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Research Article

Bacterial multiplication associated with virus infections is related to defects in in situ bactericidal (phagocytic) mechanisms of the lung. To determine whether the phagocytic defect was in bacterial ingestion and/or intracellular digestion, mice were infected with a sublethal dose of aerosolized Sendai virus and challenged 7 days later with a finely dispersed aerosol of Staphylococcus aureus. Groups of uninfected and virus-infected mice were sacrificed at 0, 6, 12, and 24 h after challenge, the lungs were perfused with formalin in situ, and the intra- or extracellular location of the bacteria was determined histologically. At 0 h, 49% and 51% of the staphylococci had an intracellular location in virus and nonvirus-infected lungs, respectively. With time, decreasing numbers of staphylococci were observed within the phagocytic cells of nonvirus-infected lungs, mostly as single organisms or in small clusters of less than four. In contrast, in focal area of virus-infected lungs, increasing numbers of phagocytic cells showed clumps of more than 25 bacteria/cell. These data demonstrate that virus-infected suppression of pulmonary antibacterial activity against S. aureus is related primarily to defects in intracellular processing mechanisms.

Defect in Intracellular Killing of *Staphylococcus aureus* within Alveolar Macrophages in Sendai Virus-Infected Murine Lungs

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ABSTRACT Bacterial multiplication associated with virus infections is related to defects in *in situ* bactericidal (phagocytic) mechanisms of the lung. To determine whether the phagocytic defect was in bacterial ingestion and/or intracellular digestion, mice were infected with a sublethal dose of aerosolized Sendai virus and challenged 7 days later with a finely dispersed aerosol of *Staphylococcus aureus*. Groups of uninfected and virus-infected mice were sacrificed at 0, 6, 12, and 24 h after challenge, the lungs were perfused with formalin *in situ*, and the intra- or extracellular location of the bacteria was determined histologically. At 0 h, 49% and 51% of the staphylococci had an intracellular location in virus and nonvirus-infected lungs, respectively. With time, decreasing numbers of staphylococci were observed within the phagocytic cells of nonvirus-infected lungs, mostly as single organisms or in small clusters of less than four. In contrast, in focal area of virus-infected lungs, increasing numbers of phagocytic cells showed clumps of more than 25 bacteria/cell. These data demonstrate that virus-infected suppression of pulmonary antibacterial activity against *S. aureus* is related primarily to defects in intracellular processing mechanisms.

INTRODUCTION

Pulmonary virus infection often predisposes to bacterial infections in the lung (1–3). 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 desquamative lesions of the bronchial epithelium and consolidation in the lung parenchyma (3–5). This pathology resembles closely that induced by influenza virus infection in man (6).

Quantitative measurements of bactericidal and physical transport 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 mechanisms of the lung, but that despite histologic evidence of destruction of bronchial ciliated epithelium, the transport mechanisms of the lung remain intact (5). Further quantitative studies have demonstrated that intrapulmonary bactericidal mechanisms are suppressed in unconsolidated as well as consolidated areas of virus-infected lung, but that bacterial multiplication is limited to the consolidated areas (7). In the series of experiments reported here, further support for a concept of a virus-induced phagocytic defect in the lung was sought by histologic examination of the location of bacteria in virus-infected lungs after aerosol challenge with *S. aureus*.

METHODS

Animals. Male Swiss albino mice (CD 1 strain, 18–20 g) were used in these experiments. The animals were housed in filter-topped cages and fed food and water ad libitum.

Infection schedule. Mice were infected by aerosol with a sublethal dose of Parainfluenza 1 (Sendai) virus by previously described methods (5).

For bacterial challenge, 3 liters of tryptic soy broth (TSB) was inoculated with *Staphylococcus aureus* (coagulase-positive FDA strain 209P, phage type 42D) and cultured at 37°C for 18 h. The bacteria were sedimented by centrifugation and resuspended in 20 ml of tryptic soy broth.

7 days after virus infection, groups of uninfected and virus-infected mice were challenged with the bacteria. The nebulizer delivered concentrated and finely dispersed particles, of which more than 95% were less than 3.5 μm in size (5). Immediately after bacterial challenge (0 h) and

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at 6, 12, and 24 h thereafter, groups of 16 virus-infected and 16-uninfected mice were sacrificed by intraperitoneal injection of sodium pentabarbital. Half the mice in each group were used for quantitation of viable staphylococci in the lungs, while the other half were used to determine the anatomic location of staphylococci.

