

Role of Immunity in Viral-Induced Bacterial Superinfections of the Lung

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Although viral illnesses are predisposing causes for pulmonary bacterial infections, the interrelationships of viral virulence and host immunity to alterations in susceptibility to bacterial infection are incompletely understood. We used two mutant strains of encephalomyocarditis virus (minimally virulent Mengo-37A and a highly virulent Columbia SK [Col-SK]) to investigate these interrelationships. Mice that had been immunized to Mengo-37A, and nonimmunized controls, were challenged with aerosols containing 10^4 plaque-forming units of Mengo-37A or Col-SK per liter. The effect of each viral infection on pulmonary antibacterial activity was assessed 3 days later by measuring the capacity of the lungs to kill inhaled radiophosphorus (^{32}P)-labeled *Staphylococcus aureus*. The degree of antibacterial dysfunction found was proportional to the virulence of the infecting virus. If the host was immune to the infecting virus, bactericidal function was not impaired by viral challenge. Neither mutant caused significant pulmonary damage; therefore: (i) viral-induced impairment in bactericidal activity reflects, quantitatively, the virulence of the virus and (ii) viral immunity protects pulmonary bacterial defenses by preventing damage to the phagocyte from the virus or its attendant metabolic abnormalities.

Influenza, as well as a few other viral illnesses, predisposes the lungs to bacterial infection (3, 8, 17, 18, 21, 22). Impairment of the normal resistance of the respiratory mucosa (28) and inhibition of the alveolar macrophage system (8, 18, 20) are two consequences of viral infections recognized to be of pathogenetic significance. Because decreases in resistance to bacterial infection have been reported in instances in which the virus produced varying degrees of pulmonary damage (8, 17, 18, 20), we studied the relationship of viral virulence, morphological damage, and host immunity to alterations in pulmonary susceptibility to bacterial infection.

Two strains of encephalomyocarditis virus (EMC), a highly virulent Columbia-SK (Col-SK) strain and a minimally virulent Mengo-37A mutant were used. When administered aerogenically the Col-SK strain is 100% lethal for mice, without causing pulmonary damage (2). Equivalent infection with the Mengo-37A mutant results in a nonfatal illness in which pulmonary damage in the early postinfection period is minimal (2, 8). In the work detailed in this report, mice that were immune to infection with EMC virus and nonimmune mice were

infected with aerosols of Mengo-37A and Col-SK virus. The effect of the viral infection on pulmonary architecture and on pulmonary bacterial defense was determined in immune and in nonimmune mice after infection with aerosols of *Staphylococcus aureus*. According to the data obtained, the degree of antibacterial dysfunction in the nonimmune host correlated with the virulence of the EMC virus and was independent of anatomical damage. If the host was immune to the infecting virus, pulmonary antibacterial function remained unimpaired, regardless of the intrinsic virulence of the infecting virus.

MATERIALS AND METHODS

Female NAMRU mice were used in all experiments (7). Animals were housed in plastic cages and fed mouse pellets and water ad libitum.

Microorganisms. Pools of Mengo-37A and Col-SK were prepared in L cells (L-929) and assayed as previously reported (1).

S. aureus strain 566 was cultured and labeled with phosphorus 32 (^{32}P) by the method of Green and Kass (13). In this procedure, the test bacteria are inoculated into 25 ml of a phosphorus-free culture media containing 1.0 mCi of ^{32}P . After 16 h of growth at 37 C in a shaker water bath, the labeled cells are removed

by centrifugation, washed, and suspended in 8.0 ml of saline. A 5-ml amount of this suspension was used for aerosolization.

Viral immunization and challenge. The aerosol unit and the experimental procedures used in the viral experiments have been described previously (24). Groups of mice were infected with aerosols of Mengo-37A and, where indicated, were challenged 21 days later with aerosols of Mengo-37A, Col-SK, or spent tissue culture medium. The concentration of airborne virus was determined by assaying impinger samples. The inhaled dose was then calculated by the method of Guyton (15). Control mice were exposed to aerosols of spent tissue culture medium.

