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Staphylococcal Clearance and Pulmonary Macrophage Function During Influenza Infection

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Direct infection of pulmonary macrophages with influenza virus in vitro does not alter macrophage functions necessary for staphylococcal clearance. To determine whether these functions are altered during viral pneumonitis, we evaluated macrophages recovered from influenza-infected mice which had undergone aerosol challenge with *Staphylococcus aureus*. Sublethal infection with influenza A/PR8 produced patchy hemorrhagic pneumonia in CF_1 mice and significantly reduced the intrapulmonary killing of staphylococci inhaled during aerosol challenge. However, only a small fraction of macrophage monolayers established from animals with influenza expressed viral hemagglutinin on their plasma membrane, and alveolar macrophages from infected mice ingested staphylococci and yeast cells in vitro at the same rate as control macrophages. The in vitro intracellular bactericidal activity against staphylococci ingested in vivo was comparable in monolayers from control and PR8-infected mice. In experiments with more severe influenza infections (mortality > 50% by day 7), a larger fraction of the staphylococci recovered by bronchoalveolar lavage appeared to be ingested in vivo during the aerosol exposure in the PR8-infected mice than in the control mice, but intracellular killing by macrophages during in vitro incubation was similar in control and PR8 monolayers. Hence, the severity of viral infection did not influence intracellular bactericidal activity. In virus-infected mice, a significantly larger fraction of viable staphylococci in the lower respiratory tract at the end of aerosol exposure was adherent to the trachea and major bronchi. In summary, PR8 infection established by intranasal inoculation impaired staphylococcal killing in the lung even though these infections did not alter in vivo ingestion rates or in vitro intracellular killing rates of macrophage populations in bronchoalveolar spaces.

Viral respiratory tract infections increase the frequency and severity of bacterial pneumonia by altering pulmonary clearance processes, and these bacterial superinfections produce most of the morbidity and mortality observed during viral epidemics. This defect in host defenses is transient and appears to involve only the respiratory tract. Jakab and Green have demonstrated that resolving Sendai virus infections suppress staphylococcal killing in the murine lung but do not alter physical transport from the lung (5). Using histological techniques, these workers have suggested that this reduction in in situ killing reflects impaired macrophage function, specifically, impaired intracellular killing (6). However, Warshauer et al. have suggested that influenza infection in mice inhibits both ingestion and intracellular killing of *Staphylococcus epidermidis* (12). We have attempted to clarify the principal defect in macrophage function by evaluating macrophages infected in vitro with influenza virus but could not detect any alteration in ingestion, phagolysosome formation, or intracellular killing (9). Since in vitro infection of macrophages with influenza virus did not alter the functions most directly relevant to bactericidal activity, it appears unlikely that the apparent in vivo dysfunction is a direct effect of virus on macrophage function. However, the inflammation associated with influenza pneumonitis could alter macrophage function without direct viral infection, or, alternatively, the defect in bacterial clearance could have resulted from an alteration in extracellular bactericidal processes which we and others have described (10; J. D. Coonrod, Clin. Res. **29**:727A, 1981). To test these possibilities, we evaluated macrophages recovered from mice infected with influenza virus after aerosol challenge with *Staphylococcus aureus*. This allowed the parallel evaluation

of pulmonary clearance, in vivo ingestion, and intracellular killing during prolonged in vitro incubation.

MATERIALS AND METHODS

Mice. Outbred female CF_1 mice (Carsworth Farm) weighing 20 to 25 g were used throughout these experiments.

Influenza virus. Influenza A/PR8/34 (HON1) virus was adapted to CF_1 mice by serial respiratory tract infections. After mouse passage 10, virus stocks were grown in 11-day-old embryonated eggs (9). Hemagglutination titers and hemagglutination inhibition titers were determined with standard microtiter techniques (9). Virus titers in lung homogenates were determined by 50% tissue culture infective dose titrations with Madin-Darby canine kidney cells and hemagglutinin release into monolayer supernatants as an endpoint (4). To establish infections for the bacterial clearance studies, mice were inoculated under light ether anesthesia with 0.05 ml of virus intranasally. Serial dilutions of the virus stock had been used in pilot studies to identify an inoculum which consistently produced pulmonary infection.