Bactericidal activity. Lungs of sacrificed mice were removed aseptically by transection of the mainstem bronchi, washed with sterile phosphate-buffered saline, and checked carefully for surface lesions. Lungs were homogenized in 3 ml of tryptic soy broth and cultured quantitatively in quadruplicate on tryptic soy agar by the standard pour plate technique in Petri X-plates.

Pulmonary bactericidal activity in each individual animal sacrificed at any time was calculated by the following formula (8): Percent bacteria remaining = bacterial count (lung)/mean bacterial count at 0 h.

The 0-h mean bacterial count of control animals and of virus-infected animals was used to calculate the percent bacteria remaining in uninfected and virus-infected mice, respectively.

Bacterial localization. The lungs of mice were perfused *in situ* with 10% buffered formalin via an intratracheal cannula. The fixed lungs were embedded in paraffin and sectioned at a thickness of 4–5 μm. The sections were stained with Brown and Brenn tissue stain for bacteria (9). This stain is a modification of the Gram stain. Both viable and nonviable staphylococci fix the crystal violet and stain blue. After staining, the sections were scanned for staphylococci at 1,000 × magnification with a Zeiss microscope (Carl Zeiss, Inc., New York). The intra- or extracellular location of 100 consecutive bacteria was determined for each lung.

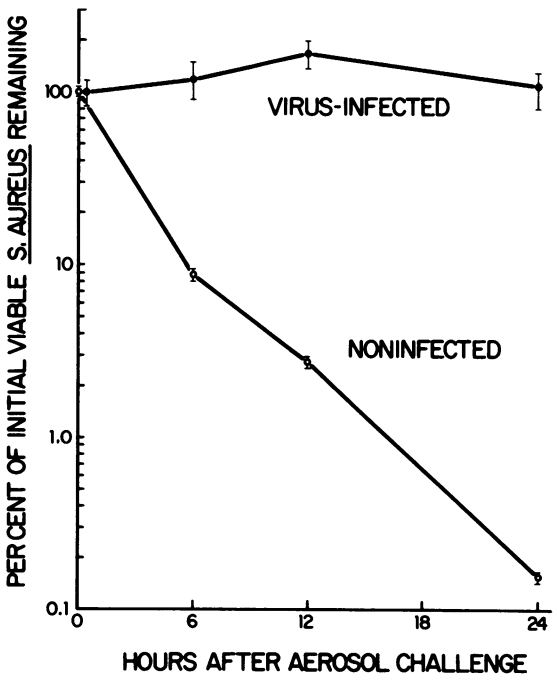

FIGURE 1 Intrapulmonary killing of *S. aureus* in Sendai virus-infected and uninfected mice.

TABLE I

Location of Inhaled Staphylococci within the Lungs of Uninfected and Sendai Virus-Infected Mice Sacrificed Immediately after Bacterial Challenge

	Intracellular bacteria per macrophage								Extracellular bacteria	Intracellular bacteria
	1	2	3	4	5	6	7	>8		
									%	%
Control animal										
1	21	6	4	0	1	0	0	0	50	50
2	29	7	0	3	0	0	1	0	38	62
3	23	4	2	1	0	0	0	0	59	41
4	14	12	2	0	1	0	0	0	51	49
5	23	8	2	0	0	0	0	0	55	45
6	25	8	4	1	0	0	0	0	43	57
Av	22.5	7.5	1.8	0.8	0.3	—	0.2	—	49±3*	51±3
Intracellular bacteria, %	45.9	30.6	11.0	6.3	3.1	—	2.8	—		
Virus-infected										
1	43	9	0	0	0	0	0	0	39	61
2	27	7	2	0	0	0	0	0	53	47
3	18	7	2	2	1	0	0	0	49	51
4	38	3	1	1	1	0	0	0	44	56
5	17	6	0	1	0	1	1	0	54	46
6	33	2	1	0	1	0	0	0	55	45
7	26	2	2	2	0	0	0	0	56	44
8	24	3	0	2	0	0	0	0	62	38
Av	28.3	4.9	1.0	1.0	0.4	0.2	0.2	—	51±3	49±3
Intracellular bacteria, %	56.9	19.7	6.0	8.0	4.0	2.4	2.8	—		

* Mean±SE.

