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Induction of Phagocytic Inhibitory Activity in Cats with Chronic *Pseudomonas aeruginosa* Pulmonary Infection

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Chronic pulmonary infection has been established in cats by repeated intrapulmonary inoculation of viable *Pseudomonas aeruginosa* enmeshed in agarose beads. In the serum of all chronically infected animals, a substance(s) developed which inhibited phagocytosis of *P. aeruginosa* by normal cat alveolar macrophages. Phagocytosis was measured by incubating macrophage monolayers (5×10^5 alveolar macrophages) for 20 min in the presence of ^{3}H-labeled bacteria and 5% serum from control or infected animals. Inhibitory activity developed 4 to 16 weeks after initial infection, and inhibition of phagocytosis of *P. aeruginosa* in the presence of infected cat serum ranged from 30 to 79%. After inhibitory activity developed, it persisted throughout the remainder of the experiment in each animal. The activity was specific for *P. aeruginosa* of the infecting serotype and did not affect phagocytosis of gram-positive organisms. Inhibitory activity was unchanged by heating serum at 56°C for 30 min. We have previously described a *P. aeruginosa*-specific, heat-stable, phagocytosis-inhibitory activity in the serum of patients with cystic fibrosis. Since inhibitory activity also develops in cats with chronic *P. aeruginosa* pulmonary infection, such activity may not be a primary intrinsic abnormality in patients with cystic fibrosis. The animal model described here offers a system for following the development of and for characterization of the *P. aeruginosa*-specific phagocytosis-inhibitory activity.

Chronic pulmonary infection with *Pseudomonas aeruginosa* is a major cause of morbidity and mortality in patients with cystic fibrosis (CF), but it is rarely associated with chronic lung disease in other individuals (10). Because of the multifactorial nature of the pulmonary disease of CF, it is extremely difficult to separate alterations induced by infection from those intrinsic to the disease. Serum from patients with CF and chronic *P. aeruginosa* pulmonary infection specifically inhibits phagocytosis of *P. aeruginosa* in vitro by alveolar macrophages from both normal individuals and patients with CF (7). This inhibitory activity is also present in serum from individuals with chronic *P. aeruginosa* bronchitis, but is not present in serum from CF patients without chronic *P. aeruginosa* infection (M. J. Thomassen, C. Demko, P. Kuchenbrod, and B. Boxerbaum, RES J. Reticuloendothel. Soc. **24**:28a, 1978). These data suggest that acquisition of inhibitory activity is secondary to chronic *P. aeruginosa* pulmonary infection. Therefore we postulated that the inhibitory activity could be found in serum from animals with such an infection. We have adapted the agarose bead model of Cash et al. (2) for the establishment of chronic *P. aeruginosa* pulmonary infection in cats. Using this model we have demonstrated the development of a substance(s) in the serum of chronically infected cats which inhibits the ability of normal cat alveolar macrophages to phagocytize *P. aeruginosa*.

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MATERIALS AND METHODS

Preparation of agarose beads. Preparation of the agarose bead slurry has been described previously (2). Briefly, strain JR-1, an international serotype 5 strain of *P. aeruginosa*, was grown at 37°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 18 h. Heavy mineral oil, warmed to 50°C, was vigorously stirred with a magnetic spin bar as 10 ml of melted agarose with 1 ml of bacteria was added. The oil-agarose mixture was cooled rapidly by placing crushed ice around the vessel. Stirring continued for 5 min as agarose droplets solidified into beads 100 to 150 μm in diameter. Beads were washed in 0.5% sodium deoxycholate in phosphate-buffered saline and then in 0.25% sodium deoxycholate in phosphate-buffered saline to facilitate removal of the mineral oil. This was followed by four washes in phosphate-buffered saline. The loosely packed beads were then suspended in an equal volume of phosphate-buffered saline to form a

bead-buffer slurry. Bacterial cell counts were determined by plate counts with homogenized slurry.