Macrophage preparation and cultivation. Macrophages were recovered from control and influenza-infected mice by bronchoalveolar lavage with phosphate-buffered saline containing 1.25 mM EDTA. Each animal was lavaged with 1.0 ml of fluid through a tracheal cannula; this volume was injected and withdrawn slowly with gentle thoracic massage three times before complete removal. The number of leukocytes in the lavage fluid was determined with a hemocytometer, and a differential cell count was performed with Cytospin preparations and Diff-Quik staining. Macrophage monolayers were then established in glass Leighton tubes and incubated in tissue culture medium (M199 plus 5% heat-inactivated newborn calf serum plus antibiotics) at 37°C in a 5% CO_2–95% air atmosphere. These monolayers were evaluated for evidence of influenza infection with an erythrocyte rosette assay which detects viral hemagglutinin on the plasma membrane (9). Pulmonary macrophages from whole lung specimens were obtained by tissue disruption (mincing and collagenase digestion) and established in monolayers by the methods described above (9).

Macrophage function assays. (i) Staphylococci ingestion. Macrophage monolayers on glass cover slips were incubated in vitro for 2 to 4 h, washed once, and incubated with suspensions of *S. aureus* 502A ($1.5 \times 10^8 \pm 0.3 \times 10^8$ colony-forming units (CFU) per ml) in our routine tissue culture medium for 60 min at 37°C. Monolayers were then washed with warm M199 three times and treated with lysostaphin (1 U/ml) for 10 min at 37°C. These monolayers were then washed again, air dried, fixed with methanol, and stained with Giemsa. Coded slides were examined by light microscopy (400× magnification), and the fraction of the monolayer which ingested one or more staphylococci was determined by counting 100 to 200 individual cells in random fields. The bacteria-to-macrophage ratio in these assays exceeded 200.

(ii) Saccharomyces ingestion. Macrophage monolayers on glass cover slips were incubated with suspensions of commercial *Saccharomyces* sp. (5×10^6/ml) in our routine tissue culture medium for 60 min, washed three times with warm M199, and examined by phase microscopy (400× magnification). The fraction of the monolayer which had ingested one or more yeast cells was determined by counting 100 to 200 individual macrophages in random fields on coded slides. The yeast-to-macrophage ratio in these experiments was 10:1. The viability of macrophages evaluated in these assays exceeded 95% by trypan blue dye exclusion criteria.

Bacterial clearance. The experimental methods necessary for measuring bacterial clearance from the lung have been described in detail elsewhere (10). In brief, control mice and influenza-infected mice (day 7) were exposed to fine-particle aerosols of *S. aureus* 502 A and sacrificed at various times after exposure for removal of whole lung specimens or for bronchoalveolar lavage. Lungs were homogenized, serially diluted, and plated on brain heart infusion agar for quantitative determination of the number of viable staphylococci. Lavage fluids were quantitatively cultured after hypotonic lysis of phagocytes to determine the total number of staphylococci and after lysostaphin treatment (1 U/ml for 10 min at 37°C) and one centrifugation-wash cycle to determine the number of intracellular bacteria. Macrophages were also collected from lysostaphin-treated lavage fluid and cultured in vitro to determine intracellular bactericidal activity. For those experiments, macrophages were collected by centrifugation after lysostaphin digestion of extracellular bacteria, washed once with M199, and resuspended in complete tissue culture medium without antibiotics. After a 1-h incubation period in glass Leighton tubes, debris and nonadherent cells were removed by two rinses with medium, and the macrophage monolayer was incubated in complete medium which contained low concentrations of penicillin (10 U/ml). Monolayers were lysed at zero time and at 20 h with distilled water and diluted for quantitative bacterial cultures. The number of macrophages per Leighton tube was determined by phase microscopy with an eyepiece grid. In some experiments, the trachea and major bronchi were dissected away from the lung parenchyma, homogenized, and quantitatively cultured to determine the distribution of staphylococci deposited in the lower respiratory tract during aerosol challenge.

