# Effect of Age on Respiratory Defense Mechanisms*

## Pulmonary Bacterial Clearance in Fischer 344 Rats After Intratracheal Instillation of *Listeria monocytogenes*

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***Study objectives:*** **To examine the lung defense mechanisms of both young and aged rats before and after pulmonary challenge with a bacterial pathogen.**
***Design:*** **Male Fischer 344 rats, either 2.5 months or 20 months of age, were intratracheally inoculated with $5 \times 10^3$, $5 \times 10^4$, or $5 \times 10^5$ *Listeria monocytogenes*, and the effects on mortality, lung inflammation, pulmonary bacterial clearance, alveolar macrophage (AM) function, and T-lymphocyte characterization were determined.**
***Measurements and results:*** **In noninfected control animals, the older rats had lower numbers of AMs on lavage and a lower percentage of total T, CD4+, and CD8+ cells. No difference was observed between noninfected young and old rats in AM function, assessing both chemiluminescence and nitric oxide (NO) production. After bacterial challenge, aged rats exhibited an increase in mortality, pulmonary infection, and edema, and lung lesions, which were more extensive than those observed in the younger rats. Interestingly, AM chemiluminescence was enhanced, while AM NO, a highly important antibacterial defense product, was abrogated in the aged rats as compared to the young rats.**
***Conclusions:*** **This study demonstrated that advanced age is associated with alterations in lung defense mechanisms and increased susceptibility to pulmonary bacterial infection marked by elevated mortality, slowed pulmonary bacterial clearance, and altered AM function, specifically a decrease in NO production. These observations are indicative of reduced pulmonary defense function in an older population of rats.** ***(CHEST 2001; 120:240–249)***

**Key words:** age; chemiluminescence; host defense; *Listeria monocytogenes*; macrophage; nitric oxide; pulmonary clearance

**Abbreviations:** AM = alveolar macrophage; bp = base pair; cpm = counts per minute; EMEM = Essential Minimum Eagle Medium; IFN = interferon; IL = interleukin; LALN = lung-associated lymph node; MoAb = monoclonal antibody; mRNA = messenger RNA; NIOSH = National Institute for Occupational Safety and Health; NO = nitric oxide; PBS = phosphate buffer solution; PCR = polymerase chain reaction; PMN = neutrophils (polymorphonuclear leukocytes); ROS = reactive oxygen species; RT-PCR = reverse transcription PCR; SE = standard error; TNF = tumor necrosis factor

Advanced age appears to be a predisposing factor for increased incidence of respiratory infection.[1–3] Pneumonia is the leading infectious cause of death in the elderly.[4] Elderly individuals are at a particularly high risk for severe consequences of pneumonia due to decreased respiratory reserve, the existence of other pulmonary diseases such as COPD, and the waning of innate and specific immunity that occurs with aging.[2] Pulmonary structural changes also occur with advanced age. Smaller airway sizes and increases in alveolar duct diameter have been reported in the aging human lung.[5] These morphologic alterations are considered to be due to changes in the relative proportions of decreased elastic tissues and increased collagen that occur with aging.

During the normal aging process, physical and biochemical changes occur which may affect the

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response of the lung to inhaled agents. Exposure to the oxidant gas $NO_2$ resulted in more epithelial damage and altered tissue repair[6] while $O_3$ increased the proliferative response[7] in the lungs of aged rats as compared to young rats. Other studies[8,9] have demonstrated that older animals have a greater lung oxidative stress potential than do younger animals. An age-associated decrease in nitric oxide (NO), an important mediator in alveolar macrophage (AM) defense against infection, has been observed when studying aged rats.[10] It has also been reported that elevated concentrations of nonheme iron ($Fe^{3+}$), a factor associated with an enhanced risk of infection, increases with age in both humans and rats.[11] In addition, elderly populations have been shown to be more susceptible to increased ambient particle levels as compared to the general population.[12]

In order to assess the pulmonary response to bacterial infection, a laboratory model using *Listeria monocytogenes* was employed. *L monocytogenes* is a Gram-positive, facultative intracellular bacterium that has been used in a number of animal studies[13–16] to assess pulmonary host defense mechanisms. The initial immune response of the host to *L monocytogenes* is marked by macrophage activation and a rapid recruitment of neutrophils (polymorphonuclear leukocytes [PMNs]) to the site of infection.[17] While the innate immune response is efficient at limiting the initial spread of infection, rapid clearance of *L monocytogenes* also depends on acquired T-cell–mediated immunity.[18] It was the goal of the current investigation to study the lung defense mechanisms of young and aged rats before and after pulmonary challenge with a bacterial pathogen. Fischer 344 rats, young (2.5 months of age) and aged (20 months of age), were instilled intratracheally with three doses of *L monocytogenes*, and the effect on mortality, lung injury and inflammation, pulmonary bacterial clearance, AM function, and T-lymphocyte characterization was determined.

## Materials and Methods

### *Animals*

Two populations of Fischer 344 rats were obtained (Harlan Sprague Dawley; Indianapolis, IN). Young adult male rats that were 2.5 months of age and weighed between 157 g and 255 g (mean ± SD age, 214.2 ± 3.02 g) comprised the "young" treatment group. Aged male rats that were ≥ 20 months of age and weighed between 353 g and 512 g (mean, 429.6 ± 4.48 g) comprised the "old" treatment group. The aged rats were in good general health, showing no clinical or histopathologic evidence of infection. They were acquired from colonies under the guidance of the National Institute on Aging (National Institute of Health, Bethesda, MD), which were maintained by Harlan Sprague Dawley, Inc. On arrival at our facility, all rats received a conventional laboratory diet and tap water *ad libitum*.

