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NEUTROPHIL RESPONSES TO INTRAVASCULAR PNEUMOCOCCAL SONICATE[1]

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Abstract—Leukopenia and pulmonary leukostasis are prominent features in patients succumbing to pneumococcal (PNC) infections. We examined mechanisms involved in recruitment of polymorphonuclear neutrophils (PMNs) into pulmonary capillaries and alveolae after PNC sonicate injection. We showed that by 15 min postinjection, PMN chemotactic activity was found in bronchoalveolar lavage (BAL) fluids and increased with time until the end point of the study at 90 min. Accompanying the increased chemotactic activity in BAL fluids was a decrease in circulating PMNs more pronounced in the femoral artery (FA) then the pulmonary artery (PA). Superoxide anion (O_2^-) production by peripheral PMNs was depressed following PNC sonicate injection, and comparison of FA and PA showed that FA PMNs produced less O_2^- than PA PMNs. PA PMNs also showed enhanced random migration when compared to the depressed random migration of FA PMNs. This study demonstrated that an intravascular challenge of PNC sonicate was associated with increased chemotactic activity for PMNs in BAL fluid. Fewer PMNs and altered PMN function resulted from passage through the pulmonary microvasculature after PNC sonicate injection.

INTRODUCTION

Pneumococcal (PNC) infections continue to cause significant morbidity and mortality in spite of immunization, appropriate use of antibiotics, and modern

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intensive care (1–3). Mortality in the first five days of treatment of bacteremic pneumococcal infections is unchanged from the preantibiotic era, even with the demonstration that antibiotics eradicate viable PNC (4). In some instances, death has been attributed to the adult respiratory distress syndrome (ARDS) and cardiovascular collapse (5). Leukopenia is noted to be a prominent feature in many patients dying from PNC infections (5, 6). We recently reported a case of death from PNC bacteremia with manifestations of leukopenia and pulmonary leukostasis found at autopsy (7). Pulmonary leukostasis, the aggregation of polymorphonuclear neutrophils (PMNs) in the pulmonary microcirculation, occurs in experimental animals after intravascular challenge with endotoxin (8), gram-positive organisms including PNC (9, 10), and during extracorporeal circulation (11). This phenomenon may be important in ARDS as PMN production of toxic oxygen radicals is thought to induce lung capillary endothelial damage and resultant increases in pulmonary vascular permeability (12, 13). The deposition of PMNs in the pulmonary capillaries may involve complement activity (13, 14), but the specific chemoattractant(s) are unknown.

The present study was designed to compare functional changes in PMNs harvested from the systemic and pulmonary vascular beds after intravascular PNC challenge in the dog, and to evaluate the recruitment of PMNs into the alveolar space after such a challenge.

MATERIALS AND METHODS

Preparation of Viable PNC and PNC Sonicate. Type 1 PNC identified with specific type 1 antisera (Statens Seruminstitut, Copenhagan, Denmark) was maintained on blood agar after three intraperitoneal passages through mice. PNC were inoculated into brain–heart infusion broth and incubated at 37°C with 5% CO_2 for 18 h. Cultures were centrifuged and the organisms washed three times and resuspended in 10 ml of sterile, nonpyrogenic saline. The resulting PNC–saline suspension contained $\geq 10^8$ colony forming units (CFU) of type I PNC.

PNC sonicate was prepared as previously described (15). The PNC–saline suspension was cultured quantitatively and heat-treated at 65°C for 20 min to kill the organisms and inactivate the autolytic enzyme. The suspension was then sonicated with a cell disrupter (model W185; Heat Systems, Ultrasonics, Inc., Plainview, New York) at 65 W for 3-min intervals in an ice bath for a total of 60 min. The sonicate vehicle and PNC were tested for endotoxin contamination by using the *Limulus* amebocyte lysate pyrogen assay (Mallinkrodt, Inc., St. Louis, Missouri). The sensitivity of this system is 0.06 ng/mg. All assays were performed in triplicate. No endotoxin was detected.

