

Pulmonary Antibacterial Defenses during Mild and Severe Influenza Virus Infection

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Severe influenza virus infections with pneumonic involvement are known to predispose the lungs to bacterial superinfections due to dysfunctions in the alveolar macrophage (AM) phagocytic system. To determine whether milder forms of influenza without pneumonic involvement have a similar outcome, pulmonary antibacterial defenses and AM phagocytosis were compared in murine models of mild and severe influenza virus A/HK/68 infections. Bactericidal activity was quantitated by the intrapulmonary killing of *Staphylococcus aureus* following aerosol challenge, whereas the functional capacity of the AMs was determined by Fc-receptor-mediated phagocytosis. With the severe virus infection, maximal suppression of bactericidal activity occurred on day 8 of infection and correlated with impairment of AM phagocytosis. A lesser but significant degree of suppression of pulmonary antibacterial defenses and AM phagocytosis was observed on the third day of the mild virus infection. The data demonstrate that mild influenza virus infections that are limited to the upper respiratory tract also impair pulmonary antibacterial defenses and may predispose the lungs to bacterial superinfections.

Secondary bacterial pneumonias often complicate respiratory virus infections (12–14). Experimental studies have demonstrated that during the virus infection, the antibacterial defenses of the lungs are suppressed through dysfunctions of the alveolar macrophage phagocytic system (1, 6, 20). The shortcoming in the experimental models of virus-associated bacterial pneumonias, however, is that the virus strains used elicit extensive pulmonary lesions, whereas influenza in humans is primarily an upper respiratory tract infection with only occasional lower lung involvement (2, 18). Consequently, it may not be reasonable to extrapolate the experimental results of bacterial superinfections with virus-associated pneumonic involvement to upper respiratory tract virus infections in humans. Furthermore, the extent to which patients with upper respiratory influenza virus infection are at risk of developing bacterial superinfections is not known.

In the present study, the antibacterial defenses of the lungs were compared in models of upper respiratory tract influenza virus infection and influenza with pneumonic involvement. To gain insights into potential mechanisms, both models were characterized further in terms of histopathologic lesions, the distribution of viral antigen in the lungs, and alveolar macrophage phagocytosis.

MATERIALS AND METHODS

Animals. Pathogen-free female Swiss mice (Hilltop Lab Animals Inc., Scottsdale, Pa.) weighing 20 to 23 g were used for all experiments. All mice were housed in filter top cages, maintained on a 12-h light–12-h dark cycle, and fed food and water ad libitum. Infected and uninfected animals were housed in separate rooms.

Virus. Low-murine-lung passage (passage 3 [P3]) and high-murine-lung passage (passage 12 [P12]) strains of influenza virus A/HK/68 (H₃N₂) were a generous gift of Philip R. Wyde (Influenza Research Center, Baylor College of Medicine, Houston, Tex.). The derivation of the P3 and P12

variants of influenza virus, isolated from a patient, has been described previously (21).

Upon arrival in our laboratory, the P3 and P12 virus stocks were propagated and titers were determined in the allantoic fluid of 10-day-old embryonated chicken eggs by previously described methods (7), divided into 0.5-ml portions, and stored at –70°C. The titers of the stock P3 and P12 virus were respectively, 10^{8.7} and 10^{9.7} median egg-infectious doses (EID₅₀) per ml. The infecting doses used for all experiments were 10^{3.1} EID₅₀ for P12 and 10^{6.1} EID₅₀ for P3. The virus was administered intranasally in 50-μl amounts unless otherwise indicated. Control animals were intranasally inoculated with 50 μl of the virus diluent (citrate and phosphate-buffered Hanks solution [CP-Hanks]).

Pulmonary virus titers. At 1 h and on days 1, 3, 5, 7, 9, 11, and 13 postinoculation, four P3-infected mice and four P12-infected mice were killed by brainstem compression and exsanguinated by cardiac puncture. The lungs were aseptically removed and homogenized in 2 ml of iced CP-Hanks containing 250 U of penicillin and 250 μg of streptomycin per ml. The homogenates from each animal were pooled, incubated for 5 min with continuous shaking at 37°C to elute the virus, and centrifuged at 2,000 × g for 5 min. The supernatants were stored at –70°C until assayed. Pulmonary virus titers were determined by inoculating 100 μl of appropriate 10-fold dilutions of the lung homogenates in CP-Hanks into the allantoic cavity of 10-day-old embryonated chicken eggs (four eggs per dilution) as described previously (7). The data are expressed as EID₅₀ per lung.

