Immune Enhancement of Pulmonary Bactericidal Activity in Murine Virus Pneumonia

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ABSTRACT Bacterial multiplication in the lung associated with murine Sendai virus pneumonia is caused by virus-induced defects in pulmonary bactericidal mechanisms. The nature of this effect has been studied in animals immunized against the challenge bacteria. Mice were immunized against *Proteus mirabilis* by intraperitoneal inoculation and by aerosol inhalation. After the development of immunity, mice were infected aerogenically with 10^4 $TCID_{50}$ of Sendai virus. 7 days later, during the height of the bronchial inflammation and pulmonary consolidation, the mice were challenged with an aerosol of viable ^{35}S-labeled *Proteus mirabilis* or ^{32}P-labeled *Staphylococcus aureus*.

Nonimmunized virus-infected animals showed marked impairment of pulmonary bactericidal activity with subsequent multiplication of the bacterial strain in the case of *Proteus mirabilis*. Immunized nonvirus-infected animals showed enhancement of pulmonary bactericidal activity for the homologous and heterologous strains in comparison with nonimmunized animals. Virus-infected animals immunized by aerosol showed enhanced bactericidal activity against the homologous but not the heterologous bacterial strain. Neither virus infection nor immunization had a significant effect on the transport of particles in the lung. The data demonstrated that the bacterial multiplication associated with the virus pneumonia was prevented by preceding immunization against the homologous challenge organism. The data suggest a mechanism for controlling bacterial multiplication associated with virus pneumonias.

INTRODUCTION

Pulmonary virus infections often predispose to bacterial infections in the lung and contribute to the morbidity and mortality of acute and chronic respiratory illnesses (1, 2). The mechanisms of these viral-bacterial interactions has been studied in a model of combined Sendai virus and staphylococcal infection in the lung. Mice infected with Sendai virus develop extensive desquamative lesions of the bronchial epithelium and consolidation in the lung parenchyma (3). This pathology resembles closely that induced by influenza virus infection in man (4).

Quantitative measurement of bactericidal and transport defense mechanisms in the lung during virus pneumonia has shown that during limited time periods of the virus infection, severe defects are produced in the *in situ* bactericidal (phagocytic) mechanism of the lung, but that despite histologic evidence of extensive destruction of bronchial-ciliated epithelium, the transport mechanisms of the lung remain intact (3, 5).

Aerosol immunization with Sendai virus protects animals against subsequent viral challenge and thus prevents the virus-induced suppression of pulmonary bactericidal activity (6). In the series of experiments reported here, further support for the concept of a virus-induced phagocytic defect in the lung was sought by examination of the effects of bacterial immunization on these virus bacterial interactions. Immunization against the challenge bacterial strain should enhance phagocytosis of the challenge organism on subsequent exposure. What effect this immunization would have in immune animals subsequently infected with Sendai virus is unknown and is the subject of the following report.

METHODS

Animals. White male Swiss mice (CBL HAM/ICR strain) weighing 18–20 g were used. Animals were housed in filter-topped plastic cages and allowed free access to food and water during the course of the experiment.

Virus. Parainfluenza 1 (Sendai) virus obtained from the American Type Culture Collection, was harvested in the allantoic fluid of 13-day old chick embryos after incubation at 37°C for 2 days, titered in cultures of rhesus

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monkey tissue cultures and stored at −70°C in small portions.

Preparation of vaccines. *Proteus mirabilis* was grown in trypticase soy broth (TS broth) at 37°C for 18–24 h. The cells were centrifuged, washed twice in 0.07 M phosphate buffer, pH 7.4, and resuspended in 1/10th the original volume. For intraperitoneal (i.p.) inoculation, this suspension was incubated for 4 h at 60°C in order to kill the organisms, checked for sterility, and adjusted to a standard opacity. Animals were injected intraperitoneally with 0.1 ml of the vaccine (approximate 10^{10} killed organisms).

For immunization by aerosol, the washed and concentrated suspension was used live. The bacterial suspension contained approximately 10^{10} organisms per milliliter. Animals were exposed to an aerosol of this suspension for 2 h in a previously described exposure chamber (3); 30 ml of the suspension was nebulized during this period and animals inhaled approximately 10^6 organisms.