Hemodynamic Measurements. Mongrel dogs (approximately 20–30 kg each) were anesthetized with 2.5% intravenous sodium thiopental, 20 mg/kg of body weight. Anesthesia was maintained with intermittent injections. The dogs were intubated with a cuffed endotracheal tube and ventilated with a Bird Mark IV pressure-cycled ventilator; end-respiratory pressure was maintained at 5 cm H_2O. Tidal volume respiratory frequencies were adjusted to maintain normal arterial blood gasses for this altitude (pH 7.38–7.42, PO_2 >70 mm Hg, PCO_2 30–36 mm Hg). A No. 7 French, balloon-tipped, flow-directed, thermodilution catheter was inserted percutaneously into the femoral vein and advanced into the pulmonary artery with pressure wave-form monitoring. Mean femoral

arterial pressures (FAP) and mean pulmonary arterial pressure (PAP) were measured with pressure transducers (Statham Instruments, Inc., Oxnard, California). Hemodynamic measurements were made preinjection and 5, 15, 45, and 90 min after injection.

Hematologic Measurements. Blood samples were drawn from the PA and FA preinjection and 5, 15, 45, and 90 min postinjection of sonicate, whole organisms, or saline. Total white blood cell (WBC) counts were determined with a model Z Coulter counter (Coulter Electronics, Inc., Hialeah, Florida) and differential counts with Wright-stained smears.

Animal Groups. Dogs were maintained in accordance with recommendations as set forth by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Three groups of dogs were used: (1) five dogs injected with 10 ml of sterile, nonpyrogenic normal saline; (2) five dogs injected with 4×10^8 CFU of PNC suspended in 10 ml sterile, nonpyrogenic normal saline; and (3) five dogs injected with PNC sonicate prepared from 4×10^8 CFU suspended in 10 ml sterile, nonpyrogenic normal saline. All injections were made via the femoral artery to prevent any particles of sonicate from lodging in the pulmonary microvasculature.

Bronchoalveolar Lavage (BAL) and Preparation of Alveolar Macrophages (AM). After intubation, a silastic catheter 25 cm long and 2 mm in diameter was introduced into the endotracheal tube and threaded through the trachea and right bronchus into a distal bronchiole. At 0, 15, 45, and 90 min postinjection, the lung was lavaged with 50–60 ml of sterile, normal saline with return of 25–40 ml. After centrifugation at 1200 *g* for 15 min, the supernatant was decanted and saved. Total cell counts/mm^3 were determined using a Coulter counter; differential counts of recovered cells were made using cytocentrifuged smears stained with Wright-Giemsa for light microscopy. Mononuclear cells were separated from the lavaged cell population by Hypaque-Ficoll density centrifugation (16); the resulting mononuclear cells were >95% viable as determined by trypan blue exclusion and contained a mean of 94% macrophages and 6% lymphocytes. The cells were further washed in Hanks' balanced salt solution (HBSS) and resuspended at a cell concentration of 1×10^7 cells/ml in HBSS and 5% fetal calf serum (FCS). All cell containing suspensions and supernatants were maintained on ice until functional studies which were performed within 2 h of lavage.

PMN Preparation. Whole blood samples from dogs were obtained in heparinized syringes. After centrifugation through Ficoll-Hypaque, the resultant cell pellet containing PMNs and red blood cells was suspended in HBSS and mixed with 1 ml of Plasmagel (HTI, Buffalo, New York) per 3 ml of blood (17). The leukocyte-rich supernatant obtained after 30 min of incubation at 37°C was then centrifuged, and the cell pellet left after decanting was mixed with distilled water (4°C) for 30 sec to lyse residual red blood cells. Cells were then washed twice and resuspended in HBSS at a concentration of 10^7 cells/ml. The final preparation contained greater than 95% PMNs.

