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Factors Influencing the Clearance of Bacteria by the Lung *

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Although much quantitative information has been accumulated on the properties of mechanisms of defense against systemic bacterial infection, relatively little parallel information exists for the defensive mechanisms of the respiratory tract. Yet, the bronchi and the pulmonary parenchyma are able to maintain a sterile environment despite virtually continuous exposure to inhaled bacteria.

Lurie, Heppleston, Abramson, and Swartz (1) and Middlebrook (2) adapted aerosol techniques developed by Wells (3) to quantitative studies of experimental pulmonary infection with tubercle bacilli. With similar techniques, Laurenzi, Berman, First, and Kass (4) have defined quantitatively the antibacterial action of the lungs of mice against inhaled staphylococci and have shown that within a few hours most of the retained bacteria can no longer be cultured. The precise mechanisms by which the rapid pulmonary defensive response occurs are not clear. As a first approach toward understanding these mechanisms the effects of certain agents on pulmonary clearance of bacteria were studied.

Because of suggested clinical associations between decreased host resistance and alcoholic intoxication, hypoxia, corticosteroids, and starvation, these agents were selected for study. It would be expected that agents associated with increased susceptibility to infection would impair pulmonary bacterial clearing if, indeed, this action

is important in maintaining host resistance to infection. Since there is much evidence to suggest that the events during the first few hours of the encounter between bacterium and host are decisive in determining the outcome of the infection (5), the early phase of pulmonary bacterial clearance was studied. The results show not only that pulmonary clearance of bacteria is inhibited by the agents studied, but also that the methods provide a sensitive bioassay for evaluating the effects of various agents on host resistance mechanisms.

Methods

The methods used to produce pulmonary infection were essentially those of Laurenzi and associates (4). White Swiss mice of either sex were exposed to a finely divided aerosol of a suspension of *Staphylococcus aureus* (FDA 209P, type 42D) in phosphate buffer. After exposure half of the animals were killed immediately and the remainder 4 hours later, and the lungs were removed and cultured quantitatively. The clearance activity of the lungs was assessed by comparing the total numbers of bacteria present in the lungs immediately after exposure with the total numbers present 4 hours later. This clearance activity was measured first in untreated animals from the colony and then in groups of animals exposed to ethanol intoxication, hypoxia, starvation, or injection of corticosteroid.

The animals were grouped according to the treatment received. In the animals that were intoxicated with ethanol, 3 ml of 5% ethanol in water was injected intraperitoneally 15 to 30 minutes before exposure to the aerosol. Hypoxia was induced in a decompression chamber in which atmospheric pressure was reduced by 380 mm Hg. Ventilation of the chamber was achieved by continuous flow of room air at a rate of 5 to 6 L per minute through the chamber. Steroid-treated animals were injected subcutaneously with 10 mg of cortisone acetate 24 and again 2 hours before exposure to the aerosol, with the intention that only the acute effects of massive doses would be studied. The starved animals were deprived of food, but allowed water at will for 24 to 48 hours before infection. Weight loss during this period ranged from 10 to 30% in the different groups of animals.

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† Postdoctoral fellow of the National Institute of Allergy and Infectious Diseases.

TABLE I

*Retention and clearance of Staphylococcus aureus in mouse lungs in individual experiments**