Sections of virus-infected lungs immediately adjacent to those used for bacterial localization were also stained with hematoxylin and eosin.

RESULTS

More than 3×10^{10} bacterial cells/ml were cultured from the aerosol nebulizer. During nebulization this concentration of bacteria produced an infectious cloud in the aerosol exposure chamber so that $8.8 \pm 0.5 \times 10^5$ and $9.4 \pm 0.5 \times 10^5$ viable staphylococci were recovered at 0 h from the lungs of virus-infected and uninfected mice, respectively.

Fig. 1 contains pulmonary bactericidal values in virus-infected and uninfected murine lungs at 0, 6, 12, and 24 h after bacterial challenge. A rapid decline in viable staphylococci was observed in the lungs of uninfected animals, as compared with relative statsis of the bacterial population at 0-h levels in the lungs of mice infected with Sendai virus 7 days previously. Since quantitation of viable bacteria and studies on bacterial localization were performed on the lungs of separate groups of mice that were both infected with virus and challenged with *S. aureus* simultaneously, these data are presumed to apply to animals used for the histologic studies as well.

At 0 h, each microscopic field at 1,000 × magnification usually contained numerous bacteria, so that the identification of 100 bacteria required in most instances the scanning of only one section per lung. The bluish-staining staphylococci were easily identified and their numbers as well as their location were readily apparent. Because the cell cytoplasm stained yellow and the cell nucleus stained pink, the bacteria were not easily confused with cellular components. Occasionally the bacteria could not be clearly delineated as being inside or outside of a macrophage. In these instances the bacteria were excluded from the numerical count.

Table I contains data on the number of intracellular and extracellular staphylococci at 0 h in the lungs of virus-infected and uninfected animals. Inspection of the tabulated data shows that intracellular bacteria were ob-

