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Factors Influencing the Immune Enhancement of Intrapulmonary Bactericidal Mechanisms

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The effect of specific immunization on the antibacterial defense mechanisms of the murine lung was assessed against *Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus aureus* (Smith), *Serratia marcescens, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa*. Immunization by aerosol inhalation significantly enhanced the intrapulmonary killing of *Pseudomonas aeruginosa* and *Proteus mirabilis* but not the remaining organisms. With *P. mirabilis*, systemic immunization induced higher titers of specific serum agglutinins as compared with local respiratory tract immunization; however, local immunization was more effective in enhancing pulmonary bactericidal activity than was parenteral vaccination. Passive immunity against *P. mirabilis* or aerogenic challenge with preopsonized *P. mirabilis* significantly enhanced intrapulmonary killing of the homologous organism. With *S. aureus*, pulmonary bactericidal activity was not accelerated by aerosol challenge with the preopsonized organism, nor was it accelerated in passively immunized mice. These data demonstrate that the immune enhancement of pulmonary bactericidal activity is governed by the bacterium used for challenge and the route of immunization. The results further demonstrate that with *P. mirabilis*, antibody-mediated mechanisms are involved in the immune enhancement of pulmonary bactericidal activity.

A complex host defense system protects the respiratory tract from foreign particles inhaled with ambient air. Intrapulmonary processing of particles deposited on the respiratory membrane depends on the physical, chemical, and biological properties of the particle which give it pathogenic potential (6). With inhaled bacteria, intrapulmonary defense mechanisms focus on the alveolar macrophage phagocytic system. Bacteria are rapidly ingested and inactivated in situ by these cells during the first few hours after infection. Later, a portion of the bacteria are removed by physical translocation mechanisms (10).

The phagocytic effectiveness of the alveolar macrophages is influenced by a variety of exogenous chemicals, particularly air pollutants (7, 12, 17), by environmental factors such as cold stress and hypoxia (11), by inflammatory processes of the lungs, such as virus infections (5, 19), and by metabolic derangements (4, 13). The effectiveness of this phagocytic system is also suppressed by treatment with irradiation (14) and immunosuppressive agents (9).

In comparison to the influences that suppress the phagocytic mechanisms in the lung, little is known about factors that may enhance pulmonary antibacterial activity. Immune mechanisms are known to accelerate phagocytic processes (26), and it has been demonstrated that specific immunization enhances intrapulmonary killing of *Proteus mirabilis* and alters the course of viral-bacterial interactions in the lungs (20).

In the present studies, further evidence for the concept of an immune enhancement of pulmonary bactericidal mechanisms was sought by asking the following questions: (i) do inherent differences exist between bacterial species in the immune enhancement of pulmonary bactericidal activity; (ii) which route of immunization induces maximal immune accelerated pulmonary antibactericidal activity; and (iii) what is the importance of antibody-mediated immune mechanisms on the enhancement of bactericidal mechanisms in the lung?

MATERIALS AND METHODS

Animals. White male Swiss mice (CD-1 strain) weighing 18 to 23 g were used in these experiments. Immunized and nonimmunized mice were housed in separate filter-topped cages. Food and water were supplied ad libitum.

Bacteria. *Proteus mirabilis, Klebsiella pneumoniae, Serratia marcescens,* and *Pseudomonas aeruginosa* were obtained from clinical isolates. Nonencapsulated *Staphylococcus aureus* (FDA strain 209P)

and encapsulated *S. aureus* (Smith, ATCC 13709) and *Streptococcus pneumoniae* (type I, ATCC 6301) were obtained from the American Type Culture Collection. The capsules on the Smith strain staphylococcus and the pneumococcus were verified by staining with india ink.

For bacterial challenge, the gram-negative organisms and both the staphylococci were labeled with ^{32}P by previously described methods (8). In brief, the individual organisms were incubated in 100 ml of phosphorus-free culture medium containing 1.0 mCi of ^{32}P. After 18 h of growth at 37°C in a rotating shaker water bath, the labeled cells were centrifuged, washed twice with phosphate-buffered saline (PBS) to remove unattached label, and resuspended in 8 ml of Trypticase soy (TS) broth. *S. pneumoniae* was grown overnight in 100 ml of TS broth and concentrated to 8 ml in TS broth.

Active immunization. *P. mirabilis* was grown in TS broth at 37°C for 18 to 24 h. The cells were centrifuged, washed twice with PBS, and resuspended in 1/10 the original volume. For intraperitoneal (i.p.) and subcutaneous (s.c.) inoculation, this suspension was incubated for 4 h at 60°C in order to kill the organism, checked for sterility, and adjusted to a standard opacity. Animals were injected with 0.1 ml of the vaccine (approximately 10^{10} killed organisms).