Preparation of bacterial aerosols. *P. mirabilis* and *Staphylococcus aureus* (coagulase-positive FDA strain 209P, phage type 42D) were labeled with ^{35}S and ^{32}P, respectively, by the method of Green and Green (7), and Green and Goldstein (8). In brief, *S. aureus* and *P. mirabilis* were incubated in 70 ml of phosphorus-free and sulfur-free culture medium containing 1.0 mCi of either ^{32}P or ^{35}S. After 18 h of growth at 37°C in a rotating shaker water bath, the labeled cells were centrifuged separately, washed twice in 30 ml of phosphate buffer (pH 7.4) to remove all unattached label, and suspended in 8 ml of TS broth.

Animal exposure. 9 days after immunization, control animals and immunized animals were infected by aerosol with a sublethal 10^4 $TCID_{50}$ of Sendai virus. 7 days later the animals were challenged for 30 min with ^{35}S-labeled *P. mirabilis* or ^{32}P-labeled *S. aureus*. Bacterial and radiotracer concentrations in the animal exposure chamber were sampled by previously described methods (3).

Bacteriologic and radioassay procedures. At 0, 1, 2, 3, 4, and 24 h after aerosol exposure, groups of six to nine mice were killed by cervical dislocation. The lungs were removed aseptically by transection of the mainstem bronchi, washed with sterile phosphate buffer, and carefully checked for surface consolidation. Lungs were homogenized in 3 ml of TS broth. 1 ml of the homogenate was diluted appropriately and cultured quantitatively in quadruplicate on Petri x-plates by the standard dilution pour-plate technique. *P. mirabilis* and *S. aureus* were cultured respectively on bismuth sulfite and phenylethanol agar.

Calculation of pulmonary antibacterial activity. Pulmonary bactericidal activity, defined as the change in the proportion of viable to total bacteria, was calculated in each individual animal by the radioactive ratio method of Green and Goldstein (8). The ratio of radioactive counts was determined for the organism in the aerosol and in the lungs. Bactericidal activity was calculated from the change that occurred between the two ratios and is expressed as a percentage of the ratio in the aerosol as follows: Percent bacteria remaining =

$$\frac{\dfrac{\text{Bact. count}}{\text{Radiotracer count}}\ (\text{lung})}{\dfrac{\text{Bact. count}}{\text{Radiotracer count}}\ (\text{aerosol})} \times 100 = \frac{\text{Ratio (lung)}}{\text{Ratio (aerosol)}} \times 100.$$

This method calculates bactericidal activity of the lung as a function independent of the number of inhaled organisms.

Calculation of physical transport activity. Physical transport of ^{35}S-labeled *P. mirabilis* from the lungs was determined by following the decline in radiotracer activity. The ^{35}S counts were expressed as the percentage of the initial radiotracer counts recovered.

Serology. Blood was collected by axillary bleeding from approximately one-third of the animals in each group. Bacterial agglutination tests for the detection of serum agglutinins against *P. mirabilis* were determined by standard methods (9).

Statistical determinations. The data were analyzed by determining the arithmetic means, standard deviations (SD) and standard errors (SE) for each group and comparisons between groups were made according to the two independent sample *t* tests.

Histology. Histologic studies on the lungs of test animals were not possible, the entire lung being used for quantitation. Separate groups of mice, concurrently exposed, were used for study of pulmonary histology. The lungs of these mice were fixed in 10% buffered formalin, sectioned at 5 μm and stained with hematoxylin and eosin.

RESULTS

Bacterial deposition. Initial intrapulmonary retention of aerosolized ^{35}S-labeled *P. mirabilis* was quantified in nonimmune animals and animals immunized aerogenically against *P. mirabilis* and subsequently infected with Sendai virus by aerosol or left uninfected as controls (Table I). As shown in Table I there was no consistent effect of either the immunization or the virus infection on the numbers of viable organisms or radiotracer labeled counts recovered immediately after the aerosol exposure ($P > 0.05$ for viable bacteria and radiotracer counts between pairs of the four groups). These data show that neither virus infection, immunization nor the combination significantly affected the initial deposition of infectious bacterial particles in the lungs.