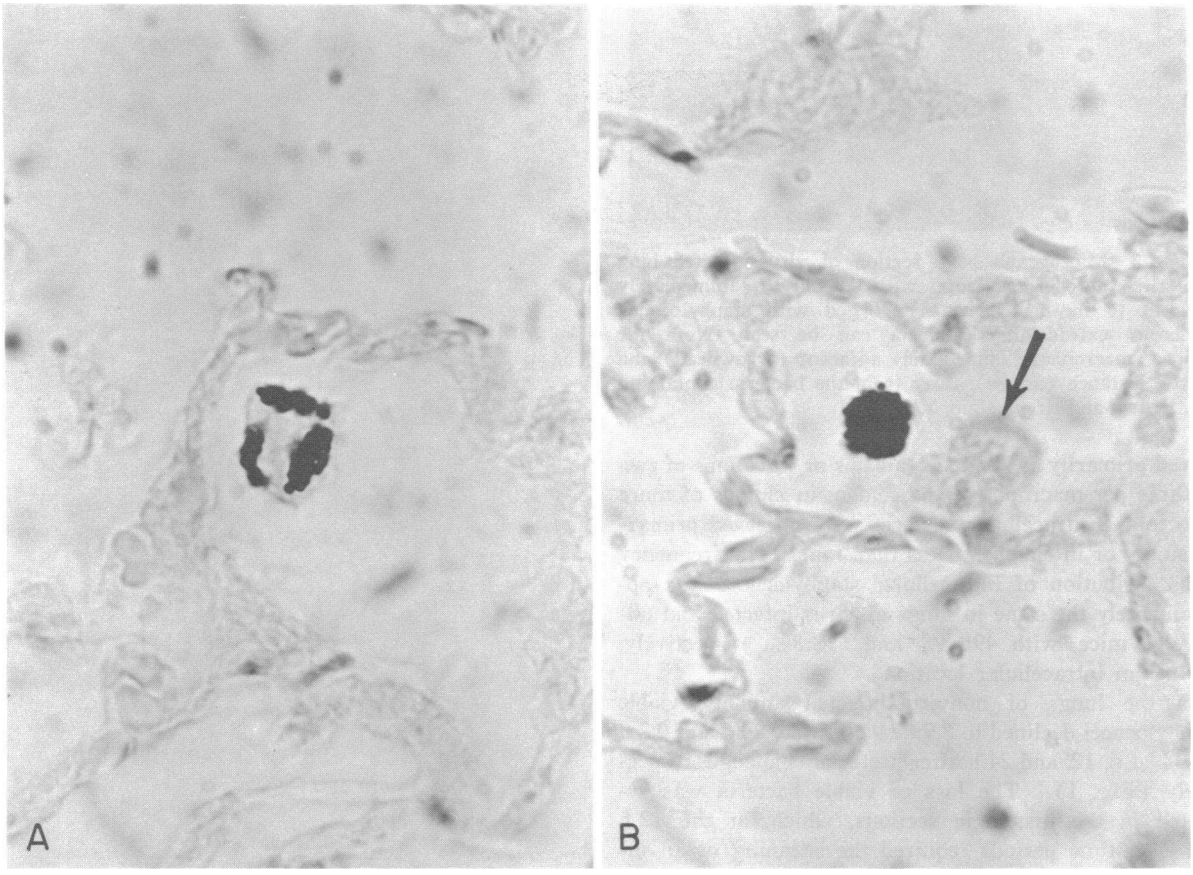

FIGURE 2 Photomicrographs of sections of virus-infected lungs 6 h after aerosol challenge with *S. aureus*. Numerous clumps of intracellular staphylococci (A) and macrophages engorged with bacteria (B) were observed. Note the macrophage containing no bacteria (B, arrow) immediately adjacent to the bacteria-laden macrophage. Brown and Brenn stain, × 1,000.

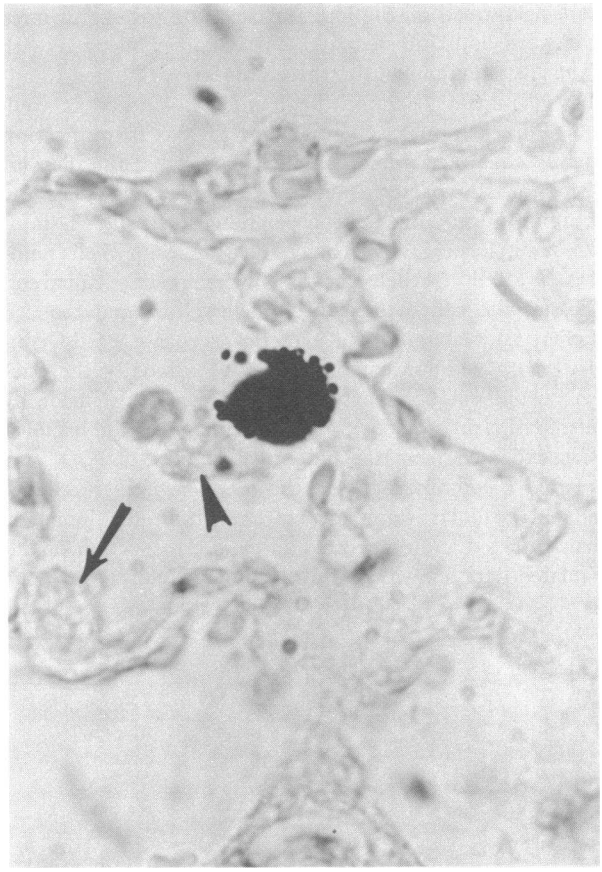

FIGURE 3 Micrograph of a section of virus-infected lung 6 h after aerosol challenge with *S. aureus*. Immediately adjacent to the macrophage engorged with staphylococci, numerous extracellular bacteria can be seen. Note the "empty" macrophage immediately adjacent (arrowhead) and a little distance (arrow) away from the bacteria containing cell. Brown and Brenn stain, × 1,000.

served primarily as single organisms or in clumps of two or three per macrophage and seldom in clumps of more than four. Extracellular bacteria were observed primarily singly or in clumps of less than four. The frequency and distribution of intracellular staphylococci was approximately the same in lungs of virus infected and uninfected mice, with 49±3% and 51±3%, respectively, having an intracellular location.