### *Intratracheal Bacteria Inoculation*

*L monocytogenes* (strain 10403S, serotype 1) was a donation from Rosana Schafer of the Department of Microbiology and Immunology at West Virginia University. *L monocytogenes* was cultured overnight in brain heart infusion broth (Difco Laboratories; Detroit, MI) at 37°C in a shaking incubator. Following incubation, the bacteria concentration was determined via spectrophotometry at an optical density of 600 nm and diluted with sterile saline solution to the desired concentrations.

The rats were lightly anesthetized by an intraperitoneal injection of 0.6 mL of a 1% solution of sodium methohexital (Brevital; Eli Lilly; Indianapolis, IN) and inoculated intratracheally with $5 \times 10^3$ (low dose), $5 \times 10^4$ (middle dose), or $5 \times 10^5$ (high dose) *L monocytogenes* in 500 μL of sterile saline solution, according to the method of Brain et al.[19] Animals in the vehicle control noninfected group were dosed intratracheally with 500 μL of sterile saline solution.

### *Mortality/Histopathology*

Animal weights and mortality were monitored daily over the course of the treatment period. Histopathologic analysis was also performed on rats from each group. The rats were killed with sodium pentobarbital, and the lungs were preserved with 10% buffered formalin by airway fixation at total lung capacity. The lobes of the lungs were removed, sectioned, embedded in paraffin, and stained with hematoxylin and eosin. Histopathologic analysis was performed by Dr. Val Vallyathan (National Institute for Occupational Safety and Health [NIOSH], Morgantown, WV), who was unaware of the experimental design and blinded to the treatment groups of the study.

### *BAL*

At 3 days, 5 days, and 7 days after bacteria instillation, the rats were deeply anesthetized with an overdose of sodium pentobarbital and then exsanguinated by severing the abdominal aorta. The left main bronchus was clamped off, and BAL was performed on the right lungs of rats from each group. Lavage was performed on their right lungs first with a 4-mL aliquot of calcium-free and magnesium-free phosphate buffer solution (PBS), pH 7.4. This first BAL fluid sample was centrifuged at 500*g* for 10 min and filtered with 0.22 μm sterile filters, and the resultant cell-free supernatant was analyzed for various biochemical parameters. Subsequently, lung lavage employed 6-mL aliquots of PBS until 50 mL was collected. These samples were also centrifuged for 10 min at 500*g*, and the cell-free BAL fluid was discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 mL of PBS buffer and evaluated.

### *Cellular Evaluation*

Total cell numbers were determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics; Hialeah, FL). Cells were differentiated using a Cytospin 3 centrifuge (Shandon Life Sciences International; Cheshire, England); $1 \times 10^5$ cells were spun for 5 min at 800 revolutions per minute and pelleted onto a slide. Cells (200 cells per rat) were identified on cytocentrifuge-prepared slides after labeling with Leukostat stain (Fisher Scientific; Pittsburgh, PA). The number of cells per lung volume was also determined by dividing the total cell number by total lung capacity. Total lung capacity equaled 12.5 mL multiplied by rat body weight in grams divided by 300.[20]

*Reverse Transcription-Polymerase Chain Reaction*

Total RNA was extracted from lung and lung-associated lymph node (LALN) tissue by guanidine isothiocyanate lysis (Trizol; Life Technologies; Rockville, MD). Tissues were processed immediately after sacrifice. Reverse transcription was performed using 5 μg total RNA, oligo(dT)12–18 primer, and Moloney murine leukemia virus reverse transcriptase (Superscript First-Strand complementary DNA Synthesis Kit; Life Technologies).

Polymerase chain reaction (PCR) was performed using complementary DNA derived from 0.4 μg RNA, primers as listed, Taq polymerase (Sigma-Aldrich, St. Louis, MO), deoxynucleoside triphosphate mix, 10X PCR buffer, $MgCl_2$, and $H_2O$. PCR conditions involved denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. The initial cycle contained a 4-min denaturation at 94°C, and the final cycle contained a 7-min extension at 72°C. Thirty-seven cycles were performed.

Primers used for reverse transcription-PCR (RT-PCR) were as follows: interferon (IFN)-γ 5′-ATCTGGAGGAACTGGCAAAA-GGACG-3′ and 5′-CCTTAGGCTAGATTCTGGTGACAGC- 3′, which amplify a 288-base pair (bp) fragment; interleukin (IL)-4, 5′-ACCTTGCTGTCACCCTGTTCTGC-3′ and5′-GTTGTGAG-CGTGGACTCATTCACG-3′, which amplify a 352-bp fragment; TNF-α 5′-TACTGAACTTCGGGGTGATTGGTCC-3′ and 5′-CAGGCTTGTCCCTTGAAGAGAACC-3′, which amplify a 295-bp fragment; IL-6, 5′-CAAGAGACTTCCAGCCAGTTGC-3′ and 5′-TTGCCGAGTAGACCTCATAGTGACC-3′, which amplify a 614-bp fragment; and G3PDH, 5′-TGAAGGTCGGT-GTCAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAG-GTCCACCAC-3′, which amplify a 983-bp fragment. With the exception of IL-4, all primer sequences were obtained commercially (Clontech; Palo Alto, CA). PCR products were visualized in ethidium bromide-stained agarose gels. Inspection of glyceraldehyde 3-phosphate dehydrogenase PCR product was used to document equal loading.

*Pulmonary Clearance of* L monocytogenes

At 3 days, 5 days, and 7 days after bacteria instillation, left lungs, which had not undergone lavage, were removed from all rats in each treatment group. The excised tissues were suspended in 10 mL of sterile water, homogenized using a Polytron 2100 homogenizer (Brinkmann Instruments; Westbury, NY), and cultured quantitatively on brain heart infusion agar plates (Becton Dickinson; Cockeysville, MD). The number of viable colony forming units were counted after an overnight incubation at 37°C.