Effect of immunization on pulmonary defenses. The effect of immunization against the *P. mirabilis* on intrapulmonary bactericidal activity and radiotracer label clearance was studied over the first 4 postexposure h. As shown in Fig. 1, immunization enhanced pulmonary bactericidal activity but had no effect on particle trans-

TABLE I

Intrapulmonary Deposition of ^{35}S-labeled P. mirabilis in Nonvirus-Infected and Sendai Virus-Infected Mice Immunized by Aerosol Inoculation with P. mirabilis

	Nonvirus infected		Virus infected	
	Viable *P. mirabilis*	^{35}S	Viable *P. mirabilis*	^{35}S
	10^2	*cpm*	10^2	*cpm*
Nonimmune	58±3*	523±20*	46±24	475±64
Immunized by aerosol	49±12	492±24	69±11	622±103

* Each value represents the mean and SE of viable *P. mirabilis* or radiotracer activity recovered from lungs of five mice sacrificed immediately after aerosol challenge with ^{35}S-labeled *P. mirabilis* ($P > 0.05$ between pairs of the four groupings).

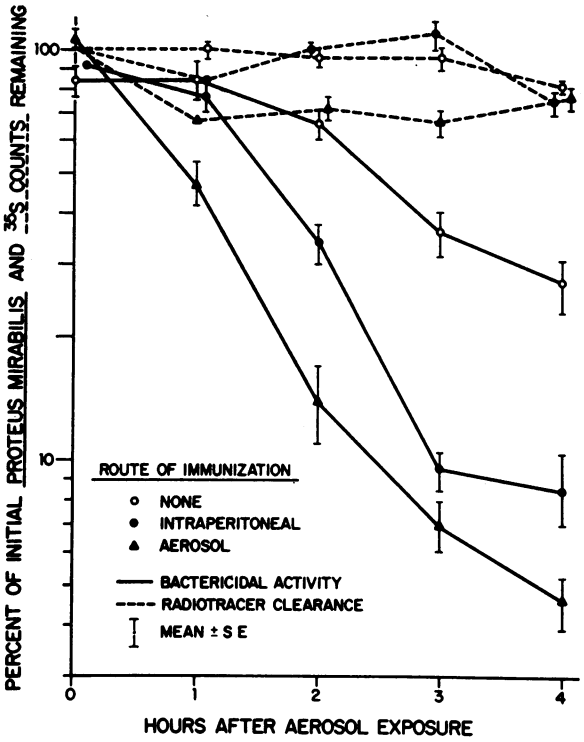

FIGURE 1 Radiotracer clearance and intrapulmonary killing of ^{35}S-labeled *P. mirabilis* in nonimmune mice and mice immunized with *P. mirabilis*. Each value represents the mean ±SE of six individual determinations.

port. The numbers of viable *P. mirabilis* declined steadily in the lungs of nonimmunized mice. However, significant enhancement of this bactericidal activity was observed both in animals immunized by the intraperitoneal route and in animals immunized by the aerosol route. The effect of aerosol immunization was significantly greater than that of intraperitoneal immunization ($P < 0.01$ at 4 h, Fig. 2). By contrast, immunization had no effect on the clearance of the radiotracer label. These data are compatible with the concept of immune enhancement of particle uptake in a phagocytic mechanism in the distal lung.

These studies were extended from 4 to 24 h in an additional set of experiments shown in Fig. 2. The same pattern of immune enhancement of early postexposure pulmonary bactericidal activity was observed although the slope of the continued bacterial killing curve was identical in all three groups. This finding is consistent with the known effects of specific immunization on particle uptake without effect on intracellular digestion. It should be noted that 0 h data for bactericidal activity are unnecessary in this model since the radiotracer label acts as a constant denominator against which the changing values of bacterial viability can be assessed; percent change in viable organisms is then calculated by comparison of these ratios with the initial ratio obtained from the infecting bacterial aerosol.

Effect of virus infection in immunized animals. Table II contains detailed data from one of the four experiments performed to determine the effect of immunization against *P. mirabilis* by aerosol or i.p. inoculation on intrapulmonary killing of ^{35}S-labeled *P. mirabilis* in Sendai virus-infected lungs. The data from this single experiment reflect the variability observed throughout the entire study and illustrate points which escape detection when the mean of each experiment is computed or the grand mean of the entire study is presented (Fig. 3).