In the lungs of nonvirus-infected animals, viable staphylococci declined to 8.9±2.9%, 2.3±0.8%, and 0.2± 0.01% at 6, 12, and 24 h after bacterial challenge, respectively (Fig. 1). The loss of viable bacteria was reflected in the histologic sections, which, at the 12-h and 24-h time periods required the scanning of an increasing number of similar sections to quantitate the location of 100 bacteria. The proportion of intracellular staphylococci increased from 51±3% at 0 h to 82±3%, 96±2%, and 98±0.5% at 6, 12, and 24 h respectively. The concentration of intracellular staphylococci per cell at these later time periods remained approximately the same as that observed at 0 h, that is, mostly as single organisms or in small cluster of less than four.

In virus-infected lungs, an ever-increasing number of staphylococci were observed in the histologic sections at the 6, 12, and 24-h time intervals. These organisms, however, were not quantified because the staphylococci occurred primarily in clumps in which the individual organisms were impossible to identify. Instead, each section was scanned in its entirety and the relative proportion of intra- and extracellular staphylococci was noted.

At 6 h after bacterial challenge, intracellular bacteria in virus-infected lung sections were observed primarily in clumps of over 10 bacteria; the actual range was estimated to be 15 to over 50 bacteria per macrophage

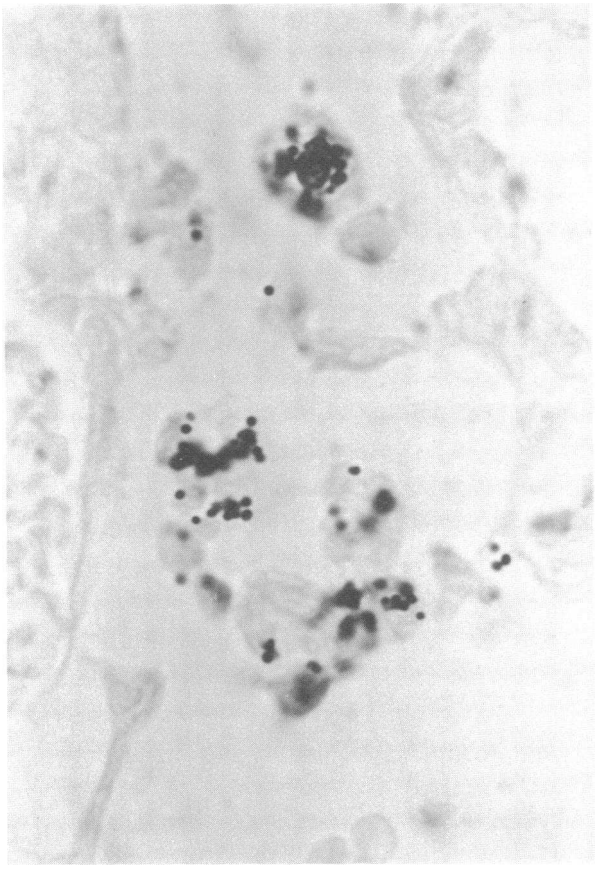

FIGURE 4 Micrograph of a section of virus-infected lung 12 h after aerosol challenge with *S. aureus*. Bacteria containing macrophages can be seen in the two adjacent alveoli. Brown and Brenn stain, × 1,000.