Superoxide Anion (O_2^-) Assay. O_2^- generation was measured by the reduction of cytochrome *c* (16). PMNs were isolated as described above. PMNs (5×10^5) were incubated at 37°C in 100 μl HBSS containing cytochrome *c* (1 mg/ml). All cell preparations were set up in duplicate. After 10 min, 100 μl of a 2 μg/ml phorbol myristate acetate (PMA) solution was added to the cells to trigger O_2^- production. This PMA concentration was previously shown to produce optimal O_2^- in this model. Controls were HBSS substituted for PMA. The amount of reduced cytochrome *c* was determined by measuring the change in optical density at 550 nm on an automated ELISA plate reader (Dynatech Laboratories, Inc., Santa Monica, California). The amount of O_2^- produced was then calculated using a molar extinction coefficient of 2.1×10^4 M/cm^2.

Random Migration. The degree of random migration of PMNs obtained from both sampling sites at all time intervals was examined by use of the migration-under-agarose technique (18). Two and one half milliliters of melted 0.75% agarose, which was supplemented by 5% FCS, were poured into 60 × 10-mm plastic culture dishes. After the medium was hardened, a series of three wells, 3 mm in diameter and 3 mm apart, were cut in each culture dish with a steel template and the agarose gel plugs were removed with gentle suction. PMNs were placed into the wells; 10 μl of a PMN concentration of 10^7 cells/ml were added to each well, and each sample was run in triplicate.

The plates were then incubated at 37°C in 5% CO_2 for 1 h. The distances the PMNs migrated were then measured using a standard microscopic grid.

Chemotactic Factors. Human C5a was prepared as previously described (19) and was obtained from fresh serum after incubation with 20 mg/ml zymosan for 60 min at 37°C in the presence of 1 M epsilon amino caproic acid (Sigma Chemical Co., St. Louis, Missouri). The chemotactic C5 fragment was then retrieved by passage of the activated serum over a Sephadex G-75 column. The C5a-containing region was pooled, concentrated, and titrated in a quantitative chemotactic assay for optimal chemotactic activity. Aliquots of this preparation were frozen at −70°C and were used as a standard source of C5a. Formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma) was stored as a 0.01 M stock solution in dimethyl sulfoxide at −70°C. Just before use, the stock solution was diluted to a previously determined optimal concentration in 0.01 M phosphate-buffered saline (pH 7.4).

Chemotaxis Assay. Agarose wells were prepared as described above for the random migration assays. Ten microliters of preparations containing 10^7 cells/ml BAL mononuclear cells, BAL supernatant, chemotactic factor (C5a or FMLP), or control media (5% FCS) were placed into a side well. Ten microliters of PMNs at a concentration of 2×10^7 cells/ml were then placed into the center well. All assays were completed in duplicate. The plates were incubated at 37°C in 5% CO_2 for 1 h. The distances the PMNs migrated were measured and the chemotactic index was then calculated using the formula:

$$\text{Chemotactic index (CI)} = \frac{\text{Distance migrated towards test well}}{\text{Distance migrated towards control well}} - 1$$

Statistical Analysis. For each parameter measured, the values for individual animals were used to calculate a group mean ± *SEM*. The mean changes for the different groups were compared at each time by a one-way analysis of variance. Dunnett's test was then used to compare individual treatment groups.

RESULTS

Hemodynamic Measurements after PNC Sonicate Injection

In the sonicate-injected dogs, there was a decrease in FAP from a mean of 110 to a mean of 70 mm H_2O and an increase in PAP from a mean of 21 to a mean of 28 mm H_2O. These pressure changes were manifested by 5 min and resolved by 15 min postinjection. No alterations in pressures were seen in the control dogs or those injected with whole bacteria.

Evaluation of BAL Fluid and Cells following Intravenous Injection of PNC Sonicate

Cellularity of BAL Fluid. At least 10^7 cells were recovered with each lavage. Before injection of the sonicate, more than 95% of cells were macrophages; PMNs constituted <2%. Eosinophils and lymphocytes made up the rest of the cell population. There was an increase over time in the percentage of PMNs found in the BAL from sonicate-injected dogs; this became statistically significant ($P < 0.05$) by 45 min postinjection (Figure 1). This was not seen

in saline- or whole-bacteria-injected dogs. There was no increase in eosinophils or lymphocytes in the BAL at any time.