Data analysis. All results are presented as mean ± standard error, and the means were compared by a *t* test. The results from microscopic assays with coded slides represent duplicate determinations from three or more separate experiments, unless indicated otherwise. Clearance of staphylococci was determined by least mean squares analysis of logarithmically transformed data, and net elimination half-lives ($t_{1/2}$s) were calculated from the slopes of the regression lines (3). Elimination $t_{1/2}$s were compared by a paired *t* test, and *P* values of ≤ 0.05 were considered significant.

RESULTS

Characteristics of influenza infections. CF_1 mice consistently developed respiratory infection and obvious clinical illness after intranasal inoculation with our strain of PR8 influenza virus. The infecting dose used for these experiments produced approximately 10% mortality

TABLE 1. Indices of viral infection in bronchoalveolar spaces

Day of infection	Influenza hemagglutinin titer[a]	% Influenza-infected macrophages[b]	No. of leukocytes[c] in:	
			Control mice	Influenza-infected mice
3	6.0	11.1 ± 5.2	4.65 ± 0.24	5.70[d] ± 0.07
5	33.6	6.7 ± 1.3	4.70 ± 0.17	5.86[d] ± 0.05
7	0	0.70 ± 0.7	4.39 ± 0.38	5.53[e] ± 0.30
11	0	0.8	4.51	5.04

[a] Hemagglutinating activity in lavage fluid supernatants, expressed as geometric mean of reciprocal titer.
[b] Percentage of glass-adherent macrophages recovered by bronchoalveolar lavage which express viral hemagglutinin activity on plasma membrane, expressed as mean ± standard error.
[c] Total number of leukocytes recovered with a 1-ml lavage per animal, expressed as log mean ± standard error.
[d] $P < 0.05$ by paired t test.
[e] $P = 0.13$ by paired t test.

by day 14, and 40 of 42 animals tested 14 days after infection developed hemagglutination inhibition antibody titers of ≥20 (geometric mean = 111). Gross inspection of the lungs revealed bilateral patchy areas of hemorrhagic consolidation, and lung homogenates from animals sacrificed on day 5 contained $10^{7.03 \pm 0.16}$ 50% tissue culture infective dose units of PR8 virus.

Bronchoalveolar lavage fluid was collected from mice sacrificed on days 3, 5, 7, and 11 of infection and evaluated for several parameters of infection (Table 1). These results indicated that these infections produce a significant leukocytosis in bronchoalveolar spaces and that a small fraction of glass-adherent macrophages recovered by lavage expresses viral hemagglutinin on their plasma membranes. Nonadherent cells collected for monolayer supernatants had a similar percentage of rosette-forming cells. In other experiments, we prepared monolayers from mice sacrificed on day 5 by using lung mincing and collagenase digestion to recover tissue macrophages; in these monolayers, 1.2 ± 0.7% and 1.8 ± 0.5% of the macrophages in vitro for 4 and 24 h, respectively, formed rosettes. Consequently, even at the height of viral replication in the respiratory tract, only a small fraction of free or tissue lung macrophages appeared to be infected with PR8 virus, although those cells are readily infected in vitro (9).

Macrophage function in vitro. Bronchoalveolar macrophages from control and PR8-infected mice were cultured in vitro for 2 to 4 h and then incubated with suspensions of either *S. aureus* 502A or *Saccharomyces* sp. for 1 h (Table 2). These results indicated that in vivo PR8 infection did not alter subsequent in vitro nonimmune phagocytosis of either *S. aureus* or *Saccharomyces* sp.