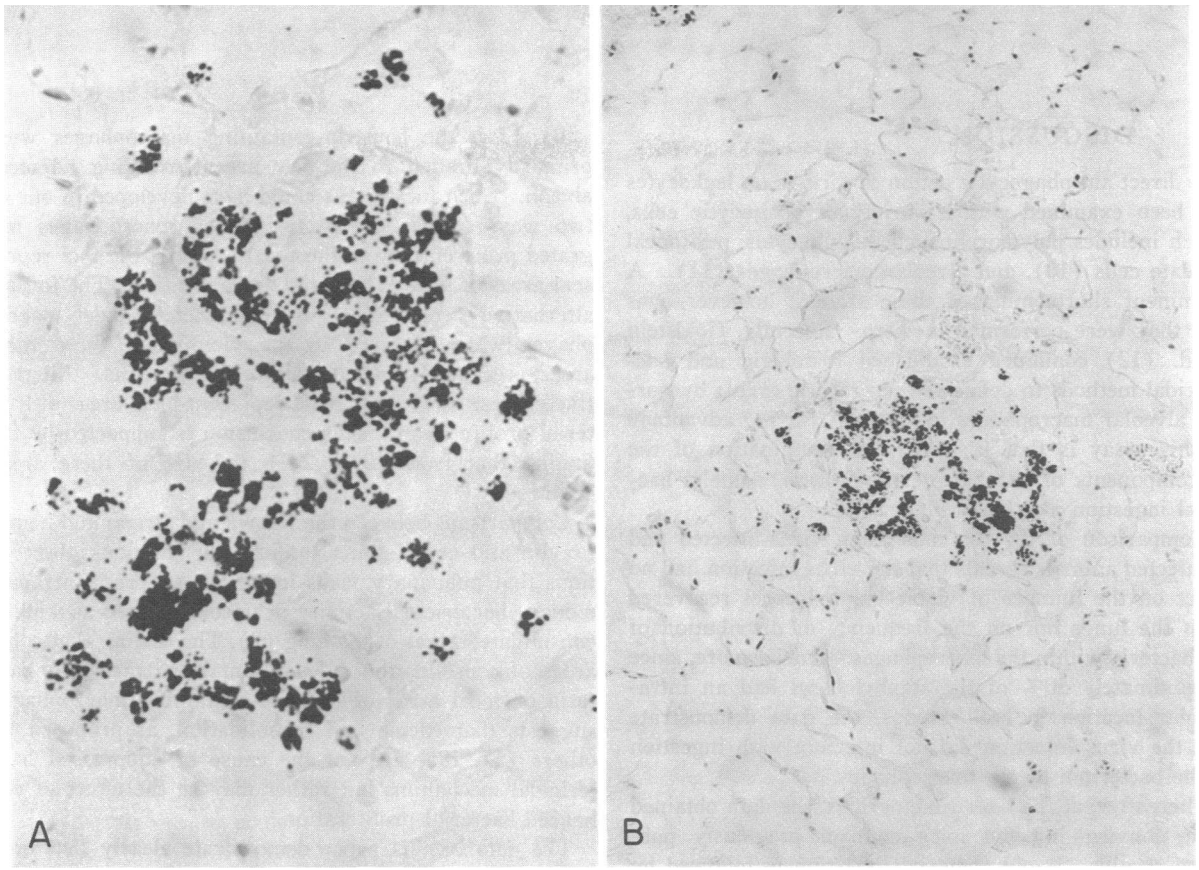

FIGURE 5 Micrographs of a section of virus-infected lung 24 h after aerosol challenge with *S. aureus*. (A) Numerous adjacent alveoli are filled with bacteria-containing macrophages. (B) The same area under lower magnification. Note the focal areas in which bacteria could be observed and the large area of the lung containing no bacteria. Brown and Brenn stain, × 450 and 100, respectively.

(Fig. 2). Such bacteria-laden macrophages were scattered throughout the entire lung section. Extracellular bacteria were observed mainly as single organisms or as occasional small clusters containing an estimated five bacteria. By far, the greater proportion of the observed bacteria had an intracellular location.

Sections of virus-infected lungs of mice sacrified at 12 and 24 h after staphylococcal challenge were also scanned in their entirety. The intracellular location of staphylococci ranged from clusters of less than 10 bacteria to macrophages engorged with an estimated more than 100 bacteria. Occasionally, a few extracellular bacteria were also observed (Fig. 3).

12 h after staphylococcal challenge, sections of virus-infected lungs showed numerous focal areas in which large numbers of intracellular bacteria could be identified. In general, such focal areas contained from one to three adjacent alveoli, each containing numerous bacteria-laden macrophages per alveolus (Fig. 4). The number of such focal areas did not appear to increase by 24 h. In contrast, however, the size of each focal area increased to include as many as 5–10 alveoli (Fig. 5). Also, within each alveolus the number of bacteria-containing macrophages increased to as many as 10 macrophages per alveolus. At both time periods a few extracellular bacteria were also noted within such alveoli, mainly immediately adjacent to the macrophages.

At both 12 and 24 h after staphylococcal challenge, observable bacteria in sections of virus-infected lungs were observed primarily in such focal areas. However, between these focal areas single macrophages engorged with staphylococci were also noted on occasion. Macrophages containing no bacteria were readily observed in the alveolar spaces.