Chemotactic Response of PMNs to BAL Cell Preparations and Supernatants. The results of the chemotactic assays in which dog PMNs were exposed to BAL mononuclear cells and supernatants are expressed in Figure 2. No changes in migration of dog PMNs were seen in response to BAL cells or

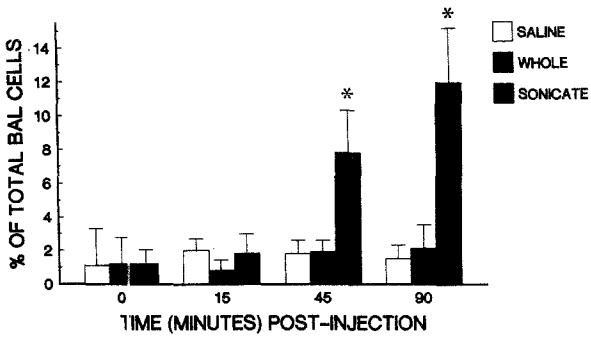

Fig. 1. Effects of PNC sonicate infusion on PMNs percentage in BAL fluid. Mean (± *SEM*) percentage of total cells in BAL fluid that were PMNs from five PNC sonicate-challenged dogs (solid bars), five dogs injected with whole PNC organisms (shaded bars), and five control dogs injected with saline (open bars). There was no difference between results using saline and whole PNC for this or any other experiments reported; therefore, only experiments using whole and sonicated PNC will be shown in the remaining figures. *Difference ($P < 0.05$) between postinjection time points and baseline percentage of total cells (time 0).$N = 5$.

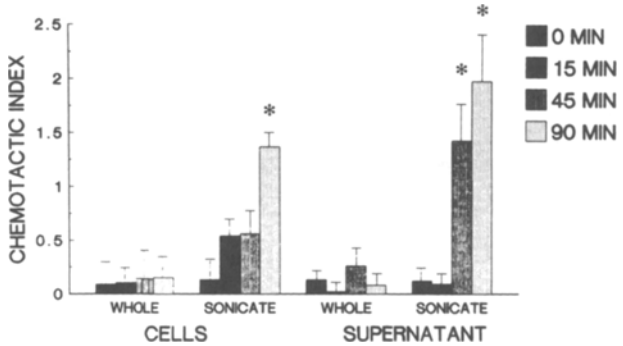

Fig. 2. Chemotactic effects of BAL cells and supernatant on PMNs. Bars represent mean (± *SEM*) chemotactic index of PMNs in response to BAL cells (first two clusters, labeled CELLS) and BAL supernatant (second two clusters, labeled SUPERNATANT) after injection with whole PNC (WHOLE) or sonicated PNC (SONICATE). Differences in shading of bars within each cluster represent different time points postinjection, as noted in the top right corner of the figure (0, 15, 45, and 90 min postinjection, respectively). *Difference ($P < 0.05$) between postinjection time points and baseline chemotactic index (time 0). $N = 5$.

supernatants obtained from dogs injected with whole organisms or saline. With increasing time after PNC sonicate injection, both the BAL cell preparation and supernatants had increased chemotactic activity for peripheral PMNs obtained from dogs.

Evaluation of PMNs in Peripheral Circulation.

Cell Counts. Mean baseline WBC count for all dogs was $6.8 \pm 1.4 \times 10^3$ cells/mm^3; mean PMN count was $4.3 \pm 0.79 \times 10^3$ cells/mm^3. These numbers did not change after injection with normal saline or whole heat-killed organisms. Mean WBC count, however, did decrease significantly ($P < 0.001$) after sonicate injection, reaching a nadir by 15 min postinjection (1.3 ± 0.54 cells/mm^3) and was accounted for totally by a reduction in PMNs (mean nadir PMN count was 0.46 ± 0.11 cells/mm^3; data not shown). As shown in Figure 3, the reduction in total of PMNs was greater in samples taken from the FA, significant by 15 min postinjection ($P < 0.01$). There were no significant changes in numbers of monocytes or lymphocytes.