Serological studies. Ten mice were exsanguinated by cardiac puncture 18 days after immunization with Mengo-37A. Hemagglutination inhibition (HAI) and neutralizing antibody assays were performed for each sera. The HAI procedure was that of Craighead and Shelokov (4), except that antigen was prepared in mouse L-cell cultures rather than in HeLa cells. L-cell monolayers were inoculated with Mengo-37A incubated 12 to 15 h at 37 C at which time a cytopathogenic effect was evident. Cultures were then frozen at -70 C, thawed, and centrifuged, and portions were stored at -70 C. The HA titer of this preparation was 1:64 with sheep red-blood cells.

Neutralizing antibody was assayed by the 50% plaque reduction (PR) technique. Serum was serially diluted in Eagle minimum essential medium and mixed with an equal volume of Mengo-37A suspension containing approximately 100 plaque-forming units (PFU)/0.1 ml. The serum-virus mixtures were incubated for 1 h at room temperature, and 0.2 ml was inoculated in triplicate onto L-cell monolayers. After a 40-min absorption period at 37 C, the monolayers were overlaid and treated as for virus assay (7). The antibody titer was expressed as that dilution which showed a 50% reduction in number of plaques compared with normal serum.

Pulmonary bactericidal activity. The methods used for the *in vivo* assessment of the bactericidal capacity of the murine lung have been published (11). Three days after the viral challenge, groups of 48 mice were infected with aerosols of ³²P-labeled *S. aureus* in an apparatus designed to produce uniform inhalation dosages of infecting bacteria. Four hours after bacterial infection the mice were killed, their lungs were removed aseptically and homogenized in 3.5 ml of broth, and measurements of radioisotope and bacterial concentrations were made in 1-ml samples of the homogenate. In order to establish the number of bacteria represented by ³²P radioactivity, an Anderson sampler was attached to the aerosol chamber during the exposure period. Quantitative measurements of radioactivity and bacterial concentrations were performed from the 1.0- to 2.0- μ m sampling plate. A ratio of bacterial concentration to ³²P concentration was computed and designated the aerosol labeling ratio, K. The mathematical expression of pulmonary bactericidal activity for an individual mouse could then be computed from the following formula: percent bactericidal activity = $1 - [\text{bacterial count at } 4 \text{ h} / \text{ } ^{32}\text{P count at } 4 \text{ h} \times K] \times 100$. The percent bac-

tericidal activity is the rate at which bacteria are killed within the lungs. The analysis of variance method was used to test for the significance of differences.

Pathological studies. Biopsy specimens of lung were randomly obtained from the various experimental groups for histological examination. The specimens were fixed by immersion in 10% buffered Formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined with a light microscope. In some experiments the lungs were fixed *in situ* by instilling 1.8% glutaraldehyde in cacodylate buffer into the trachea at 10 cm of hydrostatic pressure. A portion of these specimens was prepared for light microscopy examination in the previously described manner. A second portion was embedded in an Araldite and Epon mixture for electron microscopy examination. These sections were stained with uranyl acetate and lead citrate and examined with an A.E.I. EM6B electron microscope.

RESULTS

The data concerning the immunization of mice with Mengo-37A are shown in Table 1. The aerosol concentrations of Mengo-37A for each of the three immunizations were about 10⁴ PFU/liter of air. Calculation of murine dosage indicated that each mouse inhaled at least 10⁴ viral particles. The reciprocals of the median antibody titers, measured at 18 days by the HAI test, were 64, 64, and 32 for the three experiments. The HAI titers for control mice were less than 2.

The reciprocals of the median titers of neutralizing antibody were 160, 160, and 320. Control mice had titers of 20 or less.

Table 2 summarizes the data for the experiments in which pulmonary bactericidal activity was assessed in immunized and nonimmunized mice 3 days after viral challenge. All mice survived for at least 3 days. The nonimmunized mice that were challenged with Col-SK weighed less, were lethargic, and had poor skin turgor at the time of bacterial challenge. Members of this group that were not tested invariably died in the ensuing week. The immunized and the nonimmunized mice that were challenged with Mengo-37A or tissue culture did not lose weight and were healthy in appearance at the time of bacterial testing. Deaths did not occur in the Mengo-37A experimental groups.