Feline infection. Five adult (3 to 5.4 kg) nonconditioned cats of either sex were examined, dewormed, and observed for a minimum of 1 week for clinical evidence of respiratory disease (e.g., cough, rhinitis, fever, eye discharge, or conjunctivitis). Thereafter, four animals were given an intrapulmonary inoculation of viable *P. aeruginosa* enmeshed in agarose beads at 2-week intervals for up to 44 weeks. The fifth animal was similarly inoculated, but with sterile beads, as a control for abnormalities which might result from bronchoscopic injection of beads alone. The procedure for infection was as follows. Cats were given 0.1 mg of atropine and then anesthetized with a 1:10 mixture of acepromazine and ketamine (1 to 2 mg of ketamine per kg). The larynx and trachea were anesthetized with 1 ml of 4% topical lidocaine. A 3.5-mm flexible fiberoptic bronchoscope (Olympus BF 3C4) was used to lavage a subsegment of the right lower lobe with five 10-ml samples of normal saline at 37°C; 85 to 95% of the saline was recovered. The bronchoscope was then withdrawn to the main bronchus of the right lower lobe, and a 5-ml suspension of *P. aeruginosa* in agarose beads (0.5×10^8 to 2×10^8 colony-forming units per ml) was forcefully injected, followed by a bolus of air to disperse the beads.

To confirm the presence of *P. aeruginosa* infection, 0.1-ml samples of lavage fluid were cultured for 48 h at 37°C on blood and chocolate agar. Gram-negative rods isolated from lavage fluid were identified as *P. aeruginosa* by growth on Mueller-Hinton agar, pigment production, oxidase reaction, and, if necessary, oxidative utilization of glucose.

Preparation of cat alveolar macrophages. Normal cat alveolar macrophages were obtained by pulmonary lavage by the method of Myrvik et al. (5), as modified by Brain and Frank (1). Adult cats were sacrificed with intraperitoneal pentobarbital, and the lungs were excised and placed in a 37°C water bath. Lungs were lavaged with normal saline, and the lavage fluid was passed through a blood filter (Travenol Laboratories, Inc.) to remove mucus particles. Seven to eight lung washings from a single animal were pooled (ca. 400 ml) and centrifuged at $400 \times g$ for 10 min; the cell pellet was then washed twice with normal saline. Cell number was determined by duplicate hemocytometer count, and differential counts were made on smears stained with tetrachrome. Alveolar macrophages were plated at a density of 5×10^5 cells per 12-mm glass cover slip in 35-mm petri dishes containing McCoy 5A medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum, 1.9 mg of L-glutamine per ml, and antibiotics (gentamicin sulfate, penicillin, streptomycin, and amphotericin B). After 1 h at 37°C, nonadherent cells were removed by rinsing with Hanks balanced salt solution. The resultant monolayers (99% macrophages) were incubated in supplemented medium for 24 h at 37°C in a humidified 5% CO_2 atmosphere.

Bacteria. Cats were inoculated every 2 weeks with an international serotype 5 strain of *P. aeruginosa* (JR-1), originally isolated from the sputum of a patient with CF in San Antonio, Tex. Phagocytic assays were performed with the infecting strain (JR-1), another serotype 5 strain of *P. aeruginosa* isolated from the sputum of a CF patient in Cleveland, Ohio (P441), a non-CF clinical isolate of *P. aeruginosa* (P3, serotype 6), and *Staphylococcus aureus* Wood 46 (ATCC 10832). All strains of *P. aeruginosa* were nonmucoid with classical colony morphology when grown on Mueller-Hinton agar (8). Bacteria were radioactively labeled as described previously (7). During log-phase growth in minimal medium (Koser-Citrate) at 37°C, gram-negative bacteria were labeled with [^{3}H]leucine, and *S. aureus* was labeled with [^{3}H]thymidine. Radiolabeled bacteria were harvested by centrifugation at $3,000 \times g$ for 10 min, washed three times in saline at 4°C, and then suspended in saline to a concentration of 4×10^8 to 5×10^8 colony-forming units per ml as estimated by absorbance at 520 nm and confirmed by plate count. The specific activity of the bacteria was always greater than 10^{-2} dpm per bacterium.