Staphylococcal clearance for whole lungs and bronchoalveolar spaces. Mice were sacrificed immediately, at 6 h, and at 24 h after our standard aerosol exposure, and the number of viable staphylococci in lung homogenates or bronchoalveolar lavage fluid was determined by quantitative plate counts and used to calculate net elimination rates (Fig. 1). These results indicated that the net elimination of staphylococci was delayed in mice infected with PR8 virus, and this delay seemed to involve the bacterial subpopulation recovered by gentle bronchoalveolar lavage more than the whole

TABLE 2. Phagocytic activity

Ingestion of:	Monolayer	% Activity (mean ± SE) on the following day of PR8 infection:			
		3	5	7	11
Staphylococci[a,b]	Control	47.7 ± 11.9	69.3 ± 5.8	52.8 ± 16.3	77.0
	PR8	45.5 ± 16.6	51.5 ± 6.4	51.9 ± 12.4	84.4
Saccharomyces[c,b]	Control	ND[d]	39.5 ± 6.2	38.3 ± 8.4	ND
	PR8	ND	29.5 ± 12.4	38.3 ± 11.2	ND

[a] Percentage of macrophages in monolayers which had ingested one or more organisms during a 1-h incubation with $1.5 \times 10^8 \pm 0.3 \times 10^8$ CFU/ml.
[b] There were no significant differences at a 0.05 level identified by paired t tests.
[c] Percentage of macrophages in monolayers which had ingested one or more organisms during a 1-h incubation with 5×10^6 yeast cells per ml.
[d] ND, Not done.

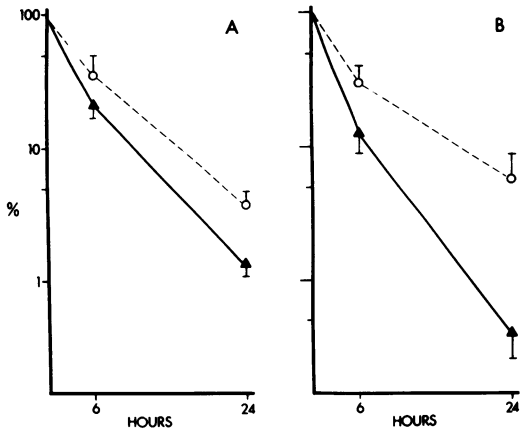

FIG. 1. Staphylococcal clearance from control mice (▲) and PR8-infected mice (○) in whole lung homogenates (A) and bronchoalveolar spaces sampled by lavage (B). Results at 6 h and 24 h are expressed as a percentage of the initial staphylococcal burden at the end of aerosol exposure. The differences at 24 h are significant at a P value of <0.05. These results are derived from 96 control mice and 77 PR8-infected mice (day 7 after virus inoculation).

lung population. In control mice the net loss of viable cocci from the bronchoalveolar spaces occurred at a faster rate than from the whole lung. When these results were analyzed by calculating the net elimination $t_{1/2}$ for each aerosol exposure, there were significant differences between control and influenza-infected mice ($t_{1/2}$ for lung clearance, 3.3 ± 0.3 h for control and 4.4 ± 0.5 h for PR8, $P < 0.05$ by paired comparison; $t_{1/2}$ for lavage space clearance, 2.5 ± 0.2 h for control and 5.4 ± 1.0 h for PR8, $P < 0.05$). We did not find net in vivo growth of staphylococci. In summary, PR8 infection produced a consistent defect in staphylococcal clearance which appeared to involve the subpopulation of bacteria in the bronchoalveolar spaces to a greater extent than the whole lung population.