Histopathologic examination. On days 1, 3, 5, 8, 10, and 15 after viral inoculation, the lungs of four P3- and four P12-infected mice and noninfected mice were removed and inflated overnight with 10% buffered Formalin at a transthoracic pressure of 20 cm of H₂O. The lungs were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Bactericidal assay. *Staphylococcus aureus* (coagulase-positive Food and Drug Administration strain 209P, phage type 42D) was incubated in 200 ml of Trypticase soy broth (TSB)

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for 20 h at 37°C in a rotating water bath. The bacteria were washed twice with phosphate-buffered saline, pH 7.1 (PBS), and suspended in 9 ml of TSB. Uninfected animals and animals infected 3 or 8 days previously with either P3 or P12 were challenged by aerosol inhalation for 30 min in an infectious-exposure chamber described previously (8). Immediately (0 h) and 4 and 24 h after staphylococcal challenge, groups of six virus-infected and noninfected animals were killed, and their lungs were aseptically removed and homogenized in 3 ml of TSB. A 1-ml portion of the homogenate was diluted appropriately in PBS and cultured quantitatively in 5% NaCl-tryptic soy agar in quadruplicate on petri X-plates. Pulmonary bactericidal activity was calculated as the percentage of bacteria remaining by the formula (16): percent bacteria remaining = (bacterial count at time *t*/mean bacterial count at time 0) × 100. With this method, each group of animals served as its own control. Three runs of each experiment were performed.

Alveolar macrophage Fc receptor-mediated phagocytosis. Alveolar macrophages were collected by inserting a Pasteur pipette into the trachea of surgically removed lungs and introducing and withdrawing 1.5 ml of sterile lavage solution (0.85% NaCl, 0.1% glucose, 0.1% EDTA, and 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]) three times, using a total of 4.5 ml of lavage solution. Direct cell counts were performed with a hemacytometer. The lavaged cells were suspended in tissue culture medium 199 (TCM 199) at a concentration of 5×10^5 cells per ml. A 200- μ l sample of this suspension was then placed on 22-mm² cover slips in plastic petri dishes (35 by 10 mm) and incubated at 37°C for 45 min. After incubation, the macrophage monolayers were gently washed with TCM 199, and Fc membrane receptor-mediated phagocytosis was assayed (19) by adding 1.5 ml of a 0.5% suspension of sensitized sheep erythrocytes (RBCs) in TCM 199 medium and incubating the resulting suspension at 37°C for 45 min. Non-ingested RBCs were hypotonically lysed by adding distilled water to the monolayers for 10 s, followed by several rinses with TCM 199 medium. The monolayers were then dried, fixed with methanol, and stained with Wright-Giemsa. The stained cell monolayers were read microscopically at 1,000 \times to quantify the percentage of macrophages containing RBCs. The number of RBCs ingested per actively phagocytic macrophage was also determined. Two hundred randomly selected macrophages were counted on each monolayer.

Viral antigen. Alveolar macrophages bearing viral antigen were assessed by indirect immunofluorescence by methods described previously (5). Briefly, monolayers of alveolar macrophages were prepared by incubating 5×10^4 cells in each chamber of four-chamber Lab-Tek tissue culture slides. After incubation for 45 min at 37°C, the monolayers were washed with PBS and then fixed with cold acetone for 10 min. A 1:30 dilution of hyperimmune rabbit antiserum against P3 or P12 was incubated with the fixed monolayers for 45 min at room temperature, and the monolayers were washed with PBS and reincubated at room temperature with a 1:30 dilution of fluorescein-labeled goat antibody against rabbit immunoglobulin G (IgG) (Kirkgaard and Perry Laboratories, Inc., Gaithersburg, Md.). After being washed with PBS, the monolayers were mounted with glycerol and examined with a Zeiss epifluorescent microscope. The presence of viral antigen was assessed in 200 randomly selected macrophages. Monolayers treated with normal rabbit serum and conjugated serum, conjugated serum only, or hyperimmune serum exhibited only minimal fluorescence and served as controls.