Serum. Blood was obtained from normal and experimental animals and allowed to clot in serum separator tubes (Becton, Dickinson, & Co., Rutherford, N.J.) at room temperature for 30 min. After centrifugation, serum was frozen in working samples at −70°C within 2 h of collection. Sera from four healthy cats were pooled, aliquoted, and stored; this pool of serum was used as a control in addition to single cat serum controls.

Phagocytic assay. After 24 h in culture, normal cat alveolar macrophage monolayers were washed with warm balanced salt solution. They were then incubated at 37°C with a reaction mixture containing 1.7 ml of McCoy 5A medium, 0.1 ml of normal or infected cat serum (final concentration of serum, 5%), and 0.2 ml of a bacterial suspension with 4×10^8 to 5×10^8 CFU/ml (final concentration, 4×10^7 to 5×10^7 radioactively labeled bacteria per ml). Phagocytosis was terminated after 20 min by the addition of cold balanced salt solution. Cells were then washed extensively to remove free bacteria and lysed with 1.0 ml of 0.2 N NaOH. The remaining radioactivity (representing cell-associated bacteria) was determined in a liquid scintillation counter (Tracor Instruments, Austin, Tex.) with Formula 963 scintillation cocktail (New England Nuclear Corp., Boston, Mass.). Assays were performed in quadruplicate, and one monolayer was fixed and stained with tetrachrome to assess bacterial adherence to glass and for determination of phagocytic index. Phagocytic index was calculated by counting 100 to 200 macrophages in at least five high-power fields of tetrachrome-stained monolayers and determining the percentage of cells with one or more cell-associated bacteria. Phagocytosis is a two-stage process consisting of an attachment and an ingestion phase. Neither measurement of uptake of radiolabeled bacteria nor phagocytic index distinguishes the two stages. Parallel experiments at 4°C also did not differentiate attachment from ingestion. The cells are relatively inactive at this temperature and events requiring substantial energy production such as ingestion will not occur. However, at 4°C radioactive uptake was less than 5% of the uptake at 37°C, indicating that both processes were being inhibited. Percent inhibition of bacterial uptake was calculated as: [1 − (^{3}H-labeled bacterial uptake in infected cat serum/^{3}H-labeled bacterial uptake in normal cat serum)] ×100%. By expressing data as percent inhibition (or enhancement) it was possible to normalize the data from different assays for phagocytosis in the presence of normal cat serum. Fourteen assays were performed on alveolar macrophages ob-