*Luminol-Dependent Chemiluminescence*

Luminol-dependent chemiluminescence, a measure of light generation representing reactive oxidant species (ROS) production, was performed with an automated Berthold Autolumat LB 953 luminometer (Wallace; Gaithersburg, MD) as described previously.[21] Resting chemiluminescence was determined by incubating $0.5 \times 10^6$ BAL cells at 37°C for 10 min in 0.008 mg/dL (weight/volume) luminol in a total volume of 0.5 mL of 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma Chemical; St. Louis, MO) followed by the measurement of chemiluminescence for 15 min. Luminol is used as an amplifier to enhance detection of the light. Nonopsonized, insoluble zymosan (2 mg/mL; Sigma Chemical) was used as a stimulant and was added to the assay immediately prior to measurement of chemiluminescence. Since PMNs do not respond to unopsonized zymosan, the zymosan-stimulated chemiluminescence produced is from AMs.[22] Measurement of chemiluminescence was recorded for 15 min at 37°C, and the integral of counts per minute (cpm) vs time was calculated. Chemiluminescence was calculated as the cpm of stimulated cells minus the cpm of the corresponding resting cells.

*AM NO Production*

BAL cells were suspended at a concentration of $1.0 \times 10^6$ cells/mL in Essential Minimum Eagle Medium (EMEM) [BioWhittaker; Walkersville, MD] supplemented with 2 mM glutamine, 100 g/mL streptomycin, 100 U/mL penicillin, and 10% heat-activated fetal calf serum, and seeded onto each well of a 24-well tissue culture plate. BAL cells were allowed to adhere to the plates for 2 h at 37°C at 5% $CO_2$. After the incubation, nonadherent cells were removed by washing three times with EMEM media. The adherent cells, which were found to be $>$ 90% AMs were then incubated in fresh EMEM for 18 h at 37°C at 5% $CO_2$. The AM-conditioned media was collected, centrifuged, and stored at − 70°C until analysis. The production of NO was determined as an accumulation of nitrite by a modified microplate assay using the Griess reagent.[23] Briefly, the samples were incubated with an equal volume of the Griess reagent at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate spectrophotometer reader (SpectraMax 250; Molecular Devices; Sunnyvale, CA). Sodium nitrite (Sigma Chemical) was used as a standard. The results were expressed as μmol nitrite/$10^6$ AMs.

*Lymphocyte Differentiation*

At 7 days after inoculation with *L monocytogenes*, LALNs were excised and homogenized in PBS to count and differentiate T cells and T-cell subsets—CD4+ helper and CD8+ cytotoxic cells. The respective cell types were labeled with an appropriate monoclonal antibody (MoAb) that was conjugated with a fluorescent probe for visualization according to the method of Luster et al.[24] Single-cell suspensions were prepared from excised lung-associated lymph nodes. The cells were collected by centrifugation and suspended in PBS, pH 7.4, containing 1% bovine serum albumin and 0.1% sodium azide to a cell density of $1.5 \times 10^6$/mL. For T-cell enumeration, anti-rat CD5RA MoAb was conjugated to fluorescein isothiocyanate. For T-cell subsets, anti-rat CD4 MoAb was conjugated with fluorescein isothiocyanate, and anti-mouse CD8 MoAb was conjugated with dipalmitoylphosphatidylethanolamine. The cells were also incubated with their respective isotype control to correct for autofluorescence. After incubation with the conjugated MoAb, the cells were washed once with the staining buffer and incubated for 5 min with propidium iodide as a viability stain. The cells were again washed and then enumerated on a Becton Dickinson FACS Vantage Flow Cytometer (Becton Dickenson). Fluorescence was gated on propidium iodide to eliminate dead cells. The values are expressed as the percent of gated live cells and the absolute number of cells staining positive for each surface marker.

*Statistical Analysis*

Results are expressed as means ± standard error of measurement (SE). Statistical analyses were carried out using a statistical program (JMP IN; SAS Institute; Cary, NC). The significance of the interaction among the different treatment groups for the different parameters at each time point was assessed using an analysis of variance. The significance of difference between individual groups was analyzed using the Tukey-Kramer *post hoc* test. For all analyses, the criterion of significance was set at $p < 0.05$.

## Results

### *Age Comparison of Noninfected Rats*

To investigate constitutive difference in lung status, the number of AMs and PMNs recovered by BAL from noninfected young and old rats were compared (Table 1). A significantly lower number of AMs was recovered from the lungs of the old rats as compared to the young rats, where as there was no difference in the lung PMN number for the two groups (data not shown). Since the old rats were slightly more than twice the weight of the young rats, we normalized cell number by dividing by the total lung capacity of each animal. There were significantly fewer AMs and PMNs per lung volume in the old rats as compared to the young rats. Lymphocytes recovered from LALN of noninfected young and old rats were counted and differentiated. There was no significant difference in the number of total T cells, CD4+ T-helper cells, and CD8+ T-cytotoxic cells when comparing the young and old rats (Table 1). However, when the percentage of T cells of recovered lymphocytes was determined, there was a significantly lower percentage of total T, CD4+, and CD8+ cells in the old rats as compared to the young rats.

In evaluating AM function for cells recovered from the lungs of untreated young and old rats, chemiluminescence and NO production were measured. No significant differences were observed in zymosan-stimulated chemiluminescence and NO production between the noninfected young and old rats.

### *Age Comparison of Rats Infected With* L monocytogenes

Following intratracheal inoculation with three different doses of *L monocytogenes* (low dose, $5 \times 10^3$ bacteria; middle dose, $5 \times 10^4$ bacteria; high dose, $5 \times 10^5$ bacteria), cumulative survival among young and old rats was determined (data not shown). The low-dose inoculum had no effect on survival for either young or old rats. One of the old rats exposed intratracheally to the middle dose of bacteria died 4 days after exposure, while none of the young rats died. In contrast, after treatment with the high dose, 40% of the old rats had died within 3 days after intratracheal inoculation with the bacteria; none of the young rats had died up to that point. Beyond 3 days, survival continued to decline with time in the old rats. By 6 days after high-dose bacteria instillation, all of the old rats had died. For the young rats, 80% of the animals were still alive at 4 days, but by 7 days postinfection, only 28% remained alive. At 8 days after high-dose bacteria instillation, all of the young rats had also died.

