Gerontology 1990;36:2–11

# In vitro Assessment of Chemotaxis by Peripheral Blood Neutrophils from Adult and Senescent C57BL/6 Mice: Correlation with in vivo Responses to Pulmonary Infection with Type 3 *Streptococcus pneumoniae*[1]

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**Key Words.** Neutrophils · Chemotaxis · Pneumococci · Pneumonia

**Abstract.** To assess the effects of advanced age on the ability of circulating neutrophils to respond to biologically relevant chemoattractants, cells were isolated from the peripheral blood of pathogen and disease-free C57BL/6 mice and evaluated in a microchemotaxis chamber. The responses of granulocytes obtained from senescent mice (26–28 months) to the chemotactic peptide, FMLP, and to leukotriene $B_4$ were similar to those found with cells from the younger animals (8–10 months). In contrast, the migration of neutrophils in response to sonicated type 3 *Streptococcus pneumoniae* was significantly greater with cells from the older animals. Similarly, the chemotactic response of neutrophils to zymosan-activated serum was greater with cells and serum from the senescent animals; however, the enhanced chemotaxis exhibited by granulocytes from the aged mice was a consequence of serum factors. Following the deposition of viable type 3 *S. pneumoniae* into the lower respiratory tract, the neutrophil influx at 24 h after challenge was significantly greater in the senescent mice; however, age-related differences in survival rates and $LD_{50}$ were not detected. Thus, in the C57BL/6 mouse, senescence is not associated with deficiencies in the response of neutrophils in vitro to chemoattractants that contribute to lung host defense against the pneumococcus; further, in this murine strain, advanced age does not result in an attenuation of the pulmonary inflammatory reaction to infection with type 3 *S. pneumoniae*.

[1] This work was supported by grants from the National Institute on Aging (AGO4907) and the Veterans Administration.

Infections of the lower respiratory tract due to *Streptococcus pneumoniae* remain a substantial problem among the elderly. For example, attack and case-fatality rates for pneumococcal pneumonia among persons 65 years of age and older have been estimated to be 3- to 5-fold greater than those experienced by younger adults [Esposito, 1988]. A number of factors are believed to contribute to enhancing the susceptibility of the senescent host to pneumococcal infection of the lungs; paramount among these factors appear to be age-related and disease-associated deficiencies in systemic immune function [Kay, 1979; Saltzman and Peterson, 1987].

The high incidence of pneumococcal pneumonia among the elderly also raises the possibility that advanced age is associated with derangements in the function of polymorphonuclear leukocytes (PMN), cells which play a central role in controlling infection due to *S. pneumoniae*. The ability of neutrophils to migrate into lungs infected with the pneumococcus by responding to locally derived chemoattractants represents one important function of these cells. Thus, the purpose of these studies was to evaluate the chemotactic activity of peripheral blood neutrophils isolated from adult and senescent C57BL/6 mice and to correlate those data with an in vivo assessment of host responses to pulmonary infection with type 3 *S. pneumoniae*.

## Materials and Methods

*Animals*

Specific-pathogen-free, adult and senescent, female C57BL/6 mice were obtained through contract with the National Institute on Aging from Charles River Breeding Laboratories, Kingston, N.Y. The ages corresponding to adult and senescent were derived from previously published strain-specific survivorship data and represent appropriate selections for comparative gerontological studies [Russell, 1972]. Adult and senescent refer to C57BL/6 mice of 8–10 and 26–28 months of age, respectively. The animals were housed in pathogen-free quarters and given sterile water and feed ad libitum. Mice with evidence of underlying disease (e.g., cachexia, tumors) were excluded from use. In preliminary experiments, we confirmed that the sera of the adult and senescent animals used in these experiments were free of antibodies to type 3 pneumococcal capsular polysaccharide (sera evaluated by radioimmunoassay, courtesy of Dr. Gerald Schiffman).