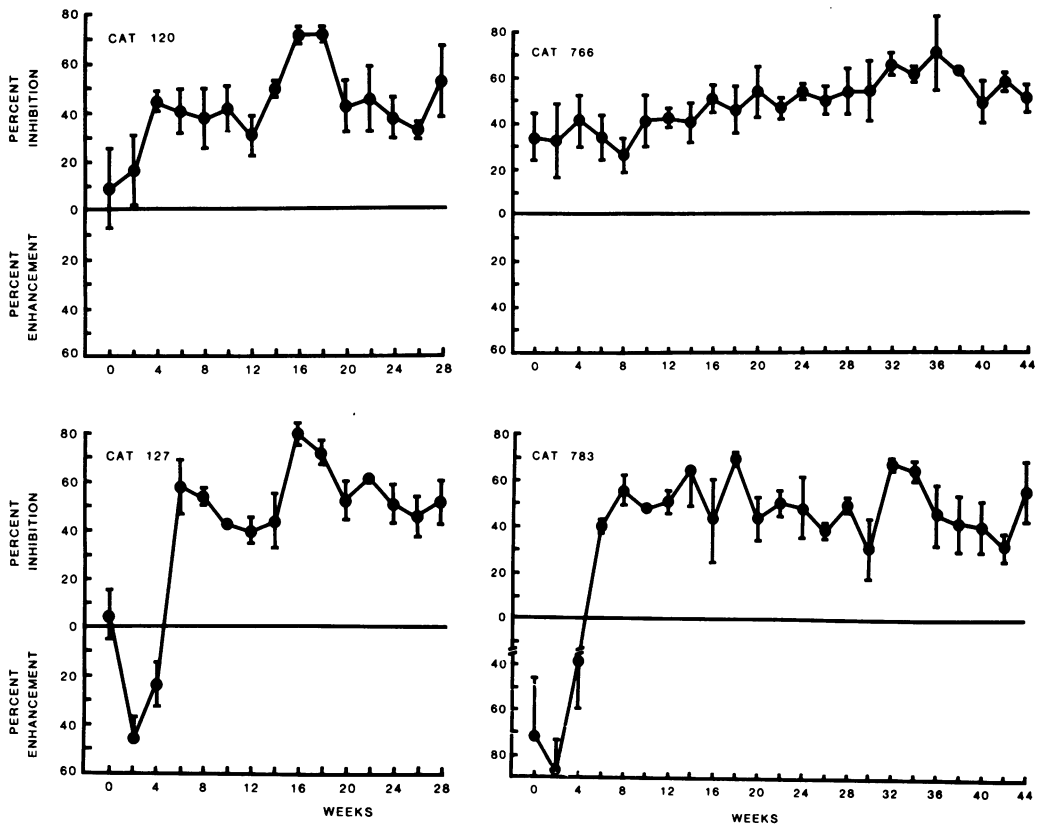

FIG. 1. Phagocytosis of *P. aeruginosa* by normal cat alveolar macrophages in the presence of infected cat serum as compared with phagocytosis in normal cat serum. All assays were carried out in triplicate. Percent inhibition (enhancement) was determined as described in the text. Each point on the graph was derived from the mean ± standard deviation of up to four experiments with that serum compared with control serum. Note that the initial point on each graph is the infected cat's preinfection serum sample compared with the control serum samples.

tained from normal cats; at least four sera from infected animals and two to four control sera were used in each assay. Incubation of *P. aeruginosa* JR-1 (without alveolar macrophages) in the same concentration of serum used in the phagocytosis assay did not have an effect on the organism as measured by viable counts. Student's *t*-test was used to compare percent inhibition of phagocytosis in preinfection serum with percent inhibition in postinfection serum samples.

RESULTS

Bacterial culture and bronchoscopy. Bacterial cultures of lavage fluid before initial infection with *P. aeruginosa* were negative for all bacteria in all animals. *P. aeruginosa* was cultured from 0.1-ml samples of bronchial washings from 30.1% (22 of 73) of lavage samples obtained from animals after initial inoculation with *P. aeruginosa*. *P. aeruginosa* was not recovered from any lavage samples from the cat inoculated with sterile beads. All cats in the infection group were reinoculated with *P. aeruginosa* JR-1 every 2 weeks to ensure chronic pulmonary exposure to the organism. Despite intermittently negative bacterial culture of small samples of lavage fluid, all four infected cats displayed erythematous bronchial mucosa and purulent secretions in the right lower lobe at bronchoscopy. The animal inoculated repeatedly with sterile beads had consistently normal bronchoscopic findings.

Phagocytosis by normal cat alveolar macrophages. Figure 1 shows the development of phagocytic inhibitory activity in the sera from infected cats. Normal cat alveolar macrophages were used in all phagocytic assays. Uptake of ^{3}H-labeled *P. aeruginosa* JR-1 in the presence of serum obtained at 2-week intervals from each infected animal was compared with uptake in serum from normal animals. The results are expressed as percent inhibition. Some variability occurred in phagocytosis of strain JR-1 in the presence of serum obtained before infection (week 0) as compared with phagocytosis in the presence of serum from normal animals. In the

TABLE 1. Percent inhibition[a] of phagocytosis of different bacterial strains in the presence of serum from infected cats

