Pulmonary Alveolar Macrophage

DEFENDER AGAINST BACTERIAL INFECTION OF THE LUNG

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ABSTRACT The rate of ingestion of inhaled bacteria by pulmonary alveolar macrophages is an important determinant of host defense. This parameter was investigated by infecting rats with finely dispersed aerosols bearing *Staphylococcus aureus* in high concentrations (about 10^8 bacteria/ft^3/min). These aerosols deposited more than 10^6 bacteria/murine lung. At 0, 2½, and 5 h after infection, bacterial clearance rates were measured in the left lung, and the intracellular or extracellular location of 100 bacteria was determined histologically in the right lung (perfused *in situ*). The clearance rates at 2½ and 5 h were 44.5% and 76.9%, respectively. The percentages of intracellular bacteria were: 0 h, 54.8%; 2½ h, 87.1%; 5 h, 91.9%. When rats were exposed for 4 h to 2.5 ppm of ozone (O_3), bacterial clearance did not occur −15.3%, although 78.7% of the bacteria were intracellular. Clumps of more than 10 bacteria—usually intracellular—were also present. These experiments demonstrate that phagocytic ingestion is an exceedingly rapid process, that in this experimental model the inactivation of inhaled staphylococci results almost entirely from phagocytosis, and that ozone-induced reductions in bacterial clearance are due to severe impairment of intrapulmonary killing mechanisms and minor impairment of bacterial ingestion.

INTRODUCTION

Bacterial inactivation by pulmonary defense systems is the principle means for maintaining the sterility of the lung (1, 2). Previous investigations have established the importance of phagocytosis by the alveolar macrophage in this initial defense against inhaled bacteria (1). Since the rate of bacterial ingestion by macrophages has not been determined, the relative significance of the phagocytic component in terms of contribution to the overall defense of the lung against bacterial invasion is unknown. An additional query relates to reductions in bacterial inactivation resulting from underlying abnormalities, like those from exposure to ozone; namely, is there injury to the component processes of phagocytosis, ingestion and intracellular killing?

Because the rate of bacterial ingestion governs the extent of phagocytosis, comparisons of rates of ingestion and inactivation of bacteria should delineate quantitatively the maximum proportion of inhaled bacteria susceptible to destruction by phagocytosis. This report details a method for measuring in vivo the rate of bacterial ingestion by alveolar macrophages. Rats are infected with concentrated and finely dispersed aerosols of staphylococci. Sufficient numbers of bacteria are inhaled to allow statistical analysis of the proportion of intracellularly located bacteria at various time intervals after infection. Comparison of these rates of phagocytic ingestion with simultaneously measured rates of bacterial inactivation allows a quantitative assessment of the importance of phagocytosis to the maintenance of pulmonary sterility.

In a second series of experiments, rats were exposed to ozone after aerosol infection with staphylococci. This noxious gas was chosen because it inhibits pulmonary bacterial inactivation and allows bacterial proliferation without altering mucociliary transport rates (3). Our results suggest that exposure to ozone causes severe defects in the intrapulmonary killing of bacteria and lesser defects in the rate of bacterial ingestion.

METHODS

Animals. Sprague-Dawley rats, free of chronic respiratory disease, weighing 120–150 g, were used in these experiments. The animals were housed two to three per cage and fed food and water ad libitum.

Infection schedules. 2 liters of tryptic soy broth were inoculated with *Staphylococcus aureus* and cultured in a shaker water bath at 37°C for 16 h. The bacteria were sedimented by centrifugation and resuspended in 20 ml of

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TABLE I

Particle Size Distribution of Staphylococcus aureus *in the Infection Chamber*

Andersen sampler stage	Aerodynamic size*	Number of particles‡	% of total
1	>8.3	10.7×10^5	0.04
2	5.0–10.5	4.3×10^5	0.02
3	3.0–6.0	64.3×10^5	0.22
4	2.0–3.5	21.3×10^7	7.79
5	1.0–2.0	22.8×10^8	83.42
6	<1.0	23.2×10^7	8.49

* From Andersen (9).

‡ 20 cubic ft of air.

saline. Approximately 5.0 ml of this suspension was aerosolized into an exposure chamber that permitted quantitative infection of the lungs of rodents (4). The nebulizer (5) delivered concentrated, finely dispersed particles, the majority of which were 1.0–3.0 μm in size. At 0, ½, 1, 2, 2½, 4, and 5 h after infection, groups of five infected rats were sacrificed with ether. The lungs of each rat were exposed aseptically. A ligature was placed securely about the left main stem bronchus and this lung was excised for determination of the numbers of viable staphylococci. The right lung was perfused with 2.5% glutaraldehyde in cacodylate buffer at 10 cm of pressure via an intratracheal cannula; this lung was used to determine the anatomic location of individual staphylococci.

Bacterial clearance rates. Bacterial clearance is the rate at which deposited bacteria are removed from pulmonary tissues or become nonviable within the lungs. Previous studies have shown that in this murine model, the inactivation of bacteria by intrapulmonary defenses is quantitatively much more important than removal by physical transport mechanisms (1).

The left lung was homogenized in a high-speed glass homogenizer containing 3.5 ml of tryptic soy broth. This method disperses the staphylococci and allows their numerical determination by standard pour-plate techniques (4). A mean bacterial count for each group of rats at each time period was calculated and bacterial clearance was expressed as the number of bacteria present immediately after exposure (N_o) minus the number present at t hours (N_t) divided by the initial number of bacteria (N_o)

$$\% \text{ bacterial clearance} = \left[\frac{N_o - N_t}{N_o}\right] \times 100$$

These data were analyzed for significance of difference by the theorem of Wilks (6, 7).