Superoxide Anion Production following Injection of PNC Sonicate. After injection with sonicate, there was a significant ($P < 0.01$) reduction in the amount of O_2^- that was produced by cells stimulated by PMA (Figure 4). This was most pronounced at 15 min and was seen in cells obtained from both the femoral and pulmonary arteries. The PMNs obtained from the FA, however, produced less O_2^- than cells from the PA. In all sonicate-injected dogs, there

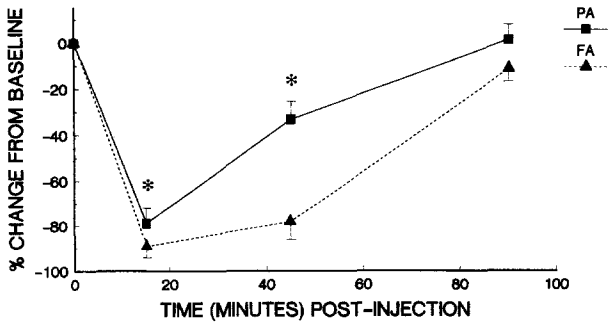

Fig. 3. Effects of PNC sonicate challenge on circulating PMN counts. Mean ($\pm$ *SEM*) percent change in numbers of PMNs harvested from the femoral artery (broken line) and pulmonary artery (solid line) in five dogs infused with PNC sonicate. *Difference ($P < 0.01$) in means between femoral and pulmonary arteries. $N = 5$. The change from baseline was significant for both femoral and pulmonary arteries at 15 and 45 min.

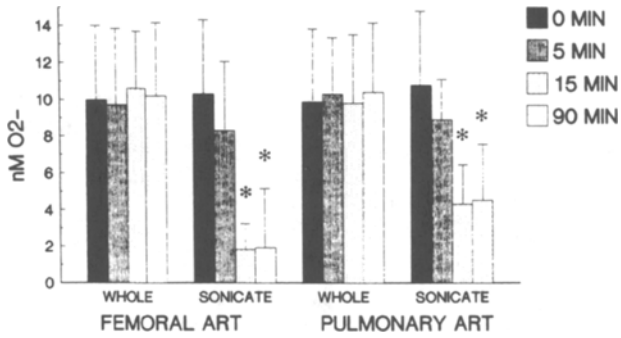

Fig. 4. Production of superoxide anion (O_2^-) by PMNs stimulated with PMA from FA and PA. Bars represent mean ($\pm$ *SEM*) production of superoxide anion (O_2^-) of PMNs obtained from femoral (first two clusters, labeled FEMORAL ART) and pulmonary (second two clusters labeled PULMONARY ART) arteries. Differences in shading of bars within each cluster represent different time points postinjection, as noted in the top right corner of the figure (1, 15, 45, and 90 min postinjection, respectively). *Difference ($P < 0.01$) between postinjection time points and baseline O_2^- (time 0). $N = 5$. There was a significant difference in O_2^- production between femoral and pulmonary arteries at 15 min.

was a positive PA–FA gradient, which was statistically significant at 15 min ($P < 0.001$). The O_2^- production was still depressed by 90 min in both FA and PA. There was no change from baseline production of O_2^- by PMNs obtained from dogs infused with saline or whole organisms.

Migration of PMNs Obtained from Dogs Injected with PNC Sonicate. PMNs from the PA showed significantly greater ($P < 0.01$) random migration at 15 min after sonicate injection as compared to preinjection migration (Figure 5). In contrast, cells from the FA showed depressed migration ($P < 0.01$) at 15 min. Random migration returned to preinjection activity by 90 min in both cell populations. No changes from baseline were seen in either FA or PA PMNs obtained from dogs challenged with normal saline or whole bacteria (mean CI = 3.45 $\pm$ 1.34).