In vivo ingestion and in vitro intracellular bactericidal activity. Leukocytes were recovered by bronchoalveolar lavage from control and influenza-infected mice sacrificed immediately after the termination of aerosol exposure, treated with lysostaphin, washed with medium, and then cultured in vitro. A total of 37.4 ± 10.6% of the leukocytes from control mice and 25.9 ± 5.4% of the leukocytes from influenza-infected mice were glass adherent after 1 h in vitro and two rinses with medium ($P > 0.2$), and these monolayers contained 2.1 ± 0.5 CFU/100 macrophages (control mice) and 4.6 ± 2.8 CFU/100 macrophages (influenza-infected mice) ($P > 0.2$). With prolonged in vitro incubation, the number of viable intracellular cocci gradually fell (Fig. 2). The net elimination $t_{1/2}$s from these experiments were 7.7 h in macrophages from control mice and 7.4 h in macrophages from animals with PR8 infection. Although the initial leukocyte preparations recovered from mice with influenza infection (day 7) and from control mice contained neutrophils (10.5 ± 3.3% for influenza-infected mice and ≤1% for control mice), these were removed from the monolayers by the rinse steps after macrophage attachment.

Severe infection. Although clearance calculations demonstrated a consistent reduction in staphylococcal killing in PR8-infected mice, there was no evidence of intrapulmonary staphylococcal replication, and, therefore, one could argue that these infections were too mild to cause macrophage dysfunction. To evaluate the effect of more severe influenza infection on macrophage function, we repeated the in vitro studies after infection of mice with a dose of PR8 which produced 53% mortality by day 7. These mice were very ill and occasionally died during the aerosol exposure. After aerosol challenge on day 7, mice were sacrificed for bronchoalveolar lavage to determine the fraction of staphylococci ingested in vivo and to establish monolayers. In control mice, 3.4 ± 1.2% of the staphylococci recovered by gentle lavage were resistant to lysostaphin and recoverable by low-speed centrifugation and, therefore, presumably were in an intracellular location (10). In PR8-infected mice, the fraction was slightly higher (9.7 ± 2.4%, $P = 0.06$), suggesting that bronchoalveolar phagocytes in influenza-infected mice ingested a larger fraction of the bacteria delivered

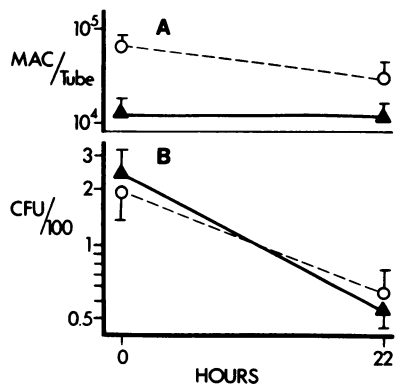

FIG. 2. Intracellular bactericidal activity against *S. aureus* 502A. Macrophages were recovered from control mice (▲) and PR8-infected mice (○) (day 7 after virus inoculation) after aerosol challenge with staphylococci. (A) Number of glass-adherent macrophages (MAC) present in Leighton tubes during in vitro incubation. (B) Reduction in viable intracellular staphylococci during in vitro incubation.

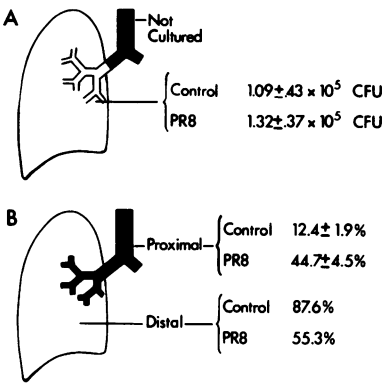

FIG. 3. Staphylococcal deposition during aerosol challenge. (A) Lung homogenates included fragments of viable airways; control and PR8-infected mice (day 7 after virus inoculation) had identical numbers of staphylococci deposited in the lower respiratory tract. (B) Trachea and major bronchi were dissected away from the lung parenchyma; in PR8-infected mice there was a significant increase in the fraction of staphylococci deposited in major airways ($P < 0.5$). Results for this figure are from mice sacrificed immediately after aerosol exposure.

during the 1-h exposure. The number of macrophages recovered from these sicker mice was similar to that recovered in the identical studies described above with mice with milder infection (Fig. 2). In addition, macrophage monolayers established from these sicker mice killed the staphylococci ingested in vivo during aerosol exposure as well as did monolayers from normal mice (82.6 ± 9.8% and 76.4 ± 6.1% reduction in viable counts in 20 h, respectively; $n = 4$ experiments). Therefore, PR8 infections which were lethal for a large fraction of mice produced no greater effect on macrophages than did milder infections.