*Reagents*

Zymosan (from *Saccharomyces cerevisiae*), n-formyl-*l*-methionyl-*l*-leucyl-*l*-phenylalanine (FMLP), n-formyl-*l*-norleucyl-*l*-leucyl-*l*-phenylalanine (FNLP), bovine serum albumin (fraction V) and dextran (500,000 molecular weight) were purchased from Sigma Chemical Co., St Louis, Mo. Purified leukotriene $B_4$ ($LTB_4$) was supplied by Amersham, Arlington Heights, Ill. Zymosan was boiled and washed, as described [Esposito et al., 1988a] and stored at −70 °C. In some experiments, zymosan-activated serum (ZAS) was employed as a source of C5a chemoattractant activity and was prepared by the method of Wexler et al. [1983].

*Bacteria*

Type 3 *S. pneumoniae* (American Type Culture Collection, Rockville, Md.) was employed throughout these experiments. The isolate was preserved at −70 °C in 90% sheep red blood cells and 10% Todd Hewitt broth (Gibco Diagnostics, Madison, Wisc.) and passaged monthly in mice to maintain virulence. In preparation for animal challenge studies, bacteria were incubated overnight in Todd Hewitt broth (37 °C), washed twice with cold phosphate-buffered saline (PBS) (pH 7.4), and resuspended in PBS to the desired concentration, which was determined spectrophotometrically and confirmed by streaking serial 10-fold dilutions on 3% sheep blood agar plates and which was expressed as colony forming units (CFU) per milliliter. To prepare pneumococcal sonicate, 8 ml of a suspension containing $10^7$ CFU/ml were placed in a 15-ml polystyrene tube which was main-

tained in an ice-alcohol bath and sonicated for 30 min (W-225 Sonicator; Heat Systems-Ultrasonics, Farmingdale, N.Y.); morphometric and culture studies confirmed complete disruption of the bacterium. The sonicate was divided into 0.5-ml aliquots and stored at −70 °C.

*Neutrophils*

Murine peripheral blood PMN were obtained employing modifications of the method of Van Dissel et al. [1986]. Animals were given 1 ml of a 100 U/ml solution of heparin in 0.9% NaCl by subcutaneous injection and exsanguinated by orbital plexus phlebotomy with heparinized Pasteur pipettes. The blood was diluted 5-fold in PBS and pooled, and the cells were isolated by centrifugation on a ficoll/sodium diatrizoate gradient (Histapaque; Sigma); erythrocytes were removed by sedimentation (3% dextran) and hypotonic lysis (0.2% NaCl). The cells were washed twice with Hanks' balanced salt solution without $Ca^{2+}$ or $Mg^{2+}$ (MHBSS) and resuspended to the desired concentration in Gey's balanced salt solution containing 2% bovine serum albumin (GBSS). Greater than 85% of the cells recovered from the mice were neutrophils (modified Wright-Giemsa stain), and >95% of the cells were viable (trypan blue exclusion). Finally, human PMN, employed as controls in some of the preliminary studies, were obtained as described in our prior work [Esposito et al., 1988a].

*Chemotaxis*

Neutrophil migration was assessed in a 48-well acrylic microchemotaxis chamber, as in our prior work [Esposito et al., 1988a]. Neutrophils from adult and senescent animals ($5 \times 10^4$ cells) were placed above 3-μm pore size nitrocellulose filters in the upper compartments of the microchambers (Neuro Probe, Cabin John, Md.); 25 μl of GBSS or chemoattractant (diluted in GBSS) were placed in the lower compartments. After incubation (37 °C) in a humidified 5% $CO_2$ atmosphere, the filters were fixed, stained and mounted, as in our prior studies [Esposito et al., 1988a], and chemotaxis was quantitated by the method of Zigmond and Hirsch [1973]. Data from 5–7 filters were pooled, and the results were expressed as the mean distance (micrometers) of cell migration. Directed migration was defined as the distance migrated towards buffer-containing chemoattractant; random migration was defined as the distance moved towards buffer alone; and net migration represents the difference between directed and random migration [Esposito et al., 1988a].