For immunization by aerosol, the washed and concentrated suspensions of all the organisms were used live except where noted otherwise. Each bacterial suspension contained approximately 10^{10} organisms per ml. Animals were exposed to an aerosol of this suspension for 2 h in a previously described exposure chamber (19, 31); 30 ml of the suspension was nebulized during this period, and animals inhaled approximately 10^6 organisms. For *S. pneumoniae* the concentration in the nebulizer was approximately 10^8 organisms/ml, with animals inhaling approximately 10^4 organisms during the immunization procedure.

Passive immunization. Rabbits were hyperimmunized for 5 weeks by weekly intravenous injection with increasing doses of killed *P. mirabilis* or *S. aureus*. Three weeks after the last injection, the rabbits were exsanguinated and the serum was separated and tested for agglutinins against *P. mirabilis* by microagglutination techniques. The reciprocal of the agglutinin titer of the pooled serum was 1:2,046. No *P. mirabilis* agglutinins were detected (<1:2) in the serum of nonimmunized rabbits. Agglutinins against *S. aureus* were tested by the method of Boger et al. (2); the reciprocal of the agglutinin titer was 1:12,800. Before use, all sera were heat inactivated for 30 min at 56°C.

Groups of mice were injected by the lateral tail vein with 0.3 ml of either the normal or *P. mirabilis* hyperimmune serum. For the in vivo bactericidal assays, the injections were made at 30 min and 18 h before aerosol challenge. In experiments to determine the titer of circulating *P. mirabilis* agglutinins in passively immunized mice, the injections were at 0-, 1-, 4-, 10-, and 18-h intervals before examination.

Bacterial opsonization. *P. mirabilis* and *S. aureus* were labeled with ^{32}P as detailed above. After being washed twice with PBS, the organisms were resuspended in 8 ml of PBS containing 2 ml of either the normal or hyperimmune rabbit serum. The mixture was gently shaken at 37°C for 30 min in a rotating water bath, centrifuged, washed twice with PBS to remove the excess antibody, and resuspended in 8 ml of TS broth. In separate experiments, mice were challenged with the preopsonized organisms or the bacteria treated with normal rabbit serum.

Bacteriological and radioassay procedures. Two weeks after immunization, control animals and immunized animals were challenged for 30 min by aerosol with the homologous organisms. Immediately after aerogenic challenge and at various times thereafter, groups of six to ten mice were killed by cervical dislocation. The lungs were removed aseptically and homogenized in 3 ml of TS broth, except where otherwise noted.

One milliliter of the homogenate was diluted appropriately in TS broth (*S. pneumoniae*) or PBS (all other organisms) and cultured quantitatively in duplicate. Sheep blood agar, bismuth silfite, and phenylethanol agar were used for *S. pneumoniae*, *P. mirabilis*, and *S. aureus*, respectively. TS agar was used for all other organisms.

Quantitative measurement of ^{32}P activity of the lungs was performed on another 1-ml portion of each lung homogenate by previously described methods (19).

Bacterial dispersal. The following experiment was performed to assure that the homogenization procedure adequately dispersed the bacteria in the lungs of mice. After aerosol challenge with *P. mirabilis*, the lungs of nonimmunized and passively immunized mice were quantitated for viable *P. mirabilis* as detailed above; that is, after homogenization in 4 ml of TS broth, the appropriate 10-fold dilutions of the homogenate in PBS were quantitated in quadruplicate on bismuth sulfite (BS) agar by conventional pour plate techniques. The homogenate was then treated to another homogenization process and quantitated as was done after the initial homogenization. After the secondary homogenization, a 1-ml portion of the homogenate was added to 9 ml of sterile distilled water. After 15 min, the dilution suspension was vigorously aspirated with a 10-ml pipette and quantitated in quadruplicate on BS agar. Thereafter, the dilution suspensions in sterile distilled water received another homogenization treatment and were again quantitated in quadruplicate on BS agar. The same end dilutions of the homogenate diluted in PBS and sterile distilled water were quantitated for colony-forming units (CFU) of *P. mirabilis*.

Calculation of pulmonary antibacterial activity. Pulmonary bactericidal activity in each individual animal was calculated by a modification of the radioactive ratio method (8, 31) as follows:

$$\text{Percent bacteria remaining} = \frac{\text{bacterial count/tracer count (lung, time } t)}{\text{mean (bacterial count/tracer count) at 0 h}} \times 100$$

where the mean (bacterial count/tracer count) at 0 h is calculated by averaging the ratio of bacteria to

tracer for the 0-h animals. Bactericidal values for control and immunized animals at time t were calculated from correspondently treated groups killed at 0 h. This method calculates bactericidal activity of the lung as a function independent of the number of inhaled organisms.

RESULTS

To determine whether immunization resulted in bacterial clumping or in rapid particle uptake with organisms remaining viable intracellularly, and to assure macrophage lysis with adequate dispersal of the bacteria, the lungs were homogenized and quantitated two consecutive times followed by quantitation after hypotonic lysis and hypotonic lysis followed by a tertiary homogenization treatment.