Experimental group	Run no.	Bacterial count of nebulizer suspension	Bacterial count in lungs		Percentage cleared† 0- to 4-hr count 0-hr count
			At 0 hrs†	At 4 hrs†	
Untreated	1	3.8×10^9	67.1 ± 4.8 [6]	7.89 ± 2.22 [5]	88.1 ± 3.2 [11]
	2	2.1×10^9	32.6 ± 3.4 [5]	4.45 ± 1.10 [5]	86.4 ± 3.6 [10]
	3	3.5×10^9	40.9 ± 4.6 [2]	7.40 ± 1.80 [2]	81.9 ± 4.9 [4]
	4	3.2×10^9	42.8 ± 5.2 [3]	5.25 ± 0.53 [3]	87.7 ± 2.7 [6]
Mean					86.0 ± 1.4
Hypoxia	1	3.0×10^9	61.3 ± 3.6 [2]	24.8 ± 2.2 [2]	59.5 ± 4.3 [4]
	2	5.5×10^9	49.2 ± 6.3 [5]	27.8 ± 2.9 [6]	43.5 ± 9.4 [11]
	3	3.9×10^9	38.4 ± 2.7 [9]	11.8 ± 1.8 [7]	69.3 ± 6.1 [16]
Mean					58.4 ± 7.6
Ethanol	1	3.2×10^9	42.3 ± 2.8 [3]	24.8 ± 10.8 [3]	41.5 ± 26.0 [6]
	2	5.6×10^9	42.4 ± 12.2 [6]	16.5 ± 2.0 [5]	61.1 ± 6.0 [11]
	3	4.1×10^9	35.2 ± 2.6 [4]	19.6 ± 1.3 [5]	44.3 ± 5.5 [9]
	4	3.3×10^9	22.6 ± 14.0 [3]	10.8 ± 3.3 [3]	52.4 ± 33.0 [6]
Mean					49.8 ± 4.7
Cortisone	1	4.5×10^9	66.2 ± 5.6 [5]	12.9 ± 1.0 [6]	80.5 ± 2.3 [11]
	2	4.4×10^9	75.8 ± 8.1 [6]	18.0 ± 2.1 [6]	76.2 ± 3.7 [12]
	3	3.5×10^9	55.7 ± 2.7 [4]	15.8 ± 3.3 [4]	71.6 ± 6.1 [8]
Mean					76.7 ± 2.6
Starvation	1	3.4×10^9	30.5 ± 3.1 [6]	5.1 ± 1.0 [7]	83.3 ± 3.6 [13]
	2	2.3×10^9	31.3 ± 6.0 [4]	12.4 ± 4.1 [3]	60.3 ± 15.0 [7]
	3	2.6×10^9	34.4 ± 3.8 [6]	12.0 ± 2.6 [6]	65.2 ± 8.4 [12]
	4	3.2×10^9	39.4 ± 3.1 [6]	13.0 ± 3.0 [6]	67.0 ± 8.0 [12]
	5	4.5×10^9	47.7 ± 3.1 [6]	11.0 ± 3.2 [6]	77.0 ± 2.6 [12]
Mean					71.4 ± 5.3

* Number in brackets is the number of animals in each calculation.

† Mean \pm SE $\times 10^9$.‡ Mean \pm SE.

Each experiment comprised a single treatment, and the effect of each treatment was evaluated in from three to five experiments. The results were analyzed in two ways. Initially, the clearance rate for each experiment was determined and compared with other experiments using the same treatment and with experiments using no treatment. A mean value of the several experiments for each treatment was then determined. It was found that the variation in the results from day to day was less than the variation among the animals on a given day. Therefore, in the computation of the clearance rates as they are presented in Table II and Figures 1 to 4, the results of all experiments using a given treatment were pooled. The mean bacterial count present immediately after aerosol exposure was related to the mean bacterial count at stated intervals thereafter. The clearance rate was expressed as the ratio of the initial minus the final count to the initial count. The formula for calculation of the standard error of the ratio was derived from theorem 9.3.1a of Wilks (6):

$$SE_R = \sqrt{\frac{1}{\bar{X}_0^2} \left[\frac{S_0^2}{N} \left(\frac{\bar{X}_t}{\bar{X}_0} \right)^2 + \frac{S_t^2}{N_t} \right]},$$

where SE_R = standard error of the ratio, \bar{X}_0 = mean initial count, \bar{X}_t = mean count at time t , S_0^2 = variance of initial count, S_t^2 = variance of count at time t , N_0 = number of animals at initial count, and N_t = number of animals at time t .