PMNs from the PA and FA were also tested for chemotactic responsiveness to the chemotactic agents FMLP and C5a. Responsiveness of cells from both sampling sites to FMLP was found to be similar at all time intervals with a mean CI of 3.05 $\pm$ 1.6. In contrast, PMNs obtained from both sampling sites responded maximally to C5a only prior to infusion of PNC sonicate (mean CI = 2.9 $\pm$ 1.56; Figure 6). In PMNs from FA sampling sites no significant chemotactic activity was demonstrated in response to C5a at any time after sonicate injection (mean CI = 0.12 $\pm$ 0.10). However, this degree of chemotactic suppression did not occur until 90 min after sonicate injection in PMNs harvested from the PA. PMNs from dogs injected with whole organisms or

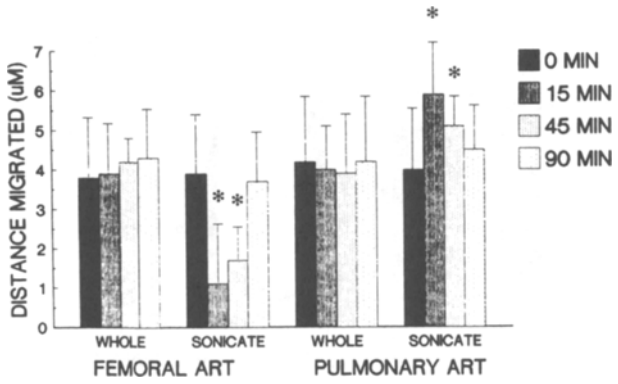

Fig. 5. Random migration of PMNs obtained from FA and PA. Bars represent mean (± *SEM*) chemotactic index of PMNs obtained from femoral (first two clusters, labeled FEMORAL ART) and pulmonary (second two clusters, labeled PULMONARY ART) arteries. Differences in shading of bars within each cluster represent different time points postinjection, as noted in the top right corner of the figure (1, 15, 45, and 90 min postinjection, respectively). *Difference ($P < 0.01$) between postinjection time points and baseline random migration (time 0). $N = 5$. There was a significant difference in random migration between femoral and pulmonary arteries at 15 and 45 min.

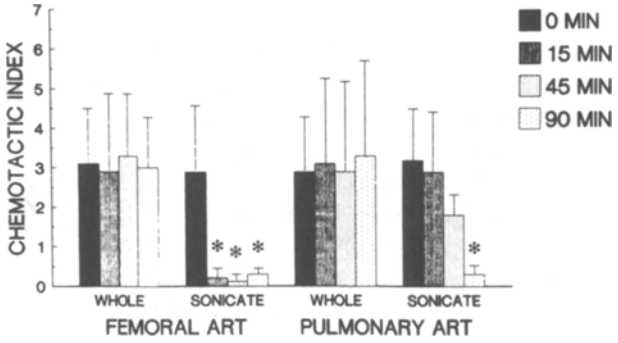

Fig. 6. Chemotactic effects of C5a on PMNs harvested from femoral (first two clusters, labeled FEMORAL ART) and pulmonary (second two clusters, labeled PULMONARY ART) arteries. Bars represent mean (± *SEM*) chemotactic index of PMNs. Differences in shading of bars within each cluster represent different time points post injection, as noted in the top right corner of the figure (0, 15, 45, and 90 min postinjection, respectively). *Difference ($P < 0.05$) in means between postinjection time points and baseline chemotactic index (time 0). $N = 5$.

saline demonstrated a mean CI of 2.7 ± 1.4 to FMLP and 3.2 ± 1.7 to C5a at all times.

DISCUSSION

In a recent survey (2), mortality in pneumococcal bacteremia was 30.5% overall, and 76% in patients with cardiopulmonary failure. The terminal events

that precede death in pneumococcal bacteremia involve cardiopulmonary dysfunction with leukopenia, pulmonary leukostasis, and profound shock (7). We have been able to reproduce this lethal combination of findings in an experimental dog model (20). In our model only PNC sonicate, not whole viable organisms, was able to induce leukopenia, pulmonary leukostasis, and shock, suggesting a requirement for cell disruption for initiation of the events leading to death. This may serve as an explanation for the clinical observation that death may rapidly ensue despite elimination of viable organisms by antibiotics (4).

Leukopenia is a prominent finding in patients with pneumococcal sepsis. We have shown previously (15, 20) that injection of PNC sonicate in the dog model induces a significant leukopenia, a finding confirmed in the present study. This appears to be due to depletion of PMNs from the peripheral circulation, possibly via entrapment of these cells in the pulmonary vasculature.