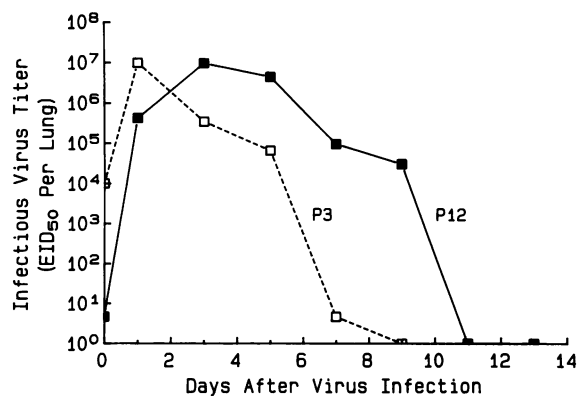


FIG. 1. Comparison of pulmonary virus titers between mice infected with P3 and P12 virus. Each value represents a pool of four lungs.

Localization of viral antigen in the lungs was performed by immunoperoxidase staining by a modification of the method of Paradis et al. (15). Lungs were prepared by injecting 0.75 ml of Tissue Tek O.C.T. compound (Ames Company, Division of Miles Laboratories, Elkhart, Ind.) into each lung. The lungs were then frozen at -27°C, sectioned with a cryostat at 8 μ m, layered onto gelatin-coated (3% gelatin, 0.05% potassium dichromate) slides, fixed with iced acetone for 10 min, rinsed in PBS for 20 min, and then incubated in 0.3% H₂O₂ to quench endogenous peroxidase. Following a 20-min rinse in PBS, the lung sections were incubated with 3% normal goat serum for 20 min and then blotted to remove the excess serum. Thereafter, hyperimmune rabbit anti-P3 or anti-P12 serum (diluted 1:500 in PBS supplemented with 0.05% Tween 20, 0.5% bovine serum albumin, and 2% normal goat serum) was placed on the slides for 30 min. The sections were then washed with PBS for 10 min, followed by a 30-min incubation with biotinylated goat anti-rabbit IgG diluted with the supplemented PBS according to the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.). After a 10-min incubation, the ABC reagent was placed on the sections and incubated for 30 min. The sections were rinsed again in PBS for 10 min, and then the color reaction product was developed for 5 min with the peroxidase substrate solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) and 0.01% H₂O₂ in PBS. The slides were then rinsed with tap water for 5 min and counterstained with Mayers hematoxylin for 5 min. After a final 5-min rinse in tap water, the sections were mounted with Cytoseal 60 (Cornwell Corp., Oak Ridge, N.J.). All incubations were carried out at room temperature unless stated otherwise.

Statistical methods. Data from the in vivo bactericidal assay were tested by one-way analysis of variance and further compared by Duncan's multiple-range tests. Alveolar macrophage phagocytosis and the number of RBCs ingested per phagocytic macrophage during the P3 and P12 infection were compared by the chi-square test and the Wilcoxon test, respectively. A value of *P* < 0.05 was considered significant.

RESULTS

Preliminary histopathologic range-finding studies revealed that an inoculum of 10^{6.1} EID₅₀ of P3 elicited minimal bronchitis which could not be obtained with a lower dose