**Table 1—*Age Comparison of Noninfected Rats**

| Lung Parameters | Young Rats | Old Rats |
|---|---|---|
| Cell count, No. ($\times 10^6$)/lung volume (mL) | | |
| AMs | 0.38 ± 0.04† | 0.15 ± 0.03 |
| PMNs | 0.07 ± 0.01† | 0.03 ± 0.00 |
| Lymphocytes, No. ($10^6$) | | |
| Total T Cells | 11.7 ± 0.80 | 12.5 ± 0.98 |
| CD4+ | 4.61 ± 0.48 | 4.81 ± 0.48 |
| CD8+ | 4.37 ± 0.20 | 4.93 ± 0.53 |
| Total lymphocytes, % | | |
| Total T cells | 46.2 ± 2.71† | 31.9 ± 1.78 |
| CD4+ | 18.2 ± 1.77† | 12.3 ± 1.13 |
| CD8+ | 17.3 ± 0.85† | 12.5 ± 1.00 |
| Macrophage function | | |
| Zym CL, cpm $\times 10^5/10^6$ AMs | 5.95 ± 0.54 | 5.93 ± 1.06 |
| NO, μmol/$10^6$ AMs | 12.5 ± 3.49 | 9.59 ± 2.90 |

*Data are presented as mean ± SE (n = 5 to 10). Zym CL = zymosan chemiluminescence.
†Significantly greater than mean values for old rats.

Histopathologic analyses were performed on the lungs of rats from each treatment group (Fig 1). Lungs appeared normal for both the noninfected young and old groups (data not shown). A severe pneumonitis, characterized by a peribronchiolar accumulation of PMNs, and the appearance of multiple granulomatous lesions were observed throughout the lungs of both the young and old rats 5 days after inoculated intratracheally with the low $5 \times 10^3$ dose of *L monocytogenes* (Fig 1, *top left*, *A*, and *top right*, *B*). Five days after intratracheal instillation of the middle $5 \times 10^4$ dose, severe edema, inflammation with significant infiltration of PMNs, and many granulomatous lesions with amorphous tissue debris were observed (Fig 1, *bottom left*, *C*, and *bottom right*, *D*). The inflammation, edema, and lesions that were observed in the old rats inoculated intratracheally with both the low and middle bacteria doses were more extensive and more pronounced than those observed in the young rats.

In assessing cell numbers after treatment with the low and middle bacteria doses, there was mostly no difference in the number of AMs recovered by BAL when comparing the young and old rats (Fig 2, *top*, *A*). However, there were significantly more PMNs recovered at both the middle and low bacteria doses at 5 days and 7 days for the old rats as compared to the young rats after *L monocytogenes* inoculation (Fig 2, *bottom*, *B*).

Lymphocytes recovered from LALNs of young and old rats 7 days after intratracheal inoculation with the low and middle *L monocytogenes* doses were counted and differentiated (Fig 3). For both doses, there were significantly more total T and CD4+ T-helper cells recovered from the LALNs of the old rats as compared to the young rats (Fig 3, *top*, *A*). There was no difference in CD8+ T-cytotoxic cell number between the young and old rats. How-

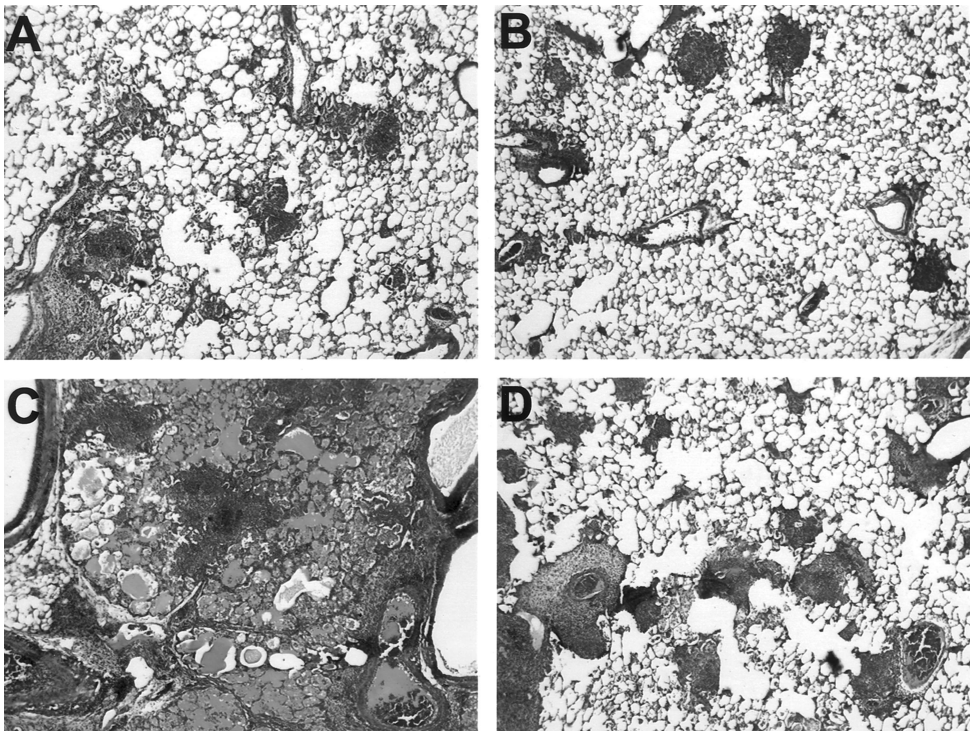


FIGURE 1. Photomicrographs of rat lungs 5 days after intratracheal inoculation with *L monocytogenes*: old rats, + $5 \times 10^3$ *L monocytogenes* (*top left*, *A*); young rats, + $5 \times 10^3$ *L monocytogenes* (*top right*, *B*); old rats, + $5 \times 10^4$ *L monocytogenes* (*bottom left*, *C*); and young rats + $5 \times 10^4$ *L monocytogenes* (*bottom right*, *D*) [hematoxylin-eosin, original × 10].

ever, there was a greater percentage of CD8+ cells for the two young groups as compared to the old groups (Fig 3, *bottom*, *B*).