*Animal Challenge*

Infection of the lower respiratory tract was induced by direct intratracheal inoculation, as detailed elsewhere [Esposito and Pennington, 1983]. In brief, animals were anesthetized with intraperitoneal methohexital sodium and suspended vertically by hanging the lower incisor teeth on a wire hook and by retaining the upper incisors with a taut rubber band. The oropharynx was transilluminated and under direct visualization the trachea was cannulated with a blunt-tipped metal spinal needle (No. 22 gauge) to which was attached a microliter syringe containing the bacterial suspension. After 50 μl of the bacterial suspension was delivered into the trachea, the cannula and syringe apparatus was removed. The animals were maintained in a vertical position for 2–4 min and then suspended at a 45° angle until awake. In each experiment, adult and senescent mice were infected in parallel.

*Pulmonary Lavage and Bronchoalveolar Cell Analysis*

The pulmonary inflammatory response to infection with *S. pneumoniae* was characterized, as in our prior studies [Esposito and Pennington, 1983]. Infected or control animals were sacrificed by $CO_2$ asphyxiation at 0 or 24 h after infection, the trachea was cannulated with polyethylene tubing, and the lungs were lavaged with 1 ml aliquots of MHBSS containing 0.05 *M* ethylenediaminetetraacetic acid (EDTA) to a total volume of 15 ml. The lavage specimens were centrifuged (4 °C) at 600 *g* for 10 min and the resulting cell pellet was washed with MHBSS without EDTA and resuspended in Hanks' balanced salt solution. The total number of cells was determined on a hemocytometer; all counts were done in duplicate. Mononuclear cells and neutrophils were identified by morphology on smears prepared with Wright-Giemsa stain; differential counts were performed on 200 cells.

To quantitate the peripheral blood neutrophil response to infection within the lungs, blood (approximately 100 μl) was obtained with a heparinized Pasteur pipette by orbital plexus phlebotomy prior to sacrifice for lung lavage. The blood was immediately transferred to a microdilution chamber (Unopette

Microcollection System; Becton-Dickinson, Rutherford, N.J.), and the total number of leukocytes was quantitated on a hemocytometer; duplicate counts were performed on duplicate specimens, and the results were recorded as the mean values. Aliquots of blood were also smeared and stained (modified Wright-Giemsa), and differential counts were performed on 200 consecutive cells; the results were reported in terms of the absolute number of neutrophils per cubic millimeter.

*$LD_{50}$ and Survival Rates*

To assess the effects of advanced age on survival, successive groups of 8–10 adult and senescent animals were challenged with increasing concentrations of the pneumococcus until a 100% mortality rate was achieved. All inocula were verified by colony counts. The animals were observed every 12 h for a total of 168 h and the interval between the time of infection and death was recorded. The median lethal inoculum ($LD_{50}$) was calculated by the method of Reed and Muench [1937].

*Data Presentation and Statistical Analysis*

To consolidate the number of tables presented, results of the chemotaxis assays were grouped together; however, the data derived with each chemoattractant were obtained in completely separate experiments. Results obtained from adult and senescent mice were analyzed with the two-tailed Student's t test; $p < 0.05$ was considered significant.

## Results

*Chemotactic Responses*

Since scant chemotaxis data are available concerning peripheral blood murine PMN, a series of preliminary experiments were necessary in order to characterize the responses of these cells to the relevant chemoattractants and to identify the optimal experimental conditions for the comparative evaluations. To conserve valuable senescent animals, cells from adult C57BL/6 mice were used in most of the preliminary studies; in addition, human PMN were employed as positive controls in some of the initial experiments.

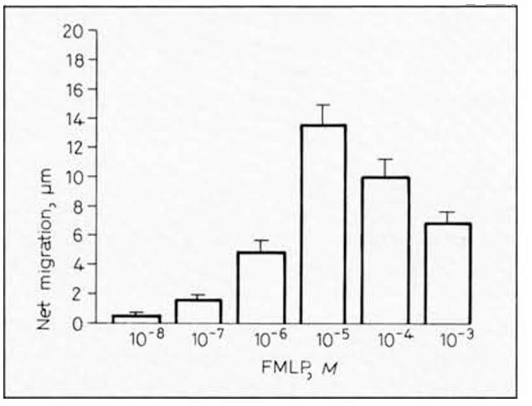


**Fig. 1.** The response of neutrophils obtained from adult C57BL/6 mice to FMLP. Peripheral blood granulocytes were isolated and chemotaxis was assessed, as described (Methods). In all experiments, the microchemotaxis chambers were incubated (37 °C) for 90 min. Data are expressed as the mean (± SEM) for 5 experiments. Maximal response noted at $10^{-5}$ *M* FMLP.