It has been demonstrated previously that pulmonary leukostasis occurs in PNC bacteremia not only in experimental animals (15, 20), but also in humans (7). This pneumococcus-induced augmentation of tissue leukostasis is specific for the pulmonary vascular bed (21). While we did not histologically demonstrate pulmonary leukostasis in the present study, several aspects of these experiments suggest that PMNs are being taken up in the pulmonary circulation after PNC sonicate injection. A positive gradient of PMNs between samples from the PA and the FA was found in all sonicate-injected dogs by 15 min postinjection. In addition, differential cell counts of BAL fluid showed a fivefold increase in the percentage of cells that were PMNs by 90 min postinjection of PNC sonicate. Although we can only hypothesize that these increased numbers of alveolar PMNs came from the peripheral circulation via the pulmonary capillaries, other studies have clearly demonstrated PMNs aggregating in the pulmonary microvasculature and entering the alveolus within 2.5 h after inoculation in experimental PNC pneumonia (22, 23).

The results from our experiments on random migration and O_2^- production suggest that functionally distinct subpopulations of PMNs exist in pulmonary and femoral arteries in this model of PNC bacteremia. These observations support prior reports that have demonstrated at least two functionally distinct subpopulations of PMNs in patients and experimental animals with pulmonary leukostasis (24, 25). Cells in one subpopulation exhibit greater functional activation and tend to adhere to the pulmonary endothelium; those in another subgroup are functionally distinct and tend not to adhere to the pulmonary endothelium.

Several studies have implicated complement activation as a key factor in the uptake of neutrophils into the pulmonary microcirculation. Till et al. (12) have characterized a major role for C5a in an experimental model of pulmonary leukostasis and ARDS. These authors demonstrated both increased serum levels of PMN chemotactic activity and an inverse relationship between the presence

of chemotactic activity and the concentration of PMNs in the blood within 5 min of complement activation. Other studies have shown a brief increase in PMN adherence within the pulmonary vasculature associated with synchronous neutropenia early in the course of experimental complement activation, followed within minutes by hypoadherence and release of PMNs into the peripheral circulation (26). Other authors have demonstrated that C5a receptors on peripheral PMNs are down-regulated after release from the pulmonary vasculature (27). Data from our study also suggest that C5a may be involved in modulation of PNC sonicate-induced pulmonary leukostasis and neutropenia. Chemotaxis of PMNs exposed in vivo to PNC sonicate was depressed in response to C5a, suggesting prior in vivo C5a exposure. PMNs from dogs injected with saline or whole organisms were normally responsive to C5a, indicating that PNC sonicate led to decreased responsiveness of these cells to C5a.

The etiology for PMN influx into the pulmonary parenchyma in response to systemic administration of PNC sonicate is not clear. AMs are known to produce chemoattractants specific for PMNs (28). The increased chemotactic activity for PMNs by the BAL mononuclear cell preparations in our experiments supports a role for this cell. Additionally, intraalveolar PMNs have been documented to produce chemoattractants for recruitment of additional PMNs into the lung after intrapulmonary deposition of PNC (29). Intraalveolar PMN production of chemotactic activity in response to the administration of intravascular PNC sonicate would also explain results previously reported from this laboratory in which complement-deficient dogs became granulocytopenic and developed pulmonary leukostasis after PNC sonicate injection (20).

In summary, this study demonstrated that an intravascular challenge of PNC sonicate was a stimulus for migration of PMNs into the lung parenchyma. The factor(s) responsible for this migration may be produced in the lungs from mononuclear cells or intraalveolar PMNs, or they may be generated intravascularly. In addition, PNC sonicate infusion produced changes in circulating PMN counts and function. Fewer PMNs with less functional activity were recovered from the circulation after passage thru the lungs (FA) compared to cells examined prior to pulmonary transit, suggesting the existence of distinct PMN subpopulations. At least a portion of PNC sonicate effects may be induced by PMN exposure to C5a, resulting in down-regulation of cellular receptors and PMN responses to this peptide.

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