Early bacterial clearance from murine lungs. Species-dependent phagocyte response.

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Research Article

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Early Bacterial Clearance from Murine Lungs

SPECIES-DEPENDENT PHAGOCYTE RESPONSE

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ABSTRACT Two sets of phagocytic cells are available to defend the lung against inhaled bacteria. Both resident alveolar macrophages and granulocytes from the circulation have been observed in pulmonary airspaces after the deposition of bacteria; their functional roles, however, have never been defined. We rendered mice selectively granulocytopenic with heterologous antiserum in order to ascertain the relative contributions of these two groups of cells in intrapulmonary bacterial killing. The clearance of *Staphylococcus aureus* was unimpaired in granulocytopenic animals, confirming the primary role of the alveolar macrophages in the killing of these organisms. In contrast, granulocytopenic animals cleared only 10.0±7.0% of an inoculum of *Klebsiella pneumoniae* compared with 33.0±4.0% clearance in normal animals ($P < 0.02$), and *Pseudomonas aeruginosa* proliferated to 513% of baseline levels in granulocytopenic animals, whereas normal mice cleared 26.8±10.6% of the inoculum. These findings indicate that circulating granulocytes play a major role in the clearance of the latter two organisms. This variation in cellular response to different bacterial species suggests that the defense of the lung against pathogenic bacteria is more complex than has been previously assumed.

INTRODUCTION

Although the lung is repeatedly inoculated with bacteria from the upper airways (1), pneumonia rarely ensues, even when the inoculum contains pathogenic bacteria (2, 3). This implies an efficient clearance mechanism capable of killing or eliminating the bacteria before their multiplication leads to clinical disease. This mechanism could be the function of the resident population of alveolar macrophages that are known to be avid phagocytes. However, large numbers of polymorphonuclear leukocytes are also locally available from a pool that marginates in pulmonary capillaries (4). Attempts have been made to define the relative participation of these two populations of cells by morphologic examination of lung tissue. The static nature of such studies, however, allows only inferences concerning the functions of these cells, and conflicting opinions have arisen as to their relative roles.

The elimination of one population of phagocytes while the other remains intact allows the contribution of each cell type to be assessed independently. We have created a granulocytopenic murine model with the use of specific antigranulocyte antibody (5), and have examined the early pulmonary response to the inoculation of three species of bacteria. By comparing the bacterial clearance of granulocytopenic mice with that of normal mice, we have found evidence that the alveolar macrophage is the principle phagocyte of *Staphylococcus aureus* during this interval, as previously suggested by morphologic studies (6–8). However the granulocyte is the principal phagocyte for the gram-negative bacilli studied within the first few hours after their deposition in the lung. Our findings indicate that the cellular defense of the lung is more complex than has been inferred from investigations using only *Staphylococcus*, and is apparently species-dependent.

METHODS

Bacteria. *S. aureus* strain FDA 209 and *Klebsiella pneumoniae* were maintained on trypticase soy agar. In preparation for nebulization each was inoculated into 1 liter of

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trypticase soy broth, incubated for 18 h at 37°C, washed twice, and resuspended in 150 ml of distilled water. *Pseudomonas aeruginosa* was maintained on blood agar. Before use it was inoculated into brain heart infusion broth, incubated for 18 h at 37°C, washed twice, and resuspended in 150 ml of distilled water.

Antisera. Antigranulocyte globulin (AGG)[1] was obtained by a method previously described (5). Briefly, New Zealand white rabbits were immunized with four weekly subcutaneous injections of $\sim 5 \times 10^7$ peritoneal exudate granulocytes emulsified with Freund's complete adjuvant. Similar booster injections were repeated at monthly intervals; blood was obtained by exsanguination at 7 mo. Serum was separated and heat-inactivated at 56°C for 40 min. Each milliliter of antiserum was absorbed for 30 min at room temperaure : (*a*) twice with 0.1 ml of packed murine erythrocytes; (*b*) twice with 10^8 splenic lymphocytes; (*c*) twice with 10^8 thymocytes; and (*d*) twice with 1.5×10^6 alveolar macrophages. An immunoglobulin G fraction was obtained from the absorbed antiserum by sodium sulfate fractionation (9) followed by column chromatography of DEAE-cellulose in 0.0175 M phosphate buffer at pH 6.9 (10). One-ninth volume of 10× phosphate-buffered saline was added, and the protein concentration was adjusted to 3 mg/ml by ultrafiltration over a PM 30 filter (Amicon Corp., Scientific Systems Div., Lexington, Mass.) This final material was passed through a sterile 0.45-μm filter (Millipore Corp., Bedford, Mass.), cultured to confirm sterility, dispensed in aliquots, and frozen at −20°C. Each lot of antisera had a different antibody activity per milligram of protein. In preliminary experiments we determined that the lot used in this study contained sufficient antibody to cause a profound neutropenia at 2 h after injection of 0.15 ml. We have previously demonstrated that this AGG is devoid of antimacrophage activity and that alveolar macrophages lavaged from animals treated with AGG are present in normal numbers, have normal viability, and retain full bactericidal capacity (5).