Neutrophils from adult C57BL/6 mice failed to demonstrate any movement in response to up to $10^{-4}$ *M* FMLP when incubated for 30 min [data not shown], and with reference to human PMN [Falk et al., 1980], these cells remained relatively unresponsive to the chemotactic peptide even following 90 min of incubation (fig. 1). Similar observations were made in a limited series of dose-response experiments with neutrophils from senescent C57BL/6 mice, adult BALB/c mice (4–6 months), and adult CD-1 mice (3–4 months) [data not shown].

Identical results were obtained when FNLP was employed as the chemoattractant with cells from adult C57BL/6 [data not shown]. The differences between the responses of murine and human neutrophils

**Table 1.** Chemotactic responses of neutrophils obtained from adult and senescent C57BL/6 mice

| Chemoattractant | Net migration, μm | |
|---|---|---|
| | adult | senescent |
| FMLP ($10^{-5}$ *M*) | 14.1 ± 1.3 | 13.9 ± 1.1 |
| $LTB_4$ (2 ng/ml) | 59.0 ± 4.3 | 54.5 ± 6.8 |
| Pneumococcal sonicate | 3.3 ± 0.5 | 9.8 ± 0.7* |
| ZAS | 27.0 ± 1.3 | 34.3 ± 1.2* |

Peripheral blood neutrophils were obtained and chemotaxis was assayed, as described (Methods). Conditions for these studies were derived from information generated in preliminary experiments (Results). In studies with ZAS, animals of the same group served as the source of both serum and cells. Data derived from 5–7 experiments with each chemoattractant and expressed as the mean ± SEM. Net migration greater with neutrophils from the senescent animals, * $p < 0.05$.

**Table 2.** Chemotactic responses of neutrophils obtained from adult and senescent C57BL/6 mice to ZAS

| Source of serum | Net migration, μm | |
|---|---|---|
| | adult | senescent |
| Adult | 26.2 ± 0.9 | 28.3 ± 1.2 |
| Senescent | 32.8 ± 1.0* | 34.9 ± 1.1* |

Peripheral blood neutrophils were obtained and chemotaxis was assayed, as described (Methods). Data derived from 7 experiments and expressed as the mean ± SEM. Net migration of cells greater with serum from senescent versus adult animals, * $p < 0.05$.

were remarkable; for example, with $10^{-7}$ *M* FMLP as the chemoattractant and following 30 min of incubation, the mean (± SEM) distances of net migration for human and murine PMN (n = 4) were 36.4 ± 3.1 and 0 μm, respectively; similar observations were made with $LTB_4$ and pneumococcal sonicate [data not shown]. A series of dose-response experiments similar to those noted with FMLP were also performed with $LTB_4$ and the pneumococcal sonicate in order to identify the concentrations that produced the optimal net migration of PMN from both study groups. The dose of pneumococcal sonicate that was associated with maximal migration and that was utilized in the subsequent studies represents a $10^{-1}$ dilution in GBSS of an initial bacterial suspension of $10^7$ CFU/ml. The ZAS was diluted 1:1 in GBSS prior to use, as in our recent studies [unpubl. observations]. Finally, in studies with FMLP, $LTB_4$ and pneumococcal sonicate, the microchemotaxis chambers were incubated 90 min; in experiments with ZAS, they were incubated 30 min.