The results of the bacterial dispersal experiments on the lungs of mice sacrificed immediately after aerosol challenge are presented in Table 1. The data are expressed as the ratio of viable bacteria per radiotracer count. The radiotracer provides a stable denominator against which the numerator of viability can be quantified; a decrease in the ratio would reflect a decrease in the number of CFU of bacteria, whereas an increase in the ratio would reflect an increase in the CFU of *P. mirabilis*. The data show that the dispersal procedures did not affect the number of CFU of *P. mirabilis* recovered from the lungs of mice killed immediately after aerosol challenge.

Table 2 presents the results of the bacterial dispersal experiments on the lungs of mice at 4 h after bacterial challenge. The bactericidal values obtained from the initial lung homogenates of nonimmunized and passively immunized mice were 53.9 ± 4.5% and 19.2 ± 1.6%, respectively. A secondary homogenization treatment of the original lung homogenate had no significant effect on the bactericidal values. However, when the secondary homogenization procedure was followed by hypotonic lysis, fewer CFU were recovered on the agar plates, which was reflected in the lower bactericidal values. Subsequent treatment of the hypotonic lysate by another homogenization process further affected the viability of *P. mirabilis* as demonstrated by still lower bactericidal values.

TABLE 1. *Effect of routine homogenization and special dispersal treatment on viable P. mirabilis recovered from lungs of mice sacrificed immediately after aerosol challenge*

Treatment of the lung	Ratio (CFU of *P. mirabilis*/radiotracer count)	
	Non-immunized	Passively immunized
Initial homogenization in TS broth	61.5 ± 3.0[a]	63.5 ± 3.5
Secondary homogenization in TS broth	59.7 ± 2.9	62.9 ± 3.6
Hypotonic lysis and aspiration	59.9 ± 2.0	61.6 ± 3.0
Hypotonic lysis and tertiary homogenization	60.9 ± 1.6	61.1 ± 3.1

[a] Each value represents the mean ± standard error of eight individual determinations.

TABLE 2. *Comparison of pulmonary bactericidal values against Proteus mirabilis in lungs processed routinely and receiving special dispersal treatment*

Treatment of the lung	Percent initial viable bacteria remaining at 4 h after aerosol challenge[a]	
	Nonimmunized	Passively immunized
Initial homogenization in TS broth	53.9 ± 4.5[b]	19.2 ± 1.6
Secondary homogenization in TS broth	55.1 ± 6.3	17.9 ± 1.3
Hypotonic lysis and aspiration	46.3 ± 6.0	14.3 ± 1.0
Hypotonic lysis and tertiary homogenization	38.8 ± 4.1	8.6 ± 1.0

[a] Zero-hour bactericidal values after initial homogenization in the lungs of nonimmunized mice and mice passively immunized against *P. mirabilis* were 100 ± 4.8 and 100 ± 5.4%, respectively.

[b] Each value represents the mean ± standard error of eight individual determinations.

Intrapulmonary killing of the selected organisms in nonimmunized mice is compared in Fig. 1. The data show the inherent differences between the organisms in infected lungs and the bactericidal activity of normal lungs; intrapulmonary killing of the selected gram-negative organisms proceeded at a slower rate in nonimmunized animals than did the killing of selected gram-positive organisms.

To determine the effect of respiratory tract immunization on pulmonary bactericidal activity, mice were immunized by aerosol inhalation of live bacteria followed at 2 weeks by challenge with the homologous organism. Table 3 presents data from representative experiments on the number of viable organisms recovered from the lungs of immunized and nonimmunized mice after challenge with each bacterium for 30 min. More than 10^5 organisms were recovered immediately after expo-

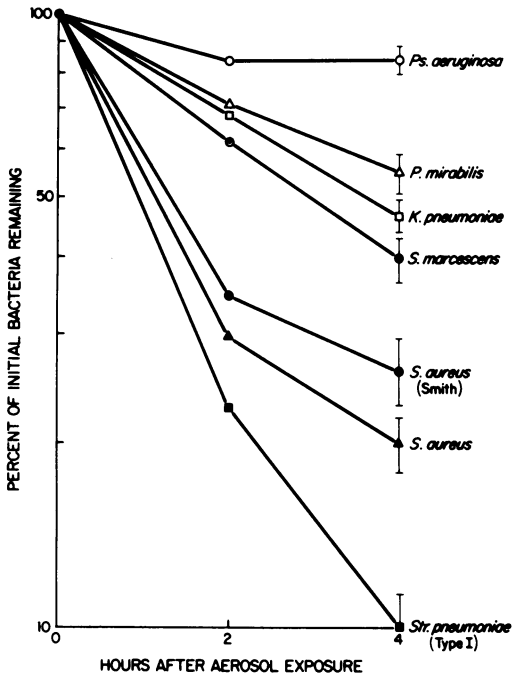

FIG. 1. *Comparison of intrapulmonary killing of selected organisms in nonimmunized mice. The data were obtained by pooling the individual bactericidal values from two to three experiments. Each point represents the mean ± standard error of 16 to 24 individual determinations. The standard error was omitted for the 2-h values because of overlapping of the error bars.*

sure from the lungs of the animals; the notable exception was *S. pneumoniae*, where approximately one-tenth of the number was recovered due to multiplication of the fastidious pneumococcus to a lower maximum population density in the growth media, which resulted in fewer organisms in the nebulizer suspension. The differences in the number of organisms recovered between immunized and nonimmunized lungs in these other experiments were not significant ($P > 0.05$).