Serum source (cat no.)[b]	Serum collection[c]	% Inhibition of phagocytosis of:			
		P. aeruginosa			*S. aureus*
		JR-1	P441	P3	
120	26	32	47	NA[d]	−56
	28	52	25	−18	−175
127	26	45	54	NA	−3
	28	51	26	6	−2
766	32	65	24	16	NA
	34	60	NA	16	NA
	42	57	NA	NA	−6
	44	50	40	3	19
783	32	68	NA	16	NA
	34	65	NA	−32	NA
	36	46	NA	NA	−13
	42	31	NA	NA	11
	44	56	57	NA	NA
Mean ± SD		52.2 ± 11.7	39.0 ± 14.2	1.0 ± 18.9	−28.1 ± 63.4
141	22	12			
	24	11			
	26	−12			
Mean ± SD		3.7 ± 4.0			

[a] Enhancement of phagocytosis is indicated by negative percent inhibition.
[b] Cats 120, 127, 766, and 783 were inoculated with *P. aeruginosa* JR-1; cat 141 was inoculated with sterile agarose beads.
[c] Weeks after inoculation.
[d] NA, Not assayed at the indicated time point.

presence of serum from one animal (cat 127) obtained 2 and 4 weeks after initial infection, enhancement of phagocytosis of strain JR-1 occurred when compared with phagocytosis in preinfection serum ($P < 0.005$). The other three animals did not show significant enhancement of phagocytosis after the initial infection. Phagocytosis-inhibitory activity developed in all animals, although the time of development varied. Cats 127 and 783 developed significantly ($P < 0.005$) increased inhibition of phagocytosis of strain JR-1 as compared with preinfection sera, beginning 6 weeks after initial infection, and in cat 120 the activity was demonstrated at 4 weeks. Once the activity developed it could be demonstrated in all subsequent serum samples (P value varied from $P < 0.05$ to $P < 0.005$) throughout the experiment. Preinfection serum from cat 766 supported low levels of phagocytosis; nevertheless, all sera obtained 16 weeks or more after initial infection ($P < 0.025$ to $P < 0.005$) were significantly more inhibitory. No inhibition of phagocytosis developed in the presence of serum from the animal repeatedly inoculated with sterile beads for 26 weeks. (Results from weeks 22, 24, and 26 are shown on Table 1).

Results were similar when single cat sera or pooled cat sera were used as controls. Heating sera at 56°C for 30 min did not destroy inhibitory activity (mean percent inhibition ± standard deviation equaled 43.3 ± 15.6 before heat inactivation versus 37.4 ± 17.3% after heat inactivation, $n = 10$). Examination of tetrachrome-stained macrophage monolayers confirmed the absence of background bacterial adherence, and determination of phagocytic indices confirmed inhibition of phagocytosis in the presence of infected cat serum.

Specificity of inhibitory activity. The phagocytosis-inhibitory activity appears to be specific for *P. aeruginosa* of the same serotype as the infecting strain, i.e., phagocytosis of other serotypes of *P. aeruginosa* was not inhibited. Phagocytosis of *P. aeruginosa* strain P441 (serotype 5), the same serotype as infecting strain JR-1, was inhibited in the presence of all sera from infected cats (Table 1). However, phagocytosis of *P. aeruginosa* strain P3 (serotype 6) was not significantly inhibited by serum which consistently inhibited phagocytosis of strain JR-1 (some variability in level of phagocytosis occurred, with some sera from infected cats showing enhancement or slight inhibition of uptake as compared with uptake in normal cat sera). Simi-

larly, phagocytosis of *S. aureus* was not inhibited. The level of phagocytosis of *S. aureus* varied, and in one case definite enhancement was observed (serum from cat 120). For comparison, results in the presence of serum from cat 141 (the animal inoculated with sterile beads) are shown. No inhibition of phagocytosis was observed.