Although random migration and directed migration tended to be greater with cells from the aged animals under all conditions studied, significant differences were not present [data not shown]. The net migration of cells from the adult and senescent animals in response to $10^{-5}$ *M* FMLP and 2 ng/ml $LTB_4$ were similar (table 1); however, the net migration of neutrophils from the senescent mice in response to the pneumococcal sonicate was greater than that observed with cells from the younger group (table 1). The response of PMN from the aged animals to ZAS was also greater than that found with cells from the younger animals (table 1). In additional experiments, granulocytes from the adult and senescent animals were exposed to serum from the same or opposite study group. These experiments demon-

strated that in the presence of ZAS derived from serum from senescent mice, the net migration of neutrophils obtained from both the adult and aged animals was significantly greater than that associated with ZAS produced from serum from the younger mice (table 2). Thus, these studies established that the age-related difference observed in neutrophil responses to ZAS (table 1) was due primarily to a serum factor(s).

*Pulmonary Inflammatory Response*

To assess the importance of the age-related differences found in vitro (table 1) to antibacterial mechanisms operant in the intact host, animals were challenged intratracheally with $10^6$ CFU of type 3 *S. pneumoniae*, and the cellular responses were characterized (Methods). A doese of $10^6$ CFU was selected since we have found in prior studies that that inoculum produces an intense host response in our animal model [Esposito, 1985]. The number of resident bronchoalveolar macrophages isolated from uninfected, aged C57BL/6 mice was significantly greater than that secured from the younger animals (fig. 2); this finding confirms a similar observation made by this laboratory in prior studies [Esposito et al., 1988b]. More importantly, at 24 h after pneumococcal challenge, the number of neutrophils present within the bronchoalveolar spaces was significantly greater in the older animals (fig. 2); comparable results were obtained at a lower inoculum ($10^{-5}$ CFU) [data not shown]. Finally, in uninfected animals, the mean (± SEM) numbers of circulating neutrophils in the adult and senescent mice (n = 6) were 868 ± 72 and 935 ± 97 $mm^{-3}$, respectively. At 24 h after infection, the number of circulating granulocytes tended to be lower in the aged mice; the mean (± SEM) numbers of circulating neutrophils in the adult and senescent animals (n = 6) were 1,979 ± 292 and 1,715 ± 243 $mm^{-3}$, respectively ($p > 0.05$).

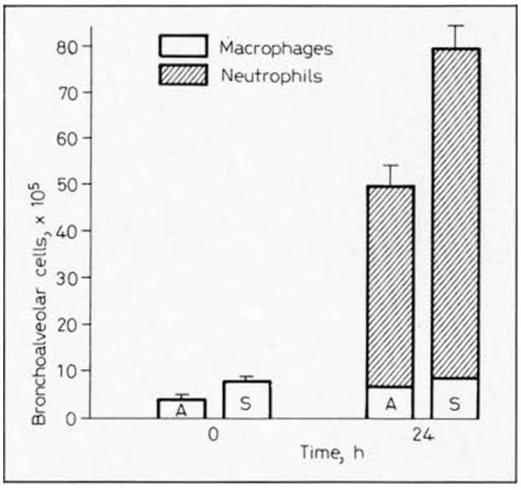


**Fig. 2.** The effect of advanced age on the generation of a pulmonary inflammatory response to type 3 *S. pneumoniae*. Adult (A) and senescent (S) C57BL/6 mice were challenged intratracheally with $10^6$ CFU of bacteria, and at 24 h, the cellular response was characterized (Methods); baseline (0 h) bronchoalveolar cell counts were performed on uninfected animals. Data derived from 7 animals/group and expressed as the mean (± SEM). At 0 h, the number of resident pulmonary macrophages was significantly greater in the senescent mice ($p < 0.05$), and at 24 h, the number of neutrophils present within the bronchoalveolar spaces was significantly greater in the aged animals ($p < 0.05$).

*Survival*

A significant age-related difference in the susceptibility of the C57BL/6 mouse to lethal infection with type 3 *S. pneumoniae* was not present (fig. 3). The corresponding $LD_{50}$, expressed as $\log_{10}$ CFU, for the adult and senescent mice were 3.30 and 3.35, respectively.