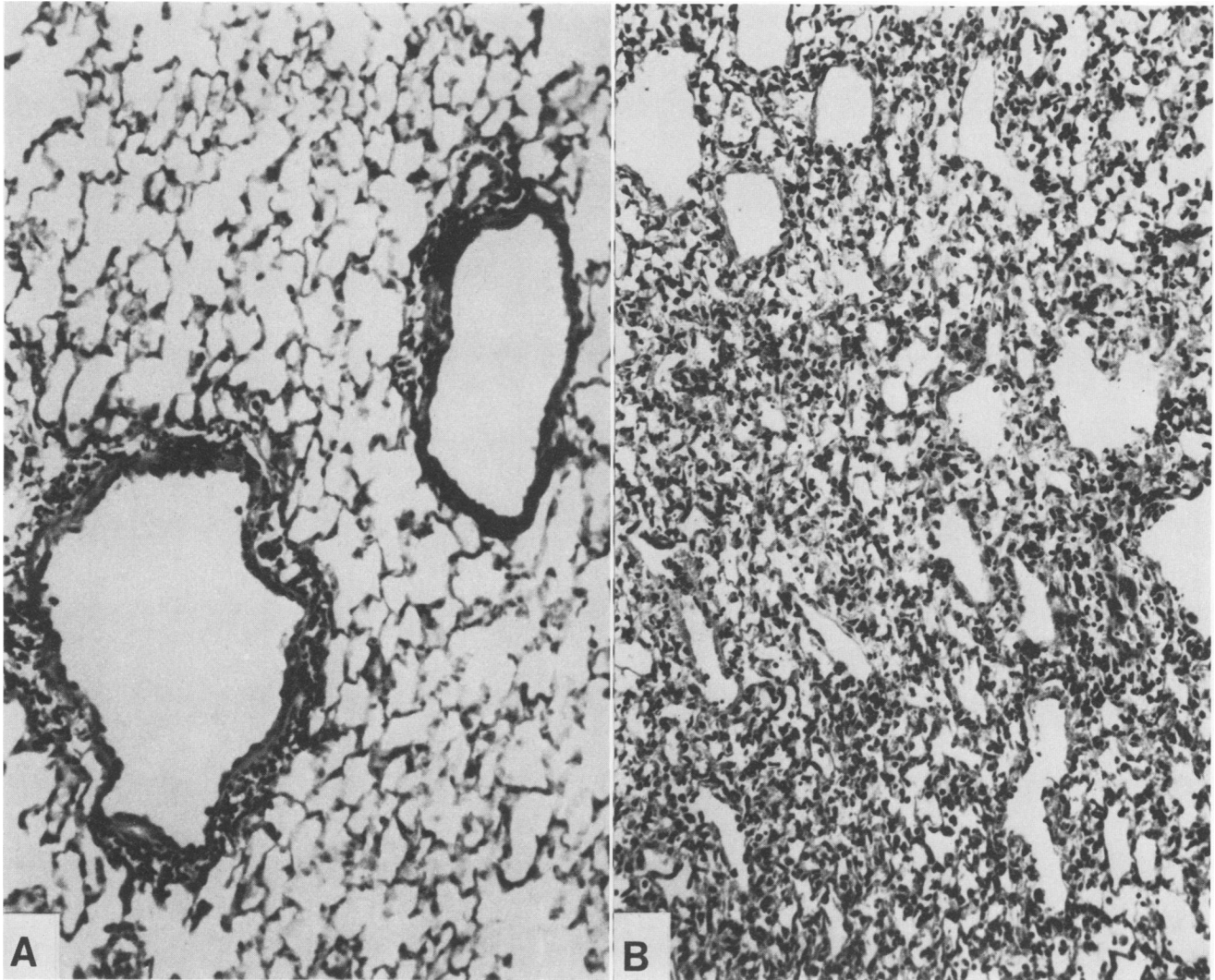


FIG. 2. Photomicrographic comparison of representative sections of lungs from mice at the height of the virus-induced lesions. (A) P3 on day 5; (B) P12 on day 8.

and which would not significantly increase in severity with a higher dose. The same dose of P12 resulted in 100% mortality by day 5 of infection. Consequently, a lower dose of P12 ($10^{3.1}$ EID₅₀) which caused severe pneumonitis without the early fatalities was chosen.

Virus proliferation in the lungs of animals infected with P3 or P12 is presented in Fig. 1. In both groups, the virus proliferated extensively to peak titers of 10^7 EID₅₀ per lung. A 1,000-fold difference was observed between the P3 and P12 virus titers 1 h after inoculation (time 0), which reflects the differences in inoculum size. Viral proliferation of P3 peaked 24 h after infection, and thereafter pulmonary virus titers declined steadily, with no infectious virus recoverable by day 9. In contrast, the P12 virus exhibited a slightly different growth pattern. Despite the smaller amount of infectious virus detected at 1 h, the P12 virus multiplied rapidly and attained a peak titer 72 h after inoculation. After this time, the viral titers declined steadily, and by day 11, no infectious virus could be recovered.