RT-PCR was used to document levels of messenger RNA (mRNA) encoding the proinflammatory cytokines tumor necrosis factor (TNF)-α and IL-6, as well as the Th1 cytokine, IFN-γ, and the Th2 cytokine, IL-4, in lung and LALN tissue (Fig 4). Seven days after infection with a low dose of *L monocytogenes* ($5 \times 10^3$ organisms), greater levels of mRNA encoding the proinflammatory cytokines TNF-α and IL-6 were detected in the lungs of old rats as compared to the young animals. Lung levels of IFN-γ and IL-4 did not differ between young and old animals at this time point. The pattern of cytokine gene expression was different in LALN tissue. In LALN tissue, TNF-α mRNA levels were greater in young animals. Other LALN tissue cytokine mRNA levels evaluated did not differ between the two groups.

The number of bacteria colony forming units was determined in left lungs and showed a significant effect of age on infection and clearance after intratracheal inoculation with *L monocytogenes.* After treatment with the middle $5 \times 10^4$ bacteria dose, there was dramatically higher counts of *L monocytogenes* in the left lungs of the old rats as compared to the young rats at 5 days and 7 days after bacteria instillation (Fig 5). For the low $5 \times 10^3$ dose, there was dramatically higher counts of *L monocytogenes* in the left lungs of the old rats at all three time points. At 7 days after *L monocytogenes* treatment, substantial increases in bacteria colony forming unit number in log-10 base units of 1.5-fold and 1.4-fold were observed for the middle- and low-dose *L monocytogenes* treatments for the old rats as compared to the young rats, respectively.

ROS production was determined by measuring chemiluminescence of AMs recovered from young and old rats. A significant increase in AM chemiluminescence was observed for the old rats as compared to the young rats at the low and middle bacteria doses at all three time points after *L monocytogenes* instillation (Fig 6). An unexpectedly significant elevation in chemiluminescence was observed for the lower bacteria dose as compared to the middle dose in the old rats 5 days after bacteria treatment. The production of NO by AMs recovered from young and old rats was also measured (Fig 7). In opposition to the chemiluminescence findings, a significant elevation in NO was observed for the young rats at the middle bacteria dose for all three time points and for the low bacteria dose 5 days and 7 days after *L monocytogenes* instillation as compared to the old rats.

The different lung parameters for both young and old rats were also measured after intratracheal inoc-

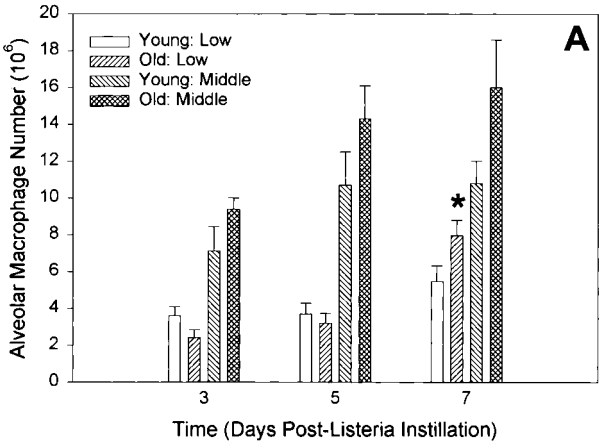


FIGURE 2. Number of AMs (*top*, *A*) and PMNs (*bottom*, *B*) recovered from the lungs of young and old rats 3 days, 5 days, and 7 days after the intratracheal instillation of the low dose ($5 \times 10^3$ bacteria) and the middle dose ($5 \times 10^4$ bacteria) of *L monocytogenes*. Values are mean ± SE (n = 5 to 9). At each time point, the old rat/low-dose group (old: low) is significantly greater than the young rat/low-dose group (young: low) [designated by *]; the old rat/middle-dose group (old: middle) is significantly greater than young rat/middle-dose group (young: middle) [designated by #; $p < 0.05$].

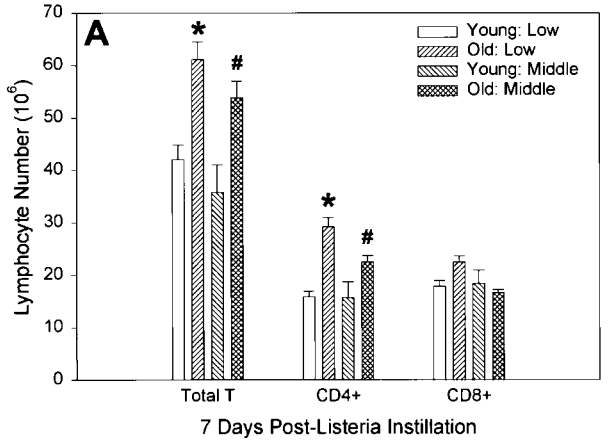


FIGURE 3. Number (*top*, *A*) and percentage (*bottom*, *B*) of T lymphocytes recovered from LALNs of young and old rats 7 days after the intratracheal instillation of the low ($5 \times 10^3$ bacteria) and middle ($5 \times 10^4$ bacteria) doses. Values are mean ± SE (n = 4). For each cell type, the old rat/low-dose group (old: low) is significantly different from the young rat/low-dose group (young: low) [designated by *]; the old rat/middle-dose group (old: middle) is significantly different from the young rat/middle-dose group (young: middle) [designated by #; $p < 0.05$].