The degree of significance of the differences between these mean ratios was then calculated by the t test.

The technical error inherent in the method of quantitative cultures (principally pipetting error) was reduced to a negligible value by obtaining a mean value of quintuplicate plates for each sample. There are two other sources of error inherent in the method: 1) variation within the animal population and 2) variation among experiments. Because the aerosol apparatus had a capacity for only 14 to 16 animals, two to four runs for each treatment group were necessary to obtain adequate data for comparison. Since the mechanical characteristics of the aerosolizing process did not permit a precise duplication of bacterial infection from day to day, there arose a run-to-run variation in the numbers of bacteria initially present in the mouse lungs. Clearance rates, however, were independent of inoculum size within the variation experienced. Animal variation can be attributed both to variation in rates of deposition and retention of bacteria in the lung and to variation in clearance rates. Both sources of error are included in the final expression for variance of the ratios.

Results

The bacterial count of the nebulizer suspensions in each experiment and the mean number of bacteria cultured from the lungs at each time period after exposure to the aerosol are presented in Table I. The number of organisms present im-

mediately after the 30-minute exposure period tended to be directly proportional to the concentration of bacteria in the nebulizer, but this relationship cannot be definitely established over such a small range of nebulizer concentrations. Clearance activity was relatively independent of the loading dose of bacteria within the range of these experiments. The initial number of retained bacteria was significantly greater in the steroid-treated than in the untreated animals and was lower in the intoxicated and starved animals. The reasons for these variations in initial retention of inhaled bacteria are not clear, but may be related to altered patterns of respiration produced by the treatment. The number of organisms remaining at 4 hours is in each case greater in the experimental group than in the untreated. To determine clearance rates, however, the 4-hour figure must be related to the number of bacteria present initially.

The rate of bacterial clearing in untreated animals is shown in Figure 1. This graph shows that removal of bacteria from the lungs follows a logarithmic curve. Sixty-five percent of the initially retained staphylococci were cleared at the end of the first 2 hours, 87% at 4 hours, 95% at 6 hours, and 99% at 24 hours. The same rapidity of clearance and reproducibility of results was seen as was found by Laurenzi and associates (4).

The clearance data for the individual experimental groups are presented in Table I and are summarized according to treatment in Table II. In Table I, the results of each experiment are

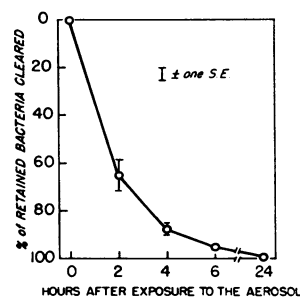


FIG. 1. CLEARANCE OF *Staphylococcus aureus* FROM LUNGS OF UNTREATED MICE. Clearance expresses the percentage of difference between the number of viable organisms cultured immediately after exposure to the aerosol and the number cultured at intervals after that initial measurement. The percentage cleared equals the initial number of cultured bacteria minus the final number divided by the initial number.

TABLE II
Retention and clearance of *Staphylococcus aureus* in mouse lungs: summary of all experiments*

Experimental group	Bacterial count		Percentage cleared† 0- to 4-hr count 0-hr count
	At 0 hrs†	At 4 hrs†	
Untreated	48,500 ± 4,400 [16]	6,200 ± 900 [15]	87.3 ± 2.2 [31]
Hypoxia	44,700 ± 3,100 [16]	19,900 ± 2,500 [15]	55.0 ± 6.8‡ [31]
Ethanol	36,900 ± 5,300 [16]	18,000 ± 2,300 [16]	51.3 ± 9.3‡ [32]
Starvation	34,100 ± 1,900 [22]	10,100 ± 1,400 [22]	70.3 ± 4.3‡ [44]
Cortisone	67,300 ± 4,000 [15]	15,500 ± 1,300 [16]	77.0 ± 2.4‡ [31]

* Number in brackets is the number of animals in each calculation.