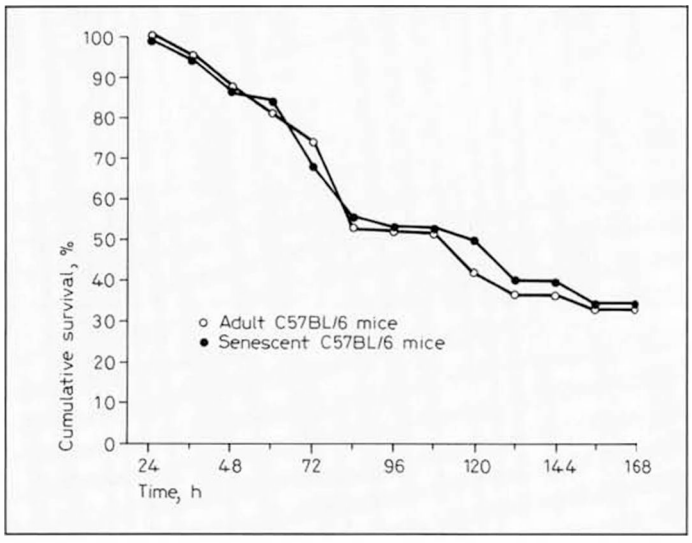


**Fig. 3.** The survival of adult and senescent C57BL/6 mice infected with type 3 *S. pneumoniae*. Paired groups of animals were challenged intratracheally with varying concentrations of type 3 *S. pneumoniae* and observed for 168 h. Data derived from 38 adult and 38 senescent animals. Significant differences not present.

## Discussion

Autopsy studies in humans and pathologic investigations in laboratory animals have shown that neutrophils are recruited into the lungs within a few hours following the onset of infection with *S. pneumoniae* [Esposito, 1985; Loosli, 1940; Wood, 1941]. Further, data from animal models have demonstrated that factors which impair the recruitment of neutrophils into lungs infected with the pneumococcus result in rapid bacterial proliferation and often death of the host; these factors include granulocytopenia [Wood and Irons, 1946] and complement depletion [Gross et al., 1978].

Contemporary investigations have identified a number of chemotactic factors that contribute to the recruitment of neutrophils into infected airspaces. First, pulmonary macrophages can liberate $LTB_4$ and other neutrophil chemoattractants [Merrill et al., 1980; Fels et al., 1982; Rouzer et al., 1982]. Second, bacterial pathogens can release materials that are chemoattractant for PMN. In culture, proliferating type 3 *S. pneumoniae* produces a low-molecular-weight chemoattractant [Ward et al., 1968], and in a rabbit model, Tuomanen et al. [1987] have recently shown that pneumococcal cell wall components and type 3 capsular polysaccharide can contribute to the induction of inflammation within the lungs. Finally, complement fragments, such as C5a, which are generated locally as a result of infection, also appear capable of amplifying the pulmonary inflammatory reaction [Gross et al., 1978].

In the current studies, we have evaluated the possibility that advanced age may be associated with changes in the capacity of neutrophils to respond to chemoattractants that appear to be important in the generation of an inflammatory response to type 3 *S. pneumoniae*. We have focused on the C57BL/6 mouse since this murine strain has been extensively employed in prior immunogerontological studies [Kay, 1979; Saltzman and Peterson, 1987]. In addition, in order to

more closely parallel the clinical situation and to avoid the use of elicited neutrophils (e.g., peritoneal exudate cells), we have utilized peripheral blood granulocytes.

We have found that the chemotactic responses of neutrophils isolated from senescent C57BL/6 mice to FMLP and $LTB_4$ were equal to those observed with cells secured from the younger animals (table 1). We have shown that the response of neutrophils from the older mice to ZAS (presumably C5a) was greater than that observed with cells from the younger animals and that the enhanced chemotaxis was due to the serum derived from the aged animals (table 2); further studies will be required to assess the contribution that C5a and other factors made to this observation. We have also demonstrated that neutrophils from the senescent mice were more responsive to pneumococcal sonicate than were cells from the younger group (table 1). Obviously, pneumococcal sonicate represents a crude preparation, containing capsular polysaccharide, cell wall fragments and other components; however, the use of the sonicate as a neutrophil chemoattractant would appear to be clinically relevant, since upon death, the pneumococcus undergoes bacteriolysis and, thus, discharges a variety of cellular constituents into the environment.