In mice that had been immunized, the pulmonary radiophosphorus, bacterial concentrations, and the bactericidal activity rates were not altered after viral challenge. Because the pulmonary radiophosphorus concentration is an index of the number of bacteria that were inhaled, these results indicate that respiration was similar in controls and in immunized mice that were infected with virus.

A comparison of the pulmonary bactericidal activity data in the nonimmunized groups of mice showed that infection with Col-SK caused severe impairment in bactericidal activity ($P < 0.01$), whereas infection with Mengo-37A resulted in significant ($P < 0.05$) but less severe abnormalities. It is noteworthy that the pulmonary radiophosphorus concentrations were reduced by one-half in mice infected with Col-SK ($P < 0.01$). This decrease in the number of inhaled bacteria probably reflects alterations in ventilatory patterns due to the diseased state of the animals. Despite the large decrease in pulmonary bacterial burden, bactericidal activity was markedly impaired. Pulmonary radiophosphorus concentrations were similar in mice exposed to aerosols of Mengo-37A or tissue culture.

The lungs of mice that were infected with Col-SK or Mengo-37A virus did not have histological abnormalities which could be attributed to the viral infections. The epithelial cells lining the bronchi were intact. Almost all of the bronchial lumens were patent. Neither intralveolar edema nor pneumonic consolidations

were present. Occasional foci of atelectasis and chronic inflammation were found with equal frequency in biopsy specimens from control and treated animals. Because foci of atelectasis were not observed in lungs that were fixed in situ, this abnormality was probably an artifact of preparation.

The only abnormality that was found by electron microscopy examination of viral infected tissues was swelling of the cytoplasm of endothelial cells. This defect was more severe in cells from mice infected with Col-SK virus than in cells from mice infected with Mengo-37A virus. The ultrastructure of alveolar macrophages, the type I and the type II pneumocyte, was normal. The nucleus of the macrophage was round or oval and contained prominent nucleoli. The cytoplasm was abundant. Mitochondria were present in adequate numbers and were not swollen. A moderate number of lysosomes were distributed throughout the cytoplasm, and the endoplasmic reticulum did not appear to be dilated.

DISCUSSION

These studies demonstrate a quantitative relationship between viral virulence and the capacity of the lungs to kill inhaled bacteria in the mouse. Aerogenic infection with Col-SK, a highly virulent strain of EMC virus, caused much more severe bactericidal dysfunction than did infection with equivalent concentrations of the minimally virulent mutant Mengo-37A. Because the two strains of EMC virus are antigenically similar (5), these differences in intrapulmonary bactericidal activity can be attributed to the disease-producing potential of each virus.

Infection with Col-SK virus caused severe impairment of pulmonary antibacterial de-

TABLE 1. Hemagglutination inhibition and neutralizing antibody titers at 18 days in mice immunized with Mengo-37A virus

Expt	No. of mice	Aerosol dose (PFU/mouse)	HAI anti-bodies ^a	Neutralizing anti-bodies ^b
1	40	2.8×10^4	64	160
2	40	3.4×10^4	64	160
3	40	3.0×10^4	32	320

^a Reciprocal of the median titer.

^b Reciprocal dilution of the median titer of serum that resulted in a 50% reduction in the number of PFU of virus.

TABLE 2. Pulmonary bactericidal activity in immunized and nonimmunized mice 3 days after infection with Mengo-37A or Columbia SK virus

Experimental group	Viral challenge ^a	Bacterial count per ml of lung at 4 h ^b	³² P count per ml of lung at 4 h ^b	Bactericidal activity at 4 h (%) ^b	Avg wt (g) ^b
Immune	Mengo-37A	2,828 ± 810	1,053 ± 135	82.1 ± 2.3	27.6 ± 0.7
Immune	Col-SK	2,631 ± 745	1,039 ± 139	82.3 ± 1.9	28.8 ± 1.1
Immune	Tissue culture medium	2,613 ± 728	1,132 ± 152	82.9 ± 1.8	29.2 ± 1.3
Nonimmune	Mengo-37A	2,861 ± 806	1,079 ± 135	76.4 ± 2.5 ^c	29.2 ± 1.4
Nonimmune	Col-SK	2,743 ± 604	583 ± 86 ^d	38.2 ± 7.9 ^d	26.7 ± 2.5
Nonimmune	Tissue culture medium	2,500 ± 740	1,044 ± 135	82.5 ± 1.6	28.7 ± 1.3

^a 24 mice were studied for each experimental group.