Examination of pulmonary bactericidal values of individual virus-infected murine lungs 4 h after bacterial challenge reveals that *P. mirabilis* had proliferated in the lungs of virus-infected nonimmune animals to 228±61% as compared with declines in i.p. and aerosol immunized animals to 85±61 and 2.05±0.65%, respectively. By 24 h, values for animals immunized by aerosol declined to 0.20±0.11%; however, three patterns of bactericidal activity were observed in the nonimmune mice: (*a*) delayed bacterial inactivation, (*b*) relative stasis at 4 h levels, and (*c*) bacterial proliferation. The same patterns were observed, although declines and proliferation were not as great, in the i.p. immunized group.

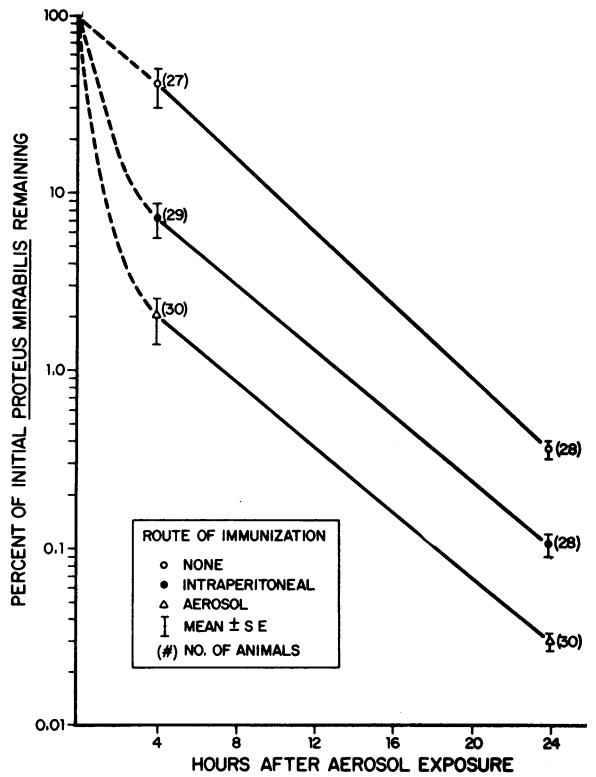

FIGURE 2 Intrapulmonary killing of ^{35}S-labeled *P. mirabilis* in nonimmune mice and mice immunized with *P. mirabilis*. Each value represents the mean ±SE.

TABLE II

Representative Single Experiment of Intrapulmonary Killing of P. mirabilis in Sendai Viris-Infected Mice Immunized by i.p. and Aerosol Inoculation with P. mirabilis

Time after exposure to ^{35}S-labeled *P. mirabilis*	Animal	Percent bacteria remaining		
		Nonimmune	i.p. immunized	Immunized by aerosol
4 h	1	174	27	5.52
	2	159	389	0.96
	3	686	34	1.10
	4	94	11	0.55
	5	112	31	2.90
	6	108	16	1.24
	7	261		2.07
	8	166		
	9	293		
Mean ±SE		228±61	85±61	2.05±0.65
24 h	1	159	229	0.05
	2	67,046	574	0.09
	3	39	92	0.05
	4	24	100	0.20
	5	360	5	0.06
	6	9,446	67	0.02
	7	886		0.97
	8	77		0.15
	9	85		
Mean ±SE		8,680±7,366	178±85	0.20±0.11

Fig. 3 compares graphically the effect of immunization on pulmonary bactericidal activity of Sendai virus-infected mice. The data was obtained by pooling individual bactericidal values from all four experiments. In nonimmune animals the mean bactericidal value at 4 h remained at approximately input levels of 106±29% in contrast to declines to 58±18% in the i.p. immunized and 3.88±1.11% in the aerosol immunized ($P < 0.001$ between nonimmune and aerosol immunized and $P < 0.01$ between i.p. and aerosol immunized). By 24 h the bacteria had proliferated to 4,464±2,483% in nonimmune animals as compared with relative stasis at 4-h values in i.p. immunized (45±22%) and a decline to 1.32±0.57% in the aerosol immunized. The degree of significance of the differences in bactericidal activity between the 24-h values could not be calculated by the *t* test due to the extensive proliferation in a portion of the nonimmune and i.p. immunized animals that consistently caused the variance to overlap zero. However, examination of data in Table II and Fig. 3 will confirm the vast differences in pulmonary bactericidal activity in virus-infected mouse lungs between nonimmune and immunized groups.