Bacterial localization. The fixed right lung was embedded in paraffin. Sections with an area of approximately 12 mm² and a thickness of 4–5 μm were cut from the medial aspect of the median lobe. These sections were stained with the Brown and Brenn tissue stain for bacteria (8). This stain is a modification of the gram stain. 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 Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N. J.). The intra- or extracellular location of 100 consecutive bacteria was determined for each lung. On occasion, when clumps of 10 or more bacteria were found, it was impossible to determine the exact number of bacterial cells. These groupings were tabulated separately. Wherever possible, the bronchial or alveolar location was noted. The proportion of bacteria that were intracellular was compared for each of the time periods studied. These data were analyzed by the Student t test.

Exposure to ozone. Groups of 15 rats were infected with aerosolized staphylococci, as in the previous experiments. Five of these animals were sacrificed immediately to determine the anatomic location and viability of the inhaled bacteria. Half of the remaining animals were exposed for 4 h in an air pollution chamber to 2.5 ppm of ozone (3). This concentration is much above ambient levels, which range from 0.1 to 0.3 ppm. The ozone was generated from oxygen by silent electrical discharge. The concentration of ozone was determined by microcoulomb ozone sensors attached to a multiple point recorder. Control animals were exposed to identical air flows containing 21% oxygen.

RESULTS

In each of the experiments, more than 10^{10} bacterial cells/ml were cultured from the aerosol nebulizer. Table I shows the particle size distribution of the aerosol for one of the 20-min infection periods. 99% of the bacterial particles were less than 3.5 μm in size, with the greatest percentage of these particles in the respirable range of 1.0–2.0 μm. Because the instrument sampled 1 ft³ air/min (9), and the infection period was 20 min, approximately 10^8 staphylococci of respirable size were present in each cubic foot of infected air.

Each microscopic section usually contained 5 or more bacteria, and some sections had as many as 40 bacteria. The identification of 100 bacteria required the scanning of similar numbers of sections at each time period studied. The bluish-staining staphylococci were easily identified and their numbers as well as their locations were readily apparent (Figs. 1 and 2). Because the cell nucleus stained pink, it was not easily confused with bacteria. The occasional problem of determining if bacteria lay on or adjacent to a phagocyte rather than within its cytoplasm was generally resolved by viewing the spatial relationship of bacteria and host cell at different focal depths. Unless the bacteria could be clearly delineated as being inside or outside of a macrophage, its location was recorded as indeterminant. Bacteria tabulated in this manner were excluded from the numerical count and statistical evaluation.

Table II contains the data from an illustrative example of the six aerosol experiments in which the rate of bacterial ingestion by pulmonary phagocytes was determined. The frequency and distribution of ingested staphylococci, the numbers of intracellularly, extracellularly, and indeterminantly located staphylococci, and the rate of bacterial ingestion are shown. Inspection of the tabulated data shows that at each time period, there is an inverse relationship between the numbers of ingested bacteria per macrophage and the frequency of