Staphylococcal deposition during aerosol exposure. To determine the effect of viral tracheobronchitis on the bacterial deposition pattern during aerosol exposure, we measured the total number of staphylococci retained in the lung during aerosol exposure and the relative distribution between lung parenchyma and major visible airways. Animals sacrificed immediately after the termination of aerosol exposure had $1.09 \times 10^5 \pm 0.43 \times 10^5$ (control mice) and $1.32 \times 10^5 \pm 0.37 \times 10^5$ (PR8-infected mice) CFU per lung ($P > 0.1$ by paired comparison) (Fig. 3A). In several additional experiments, we determined the number of CFU deposited in the trachea and major bronchi and in the residual parenchyma and found significant differences between control and infected mice in the pattern of deposition (Fig. 3B). In these experiments the residual lungs contained $1.29 \times 10^5 \pm 0.33 \times 10^5$ CFU (control mice) and $0.5 \times 10^5 \pm 0.12 \times 10^5$ CFU (PR8-infected mice), and this difference approached statistical significance ($P = 0.07$ by paired comparison). This increase in staphylococci in proximal airways was also observed in PR8-infected mice sacrificed 6 h after aerosol exposure (control mice, 4.2 ± 1.7% of total CFU in airways; PR8-infected mice, 22.4 ± 2.0% of total CFU in airways; $P < 0.05$). Consequently, influenza tracheobronchitis altered the pattern of staphylococcal deposition, and lungs routinely removed with bronchial fragments for clearance studies contained large numbers of cocci in the major airways.

DISCUSSION

This study demonstrated that influenza infections established by intranasal inoculation suppress staphylococcal killing in the lung and delay bacterial clearance from bronchoalveolar spaces. However, the staphylococcal ingestion by macrophages during aerosol exposure and the intracellular bactericidal activity measured in vitro against the bacteria ingested in vivo were similar in control and influenza-infected mice. Thus, it appears that the reduction in bacterial clearance during influenza infection cannot be completely explained by alterations in macrophage function. These studies also demonstrated that an increased fraction of inhaled staphylococci were deposited in the trachea and proximal bronchi of influenza-infected mice.

We have reported that most staphylococci recovered from normal mice by gentle bronchoalveolar lavage during the first 6 h after aerosol exposure do not sediment with macrophages during centrifugation and are sensitive to the extracellular lytic enzyme lysostaphin, that these extracellular staphylococci are cleared from the bronchoalveolar spaces at the same rate as the total lung bacterial population, and that intracellular bactericidal activity against staphylococci is too slow to explain observed intrapulmonary killing (10). These results suggested that extracellular killing must occur during the initial phase of staphylococcal clearance. In influenza-infected mice, alveolar macrophages appear to play a similar role in staphylococcal clearance. However, in contrast to control mice, the subpopulation of staphylococci in the bronchoalveolar spaces in influenza-infected mice was cleared more slowly than the whole lung population. Although this difference in the relative clearance rates of the two populations of staphylococci has several possible explanations, these results are most readily explained by an influenza-induced alteration in extracellular killing processes. The mechanism for extracellular killing of staphylococci is unknown, but secretory products of the airway mucosa and alveolar cells, such as peroxidase (2) and reactive oxygen

metabolites (8), have antimicrobial activity and could be involved.