^b Mean ± SE.

^c $P < 0.05$.

^d $P < 0.01$.

fenses without producing pathology such as necrosis, inflammation, or edema. In previous investigations of viral-bacterial interrelationships, the viral agents that were studied have induced morphologically evident damage, thereby obscuring the possibility of nonanatomically related antibacterial defects (8, 17, 18, 20). The absence of significant pulmonary damage after aerogenic infection with Col-SK is in accordance with earlier reports indicating that the lungs are not damaged as a consequence of lethal infection with Col-SK virus (11).

The mechanisms by which viruses enhance pulmonary susceptibility to bacterial infection include: (i) damage to tracheobronchial mucosa which impairs mucociliary function and allows local bacterial proliferation (23, 28); (ii) the induction of intra-alveolar edema which hinders phagocytic function (16); and (iii) a possible cytotoxic effect on the macrophage itself (14, 23). It is unlikely that abnormalities in bacterial transport or edema formation were factors in our experiments. Jakab and Green, by using a similar murine model to investigate viral-bacterial interactions, failed to show impairment of mucociliary transport mechanisms; in their view, the reduction in bacterial defense was the result of inhibition of intrapulmonary bacterial killing (18). Because edema was not found in the histological and ultrastructural examinations of infected lungs, it is unlikely that this abnormality was responsible for the defect in bacterial defense.

The pathogenesis of the viral-bacterial relationship is consistent with a toxic action of the virus on the phagocyte itself or an indirect effect of the metabolic abnormalities consequent on the viral disease. The ability of viruses to resist killing mechanisms of phagocytes and possibly to replicate within these cells appears to be an important mechanism for virulence (14, 23, 25-27). As a result of this interaction, certain viruses, such as influenza and ectometrelia, are able to inhibit phagocytosis of bacteria (6, 14, 26). Although we are unaware of data concerning the interaction of EMC viruses with macrophages, the unchecked proliferation of Col-SK virus within the lungs is consistent with diminished phagocytic function. Alternatively, the transient nature of the proliferative phase of the minimally virulent Mengo-37A strain indicates that this mutant fails to overcome cellular defenses and, hence, it is less likely to alter phagocytic function. If cellular damage was in fact responsible for the impairment in phagocytic function, the defect would be at the biochemical level because there was no struc-

tural damage on electron microscopy examination.

Host defenses may also have been impaired by metabolic abnormalities which resulted from the viral infection. Previous experiments have demonstrated that starvation (12), metabolic acidosis (10), or renal failure (9) inhibit pulmonary defense mechanisms, presumably by inhibiting phagocytic function. The mice that were infected with Col-SK were critically ill with weight loss, dehydration, and ventilatory disturbances at the time of bacterial testing; their defective bacterial resistance may have been an indirect consequence of these metabolic abnormalities.

The protective effect of immunization can be attributed to prevention of systemic illness. The Col-SK virus was restrained from proliferation within the lungs as well as other organs of immunized, infected mice (2). In immune mice, the curve relating viral concentration within an organ to time is nearly identical to the curve obtained with infection by the minimally virulent Mengo-37A strain (2). Therefore, if toxicity is related to biochemical alterations that result from the unrestricted proliferation of Col-SK virus, immunity would block this pathogenetic mechanism. Additionally, because mice that were immune did not become ill after infection with Col-SK virus, immunization was protective against secondarily occurring metabolic abnormalities. Although less striking, immunization also protected mice from the less severe diminution of bactericidal activity which followed infection with Mengo-37A virus. This salutary effect was probably mediated by the same mechanisms which prevented bactericidal impairment from the more virulent Col-SK virus.

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