† Mean ± SE.

‡ $p < .01$ against untreated animals.

presented separately, and the variance of the clearance ratio represents animal variation in that experiment. The mean clearance figure represents the mean result of the three to five individual clearance figures. The variance of that mean represents run-to-run variation, which is in general smaller than animal variation. The data in Table II were obtained by pooling the several experiments in each treatment category. The data show the effects of the experimental treatments on the proportion of bacteria remaining in a culturable state in the lungs 4 hours after exposure to the bacterial aerosol. These proportions are a measure of the efficiency of the bronchopulmonary mechanisms of defense. Each of the experimental treatments impaired these mechanisms as compared with untreated animals. It is readily apparent that this inhibiting effect of treatment enlarges the variation among the animals, indi-

cating that animals vary in their response to the experimental agents. Although the magnitude of impairment in clearing differs among the agents used, each agent caused a significant ($p < 0.01$) reduction.

Corticosteroid injection had the least effect, lowering the clearance rate by only 10%. When steroid injections were continued for 3 days, there was no enhancement of this effect. No long-term steroid trials were performed.

Acute hypoxia, induced by simulated high altitude, caused a marked depression of bacterial clearance, the rate decreasing to 55% in 4 hours. These animals were generally less active at the lowered barometric pressure, but otherwise appeared to suffer no damaging effect. Additional animals were placed at lowered barometric pressure 24 or 48 hours before infection. Immediately after infection the animals were distributed

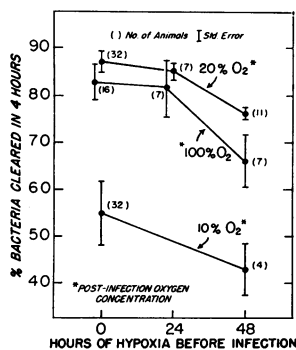


FIG. 2. EFFECT OF PRE-EXPOSURE TO HYPOXIA ON BACTERIAL CLEARANCE.

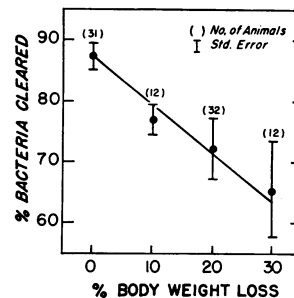


FIG. 3. EFFECT OF ACUTE STARVATION ON BACTERIAL CLEARING. Clearance was measured over the first 4 hours of infection for each group of animals. The starvation period was 24 to 48 hours.

to be at lowered pressure, ambient pressures, or in 100% oxygen at ambient pressure. As shown in Figure 2, 24 hours of hypoxia had no appreciable effect on clearance. There was a decline in clearance at all oxygen levels after 48 hours of hypoxia, but only in animals at ambient pressure after infection was this decline of statistical significance. These animals failed to gain weight as compared with controls, and oxygen failed to influence the observed decline in clearance activity. These observations suggest that the impairment of clearance may have been due to a general deterioration of the animals. The pulmonary clearance mechanisms failed to adapt to hypoxia in that they were as sensitive to the postinfection

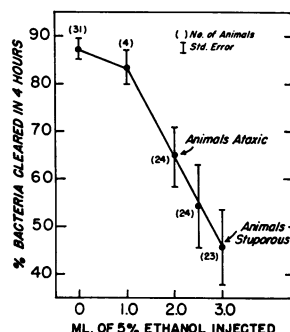


FIG. 4. EFFECT OF DOSE OF ETHANOL ON BACTERIAL CLEARANCE IN THE LUNG. Five per cent ethanol in water was injected intraperitoneally in doses calculated to produce tissue levels and neuromuscular dysfunction comparable to those seen in human intoxication.

hypoxic stress as those of animals that had no previous exposure to simulated high altitude.