DISCUSSION

P. aeruginosa, a ubiquitous organism, is not usually pathogenic for healthy individuals, but it is associated with chronic pulmonary infection in patients with CF. Extrapulmonary infection in CF is rare (7), suggesting that the unusual susceptibility to *P. aeruginosa* pulmonary infection results from defective local pulmonary defense mechanisms. Since mucociliary transport in most CF patients is impaired (3), clearance of bacteria becomes more dependent on efficient action of phagocytic cells. A heat-stable substance(s) in CF serum specifically inhibits the ability of normal human and rabbit alveolar macrophages to phagocytize *P. aeruginosa* (7). Recently, it was shown that immunoglobulin G from CF serum binds to *P. aeruginosa*, but prevents attachment of the organism to the Fc receptor on the macrophage surface, thereby inhibiting phagocytosis (4). It was suggested that this *P. aeruginosa*-specific, nonopsonizing antibody was unique to patients with CF. However, in this paper we describe the development of an inhibitory activity in serum of cats with chronic *P. aeruginosa* pulmonary infection which is similar to the activity in CF serum. Decreased phagocytosis of *P. aeruginosa* by normal cat alveolar macrophages was observed in the presence of serum from infected animals beginning as early as 4 weeks after the initial infection; inhibition was significant as compared with preinfection serum in all animals by 16 weeks after initial infection. This activity was specific for the serotype of *P. aeruginosa* used for infection and, like the human activity, did not extend to gram-positive organisms. Furthermore, the activity was not affected by heating at 56°C for 30 min. The specificity of the activity for inhibition of *P. aeruginosa* phagocytosis, its development and persistence during chronic *P. aeruginosa* infection, and its heat stability suggest that the inhibitory activity is antibody mediated. Furthermore, initial fractionation of infected cat serum on Sephacryl 200 has shown the activity to be associated with the immunoglobulin G fraction (G. Winnie, J. D. Klinger, P. Cheng, J. Sherman, and M. J. Thomassen, Cystic Fibrosis Club Abstr. **21**:71, 1982).

The sequence of events involved in the development of inhibitory activity, presumably a nonopsonizing antibody, is not clear. The distribution and level of antibody classes in response to an antigen vary and may be determined by the nature of the antigen, size of the antigenic load, duration, frequency and site of antigen deposition, and properties of the host. Host defense mechanisms, including antibody response, may be altered by chronic antigenic exposure such as that present in both CF patients and in cats with chronic *P. aeruginosa* pulmonary infection. Although the number of animals is small, all four infected animals in this study developed significant *P. aeruginosa*-specific, phagocytosis-inhibitory activity similar to that found in CF patients. The level of inhibitory activity and exact time sequence of its development were variable. An initial enhancement of phagocytosis occurred in the presence of serum from one animal (cat 127); it may have not been detected in the other animals because serum was sampled only every 2 weeks. Additional animals must be studied and more frequent serum samples must be obtained to determine more precisely the sequence of development of enhancement and inhibition of phagocytosis. Although inhibitory activity has been demonstrated only in serum at this time, it may be important in local pulmonary defense in the presence of chronic infection because transudation of serum components and antibody into the lower respiratory tract has been shown to occur (6).

Acquisition of the phagocytosis-inhibitory activity in the serum of cats occurs after prolonged intrapulmonary exposure to *P. aeruginosa* and may be antibody mediated. Further characterization of the activity is proceeding in our laboratory. The animal model described here offers a system for following the development and for characterization of *P. aeruginosa*-specific, phagocytosis-inhibitory activity in serum and bronchoalveolar lavage fluid. It may be applied to other bacteria, i.e., *S. aureus* and *Hemophilus influenzae*. Furthermore, the pulmonary cellular response to chronic exposure to a bacterial agent can be followed sequentially. Finally, this model of chronic *P. aeruginosa* pulmonary infection can be used to help differentiate pathophysiological alterations in the lung which occur as a result of chronic infection from those alterations which are intrinsic to CF pulmonary disease.

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