Comparison of bactericidal values between nonimmunized and immunized animals using the above organisms is presented in Fig. 2. Enhancement of pulmonary bactericidal mechanisms by specific immunization was observed with the gram-negative organisms. The extent of the immune enhancement depended on the organism. Thus, with *P. mirabilis* the bactericidal values from immunized animals were 40% lower ($P < 0.01$) at 4 h than were the corresponding nonimmunized bactericidal values; with *P. aeruginosa* the difference was 30% ($P < 0.05$), with *K. pneumoniae* it was 15% ($P > 0.05$), with *S. marcescens* it was 13% ($P > 0.05$), and for the gram-positive organisms it was <5% ($P > 0.05$).

To determine the contribution of humoral versus cellular immunity to the enhancement of bactericidal activity, passive opsonization of bacteria with normal and specific hyperimmune serum was performed. Treatment of *P. mirabilis* with normal serum appeared to have a slight but insignificant ($P > 0.05$) effect on enhancing pulmonary bactericidal activity (55 ± 4% with nontreated bacteria as compared with 45 ± 3% with bacteria treated with normal serum). In contrast, pretreatment of *P. mirabilis* with specific hyperimmune serum significantly ($P < 0.01$) accelerated intrapulmonary killing of the homologous organisms (Fig. 3a) and appeared to reproduce the total effect of live aerosol immunization (see Fig. 2). In similar opsonization experiments performed with *S. aureus*, pretreatment of the organism with specific hyperimmune serum had no demonstrable effect on pulmonary bactericidal mechanisms (Fig. 3b).

To further investigate the effect of humoral mechanisms on the immune enhancement of pulmonary bactericidal activity, mice were passively immunized with rabbit anti-*P. mirabilis* hyperimmune serum or injected with normal rabbit serum. Immediately after injection (0 h) and at 4, 10, and 18 h thereafter, groups of six mice were exsanguinated and their sera were tested for circulating *P. mirabilis* antibody; the geometric mean serum antibody titers at these intervals were 1:110, 1:128, 1:179, and 1:128, respectively.

Passive immunization immediately before (0 h) aerogenic challenge had no effect on accelerating bactericidal mechanisms despite the pres-

TABLE 3. *Intrapulmonary deposition of bacteria in nonimmunized mice and mice immunized by aerosol inhalation with the homologous organism*

Organism	Viable bacteria recovered from lungs	
	Nonimmunized ($\div 10^3$)	Immunized ($\div 10^3$)
Proteus mirabilis	192 ± 24[a]	213 ± 21
Pseudomonas aeruginosa	495 ± 48	384 ± 21
Serratia marcescens	225 ± 27	249 ± 36
Klebsiella pneumoniae	741 ± 60	756 ± 75
Staphylococcus aureus	300 ± 30	279 ± 21
Staphylococcus aureus (Smith)	294 ± 45	222 ± 27
Streptococcus pneumoniae (type I)	25 ± 2	23 ± 3

[a] Each value represents the mean ± standard error of 8 to 12 individual determinations.

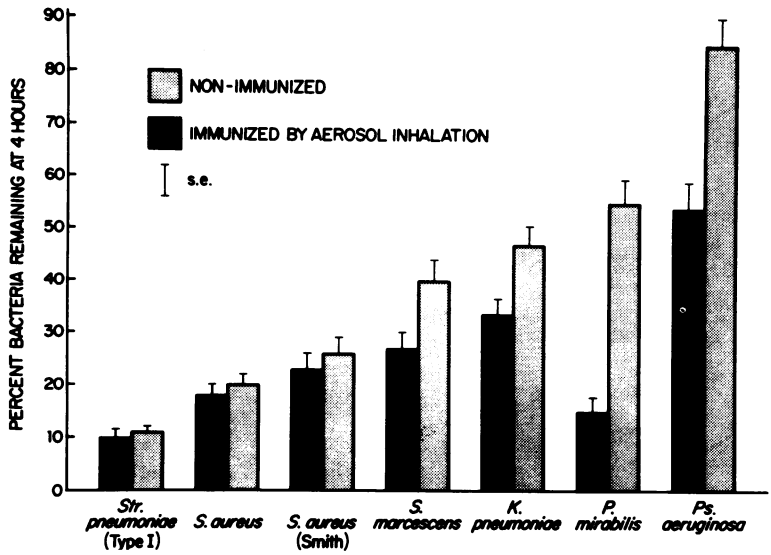

FIG. 2. *Comparison of intrapulmonary killing of selected organisms between nonimmunized mice and mice immunized by aerosol inhalation against the specific challenge organism. Each bar represents the mean ± standard error of 16 to 24 individual bactericidal values pooled from two to three experiments.*