ulation with the high $5 \times 10^5$ *L monocytogenes* dose (Table 2). There were significantly more AMs, PMNs, elevated resting and zymosan-stimulated chemiluminescence, and a greater number of bacteria in the left lung of the old rats as compared to the young rats 3 days after bacterial challenge. The other time points of 5 days and 7 days after bacteria instillation were also determined for both groups, but due to the elevated mortality for the old rats after exposure to the high $5 \times 10^5$ dose, there were not enough living old rats for statistical comparisons with the young rats.

## DISCUSSION

Epidemiology has identified elderly populations as being more susceptible to respiratory illness as compared to the general population.[2] The mechanisms for this observed increase in susceptibility to pulmonary infection have not been well characterized. The objectives of this current investigation were as follows: (1) to establish an aged animal model to assess pulmonary responses; (2) to compare the lung defense responses of aged and young rats before and after pulmonary challenge with a bacterial pathogen; and (3) to define the mechanisms by which aged animals may be more susceptible than young animals to respiratory illness.

We found that the noninfected old rats had a significantly lower number of lavagable AMs as compared to the noninfected young rats. When the number of cells per lung volume was determined because the lungs of the aged rats were nearly twice the size of the lungs of young rats, there was a

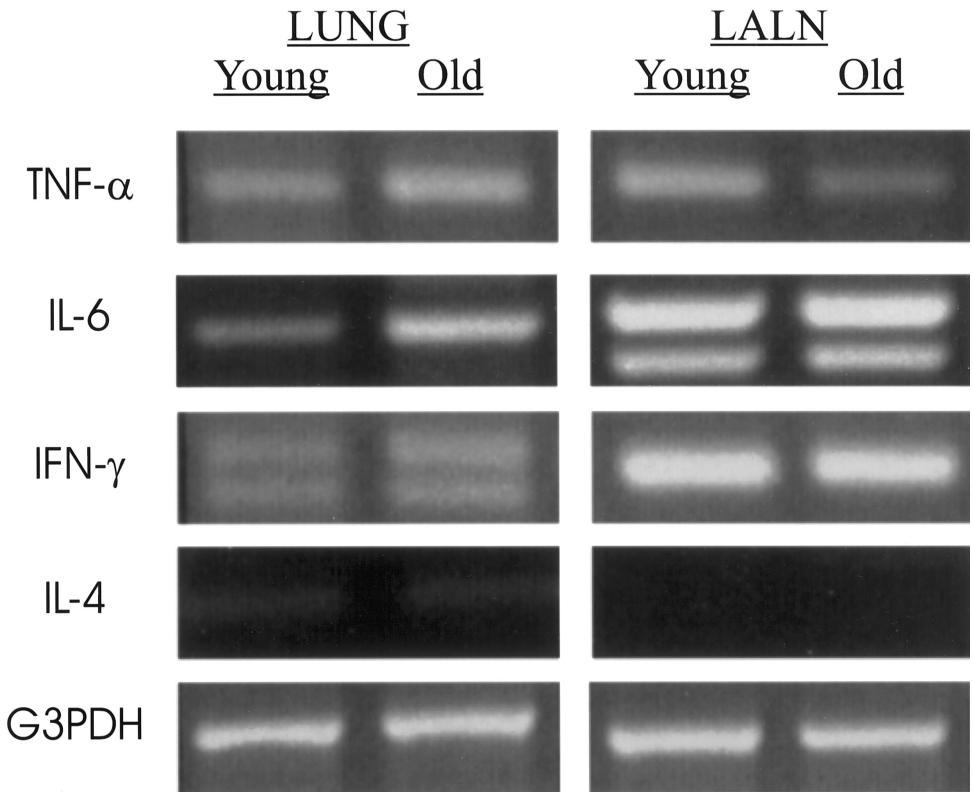


FIGURE 4. RT-PCR evaluation of cytokine mRNA levels in lungs and LALNs of young and old rats after intrapulmonary *L monocytogenes* infection. Individual RT-PCR reactions were performed using lungs and LALNs obtained from four young rats and six old rats. Equal parts of PCR product from each animal were pooled, subjected to agarose gel electrophoresis, and amplified fragments of appropriate size visualized by ethidium bromide staining. In lung, TNF-α and IL-6 reactions produced bands of greater intensity in old animals. IFN-γ primers produced dimeric product in lung, and IL-6 primers produced dimeric product in LALNs; these did not differ between young and old animals. G3PDH = glyceraldehyde 3-phosphate dehydrogenase.

significant decrease in both AMs and PMNs in the older rats. It might be expected that due to the larger lungs of the older animals, more pulmonary cells would be recovered. However, decreased retrieval of pulmonary cells by BAL in aged animals have been observed in a previous study.[25] Likewise, age-related decreases in AMs were observed after exposure to ozone.[26] Also, Clarke et al[27] observed a decrease in total cells and AMs retrieved by BAL in aged rats as compared to young rats. This observation of decreased pulmonary cells in aged rats suggests that these animals have suppressed lung defenses because AMs play a key role in the clearance of pathogens from the lungs, so altered AM number or function might well contribute to impaired clearance of pulmonary infection. It has been reported that impaired mucociliary clearance from the airways of aged individuals may be another factor that might predispose elderly to respiratory infection.[28]