The effect of aerogenic immunization with *P. mirabilis* on intrapulmonary bactericidal activity against a heterologous organism, *S. aureus*, was also studied in virus-infected mice assayed at 0, 4, and 24 h after challenge with ^{32}P-labeled *S. aureus*. As shown in Fig. 4, immunization with *P. mirabilis* did not significantly ($P > 0.05$) enhance intrapulmonary killing of *S. aureus* in nonvirus-infected animals. Furthermore, immunization with *P. mirabilis* did not prevent the virus-induced inhibition of pulmonary bactericidal activity against a *heterologous* organism in Sendai virus-infected animals. These data demonstrate that the prevention of the virus-induced defect produced by prior aerosol immunization against the challenge bacterium was effective against the homologous, but *not* the heterologous, organism.

Physical removal. In all experiments ^{35}S activity in each individual group declined significantly between 4 and 24 h (P either < 0.01 or < 0.001). This decline was to approximately 50% of the 4-h values and was unaffected by prior immunization or by virus infection (Table III). Radiotracer values at 24 h in virus-infected animals were 48±3, 59±4, and 54±3% (columns B, D, and F, Table III) respectively for nonimmune, i.p. and aerosol-immunized animals ($P > 0.05$ between nonimmune and immunized values) and 47±2, 52±2, and 57±1% for parallel control groups not infected with the virus (columns A, C, and E, Table III, $P > 0.05$ between noninfected and virus-infected values).

Antibody determination. Sera obtained from nonimmunized and aerogenically immunized mice contained

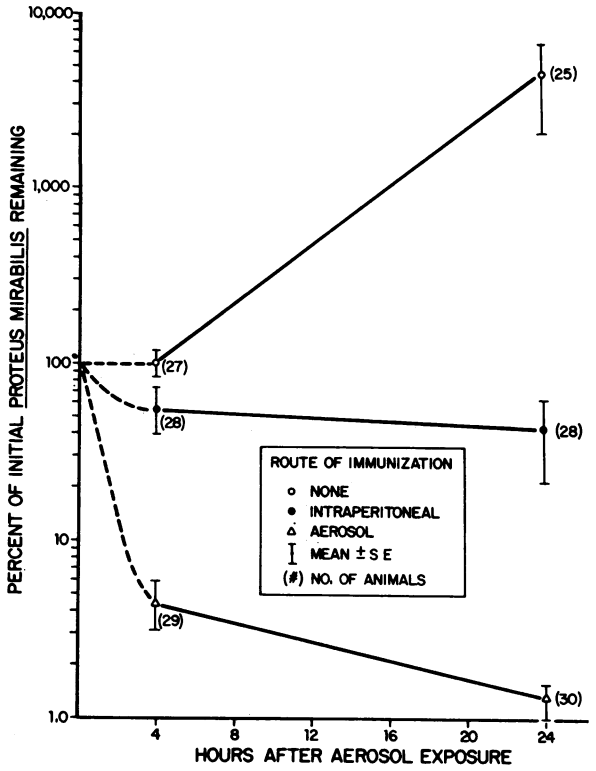

FIGURE 3 Intrapulmonary killing of ^{35}S-labeled *P. mirabilis* in nonimmune and *P. mirabilis*-immunized mice infected with Sendai virus. Each value represents the mean ±SE.