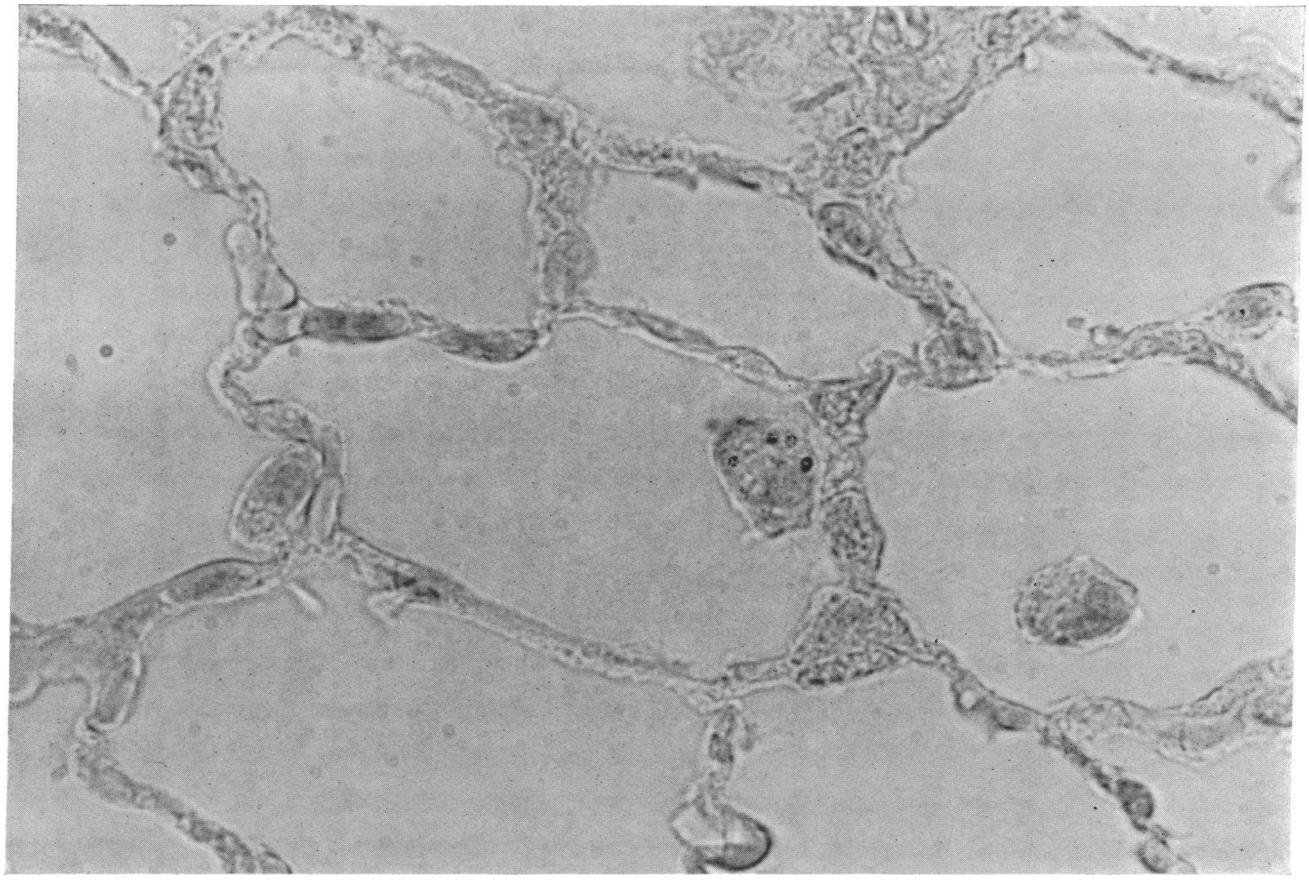

FIGURE 1 An alveolar macrophage from a normal rat with four intracytoplasmic staphylococci. One of the staphylococci is at a slightly different depth. Brown and Brenn stain × 1,000.

macrophages containing this same number of bacteria. The majority of phagocytic cells contained from one to three bacteria. Macrophages with more than 7 ingested bacteria or with uncountable clumps of 10 or more bacteria were uncommon. The intra- or extracellular location of the inspired bacteria was determined in 99% of instances.

Rats sacrificed at 2½ and 5 h after infection almost always had more phagocytes in each of the distribution categories than did rats sacrificed at 0 h. This increase in phagocytic number was most pronounced for phagocytes that contained one, two, or three ingested bacteria. The average numbers of phagocytes within each distribution category were similar for animals sacrificed at 2½ and 5 h after infection.

Because more bacteria were located intracellularly at 2½ and 5 h after infection (88.0% and 93.6%, respectively), the percentage of ingested bacteria for each distribution category is also shown in Table II. According to these data, similar percentages of ingested bacteria were present within each category at each time period. Approximately 25% of ingested bacteria were found singly within macrophages, 25% were present as two intracellularly located microorganisms, and progressively smaller percentages were present as three or more ingested bacteria.

Table III shows the percentage of phagocytic cells with one or more ingested bacteria for all experiments. Although some variation was noted, the percent of phagocytes in each bacteria-to-phagocyte category tended to be similar at each period. One-third to one-half of the phagocytes contained a single staphylococcus. Approximately one quarter of the macrophages had ingested two microorganisms. Progressively smaller percentages of phagocytes had ingested three or more bacteria.

Table IV is a comparison of bacterial ingestion and clearance rates at all periods in the six control experiments. In each experiment, more than 10^{10} staphylococci/ml were present in the nebulizer suspension and more than 10^{7} bacteria/ml were cultured from the 1.0–2.0 μm particle-sizing chamber of the aerosol sampler.

Approximately 10^{6} bacteria were cultured from the left lung of infected rats at the conclusion of exposure to the aerosol (0 h). Progressively fewer bacteria were cul-