Although these in vivo experiments and previous in vitro experiments (7, 9) have not identified any defect in macrophage antistaphylococcal activity during influenza infection, other workers have presented evidence for myxovirus-induced macrophage defects. Jakab and Green used histological techniques to evaluate the location and integrity of intraalveolar staphylococci in control and Sendai virus-infected mice and concluded that ingestion was normal but that intracellular bacterial digestion was inhibited in virus-infected mice (6). Warshauer et al. used similar methods with influenza-infected mice and concluded that both ingestion and digestion were impaired in virus-infected animals (12). However, the methods used did not allow for determination of the viability of observed intraalveolar cocci, and actual viability measurements depend on plate counts from lung homogenates. Since we could recover large numbers of extracellular cocci in both control and influenza-infected mice with a gentle nonexhaustive lavage procedure, we doubt that microscopic examination of fixed lung tissue has provided a complete overview of the site of bactericidal activity in the lower respiratory tract.

This conclusion is supported by data from Silverberg et al. (11), who used electron microscopy to study macrophages recovered from Sendai virus-infected mice which had been exposed to aerosols of *S. aureus*. They reported that 15 and 30% of macrophages from control and infected animals, respectively, had ingested staphylococci during the aerosol procedure with identical means of 1.86 cocci per macrophage and that the fraction of macrophages with intracellular bacteria fell with time after aerosol exposure. Extracellular bacteria were present in both groups at all time points. Intracellular digestion (i.e., morphological changes) of staphylococci was apparently impaired in macrophages from Sendai virus-infected mice. Therefore, these results indicate that a minority of macrophages ingest staphylococci during aerosol exposure and that the ingestion rate in virus-infected mice actually exceeds that of control mice (11). These results, obtained by lavage techniques, differ from the earlier histological studies of these workers (6), which demonstrated that the fraction of intracellular bacteria increases with time in both control and infected mice. Their demonstration of a persistent population of extracellular bacteria and of increased ingestion capacity of phagocytes from virus-infected mice is in agreement with our quantitative culture results in mice with severe pneumonitis.

Viral tracheobronchitis also increased the deposition of staphylococci in the proximal bronchi and trachea during these aerosol challenges. Since spontaneous changes in the tracheal flora appear to occur during murine influenza infections (K. Nugent and E. Pesanti, Am. Rev. Respir. Dis. **125**:173, 1982), virus-induced upper-airway disease could act in concert with alterations in bactericidal activity to produce the observed predisposition to bacterial complications associated with viral infections. For example, bacteria adherent to the damaged trachea are probably not cleared as efficiently by the mucociliary apparatus, and therefore nasopharyngeal flora could colonize the major airway more readily after minor aspiration. This would increase the total respiratory tract bacterial burden and the associated inflammatory response. In addition, microaggregates of bacteria on the trachea may act as a source of inocula to seed damaged lungs and thereby establish pneumonia. Finally, since bacterial clearance from circumscribed regions of healthy lungs is influenced by the size of the bacterial inocula (Onofrio et al., Am. Rev. Respir. Dis. **123**:216, 1981), viral tracheitis with spontaneous colonization could potentially contribute to the development of bacterial pneumonia in the absence of any alteration in bacterial clearance processes in the lung parenchyma.

These studies utilized an aerosol challenge technique which deposits modest numbers of staphylococci in the lower respiratory tract and evaluates the early phase of bacterial clearance from the lung. If a bacterial challenge overwhelms intrinsic pulmonary defense processes, then additional phagocytes (primarily neutrophils) migrate into the lung. Abramson et al. have recently demonstrated that influenza virus can inhibit neutrophil oxidative metabolism and bactericidal capacity in vitro (1). If viral infections inhibit these neutrophil functions in vivo, then this could further compromise pulmonary clearance processes and would identify another factor in the analysis of virus effects on pulmonary defense responses. Viral respiratory tract infections may increase the susceptibility to bacterial infection or the severity of bacterial pneumonia or both, and these important distinctions require additional evaluation.

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