Aerosol exposure. In each study, 66 BALB/c mice weighing 18–22 g (Charles River Breeding Laboratories, Wilmington, Mass.), were exposed to a bacterial aerosol generated by a Collison nebulizer; 90% of the aerosol droplets generated by this technique were <2 μm Diam (11). 2 h before the beginning of the exposure to aerosol, half of the animals were injected with 0.15 ml of AGG i.p.; control animals received an injection of normal saline. This time interval was chosen because it corresponds to the nadir of the granulocytopenic response (5). The animals were then housed in a special holder previously described (12), and exposed for 30 min in a modified Henderson exposure chamber (13) to the bacterial aerosol. At the termination of the exposure period half of the AGG-treated animals and half of the saline-treated controls were bled for leukocyte counts and then were killed by cross-clamping of the neck to prevent agonal aspiration. The lungs were aseptically removed at the hila, homogenized in 4 ml of sterile water using a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.), followed by hand grinding in TenBroeck sintered glass grinders (Corning Glass Works, Science Products Div., Corning, N. Y.). Serial 10-fold dilutions were made of the homogenate, plated on appropriate agar, incubated at 37°C, and counted at 24 h. Bacterial counts were made on plates containing 30–300 colonies, corrected for dilution, and expressed as total number of bacteria per animal. This procedure was repeated with the remaining animals 4 h after the end of the exposure period.

Quantative cultures of the nebulizer slurry were obtained before and after aerosolization by serial dilution on appropriate agar. Aerodynamic mean diameter of each bacterial aerosol was examined in mid-run with an Andersen air sampler incorporating a 10% gelatin medium on each of the six stages, which were melted at 40°C and quantitatively cultured (14).

Hematologic studies. Because of the inherent inaccuracies of intraperitoneal injections (15), leukocyte parameters of AGG-treated mice were checked, and animals whose polymorphonuclear leukocyte (PMN) counts were greater than 1.65 standard deviations above the group mean were excluded from analysis. Leukocyte counts were done by hand in a hemocytometer, and 200-cell differentials were performed on Wright-stained cover glass preparations of orbital sinus blood.

Statistics. Clearance of bacteria was calculated using the group mean method previously described (16), and expressed as percent of originally deposited bacteria remaining at 4 h after deposition. Data were analyzed using the Mann-Whitney test nonparametric analysis (17). A probability value of <0.05 was considered significant.

RESULTS

Granulocyte counts. The normal granulocyte count for BALB/c mice in our laboratory is 1,339±39 (SE) cells per microliter. Table I lists the granulocyte counts for each group of animals at the time periods studied. Animals receiving AGG became significantly granulocytopenic during the time of the study. Although administration of AGG did not totally eliminate circulating granulocytes, we have previously demonstrated that those that remain in the circulation are restricted in their ability to enter the lung in response to a bacterial challenge (5).

Half of the 66 animals in each of three aerosol exposure experiments received AGG. Of these 99 animals receiving AGG, 8 animals were excluded from bacteriologic analysis because of PMN counts >1.65 times the group mean. This finding agrees with previous reports that ~10% of intraperitoneal injections are misplaced and ineffective (18, 19). In addition, two control animals were excluded from analysis because blood samples were contaminated with material from the retro-orbital lymphatics, and two died during handling before exposure to the bacterial aerosol.

In response to intraperitoneal saline injection, the stress of confinement, and the exposure to the bacterial aerosol, control animals uniformly exhibited a granulocytosis at zero hour. This was sustained at the 4-h time period in mice exposed to *S. aureus* and *K. pneumoniae*. Among the control animals exposed to *P. aeruginosa*, however, granulocyte counts spontaneously declined between the zero hour and 4-h time periods to a level substantially lower than those seen in control animals exposed to the other two bacterial species.

Bacterial clearance. The initial bacterial inocula recovered from the homogenized lungs at the end of the aerosol exposure, time zero, are reported in Table II. There was no significant difference in the number

[1] *Abbreviations used in this paper*: AGG, antigranulocyte globulin; PMN, polymorphonuclear leukocyte.