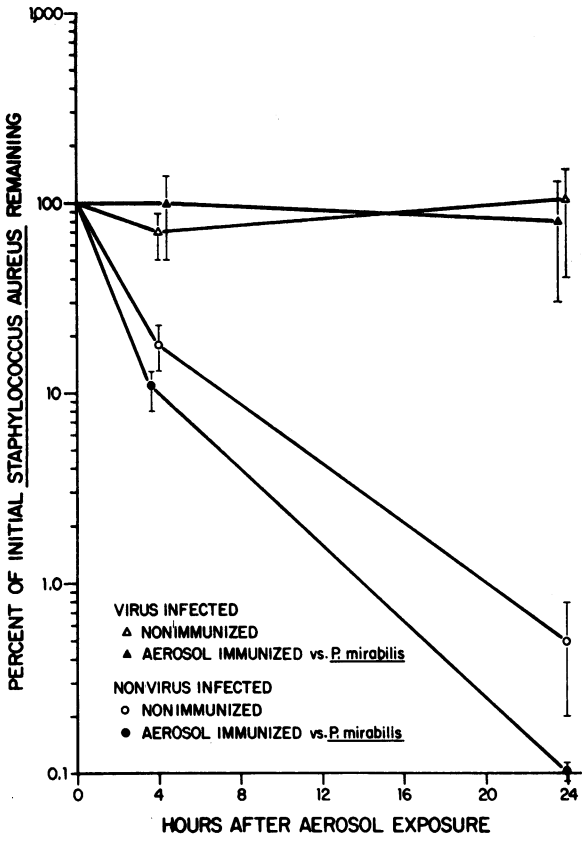

FIGURE 4 Intrapulmonary killing of ^{32}P-labeled *S. aureus* in nonimmune and *P. mirabilis*-immunized mice infected or noninfected with Sendai virus. Each value represents the mean ±SE of six to nine individual determinations.

no detectable agglutinins against *P. mirabilis* (< 1:20); serum agglutinin titers in i.p. immunized animals ranged from 1:40 to 1:320, the geometric mean for the entire group being 1:80.

Pathology. On gross inspection, all virus-infected animals had visible consolidation of approximately 25–50% of the lung 7 days after Sendai virus infection. The extent of these pulmonary changes was similar in nonimmunized and immunized mice, and within the same lung, ranged from partial to essentially complete consolidation involving parts or the whole of one or more lobes. 24 h after aerosol challenge with *P. mirabilis,* surface consolidation in nonimmunized animals increased approximately twofold over that observed in lungs of animals sacrificed at 4 h after bacterial challenge. No such increases in the total amount of surface consolidation was observed in immunized animals.

The microscopic pathology of the lungs 7 days after virus infection ranged from areas of seemingly uninvolved lung tissue to areas of bronchial epithelial cell desquamation, interstitial pneumonitis, and dense pneumonic consolidation. No discernible differences in microscopic pulmonary changes were observed between lung sections of nonimmunized and immunized animals.

DISCUSSION

Previous studies have shown that virus infection paralyzes pulmonary bactericidal activity in the first 4 h after infection (3). The data in Fig. 3 show that the result of this paralysis in nonimmunized animals is bacterial multiplication. By contrast, animals immunized by the aerosol route showed essentially normal bactericidal activity. By comparing the ratio values in the animals killed at 24 h with the ratio values from the initial infective aerosol, it was determined that about 1% of the original challenge inoculum remained viable in the virus-infected immunized animals at 24 h. The findings in the i.p. immunized animals were of interest in that although variation was considerable in this group the mean result was that no net change in bacterial numbers took place from 4 to 24 h. This finding suggested a bacteriostatic effect in the lung in which either bacteria neither multiplied nor were killed or multiplication and killing were evenly balanced.

Influenza (5), Reovirus (10), and Sendai virus (3) infections of mice are known to suppress pulmonary antibacterial activity 6–10 days after inoculation of the virus. This impairment of bactericidal activity is temporally associated with: (*a*) rapidly declining pulmonary virus titers, (*b*) the appearance of serum antibody, and (*c*) pulmonary histologic changes marked by bronchial epithelial cell destruction, edema, and consolidation. A recent finding from this laboratory shows that this impairment of host resistance during virus infection is mediated through a defect in *in situ* bactericidal mechanisms rather than through a defect in the mucociliary or alveolar transport systems (3). The data in this report indicate that virus-induced depression of bactericidal ac-

TABLE III

Effect of Sendai Virus Infection and Specific Immunization on Loss of Radiotracer from Lungs of Mice Aerosol Challenged with ^{35}S-labeled P. mirabilis

	Percent of 4 h radiotracer remaining at 24 h					
	Nonimmune		i.p. immunized		Immunized by aerosol	
Experiment no.	Non-infected A	Virus infected B	Non-infected C	Virus infected D	Non-infected E	Virus infected F
1	47*	44	63	71	69	70
2	49	53	55	62	57	58
3	47	44	51	39	52	48
4	45	50	45	63	51	39
Grand mean ±SE	47±2	48±3	52±2	59±4	57±1	54±3

* Each value is the mean of six to nine individual determinations.

tivity can be prevented by specific immunization against the challenge bacteria. This observation suggests that suppression of bactericidal activity in virus-infected lungs is caused by a functional defect in *in situ* bactericidal mechanisms rather than by destruction of the mechanism by extensive anatomic damage, inflammation, edema, etc.