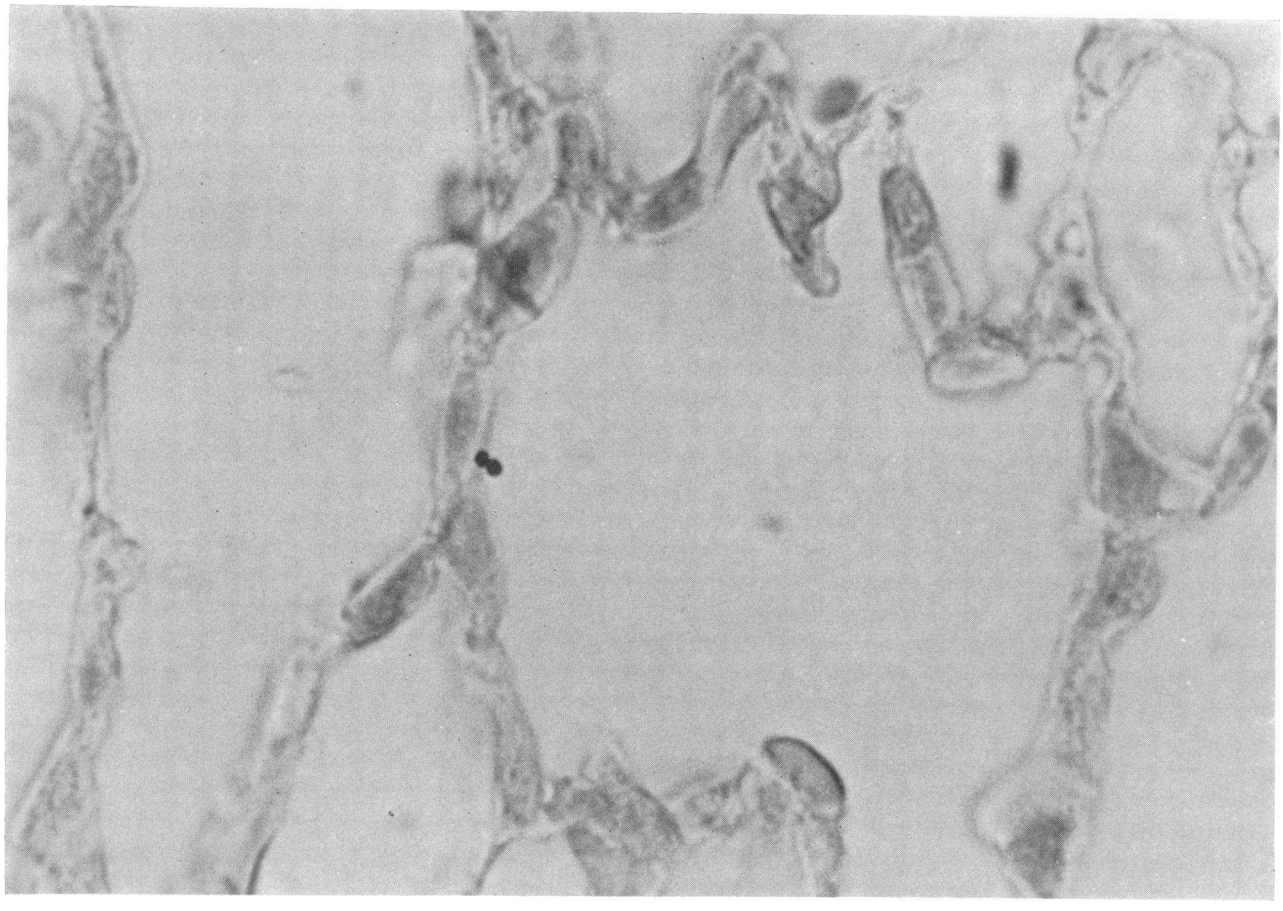

FIGURE 2 Staphylococci that have been inhaled and are located extracellularly within an alveolar space. Brown and Brenn stain × 1,000.

tured at each succeeding period. The bacterial clearance rates for the various experiments were 23.2% at ½ h, 22.5 and 31.6% at 1 h, 59.3 and 29.6% at 2½ h, 69.0 and 55.5% at 4 h, and 87.0 and 66.8% at 5 h. The corresponding rates of bacterial ingestion were 54.9% average value at 0 h, 64.4% at ½ h, 72.4 and 82.0% at 1 h, 88.0 and 86.2% at 2½ h, 95.6 and 93.2% at 4 h and 92.6 and 90.2% at 5 h. In these experiments the percent of indeterminantly located bacteria varied from 2 to 4%. Fig. 3 illustrates the curves of bacterial clearance and ingestion obtained when these data were combined. Inspection of the two curves shows that the rate of bacterial ingestion exceeded the rate of bacterial clearance for each time period.

The data for the experiments in which rats were infected with staphylococci and then exposed for 4 h to 2.5 ppm of ozone are shown in Table V. Exposure to ozone reduced the pulmonary bacterial clearance rate from a control value of 63.6% to a treatment value of −15.3% ($P < 0.001$). A significant but much smaller decrease in the percentage of bacteria located intracellularly was found in ozone-exposed rats (78.7% vs. 94.4%; $P < 0.05$).

Table VI contains pooled data from the two experiments in which the effect of a 4-h exposure to 2.5 ppm of ozone on the staphylococcal ingestion pattern of pulmonary macrophages was determined. Exposure to ozone caused a decrease in the number of macrophages that contained one or two intracellularly located staphylococci. Similar numbers of macrophages were found with three or more ingested staphylococci in the ozone-treated and control groups. In both experimental groups, there was an inverse relationship between the numbers of ingested bacteria per macrophage and the frequency of occurrence of macrophages with this number of bacteria. A statistically significant increase in the number of clumps of uncountable bacteria was observed in the lungs of the ozone-treated animals: 2.5 clumps/test animal as compared to 0.25 clumps/control ($P < 0.01$). These uncountable groupings were almost always intracellular (Figs. 4 and 5).

TABLE II

Location of Inhaled Staphylococci within the Right Lung of Rats at Various Times after Exposure to Bacterial Aerosols

Time	Animal	Number of intracellular bacteria per macrophage										Clumps	Extracellular bacteria	Indeterminant bacteria	Bacterial ingestion
		1	2	3	4	5	6	7	8	9	10				
h															%
0	1	12	10	1	3	0	3	0	1	0	0	0	27	0	73
	2	11	7	1	0	1	3	0	1	0	0	0	41	1	59
	3	19	6	1	0	0	0	0	0	0	0	1	66	0	34
	4	8	4	1	0	2	1	0	0	0	0	0	65	1	35
	5	17	9	4	3	0	0	1	0	0	0	0	34	2	66
	Av.	13.4	7.2	1.6	1.2	0.6	1.4	0.2	0.4			0.2	46.4±8.0*	0.8±0.4	53.4±8.0*
	Bacteria ingested, %	25.1	27.1	9.0	8.8	5.5	15.6	2.6	6.0			—			
2½	6	25	13	7	3	1	1	0	0	0	0	1	5	0	95
	7	28	10	6	2	0	1	1	0	0	0	0	13	1	87
	8	16	7	7	4	0	2	1	0	0	0	0	14	3	86
	9	10	5	2	2	5	1	0	0	1	1	0	16	0	84
	10	19	6	9	2	1	0	1	0	0	1	2	12	2	88
	Av.	19.6	8.2	6.2	2.6	1.4	1.0	0.6	0	0.2	0.4	0.6	12.0±1.9*	1.2±0.6	88.0±1.9*
	Bacteria ingested, %	22.3	18.6	21.1	11.8	8.0	6.8	4.8	0	2.0	4.5	—			
5	11	12	11	4	4	3	0	0	2	0	0	1	7	1	93
	12	21	14	4	1	0	1	1	1	1	0	0	5	1	95
	13	26	14	7	2	2	1	0	0	0	0	0	1	0	99
	14	13	9	10	1	0	3	0	2	0	0	1	1	2	99
	15	47	11	2	0	0	0	1	0	0	0	1	18	1	82
	Av.	23.8	11.8	5.4	1.6	1.0	1.0	0.4	1.0	0.2	0	0.6	6.4±3.1*	1.0±0.3	92.6±3.1*
	Bacteria ingested, %	25.4	25.2	17.3	6.8	5.3	6.4	3.0	8.5	1.9	0	—			