The pathologic features associated with the P3 infection were minimal compared with the P12 infection. Gross examination of lungs showed that surface consolidation steadily

increased in P12-infected animals until day 8, at which time approximately 75% of the surface area was consolidated. Thereafter, the consolidation declined slowly, but large areas were still evident on day 15. In contrast, less than 15% of the lung surface area was consolidated on day 5 of the P3 infection and declined to unobservable levels by day 10. Histopathologic examination of P3-infected lungs showed that the virus-induced lesion was limited to bronchial epithelial cell desquamation and peribronchial infiltration with mononuclear cells (Fig. 2A). In contrast, the P12 infection resulted in fulminant interstitial pneumonia (Fig. 2B).

To determine whether the histopathologic lesions were related to the site of virus proliferation, the location of viral antigen in the lungs of P3- and P12-infected animals was assessed. On the first day of both infections, viral antigen was only detected in the epithelial lining of a few of the larger airways. By the third day of the P3 infection, viral antigen was still localized in the pyknotic cells lining the larger bronchi and in the cell debris found in the bronchial lumen (Fig. 3A). In contrast, in P12-infected lungs the viral antigen was present in large areas of the lung parenchyma as well as the lumen of the airways. High-power examination of the

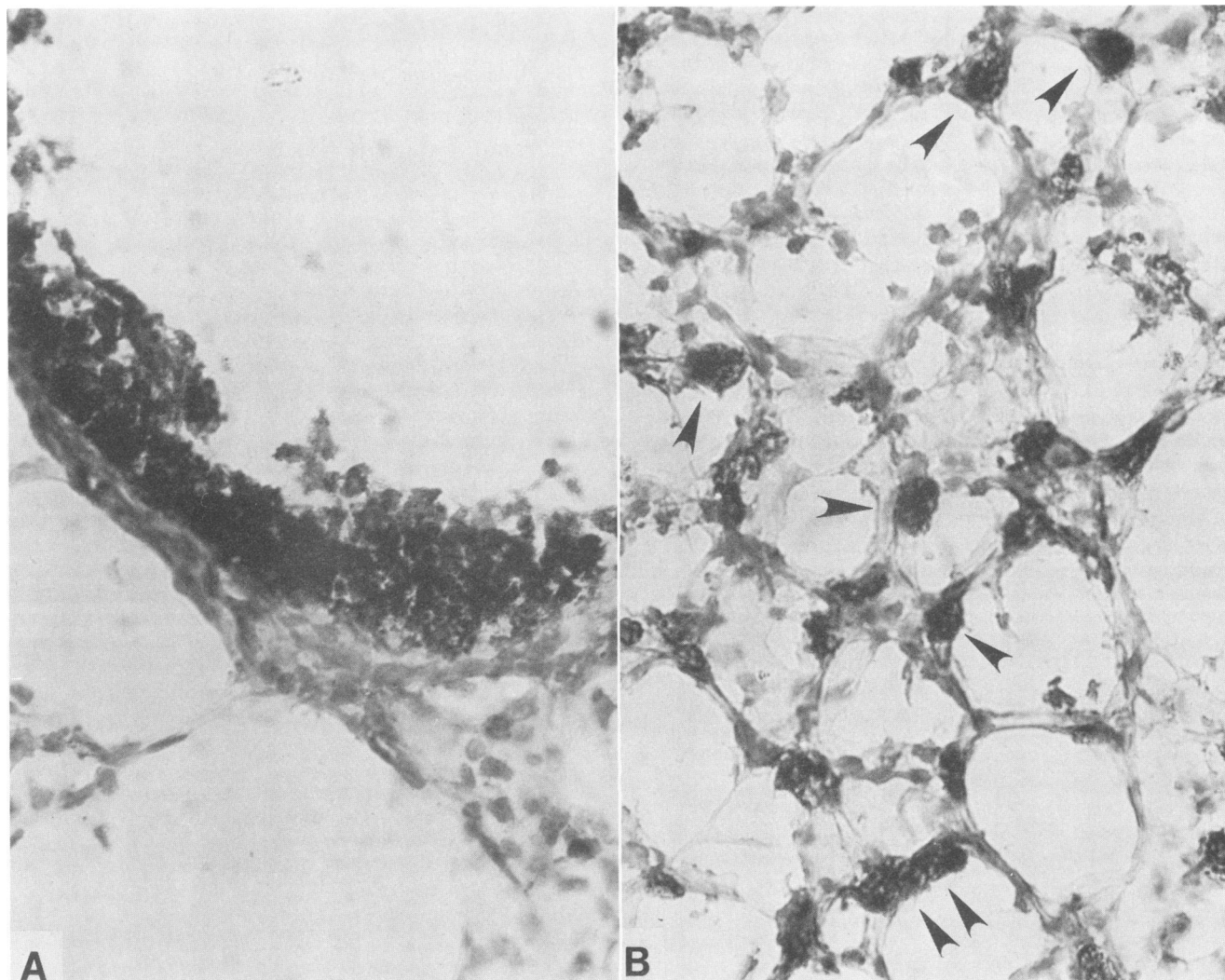


FIG. 3. Photomicrographic comparison of the distribution of viral antigen in the lungs of mice during infection with P3 (A) and P12 (B). Arrowheads point to alveolar macrophages bearing viral antigen.

affected parenchymal areas of the P12-infected lungs revealed that viral antigen was also present in alveolar macrophages (Fig. 3B). By day 5, the location of the viral antigen in the lungs of P3- and P12-infected mice did not appear to differ from that observed on day 3; however, the intensity of staining had declined markedly. By day 8, no viral antigen could be detected in lung sections of P3- or P12-infected animals.