TABLE I
PMN per Microliter of Blood

	AGG-treated		Saline-treated		*P*
	n	*mean±SE*	*n*	*mean±SE*	
0 h					
S. aureus	15	374±63	15	3,742±187	<0.01
K. pneumoniae	15	268±33	17	2,241±226	<0.01
P. aeruginosa	15	551±81	16	2,837±213	<0.01
4 h					
S. aureus	14	455±48	16	3,128±179	<0.01
K. pneumoniae	16	637±100	16	3,541±220	<0.01
P. aeruginosa	16	337±49	15	766±169	<0.05

of bacteria in the lungs of the AGG-treated animals compared with the saline-treated controls. Mean aerodynamic diameter of the aerosolized bacteria was similar in each run and similar to previous reports from our laboratory (12).

Fig. 1 depicts the percent of originally deposited bacteria remaining in the lungs at 4 h. In the animals aerosolized with staphylococci, control animals had 20.6±2.0% (SE) of the initial load remaining, compared with 19.3±2.0% for the granulocytopenic animals (P = NS). This clearance is consistent with values obtained in previous studies (20–23), and indicates that the bactericidal mechanisms for *Staphylococcus* are unimpaired in the presence of severe granulocytopenia.

By contrast, the granulocytopenic mice were significantly impaired in their ability to remove *Klebsiella* from the lungs. In control animals 67.0±4.0% of the initial inoculum remained at 4 h, compared with 90.0 ±7.0% for the granulocytopenic animals ($P < 0.02$).

Among the control animals aerosolized with *Pseudomonas* the mean number of bacteria remaining was 73.2±10.6%. This stands in marked contrast to the AGG-treated animals, in each of which *Pseudomonas* proliferated, reaching a mean level of 513±51.3% of original values.

DISCUSSION

Bacteria aspirated from the oropharyngeal region or artifically introduced into the tracheobronchial tree are rapidly cleared from the lungs by a combination of physical transport and intracellular destruction within phagocytic cells (24, 25). The nature of the phagocytic cell response has been the subject of some controversy. Early studies using bacteria presented as a bolus to the distal lung uniformly reported the presence of PMN in the alveoli within a few hours of the introduction of the bacteria (26–29). Some investigators felt that the granulocytes were primarily responsible for the killing of the bacteria (30, 31), whereas others attributed this function to the alveolar macrophage (32). More recent research (33–35) has focused on bacteria presented to the lungs in the form of a diffuse aerosol rather than a bolus, allowing large numbers of animals to receive a uniform dose of bacteria and permitting meaningful statistical evaluation of bacterial clearance kinetics. Using the aerosol method, several histologic studies have indicated that the alveolar macrophage alone is responsible for the clearance of *S. aureus* from the lung (6–8).

However, after presentation of other species of bacteria to the lungs by aerosol, the presence of granulocytes has been demonstrated. A marked granulocytic inflammatory response has been noted histologically after exposure of animals to several strains of *P. aeruginosa* (36). Increased numbers of granulocytes have been seen in the alveoli of mice 2 and 4 h after exposure to aerosols of *Escherichia coli* and *K. pneumoniae*, though not after exposure to *S. aureus* (37).

TABLE II
Bacterial Inocula at Zero Hour

	AGG-treated		Saline-treated		*P*
	n	*mean±SE* $\times 10^6$	*n*	*mean±SE* $\times 10^6$	
S. aureus	15	3.82±0.24	15	4.09±0.31	>0.2 NS*
K. pneumoniae	15	0.83±0.05	17	0.89±0.06	>0.4 NS*
P. aeruginosa	15	1.54±0.08	16	1.55±0.09	>0.2 NS*

* NS indicates probability value of >0.05.