* Mean±SE.

The percent of ingested bacteria present either as single intracellular microorganisms or as groups of two or more microorganisms was similar for the ozone-treated and control rats. The percent of macrophages in each bacteria-to-phagocyte category was also similar for the two experimental groups. Approximately half of the phagocytes had ingested a single bacterium, one-quarter had ingested two bacteria, and lesser percentages of phagocytes had ingested three or more bacteria. Hence, exposure to ozone was associated

TABLE III

Percentage of Macrophages with One or More Ingested Bacteria at Various Periods after Infection with Staphylococcus aureus

Bacteria per macrophage	Time in h						
	0	½	1	2	2½	4	5
				%			
1	54.6	44.9	39.9	33.5	54.0	47.8	55.3
2	25.9	28.6	26.9	20.6	22.0	28.9	24.8
3	9.5	9.5	14.4	15.5	12.7	8.5	9.2
4	4.4	8.8	6.2	10.3	4.7	6.5	3.5
5	2.3	2.0	3.1	5.2	2.2	3.5	2.5
6	1.6	2.7	3.7	8.4	2.4	1.1	1.5
7	1.1	1.4	1.1	1.9	0.7	0.9	0.8
8	0.5	1.4	0.8	2.6	0.0	1.1	1.9
9	0	0.0	0.0	0.6	0.4	0.9	0.2
10	0	0.7	0.6	1.3	0.4	0.2	0.0

TABLE IV

Comparison of Murine Rates for Staphylococcal Clearance (Left Lung) and Ingestion (Right Lung) at Various Times after Exposure to Bacterial Aerosols

Set	Time after exposure*	Bacterial count left lung‡	Bacterial clearance§	Bacterial ingestion§
	h		%	%
1	0	1362±114	—	46.0±8.4
	½	1045±139	23.2±12.0	64.4±5.2
	1	1049±80.5	22.9±8.7	72.4±3.7
2	0	1925±362	—	61.2±6.8
	1	1317±373	31.6±23.3	83.0±3.7
3	0	2072±419	—	56.8±5.5
	4	642±137	69.0±9.0	95.6±1.5
4	0	1392±159	—	54.8±4.5
	4	620±103	55.5±9.0	93.2±0.94
5	0	1722±245	—	53.4±8.0
	2½	701±81.5	59.3±7.5	88.0±1.9
	5	224±30.9	87.0±2.6	93.6±3.1
6	0	743±79.5	—	56.4±5.2
	2½	523±74.4	29.6±12.5	86.2±4.4
	5	247±32.9	66.8±5.7	90.2±4.9

* Five rats were studied at each time period.
‡ Mean±SE × 10^3.
§ Mean±SE.

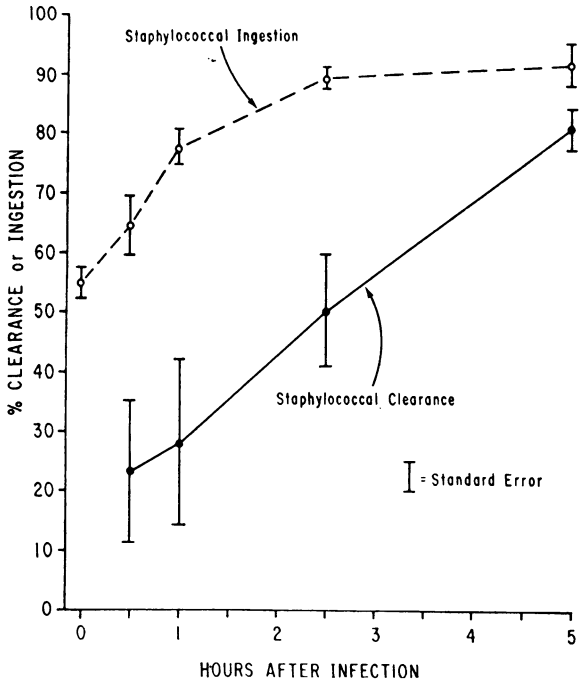

FIGURE 3 Comparison of murine rates for staphylococcal ingestion (right lung) and staphylococcal clearance (left lung) at various times after exposure to bacterial aerosols.

with a decrease in the number of macrophages that contained one or two ingested staphylococci, an increase in the number of uncountable clumps of bacteria, and an otherwise normal distribution pattern of phagocytic activity.

The only histologic abnormality found in the lungs of rats exposed to ozone was hyperemia. The bronchial and alveolar architecture was normal. Neither inflammation nor interstitial edema were present.