ence of high titers of specific circulating antibody. In contrast, when the interval between passive immunization and aerosol challenge was 18 h, significant enhancement of pulmonary bactericidal mechanisms was observed (Fig. 4a). Passive immunization with *S. aureus* hyperimmune serum 18 h before aerogenic challenge had no effect on enhancing intrapulmonary killing of staphylococci (Fig. 4b).

Since maximal immune enhancement of pulmonary antibacterial activity against the selected organisms was observed with *P. mirabilis*, routes of immunization other than local (pulmonary) administration of the immunogen were investigated with this organism. The effect of specific immunization by various routes on pulmonary antibacterial activity against *P. mirabilis* at 2 weeks is presented in Fig. 5.

Immunization by i.p. inoculations and aerosol inhalation (aer) of live and killed organisms significantly enhanced pulmonary bactericidal activity against the homologous challenge organism ($P < 0.01$). Furthermore, local immunization with the live bacterium significantly enhanced ($P < 0.05$) intrapulmonary killing of *P. mirabilis* as compared with systemic immunization. Local immunization with the killed organism appeared to be more effective than i.p. immunization but not as effective as local immunization with live *P. mirabilis* on the immune enhancement of pulmonary antibacterial mechanisms. Subcutaneous immunization had a negligible effect on accelerating intrapulmonary killing of *P. mirabilis*.

Attempts to enhance pulmonary antibacterial mechanisms over the enhancement attained by a single aerogenic exposure to live *P. mirabilis* were all negative. These attempts included primary immunization by multiple routes (i.p. + aer and s.c. + aer) and primary immunization with booster immunization at 2 weeks followed 2 weeks later by challenge. The latter combinations included: aer → aer, i.p. → i.p., i.p. + aer → i.p. + aer, i.p. + aer → aer, and i.p. + aer → i.p. Immune enhancement of pulmonary antibacterial activity by aerogenic immunization was still observed undiminished at 3 months, at which time the experiment was terminated.

Circulating titers of *P. mirabilis* agglutinins after primary and secondary immunization are presented in Fig. 6. At the time of aerosol challenge, nonimmunized mice contained no detectable (<1:2) levels of agglutinins against *P. mirabilis*. Immunization by aerosol inhalation induced lower titers of systemic antibody as compared with i.p. immunization; secondary immunization by multiple routes induced the highest levels of circulating anti-*P. mirabilis* agglutinins.

DISCUSSION

Before the data from these experiments are evaluated, certain aspects of the methods used for quantitating intrapulmonary killing of bacteria merit consideration. CFU of bacteria recovered from lung homogenates may be formed by one or more bacteria. Thus, if intrapulmo-

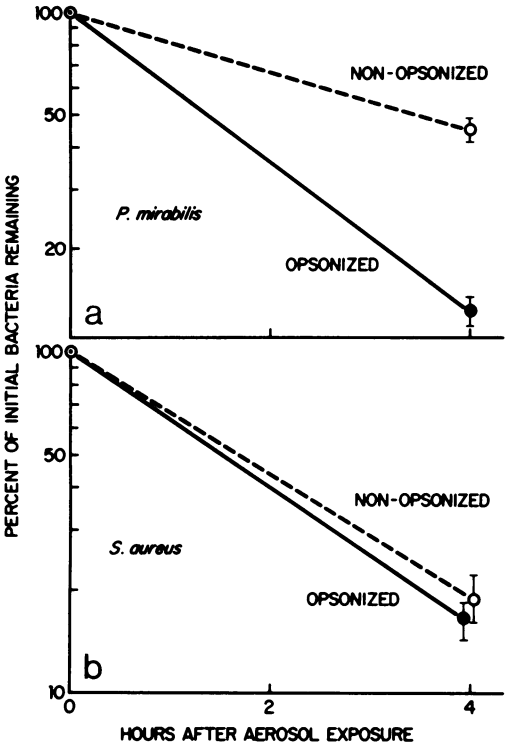

FIG. 3. *Comparison of intrapulmonary killing of P. mirabilis (a) and S. aureus (b) between mice challenged with the organism treated with specific hyperimmune serum or normal serum. Each point represents the mean ± standard error of 16 to 24 individual bactericidal values pooled from two to three experiments.*