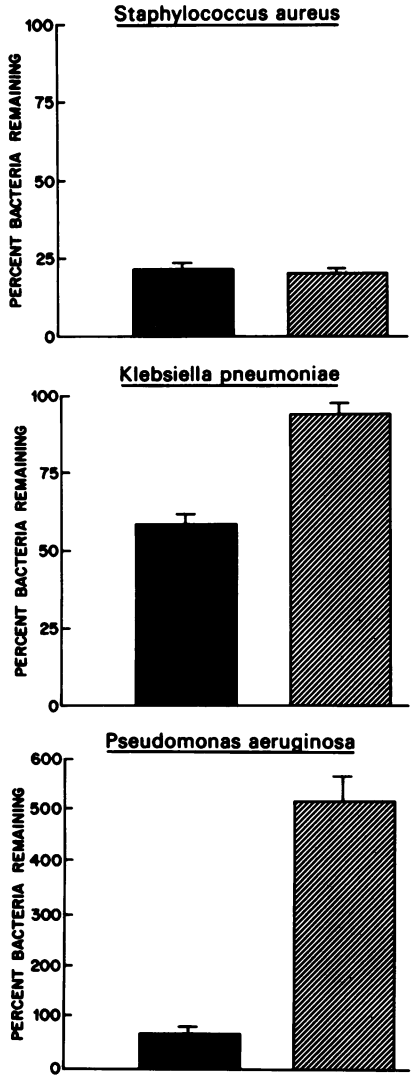

FIGURE 1 The percentages of the originally deposited bacteria remaining at 4 h are demonstrated on the vertical axis for control and granulocytopenic animals. Brackets represent 1 SEM. Note change of vertical axis in lowest panel. ▤, control; and ▧, AGG-treated.

Further, granulocytes have been recovered in lung lavage fluid from animals that had been exposed to aerosols of gram-negative bacilli (38, 39).

A brisk granulocyte recruitment to alveolar spaces may be easily visualized when one considers that approximately half of the body's store of granulocytes is sequestered in the marginal granulocyte pool (40). Many of these cells reside in the capillaries of the lung (4) and by direct observation ~300–1,000 PMN are within each square millimeter of alveolar wall (41).

All previous studies assessing the phagocytic cell response to bacteria have relied on morphologic observations, and the functional role of the granulocyte relative to the alveolar macrophage could not be definitively ascertained. To determine the degree of bacterial killing by each set of phagocytes, we have used an animal model of granulocytopenia that leaves alveolar macrophages numerically and functionally intact (5). AGG lowers circulating granulocyte levels and depletes the marginal pool, thus abrogating the intrapulmonary neutrophilic response to a bacterial aerosol (5). Using this model, the clearance of intrapulmonary bacteria in granulocytopenic animals can be attributed predominantly to alveolar macrophage function and mucociliary clearance, and an accelerated clearance in normal animals represents the contribution of granulocytes.

We have demonstrated in an earlier study (38) that an aerosol inoculum of *S. aureus* of a magnitude similar to that used in this study is cleared from the lung at a rate comparable to smaller inocula (21–23). Further, this inoculum does not cause a recruitment of granulocytes to the lung, as estimated by cells recovered in lung washes. Those data, in conjunction with the previously noted histological studies, have strongly suggested that aerosol inocula of up to ~4×10^6 staphylococci are cleared from murine lungs by alveolar macrophages. The present study provides the most definitive information that such is the case, and that granulocytes do not participate in the early defense of the lung against this organism under the conditions of this model. In addition, the maintenance of normal clearance mechanisms during granulocytopenia confirms that AGG has no deleterious effect on the function of alveolar macrophages or on mucociliary clearance.

The clearance of *Klebsiella*, however, was markedly impaired in neutropenic animals. At 4 h the lungs contained only 10% fewer bacteria than the original inoculum. Because mucociliary clearance alone would be expected to remove ~25% of the bacteria during this interval (12), bacterial multiplication must have exceeded total killing, indicating that alveolar macrophages alone were inefficient in killing *Klebsiella*. With the granulocyte function intact in the normal animals, clearance was increased to 33% of the original inoculum. Thus, granulocytes must participate in early lung defense against this organism.

Our data indicate an even greater necessity for granulocytes in the defense of the lung against *P. aeruginosa*. Normal animals cleared 26.8% of deposited *Pseudomonas* within 4 h; this was associated with a decline in circulating PMN levels, which has been previously noted (42, 43), and appears to represent margination and recruitment of available PMN to the lungs. Granulocytopenic animals in which this defense was absent were unable to inactivate *Pseudomonas*, and the bacteria proliferated to 513% of baseline levels despite a normally functioning macrophage system. Thus granulocytes from the circulation repre-

sent a major component of the normal defense against this bacterium. These interpretations are supported by the observation that *Pseudomonas*-inoculated irradiated dogs exhibit improved survival after receiving supplemental granulocyte transfusions (44).

We have not studied clearance at multiple inocula, and macrophages and granulocytes may be variably important depending on the initial bacterial phagocyte ratio. Rather, we have confined our observations to inocula that have previously been used in this model. Our data, nevertheless, are the first to clearly demonstrate a requirement for granulocytes in optimal early clearance of pathogenic bacterial species from the lung. The killing of staphylococci at this inoculum size can be attributed entirely to the alveolar macrophage, whereas for some gram-negative bacilli granulocytes are recruited from the circulation to aid in this early clearance of bacteria. Thus, factors controlling the cellular response to intrapulmonary bacteria appear to be dependent on bacterial species. Further investigations are necessary to determine what chemotactic factors determine the type of cellular response to inhaled bacteria.

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