DISCUSSION

Before the data from these experiments are evaluated, certain aspects of the histologic technique merit consideration. An intratracheal method of fixation was chosen to prevent the lungs from collapsing. In our experience, the intra- or extracellular location of individual can be assessed most accurately when alveoli are patient. Lungs fixed by immersion have atelectatic areas and it is exceedingly difficult to determine the cellular relationships of bacteria present in the midst of these contiguous tissues.

Although intratracheal perfusion probably changes the anatomic position of bacteria and bacteria-containing phagocytes, the following arguments suggest that these alterations do not significantly affect the microscopically determined ratios of intra- to extracellular bacteria. The cellular status of the bacteria is determined after the infection, and this relationship should be independent of the fixation method. Because very little of the perfusing fluid escapes from around the cannula, it is unlikely that many bacteria are removed from the lungs. The perfusion probably displaced some bacteria and bacteria-containing phagocytes into the more distal alveolar regions. If these displaced cells maintained their ratio of free to ingested bacteria, the microscopic count would be unchanged. If, on the other hand, primarily uningested bacteria were displaced, the microscopic count would show falsely high numbers of extracellular bacteria. Because fewer than 10% of the observed bacteria were extracellular at the later time periods, this kind of error must have been small, if it occurred at all. The reproductibility and small standard errors of the ingestion curve and its similarity to the clearance curve are further evidence that the visualized bacteria truly reflect the intrapulmonary bacterial events.

These experiments demonstrate that phagocytosis by

TABLE V

Effect of a 4-h Exposure to 2.5 ppm of Ozone on the Murine Rates of Pulmonary Clearanee and Phagocytic Ingestion of Staphylococcus aureus

Experimental group	Bacterial clearance*	Bacterial ingestion*‡
	%	%
Control	63.6±6.9	94.4±0.9
Ozone	−15.3±21.1§	78.7±5.2‖

* Mean±SE.

‡ Clumps of uncountable bacteria: controls, 4; ozone 25; $P < 0.01$.

§ $P < 0.01$.

‖ $P < 0.05$.

TABLE VI

*The Effect of a 4-h Exposure to 2.5 ppm of Ozone on the Staphylococcal Ingestion Pattern of Pulmonary Macrophages**

Bacteria per macrophage	Number of macrophages		Ingested bacteria		Phagocytizing macrophages	
	Control	Ozone	Control	Ozone	Control	Ozone
			%		%	
1	21.7	15.0	23.1	19.7	47.8	44.9
2	13.3	8.7	27.7	22.9	28.9	26.0
3	3.9	2.8	12.4	10.7	8.6	8.4
4	3.0	2.6	12.7	13.4	6.6	7.9
5	1.6	2.5	8.5	12.8	3.5	6.0
6	1.0	1.4	3.2	10.8	1.1	4.2
7	0.4	0.0	3.0	0.0	0.9	0.0
8	1.0	0.6	4.3	6.1	1.1	1.8
9	0.4	0.0	3.9	0.0	0.9	0.0
10	0.1	0.3	1.1	3.8	0.2	0.9
Clumps	0.25‡	2.5‡	—	—	—	—

* Each value in the table is the average obtained after pooling the individual data from 10 control and 10 ozone-treated rats.

‡ Comparison of number of clumps: control vs. ozone, $P < 0.01$.