Since areas of consolidation could not be easily discerned on sections of virus-infected lungs stained by the Brown and Brenn method, side-by-side comparisons (with two microscopes) were made on adjacent sections, one stained with Brown and Brenn and the other with hematoxylin and eosine. Such comparisons revealed that at 24 h the focal areas of alveoli filled with bacteria-laden macrophages were not limited to large areas of consolidation. Conversely, areas of consolidation did not necessarily contain such focal areas either, in that an occasional large area of consolidation was identified on the hemotoxylin and eosin section where no focal areas could be found on the corresponding Brown and Brenn section.

DISCUSSION

The direct antiphagocytic action of viruses on leukocytes has been examined with a variety of phagocytic cells, which includes polymorphonuclear leukocytes, peritoneal exudate cells (10), and alveolar macrophages (11). A prominent shortcoming of these studies, however, was that they were performed in vitro. Recently, Goldstein et al. (12) combined established histologic and bactericidal methods to define the phagocytic events by normal alveolar macrophages in vivo. A further advantage of this assay is that it allows the examination of the subcomponents of the phagocytic process, namely, bacterial ingestion and intracellular killing.

Comparison of the 0-h data from virus-infected and uninfected animals reveals that the virus infection had no effect on the number of viable staphylococci recovered from the lungs nor on the frequency of distribution of the bacteria within the macrophages. Furthermore, since approximately 50% of the staphylococci had an intracellular location in both groups, the data demonstrate that the virus infection did not interfere with ingestion of the bacterium by the macrophages.

Thereafter, at the later time periods, the data obtained from nonvirus-infected mice confirm previously published results. Rapid bacterial ingestion is followed by an equally rapid loss of culturable bacteria because of the function of the bactericidal mechanisms of the lung (13). The decline in the number of observable bacteria at the 12- and 24-h time periods is attributed to loss of the staphylococci to staining, as the intracellular digestive processes of the macrophages break down the bacterial cell walls (14–16).

In virus-infected animals an increase in the total number of intracellular bacteria was observed with time. Whether these bacteria had multiplied extracellularly and then were ingested, or whether multiplication followed ingestion, or whether both events occurred cannot be determined with certainty. That extracellular multiplication had occurred in virus-infected lungs is suggested by the 6-h observations. At this time period it was evident that the total number of staphylococci had increased over those observed in the 0-h sections. Furthermore, the 6-h sections also contained extracellular bacteria either as single organisms or as small clusters. The extracellular clustering of the staphylococci and the greater number of total bacteria observed at 6 h indicate a net increase in the number of extracellular organisms. On the other hand, the data also argue for an intracellular site of bacterial multiplication, since bacteria-engorged macrophages were observed immediately adjacent to macrophages containing no bacteria (Fig. 2B and 3). Had extensive extracellular multiplication occurred, one would expect that the ingested bacteria would be more equally distributed among such adjacent macrophages.

By 12 h the bacteria-containing macrophages were primarily limited to the few areas involving adjacent alveoli. Such focal areas could have developed in one of two ways; either the bacteria-laden macrophages migrated preferentially to the focal areas or the foci represent areas of intense bacterial multiplication. The former alternative seems unlikely since no bacteria-laden macrophages were observed in the vicinity of these focal areas, presumably migrating toward such areas. Alternatively, these focal areas must represent local areas of bacterial proliferation. This conclusion is supported by the finding that from 12 to 24 h the size of these areas enlarged.

Comparisons between the Brown and Brenn and hematoxylin and eosin stains confirm our previous observations that pulmonary virus infections suppress intrapulmonary bactericidal activity in unconsolidated as well as consolidated areas of the lung (7). The finding that some areas of consolidation did not contain extracellular bacteria or focal areas of bacteria-containing macrophages suggests that edema and consolidation, as proposed by others (17, 18), are not the cause of suppressed bactericidal mechanims but rather may be the effect of enhanced bacterial proliferation.

The data of this paper demonstrate clearly that host resistance to bacterial infection with *S. aureus* in the murine lung during Sendai virus infection is impaired through a defect in the intracellular processing mechanisms of the alveolar macrophage and not during the ingestion phase of the phagocytic process.

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