The best available experimental data indicate that the bactericidal functions of the lung depend on the phagocytic capacity of the alveolar macrophages to kill, inactivate, or limit the growth of infectious organisms (11). In the experiments reported here, immunization by parenteral and respiratory routes against the homologous challenge organism significantly enhanced the pulmonary bactericidal activity. This immune-induced accelerated bactericidal activity further supports the hypothesis that phagocytosis is responsible for bactericidal activity since phagocytic enhancement is a known effect of specific immunization (12), whereas no mechanism of immune enhancement of bronchopulmonary transport systems has been described. Although immune enhancement of phagocytosis has been demonstrated primarily with peritoneal macrophages, recent studies suggest that particle uptake by alveolar macrophages is also accelerated by previous immunization (13, 14); however, these latter observations require further documentation.

Immunologic measurements were limited to serum antibody titrations during the course of these studies. The lack of a nonspecific effect against a heterologous organism, however, indicates that specific immunologic mechanisms were involved in the enhancement of intrapulmonary killing and the prevention of the virus-induced inhibition of pulmonary bactericidal activity. The lack of a nonspecific effect against *S. aureus* and the magnitude of the specific effect against *P. mirabilis* rules out the possibility that enhancement of pulmonary bactericidal activity was due to a nonspecific effect of particulate inhalation (15) brought about by the aerogenic immunization procedure.

The mechanism of immune enhancement of pulmonary bactericidal activity is not known; however, we speculate that it relates to the development of local antibody and cellular immunity. The induction of a state of local immunity in aerogenically immunized mice is suggested by the enhanced bactericidal activity in the absence of serum antibody. Resistance to virus infection correlates positively with immunoglobulin titers in bronchial washings, but varies independently of serum antibody titers (16–18). With bacteria, local and parenteral vaccination of rabbits with *Pseudomonas aeruginosa* has been shown to induce IgG in bronchial secretions. These globulins opsonize *P. aeruginosa* in vitro and enhance particle uptake but have no effect on intracellular digestion. By analogy, the presence of opsonizing antibody in the lung, at the site of alveolar macrophage activity, should be very effective in enhancing the phagocytic activity of the pulmonary macrophage. The enhanced activity of aerosol immunization over i.p. immunization may be due to the presence of higher opsonin titers in the lungs of aerogenically immunized mice.

Recent studies also indicate that cellular immunity may be important in resistance to aerogenic infection of the lungs (19–21). Intranasal immunization has been demonstrated to induce cellular immunity in the lungs (22, 23) and cellular immunity is known to lead to macrophage activation (24, 25). On the other hand, the induction of pulmonary cell-mediated immunity by parenteral immunization has been found to be dose dependent (22) and vary with the route of immunization (26). Whether a localized cellular immune response was elicited by i.p. immunization with killed *P. mirabilis* remains to be determined. However, the studies performed in nonvirus-infected mice suggest that local immunity, either humoral or cellular or both, plays an important role in the immune enhancement of pulmonary bactericidal activity.

These studies, although applied to an experimental model, are important as a contribution to the relatively undeveloped area of local immunity to respiratory infections. The role of secretory antibodies in respiratory disease has received much attention lately (27). However, these studies have primarily stressed viral vaccines (28, 29). On the other hand, studies on local cellular immunity have been performed primarily with intracellular parasites such as *Mycobacterium tuberculosis* (30, 31) and *Listeria monocytogenes* (20), but local immunity against potential bacterial pathogens, whether humoral or cellular, has not received equal attention. Our experiments with *P. mirabilis* were performed primarily to determine the specific and nonspecific effects of immunization on viral-bacterial interactions in the lung. These studies clearly demonstrate that local immunization prevented the virus-induced defect in pulmonary bactericidal mechanisms against the homologous challenge organism.

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