In the assessment of AM function, we observed no difference in chemiluminescence and NO production when comparing noninfected aged and young rats. Wyde et al[29] also noted no differences in the ability of phagocytes from young or aged noninfected animals to phagocytize or kill bacteria *in vitro*, suggesting that no innate deficit in function exists with an increase in age. Others have concluded that macrophage function appears to be largely unaffected by aging.[2] Elder et al[9] have shown that the respiratory burst activity of recovered BAL cells as measured by chemiluminescence production is actually elevated in old rats as compared to young rats. However, the respiratory burst activity of the PMNs was also included in their measurement, which likely accounted for the observed increased activity. PMN respiratory burst activity has been reported to be impaired with aging.[30]

There were no significant differences in the number of total T, CD4+ T-helper, and CD8+ T-cytotoxic cells recovered from the noninfected aged and young rats; however, the LALNs from the older rats were slightly bigger. In an attempt to normalize the data, we calculated the percentage of T cells in the total lymphocytes recovered. We found that

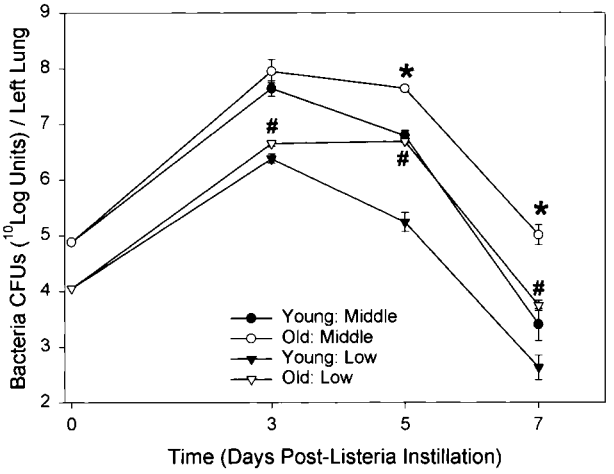


FIGURE 5. Number of bacteria colony-forming units (CFUs) in the left lung of young of old rats 3 days, 5 days, and 7 days after the intratracheal instillation of the low ($5 \times 10^3$ bacteria) and middle ($5 \times 10^4$ bacteria) doses of *L monocytogenes*. Values are mean in log-10 base units ± SE (n = 5 to 9). The old rat/middle-dose group (old: middle) is significantly greater than the young rat/middle-dose group (young: middle) [designated by *]. The old rat/low-dose group (old: low) is significantly greater than the young rat/low-dose group (young: low) [designated by #; $p < 0.05$].

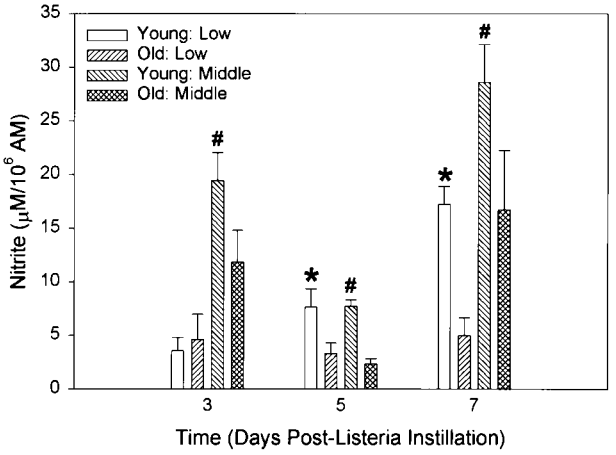


FIGURE 7. NO production by AMs recovered from the lungs of young and old rats 3 days, 5 days, and 7 days after the intratracheal instillation of the low ($5 \times 10^3$ bacteria) and middle ($5 \times 10^4$ bacteria) *L monocytogenes* doses. Values are mean ± SE (n = 5 to 9). The young rat/low-dose group (young: low) is significantly greater than the old rat/low-dose group (old: low) [designated by *]; the young rat/middle-dose group (young: middle) is significantly greater than the old rat/middle-dose group (old: middle) [designated by #; $p < 0.05$].

there was a significantly lower percentage of total T, CD4+, and CD8+ cells recovered in the aged rats as compared to the young rats. Cell-mediated immunity has been shown to be highly vulnerable to the effects of aging as evidenced by involution of the thymus with aging, diminished delayed-type hypersensitivity response due to decreased T-cell proliferation, a reduction in CD4+ cells, and defects in signal transduction among various protein kinases in senescent T cells.[30,31] Therefore, the decrease in the percentages of T cells and T-cell subsets we observed in the aged rats suggests the possibility of an impairment of cellular immune function.

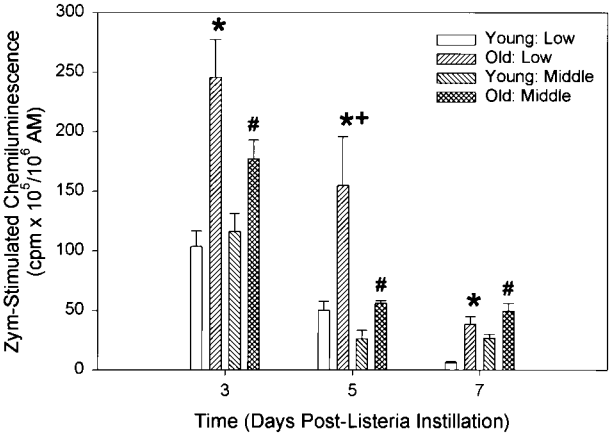


FIGURE 6. Zymosan-stimulated chemiluminescence of AMs recovered from the lungs of young and old rats 3 days, 5 days, and 7 days after the intratracheal instillation of the low ($5 \times 10^3$ bacteria) and middle ($5 \times 10^4$ bacteria) *L monocytogenes* doses. Values are mean ± SE (n = 5 to 9). At each time point, old rat/low-dose group (old: low) is significantly greater than the young rat/low-dose group (young: low) [designated by *]; the old rat/middle-dose group (old: middle) is significantly greater than the young rat/middle-dose (young: middle) [designated by #]; the old rat/low-dose group (old: low) is significantly greater than the old rat/middle-dose group (old: middle) [designated by +; $p < 0.05$].