Intrapulmonary killing of *S. aureus* in animals infected either 3 or 8 days previously with the P12 virus is presented in Fig. 4A. In uninfected lungs, viable bacteria declined rapidly so that the percentage of bacteria remaining at 4 and 24 h was 14.1 ± 2.1 and $0.14 \pm 0.05\%$, respectively (mean \pm standard error of the mean). In contrast, pulmonary bactericidal activity was significantly suppressed in virus-infected lungs by day 3, and by day 8, the bactericidal defect became even more pronounced. In P3-infected mice, pulmonary antibacterial defenses were significantly suppressed by day 3 (Fig. 4B), with 38.3 ± 3.3 and $1.30 \pm 0.20\%$ of the bacteria remaining at 4 and 24 h, respectively. By day 8, the lungs of the P3-infected animals started to regain their defenses, yet the bactericidal capacity was still significantly lower than in the lungs of non-infected animals.

To evaluate the phagocytic activity of alveolar macrophages during the course of the P3 and P12 infection, the macrophages were tested in vitro for their capacity to ingest sheep RBCs via the Fc membrane receptor. During the course of these experiments, approximately 90% of the alveolar macrophages lavaged from the lungs of uninfected animals were phagocytic, with each phagocytic macrophage ingesting approximately 4.3 RBCs. Alveolar macrophage phagocytosis was significantly suppressed through the acute stages of both viral infections (Fig. 5A). During the P3 infection, the phagocytic defect was maximal on day 3. Thereafter, the percentage of phagocytic alveolar macrophages steadily increased. In contrast, during the P12 infection, the phagocytic impairment was maximal on day 8.

The number of RBCs ingested per phagocytic alveolar macrophage during the course of P3 and P12 infection is presented in Fig. 5B. On day 3, alveolar macrophages from P3- and P12-infected animals ingested 40% fewer RBCs than those retrieved from uninfected lungs. Thereafter, no significant differences were found between the number of RBCs ingested by macrophages from P3-infected lungs. In contrast, macrophages from P12-infected animals ingested fewer