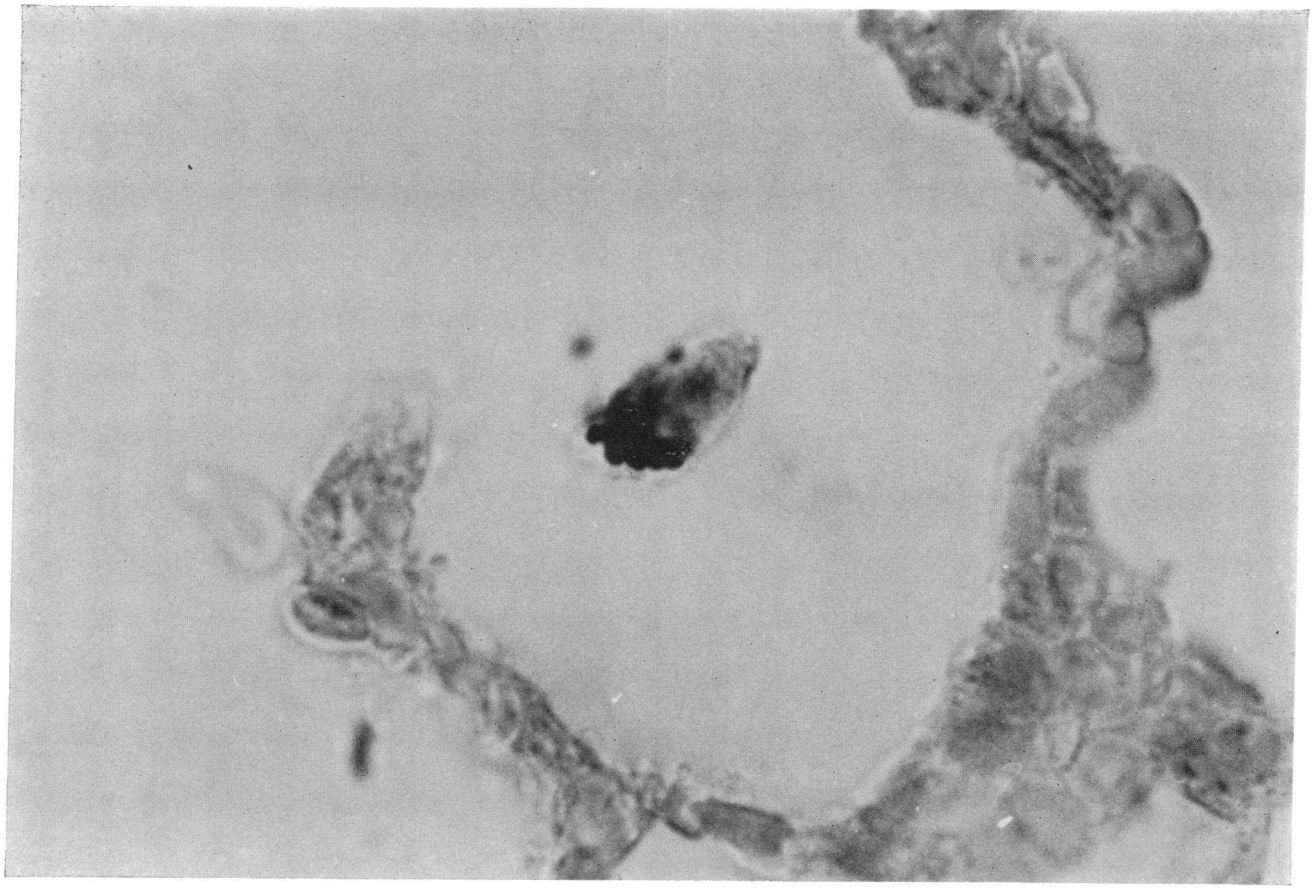

FIGURE 4 A clump containing numerous staphylococci within the cytoplasm of a macrophage from a rat exposed to 2.5 ppm of ozone for 4 h. A single extracellularly located staphylococcus is also present. Brown and Brenn stain × 1,000.

the alveolar macrophage is the principle mechanism by which the murine lung protects itself against inhaled staphylococci. Approximately 50% of inspired bacteria were ingested within minutes after they reached the lungs, 80% were ingested within the 1st h of residence, and 90% were ingested at 2½ h. Because this rate of bacterial ingestion exceeded the rate of intrapulmonary bacterial inactivation, phagocytosis may have entirely accounted for bacterial death.

These studies also indicate that nonphagocytic defense mechanisms were relatively unimportant in the initial defense of the lung against bacterial invasion. Because 50% of inhaled bacteria were present within phagocytes at 0 h, extracellular killing can have been of significance only if: (*a*) dissolution of large numbers of extracellular bacteria had occurred before the microscopic examination at 0 h; or (*b*) the ingested bacteria were rendered nonviable before phagocytosis. These possibilities are unlikely. Previous studies with radiophosphorus-labeled staphylococci have shown that the ratio of viable bacteria to radiophosphorus counts of the infected lung at 0 h is only slightly less than the ratios of the nebulizer suspension (10, 11) or the aerosol (12). These findings prove that most of the inhaled bacteria are alive at the termination of the 20-min period of infection.

Staphylococcal multiplication is probably not an important factor in these studies. Levy and Green have shown that in normal mice, staphylococcal proliferation (birth) is small when compared with staphylococcal death (13). The similarity of bacterial clearance rates for mice and rats suggests that this relationship of staphylococcal birth to death would also be true for rats.

That phagocytosis is the principle mechanism of pulmonary bacterial defense is in accordance with our present knowledge of the macrophage system. Phagocytes exhibit positive chemotaxis towards bacteria (14, 15). They can ingest bacteria in periods as brief as 1 h (16, 17), and they appear to be sufficiently numerous to in-