After intratracheal instillation with the bacterial pathogen *L monocytogenes*, there was an increase in mortality and pulmonary inflammation for the aged rats. Lung edema and lesions observed in this group were more extensive and more pronounced than those observed in the young rats. There were significant elevations in PMNs, total T cells, and CD4+

**Table 2—*Age Comparison 3 Days After Infection With the High Dose of* L monocytogenes**

| Lung Parameters | Young Rats | Old Rats |
|---|---|---|
| Cell count, No. ($\times 10^6$) | | |
| AMs | 26.9 ± 5.01† | 63.0 ± 8.47 |
| PMNs | 11.9 ± 1.20† | 35.9 ± 5.37 |
| CL, cpm $\times 10^5/10^6$ AMs | | |
| Resting CL | 2.80 ± 0.16† | 4.42 ± 0.23 |
| Zymosan CL | 29.4 ± 4.21† | 56.6 ± 3.34 |
| Bacterial clearance | | |
| Left Lung CFUs ($10^8$) | 2.37 ± 0.31† | 5.15 ± 0.78 |

*Data are presented as mean ± SE (n = 6 to 10). CL = chemiluminescence; see Figure 5 legend for expansion of abbreviation.

†Significantly less than mean value for old rats.

cells, and in some cases AMs, recovered from the aged animals. Also, pulmonary clearance mechanisms had become significantly compromised in the aged rats after bacterial exposure. With the severity and extent of lung injury observed in the aged group, this observation is not surprising. Lesions were seen throughout the pulmonary interstitium, and the alveoli had become flooded with fluid, indicating possible lymphatic obstruction and inhibition of lung liquid clearance. A similar result was also observed in mice,[29] using doses comparable to the ones chosen for this current study. It was shown that a significantly greater number of two different species of bacteria was isolated from the lungs of aged animals when compared with young animals.

It is also important to note that at baseline, there were greater proportions of T lymphocytes, CD4+ cells, and CD8+ cells in noninfected younger animals as compared to the old rats. But after infection, the older animals had influxes of T cells capable of equalizing the proportions of T lymphocytes and CD4+ cells, but insufficient to equalize the proportions of CD8+ cells. Thus, the older rats appeared to be better able to mount a CD4+ response than a CD8+ response. Given the important role of CD8+ cells in combating infection due to intracellular pathogens, this potentially is of mechanistic importance in explaining the impairment in ability of the aged rats to clear the intrapulmonary *L monocytogenes* infection.

With regard to RT-PCR results, increased levels of proinflammatory cytokine gene expression in the lungs of older rats at 7 days after infection most likely reflects continued pulmonary infection and resulting inflammation in the older animals. More complete clearance of infection in the younger animals is associated with lower levels of TNF-α and IL-6 mRNA in the lungs of these animals. IFN-γ plays a key role in host defense against intracellular pathogens.[32,33] The presence of similar IFN-γ mRNA levels in the lungs and LALN of young and old rats suggests that delayed clearance of infection in the older animals is not primarily due to impaired IFN-γ response. Additionally, the presence of similar IL-4 mRNA levels suggests that a switch toward "Th2-like" cytokine production also fails to explain impaired clearance of *L monocytogenes* from the lungs of older rats.

While lavage reveals a lower number of AMs present in the lungs of aged rats, these AMs appear to be more responsive after *in vivo* challenge with bacteria than AMs collected from young rats. A significant elevation in ROS production as measured by chemiluminescence was observed for the AMs recovered from the old rats. This response was highly consistent for each dose and at each time point after *L monocytogenes* inoculation. This indicates that the AMs may have adapted to an acute challenge with a pulmonary toxicant to improve the survivability of the aged animals. Elder et al[9] have demonstrated that aged mice and rats undergo adaptation after inhaling low doses of lipopolysaccharide subsequent to a high-dose challenge. Even with this elevation in the production of these highly ROS, there seemed to be little effect on bacterial killing. However, this elevation in oxidant generation may be responsible in mediating pneumonia-associated lung injury that led to the excess in morbidity and mortality observed in the older rats. It has been well established that activation of AMs and the subsequent release of ROS is one of the primary mechanisms by which inhaled substances damage the lungs.[34]

Interestingly, NO production by AMs recovered from the aged rats was significantly decreased as compared to the AMs of the young rats. NO is a highly reactive short-lived radical secreted by AMs and plays an important role in AM-mediated defense against infections.[35] NO has been shown to promote the cytotoxic and mitochondrial activities of AMs and modulate cell-mediated immunity.[36] NO has been demonstrated to react with superoxide anion to form the highly reactive substance peroxynitrite.[37] Hickman-Davis et al[38] have concluded that peroxynitrite generation by AMs is necessary for the killing of bacterial pathogens in the lungs. The suppression in pulmonary clearance of the bacteria observed in our current study then may be partially explained by the decrease in NO production by AMs recovered from aged rats after *L monocytogenes* challenge. Koike et al[10] demonstrated that AM NO production activated by cells from lymph nodes in BAL fluid of old rats was significantly decreased when compared with cells in BAL fluid of young rats, concluding that inhibition in NO production suppresses defense against infections in the elderly.

In summary, the present study demonstrated that aged rats have altered lung defenses, making them more susceptible to lung injury and inflammation after pulmonary bacterial challenge. There appears to be a dramatic alteration in pulmonary clearance mechanisms of aged rats as compared to young rats, which involves a decrease in NO production by AMs. Also, this work established an aged animal model that will be used in future studies to assess the effect of aging on pulmonary-infection responses in the lung.

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