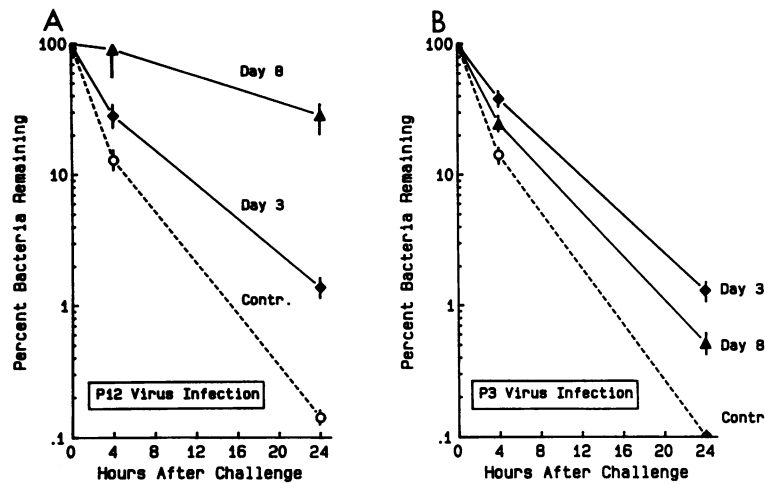


FIG. 4. Comparison of intrapulmonary killing of *S. aureus* in mice infected with P12 virus (A) or P3 virus (B) 3 or 8 days previously, respectively. Each value represents the mean \pm SEM of 15 determinations.

RBCs than those from uninfected controls until day 8 of the virus infection.

To determine whether the observed differences in bactericidal and phagocytic dysfunctions between P3 and P12

infection were related to alveolar macrophages bearing viral antigen, the percentage of macrophages with viral antigen was quantitated during the course of infection. One hour after inoculation, 2.5% of the alveolar macrophages lavaged from P3-infected lungs expressed viral antigen (Fig. 6). This value increased to 12.5% by day 5 and persisted at approximately 10% until day 14. In contrast, 17% of the alveolar macrophages recovered from P12-infected lungs expressed viral antigen 1 h after inoculation. This value increased to 42% by day 1 and peaked at 52% on day 8. By day 14, 25% of the macrophages continued to express viral antigen.

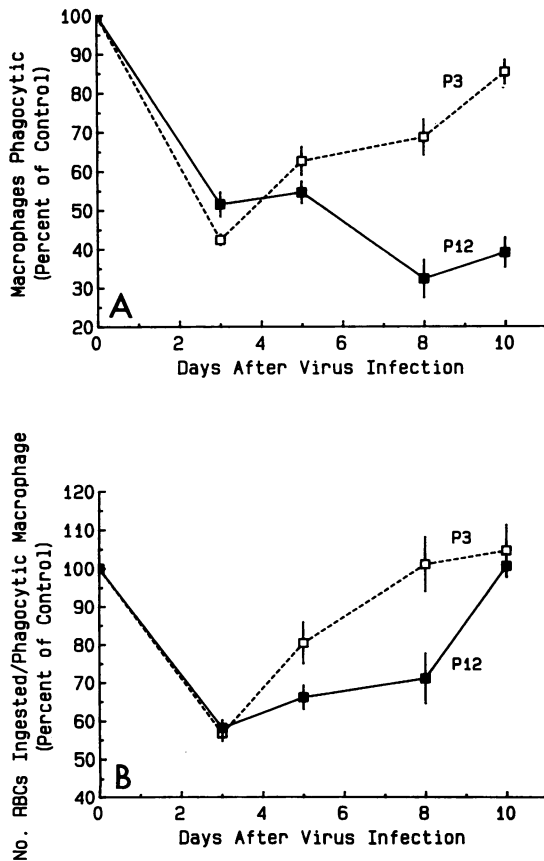


FIG. 5. Comparison of alveolar macrophage Fc receptor-mediated phagocytosis during the course of P3 and P12 infection. The data are expressed as a percentage of the daily uninfected-control values. (A) Percentage of alveolar macrophages that were phagocytic. (B) Number of RBCs ingested per phagocytic alveolar macrophage. Each point represents the mean \pm SEM of six to nine determinations.