nary deposition of bacteria occurs in clumps of organisms or if alveolar macrophages internalize more than one bacterium and the homogenization procedure does not adequately disperse the bacteria, the resulting bactericidal values may be artificially low. The lower bactericidal values in immunized mice as compared with nonimmunized mice then may only represent a technical artifact instead of a true enhancement of intrapulmonary killing of the organism. The following considerations argue against the possibility that the observed immune enhancement of pulmonary bactericidal activity was due to a methodological artifact: (i) histological studies have demonstrated that bacteria are deposited in the lung parenchyma primarily as single organisms with occasional clusters of more than four bacteria (G. J. Jakab and G. M. Green, J. Clin. Invest., in press); (ii) the overall bacterial particle burden in the experiments detailed herein was light ($<10^6$ organisms were deposited in the lungs containing many times that number of alveolar macrophages); and (iii) comparisons of bactericidal values in nonimmunized and passively immunized mice between the same lung homogenates assayed in the routine manner and assayed after secondary homogenization treatment indicate that the routine assay is adequate for the dispersal of bacteria. These observations do not demonstrate that intrapulmonary bacteria were dispersed to single organisms and that each CFU quantitated originated from a single bacterium. The data do, however, set some limits on the rigors of dispersal treatments before bacterial viability is affected.

All vaccination routes accelerated the intrapulmonary killing of *P. mirabilis*; the degree of enhancement was dependent on the route used, with local immunization being more effective than systemic immunization. Furthermore, aerogenic immunization with live organisms significantly enhanced immune-accelerated killing of the bacterium as compared with i.p. vaccination despite the higher levels of specific

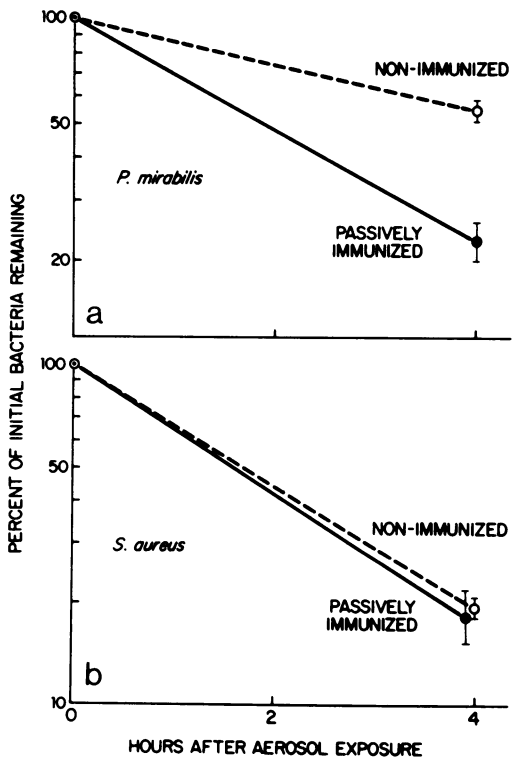

FIG. 4. *Comparison of intrapulmonary killing of P. mirabilis (a) and S. aureus (b) between nonimmunized and passively immunized mice. Each point represents the mean ± standard error of 12 to 18 individual bactericidal values pooled from two to three experiments.*

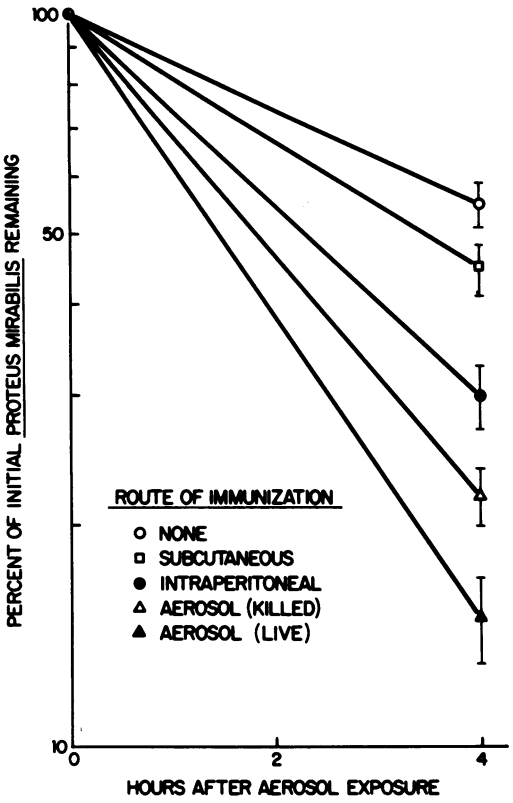

FIG. 5. *Intrapulmonary killing of P. mirabilis in mice immunized against P. mirabilis by various routes. Each point represents the mean ± standard error of 16 to 24 individual bactericidal values pooled from two to three experiments.*

circulating antibody in the i.p.-inoculated mice. These data indicate that the immune enhancement of pulmonary antibacterial mechanisms varies independently of the serum antibody titers. That pulmonary antibacterial defenses after immunization correlate with immunoglobulin titers in the lung is suggested by the studies of Reynolds et al. (27, 29).

