytogenes* in conventional mice (11). The similarities in observations made by Hahn (11) and the experiments reported here support the view that the effector cells responsible for heightened resistance of nude mice are fixed tissue macrophages of the major reticuloendothelial organs.

Abrogation of the anomalous resistance of nude mice to infection by thymus grafting was also demonstrated. This fact strongly suggests that the absence of T-lymphocytes and the development of activated macrophage populations in nude mice are causally related; such a conclusion is substantiated by other investigators who have observed increased host survival and slower growth of bacteria in reticuloendothelial organs of adult thymectomized, X-irradiated, bone marrow-reconstituted mice as compared with normal mice (33, 34). One observation not consistent with reports linking T-cell deficiency and resistance to bacterial infection is that antithymocyte serum treatment of normal mice appears to increase susceptibility to listeria infection (34).

The presence of activated macrophages in nude mice coincident with a lack of functional T-lymphocytes requires an explanation in immunological terms. Perhaps, the simplest mechanism that can be invoked is to speculate that there is an absence of T-lymphocyte suppressor cell populations normally present in immunocompetent mice. This hypothetical suppressor cell may function in immunologically normal animals by maintaining tissue macrophages in a quiescent state in the absence of sustained and appropriate antigenic stimuli. Upon induction of a cell-mediated response to infection in an immunocompetent host, suppressor function may cease or be overridden by macrophage-activating lymphokines synthesized by sensitized T-lymphocytes. In nude mice, however, a lack of T-lymphocyte suppressor cells may allow macrophages to attain a quasi-permanent state of activation with minimal antigenic stimulation. This proposition would suggest that all of the macrophage populations of nude mice should be in the activated state. However, we have shown that peritoneal macrophages of nude mice used in these experiments were not activated, as judged by phagocytic, bactericidal, and tumor cell killing potential. Macrophages may be activated by mechanisms other than lymphokines of specifically sensitized T-cell origin. *C. parvum* (4), gram-negative bacterial lipopolysaccharide (12), and bacterial phospholipids (7) are thought to induce a direct activation of macrophages in vivo. Recently, it has been shown that B-lymphocytes may produce lymphokine-like products in vitro after stimulation with B-cell mitogens, including lipopolysaccharide (32) and *C. albicans* (24). The origin of such stimulating substances in nude mice used in our study may be subclinical bacterial or viral infections. Nomura et al. (16) demonstrated that conventionally raised or specific pathogen-free nude mice have a considerably higher incidence of aerobic and anaerobic subclinical infections in lymph nodes and other organs than do their immunocompetent littermates. Nude mice that have been shown to possess high natural resistance to infection have invariably been raised and housed under conventional conditions. Organisms responsible for low-grade, chronic infection may arise from penetration of mucosal surfaces by intestinal flora (13), which in nude mice may occur to a greater than normal degree because of an underdevelopment of Peyer's patches (10) and, perhaps, other immunoregulatory abnormalities associated with the T-lymphocyte deficiency. The combined effect of athymia and X-irradiation, which leads to considerable destruction of the intestinal mucosal lining (1), would increase the potential for gut-derived infections. This would provide an even stronger microbial antigen stimulation than that which occurs in normal nude mice and thus might explain the greater resistance of X-irradiated, bone marrow-reconstituted nude mice to resist listeria infection when compared with untreated nude mice (Fig. 4). The presence of a normal microbial flora may also stimulate the functions of macrophages in conventional mice (8, 29). This association of microbial flora and macrophage function may explain the increased susceptibility of both nude and heterozygote mice to listeria infection observed after the elimination of intestinal bacteria flora with antibiotics before bacterial challenge. The persistence of partially enhanced resistance of nude mice to infection after antibiotic removal of bacterial flora may be due to an immunological stimulation by agents other than bacteria. Virus and yeast forms associated with nude mice used in this study are unaffected by antibiotics and, thus, may have served as immunological stimuli.

Evidence that microbial flora or other sources of infection are responsible for macrophage activation in nude mice has also been presented by Meltzer (15). He observed that peritoneal

macrophages of conventionally raised nude mice exhibit cytotoxicity for tumor cells. However, peritoneal macrophages of conventionally raised heterozygote or germfree nude mice did not possess significant tumoricidal activity. In addition, Rama Rao et al. (22) observed suppression of vaccinia virus replication in peritoneal macrophages of conventionally raised nude mice. Peritoneal macrophages of conventionally raised heterozygote or germfree nude mice, in contrast, supported viral replication.

The absence of activated macrophages in nude mouse peritoneum simultaneously with activated tissue macrophages as we suggest can be accounted for by a lack of sufficient antigenic stimulation locally. Blanden et al. (2) showed that after a small primary BCG challenge, the macrophages of the major reticuloendothelial organs of the mice were activated, as judged by phagocytic and bactericidal abilities, whereas peritoneal macrophages were not activated. Larger challenge doses of BCG, however, induced the activation of tissue and peritoneal macrophage populations. The nude mice used by investigators reporting that resident peritoneal macrophages are normally activated may have been subjected to more extensive antigenic stimuli than were the nude mice used in our studies.

Although the explanation of activated tissue macrophages accounting for the resistance of athymic mice is consistent with our data, the mechanism(s) remains unresolved. Conceivably, other factors, both cellular and humoral, may also contribute to the phenomenon.

ACKNOWLEDGMENTS

A. C. Allison of the Clinical Research Centre, Harrow, England, provided kind hospitality and contributed significantly in the formulation of the initial experiments done while P.F.B. was a visitor in Dr. Allison's laboratory during part of 1975. We are obliged to Dwight Stinnett, Department of Surgery, University of Cincinnati Medical Center, for performing the tumor cell killing assays. It should also be acknowledged that Robert E. Baughn, Department of Dermatology, Baylor University, Houston, Texas, made the critical observation in 1974 that led us to initiate this investigation; he observed and confirmed that athymic mice cleared *L. monocytogenes* from the blood and tissues more rapidly than did immunocompetent mice.

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