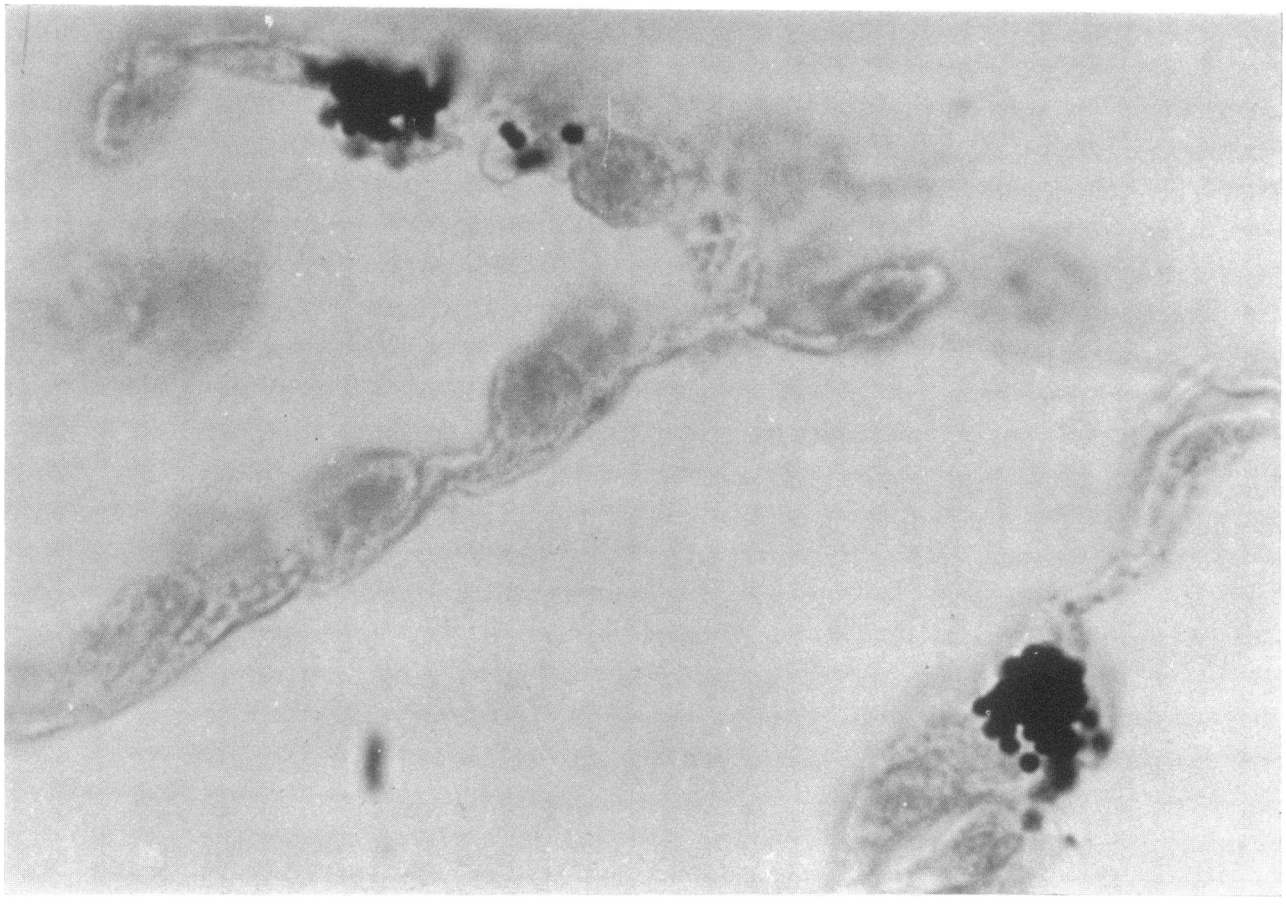

FIGURE 5 Two large clumps of staphylococci that appear to be growing out from unidentified cells of the pulmonary alveolar region. The specimen is from a rat exposed to 2.5 ppm of ozone for 4 h. Three other intracellularly located staphylococci are also present. Brown and Brenn stain × 1,000.

sure the likelihood of proximity to inhaled bacteria. Quantitative studies from lung-lavaged rats have shown that there are more than 5×10^6 free phagocytes/g of lung tissue (18, 19). The actual number of pulmonary macrophages is probably much higher, because a fraction of the cells actually present appear to be removed by washing. Hence, even though 10^6 bacteria were deposited within the lung, this bacterial burden is unlikely to overload the phagocytic system.

The observation that the percent of phagocytes with one or more ingested bacteria remained constant throughout the 5-h experimental period is consistent with random encounters and ingestion in this experimental model. If a phagocyte that had ingested one staphylococcus was more likely to ingest a second staphylococcus than a staphylococcus-free macrophage, there ought to have been an increase in the percent of phagocytes with two or more ingested staphylococci at later periods. Because such increases did not occur, either phagocytes that had ingested a staphylococcus early in the experiment were not prone to engulf additional bacteria, or the distribution of bacteria precluded ready access to additional staphylococci. In either circumstance, bacterial ingestion would be independent of preceding phagocytic events.

The number of ingested staphylococci per macrophage appears to have been determined by the particle size distribution of the bacterial aerosol. 83% of the aerosolized bacterial particles measured 1.0–2.0 μm in diameter, and 99% measured less than 3.5 μm in diameter. Particles of this size are likely to reach the bronchiolar and alveolar regions of the murine lung (20). They are distributed relatively uniformly throughout the lung (21). Because the diameter of a staphylococcus varies from 0.7 to 1.2 μm (22), most of the aerosolized particles probably contained from one to three staphylococci. Hence, the inhaled microorganisms can be expected to deposit diffusely throughout the lung in a pattern that corresponds to the distribution of particle sizes in the

aerosol. The histologic studies confirm this postulated pattern of bacterial distribution. The sections usually contained fewer than 15 widely dispersed bacteria, 85% of which were present as one, two, or three intracellularly located microorganisms. Larger numbers of ingested staphylococci were observed less often; the infrequency of occurrence corresponded to the frequency of bacterial aggregates 3.5 μm and larger. If we assume that there are many more macrophages than inhaled bacteria and that the macrophages are distributed uniformly throughout the lungs, it follows that individual phagocytes will tend to ingest only one staphylococcal group.

The experiments with ozone demonstrated inhibition of intrapulmonary killing of ingested staphylococci; bacterial clearance did not occur despite the ingestion of 70% of the inhaled staphylococci. Because clumps of 10 or more staphylococci were much more prevalent in ozone-treated than in control rats, these microcolonies probably accounted for the differences in bacterial clearance. 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. Since clumps of bacteria were found extracellularly, it seems likely that these microcolonies accounted for some of the additional staphylococci. Either their size or an ozone-induced defect in membrane function may have prevented phagocytic ingestion. Because most of the bacterial clumps were within macrophages (Figs. 4 and 5), and because on occasion these microcolonies extended beyond the cell membrane (Fig. 5), these intracellular bacterial groupings may also have represented multiplying bacteria.

Although of lesser importance, exposure to ozone also decreased the rate of bacterial ingestion. The increase in the number of extracellular staphylococci corresponded to a decrease in the number of phagocytes with one or two ingested bacteria. That is, there may have been an ozone-induced inhibition in phagocytic chemotaxis and/or ingestion. The pathogenesis of this impairment is unclear. Ozone causes edema formation (23), and previous studies suggest that phagocytic mobility is hindered by intra-alveolar fluid (24). Because the histologic assessment of edema is a crude measurement, it is possible that sufficient amounts of undetected edema fluid were present to hinder phagocytosis.

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