Maximal enhancement of intrapulmonary killing of *P. mirabilis* could be attained by aerosol inhalation with the live organism. The failure to accelerate pulmonary bactericidal mechanisms further with vaccination by multiple routes or with booster immunization indicates the necessity for local lung tissue immunization for the full protective effect of bacterial immunization. Comparisons of the effect of immunization with live and killed *P. mirabilis* suggest that the live organism may be the more potent immunogen in enhancing pulmonary antibacterial defenses.

Experiments comparing the effect of specific aerogenic immunization on the enhancement of intrapulmonary killing of selected bacteria demonstrated the importance of the challenge organism. Specific immunization with the pneumococcus and the encapsulated and nonencapsulated staphylococci had no demonstrable effect on enhancing pulmonary bactericidal mechanisms. On the other hand, specific aerogenic immunization accelerated intrapulmonary killing of the selected gram-negative organisms in the following order: *P. mirabilis* > *P. aeruginosa* > *K. pneumoniae* > *S. marcescens*. Possible explanations for the observed differences would include that the animals were already immune to the gram-positive organism so that further immunization would not enhance intrapulmonary killing. Serological determinations were limited to *S. aureus* and *P. mirabilis*, with no serum antibody detected against these two organisms in nonimmunized mice. Such determinations, however, would be of questionable value since circulating antibody levels do not accurately reflect pulmonary antibacterial immunity.

Alternatively, the more rapid intrapulmonary killing rates of the selected gram-positive organisms in nonimmunized mice may reflect previous local respiratory tract stimulation with the bacterial antigens. This explanation, however, appears unlikely since the probability seems remote that the young mice used in these studies were exposed to the capsular material of the Smith strain *S. aureus* or the specific pneumococcal polysaccharide (type I). It would be

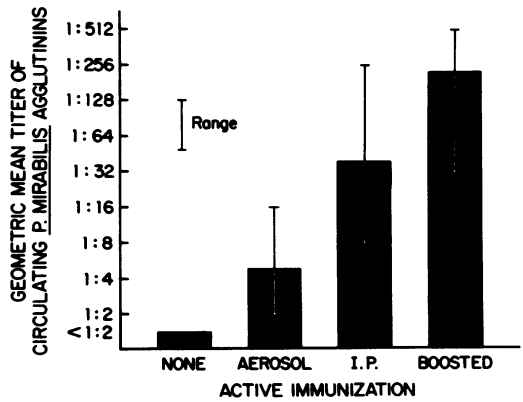

FIG. 6. *Serum anti-P. mirabilis agglutinin titers at 2 weeks in animals immunized against P. mirabilis by aerosol inhalation and intraperitoneal injection. The boosted group was immunized initially by aerosol inhalation and intraperitoneal injection followed 2 weeks later by secondary immunization by the same multiple routes; the serum was collected 2 weeks later. The data in each group represent five individual determinations.*

more likely that the nonimmunized mice were previously exposed to some of the selected gram-negative organisms such as *P. mirabilis* and *P. aeruginosa*, which are more ubiquitous in the animal's environment. However, it was precisely against these two organisms that maximal enhancement of pulmonary bactericidal activity by aerogenic immunization was demonstrated.

Although the possibility of partial immunity cannot be ruled out, there may be alternative explanations for the observed differences between the organisms in the degree of immune acceleration of pulmonary bactericidal activity. One of such alternatives would include the relative susceptibility of the organism to the bactericidal action of the alveolar macrophage phagocytic system. Examination of Fig. 1 reveals that the bactericidal values at 4 h are the lowest for *S. pneumoniae* and sequentially increase with *S. aureus*, *S. aureus* (Smith), *S. marcescens*, *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa*, respectively. This diversity in the response manifested by pulmonary bactericidal mechanisms of the murine lung toward different bacterial species was first demonstrated by Green and Kass (11), who showed that *S. albus* was killed more rapidly than *S. aureus*, which, in turn, was killed more rapidly than *P. mirabilis*. In similar aerosol challenge experiments with a variety of gram-negative bacilli, Jackson et al. (18) demonstrated the order of increased killing in normal murine lungs to be: *Herellea* sp. > *Flavobacterium* sp. > *P. mirabilis* > *Escherichia coli* > *Pseudomonas* sp. The latter laboratory extended these observations to demonstrate that in addition to bacterial genera differences, strain differences existed in the intrapulmonary killing pattern of *P. aeruginosa* in mice (32). The exceedingly rapid rate of intrapulmonary killing of *S. pneumoniae* and *S. aureus* during the initial hours after aerosol challenge has been confirmed by a number of laboratories (1, 22, 25).