To our knowledge, this work represents the first attempt to characterize the influence of advanced age on the chemotactic responsiveness of circulating murine neutrophils. Thus, a comparison of our chemotaxis data with that of other investigators is not possible. It should be noted, however, that the best controlled in vitro studies of human PMN function have not revealed age-associated derangements in neutrophil chemotaxis [Corberand et al., 1986].

To assess the relative importance of our in vitro findings, we performed in vivo studies in a pneumococcal pneumonia model [Esposito, 1985]. We found that following the deposition of viable type 3 *S. pneumoniae* into the lungs, the inflammatory response was significantly greater in the senescent animals (fig. 2). A number of factors likely contributed to this finding. First, as we have shown in vitro, the responsiveness of neutrophils to pneumococcal sonicate was greater with cells from the aged animals, and the migration of granulocytes to ZAS was enhanced with cells and serum from the senescent mice. Second, as noted in these studies (Results; fig. 2) and in prior investigations [Esposito et al., 1988b], larger numbers of resident lung macrophages were present in the older animals. Although we have demonstrated in vitro that the capacity of C57BL/6 lung marcrophages to release $LTB_4$ is comparable with cells from adult and senescent mice [Esposito et al., 1989], the larger number of macrophages within the airspaces of the older animals gives that group the potential to liberate greater quantities of chemoattractants. Of note, in prior experiments with *Staphylococcus aureus* and *Klebsiella pneumoniae*, we also observed an enhanced pulmonary inflammatory response in aged C57BL/6 mice [Esposito and Pennington, 1983].

Our studies of survival failed to demonstrate an age-related difference in the susceptibility of the nonimmune, C57BL/6 mouse to lethal infection with type 3 *S. pneumoniae* (fig. 3). Since in previous experiments in our animal model we could not detect age-associated differences in the survival of the C57BL/6 mouse to pulmonary infection due to *S. aureus* or *K. pneumoniae* [Esposito and Pennington, 1983], the results of the current

work parallel the previous data. Similar observations in C57BL/6 mice have recently been made by Rothstein et al [1987] in a model of *Escherichia coli* pneumonia. The absence of age-related differences in susceptibility to fatal bacterial pneumonia in each of these prior studies suggests that factors beyond senescence, such as the presence of underlying diseases, play very important roles in producing the high-case fatality rates observed among geriatric patients infected with these pathogens.

Although we have demonstrated in the current work and in prior investigations [Esposito and Pennington, 1983] that the local inflammatory reaction to the presence of bacteria within the lower respiratory tract is greater in the senescent versus adult C57BL/6 mouse, we have not found that the augmented granulocyte influx exerts any influence on susceptibility to lethal infection. The reason for this observation is not known; we suspect that the enhanced neutrophil response represents a compensatory mechanism for one or more age-associated defects in lung or systemic host defenses. Indeed, since we did not evaluate neutrophil microbicidal activity in the current studies, the possibility exists that the pneumococcidal activity of these cells in the aged animal is impaired.

The data from the current studies lead us to conclude that in contrast to other parameters of immune competence, especially T lymphocyte function [Kay, 1979; Saltzman and Peterson, 1987] normal aging is not associated with an attenuation in the capacity of murine neutrophils to respond to chemoattractants or to participate in the local host response to pulmonary infection with the pneumococcus. Further, our survival studies indicate that in the nonimmune, normal C57BL/6 mouse, advanced age does not enhance susceptibility to lethal infection with type 3 *S. pneumoniae*. Thus, these findings and the results of our complementary studies of murine bronchoalveolar macrophage function [Esposito et al., 1988b; Esposito et al., 1989] indicate that factors beyond advanced age must play prominent roles in predisposing the aged host to pneumococcal infection of the lower respiratory tract and to increasing the likelihood of a fatal outcome.

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Received: February 23, 1989
Accepted: September 12, 1989

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