DISCUSSION

Severe influenza virus infections with pneumonic involvement impair pulmonary antibacterial defenses, which in turn can result in bacterial superinfections (4, 13, 14, 18). The majority of influenza virus infections in humans, however, are limited to the upper respiratory tract (2, 18), and the effect of these milder infections on lung defenses is not known. In the present study, the antibacterial defenses of the lungs were compared in an influenza virus model of upper respiratory tract infection (P3) and an influenza virus model of pneumonic involvement (P12) to determine whether the milder form of influenza virus infection also

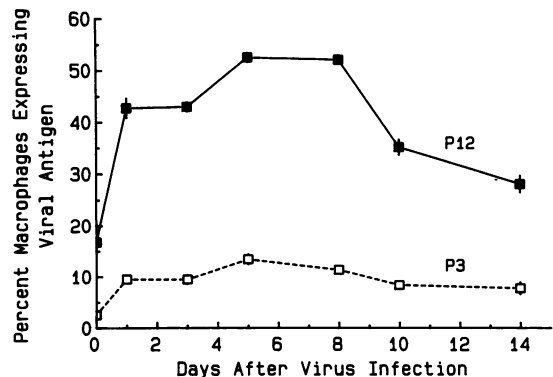


FIG. 6. Comparison of alveolar macrophages expressing viral antigen during the course of P3 and P12 infection. Each value represents the mean \pm SEM of four determinations.

affected the antibacterial defenses of the lungs. Bactericidal activity was maximally suppressed 3 days after infection with P3 and 8 days after infection with P12. The phagocytic impairment of alveolar macrophages was also maximal on day 3 of the P3 infection and day 8 of the P12 infection. These data demonstrate that the impairment in alveolar macrophage phagocytosis correlates with suppression of pulmonary antibacterial defenses in both models of virus infection.

To determine whether the suppression of pulmonary bactericidal activity and alveolar macrophage phagocytosis was associated with viral proliferation in the lungs, the infectious virus titers were quantitated throughout each infection. The results show that the impairment in lung defenses did not correlate with virus proliferation in the lungs but rather with declining virus titers. These observations are consistent with previous findings which demonstrated that the growth of virus in the lungs was not accompanied by a decrease in pulmonary bactericidal activity (4, 17).

The P12 infection suppressed the antibacterial defenses of the lungs to a greater extent than the P3 infection, which in turn correlated with the severity of the pathologic changes. The more extensive lung damage would prevent a greater proportion of the alveolar macrophages from clearing the lungs of the cell debris. It is known that ingestion of large amounts of material will block subsequent phagocytic function (11). Therefore, the macrophages which are actively processing ingested cell debris may have a reduced capacity to ingest additional material (i.e., bacteria). This contention is supported by the observation that during the P12 infection, not only were fewer alveolar macrophages phagocytic, but also each phagocytic macrophage ingested fewer RBCs.

In the case of lower respiratory tract infection, such as that induced by the P12 virus, specific immune mechanisms play a role in mediating phagocytic dysfunction (5, 6, 10). For example, phagocytic dysfunction is induced when antiviral antibody or specifically sensitized lymphocytes are added to alveolar macrophages infected *in vitro* (9, 10). Our results demonstrate that greater than 50% of the alveolar macrophages expressed viral antigen, and a similar percentage were nonphagocytic on the eighth day of the P12 infection. Consequently, the decrease in phagocytosis observed on day 8 of the P12 infection may be related to the presence of viral antigen in the alveolar macrophages. In contrast, only 15% of the alveolar macrophages expressed viral antigen at any given time during the P3 infection. Yet up to 62% of the alveolar macrophages from P3-infected animals were nonphagocytic on day 3. This would indicate that the presence of viral antigen in the macrophages is not a prerequisite for phagocytic dysfunction during the P3 upper respiratory tract infection and that the phagocytic dysfunctions observed during the P3 and P12 infections may be mediated by different mechanisms.

Although the mechanisms involved in the suppression of pulmonary bactericidal activity during the experimental P3 infection remain to be elucidated, the data provide compelling evidence that during the course of P3 infection the antibactericidal defenses of the lungs are also compromised. Extrapolation of these results to humans implies that even mild influenza virus infections of the upper respiratory tract may predispose the host to secondary bacterial infections.

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