Recently Goldstein et al. (15) demonstrated that the inhaled staphylococci are rapidly ingested by alveolar macrophages and killed as the bacterial mechanisms of the pulmonary phagocyte operate. The rapid ingestion and intrapulmonary bacterial inactivation rates, confirmed in this laboratory (Jakab and Green, in press), indicate that the alveolar macrophage is extremely efficient in phagocytizing and killing staphylococci. Active immunization against *S. aureus*, *S. aureus* (Smith), or *S. pneumoniae* would not improve the efficiency of the alveolar macrophages' bactericidal mechanisms against such bacteria that are already rapidly handled. This contention is supported by the experiment with *S. aureus*, where preopsonization of the challenge organism or specific passive immunization had no demonstrable effect on accelerating the intrapulmonary killing of staphylococci. On the other hand, the selected gram-negative organisms used in this study are less susceptible to the phagocytic action of the alveolar macrophages (i.e., less efficiently handled; 40% or more of the selected gram-negative bacilli remain in murine lungs at 4 h as compared with <30% for the selected gram-positive organisms).

Specific immunization enhances the efficiency of the alveolar macrophage phagocytic system against the selected gram-negative bacilli. Since opsonization of bacteria is known to enhance the internalization of bacteria by phagocytes (26), including alveolar macrophages (27, 29), with no demonstrable acceleration of intracellular killing, the opsonization and passive immunity experiments with *P. mirabilis* indicate that the immune acceleration of intrapulmonary killing of *P. mirabilis* can be explained almost completely by more efficient bacterial ingestion rates. Once internalized by the alveolar macrophage, intracellular processing mechanisms rapidly inactivate the organism. The opsonization and bacterial dispersal experiments indicate that bacterial uptake is the rate-limiting process in the intrapulmonary killing of *P. mirabilis* since no increment in viable bacteria occurs after dispersal cell lysis. This view is supported by our previous observation that immune enhancement of intrapulmonary killing occurs during the initial hours after aerosol challenge, and that after the first 4 h the rate of killing of *P. mirabilis* is identical in the lungs of nonimmunized and i.p.- and aerosol-immunized mice (20). It would then follow that in vivo ingestion and digestion rates of the rapidly handled *S. aureus* by alveolar macrophages would be approximately the same. Data by Goldstein et al. (15) and Kim et al. (24) support that position, and our failure to demonstrate an immune enhancement of intrapulmonary killing of *S. aureus* confirms that hypothesis. Finally, assuming that specific immunization by aerosol inhalation of live bacteria also enhances pulmonary bactericidal mechanisms to maximal efficiency against all the organisms used in this study, the differences between the total immune killing rates strongly suggest inherent differences in the capacity of the alveolar macrophages to inactivate the bacteria intracellularly.

Previous studies on specific antibacterial mechanisms of the respiratory tract have focused primarily on analyzing the humoral and cell-mediated immune responses induced in the lungs of the rabbits after local challenge with bacterial antigens (3, 16, 23, 27–30). For exam-

ple, immunization with *Pseudomonas* induced specific antibodies in the lower respiratory tract that opsonized the bacterium and thereby facilitated the in vitro phagocytic ingestion of the organism by alveolar macrophages (27, 29). Respiratory tract cell-mediated immunity, as evaluated by assays of migration inhibitory factor production, was also induced by local immunization. This capacity lasted only 2 to 3 weeks, suggesting that respiratory cell-mediated immunity is transient and may not play a major role in the specific antibacterial mechanisms of the respiratory tract against *Pseudomonas* (30).

In contrast to the above studies, which focused on the specific antibacterial immune responses of the lung, the experiments detailed herein have examined the functional response of local immunization on pulmonary antibacterial defenses. The data demonstrate clearly that humoral factors play a major role in the immune acceleration of pulmonary bactericidal activity.

Whether cell-mediated immunity was also induced in murine lungs by local immunization against the organisms used in this study is not known. Extrapolations from investigations in other models of the effect of local immunity on the presence of migration inhibitory factor in the respiratory tract after immunization are difficult to make. The considerations would include the following. (i) The effect of the presence of migration inhibitory factor in the respiratory tract on intrapulmonary killing of homologous bacteria has not been established; (ii) cell-mediated immunity is not considered the predominant immune mechanism in resistance to pyogenic bacteria; and (iii) the rate of intrapulmonary killing of the same organism may also be host dependent (21).

The data in these experiments clearly demonstrate that the immune enhancement of bactericidal activity in the murine lung is governed by factors which include (i) the bacterial genera used for specific immunization and challenge and (ii) the route of immunization. Vaccination by aerosol inhalation was most efficient in enhancing the bactericidal response of the lungs. The data also show that antibody-mediated mechanisms are involved in the immune acceleration of intrapulmonary killing of bacteria, but that the titer of specific serum antibody does not correlate with maximal enhancement of pulmonary antibacterial defense mechanisms.

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