In the total group of acutely starved animals, clearance was depressed to 70%. The degree of depression, however, was not uniform in all the groups. As seen in Figure 3, the extent to which starvation slowed clearing appeared to be related in a linear fashion to the percentage of body weight lost during the period of starvation, although all the animals were starved only 24 to 48 hours.

Alcoholic intoxication had the most striking effect on bacterial removal. This inhibiting effect was linear from doses producing ataxia to doses producing stupor (Figure 4). The doses given ranged from 2.3 to 6.9 mg of ethanol, distributed in 22- to 27-g mice. No animals were killed by the ethanol, although in the higher doses altered

TABLE III
Effect of oxygen on ethanol-induced depression of bacterial clearance

Ethanol injected	% Ambient oxygen		
	20	40	100
Bacterial count of lungs*			
ml			
0	87.3 ± 2.2	79.3 ± 7.8	83.0 ± 3.7
2	59.7 ± 14.8	57.6 ± 17.6	60.2 ± 16.6
3	51.3 ± 9.3	51.9 ± 14.0	57.1 ± 33.0

* Mean ± SE × 10³.

patterns of respiration were seen. To determine whether alcohol acted through hypoxia produced by respiratory depression, animals at each dose level were placed in graded concentrations of oxygen after exposure to the bacterial aerosol. Table III shows that even 100% oxygen failed to reverse the effects of alcohol.

Finally, several control studies were performed to show that bacterial numbers do not decline spontaneously in excised infected mouse lungs or in phosphate buffer suspensions of washed bacteria (Figure 5). The staphylococci showed active multiplication in the excised lungs as early as 2 hours after incubation. By contrast, none of the animals at any time developed evidence of active disease, indicating that these organisms were nonpathogenic under the conditions of the experiments. Histologic studies of the clearance phenomenon are described in a separate report (7). In brief, there was no consolidation or exudation in the lungs. Bacteria were phagocytosed by the mononuclear macrophages of the alveoli and distal bronchioles. By fluorescent antibody studies, specific antigenic material of bacterial origin was localized in the macrophages.

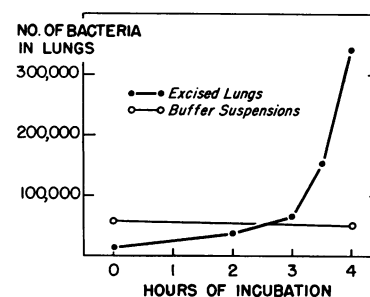


FIG. 5. INCUBATION OF *Staphylococcus aureus* IN EXCISED INFECTED LUNGS AND BUFFER SUSPENSIONS.

Discussion

Of the various methods used for introducing bacteria into the lungs, inhalation of a bacterial aerosol most closely simulates the natural mode of infection by droplet nuclei. Quantitative studies of the interaction between inhaled organisms and the defense mechanisms of the lungs are possible with relatively simple methods. The system proved to be very sensitive in detecting alterations in these defense mechanisms. The use of bacteria that were nonpathogenic for the animals under the conditions of the experiments permitted an analysis of changes in the host defenses without the intervention of the cellular and humoral alterations accompanying disease processes. The mechanisms responsible for the observed phenomena were resident in the normal animal lung and presumably operate in the day-to-day removal of inhaled material. The enormous capacity of these mechanisms is indicated by findings in animals exposed for 4 hours to the aerosol instead of the usual period of 30 minutes. Despite the addition of a calculated 10,000 organisms every 5 minutes, the animals cleared the inhaled organisms at rates similar to those found in controls.

The observation that bacterial numbers did not decline in phosphate buffer suspensions and in incubated excised mouse lungs shows that the decrease in bacterial numbers seen *in vivo* was a property of the intact animal and was not a function of the presence of preformed antibacterial substances or of some mechanical factor such as clumping. The effects of alteration in both host and environment were evaluated for a single species of gram-positive coccus. Studies to be reported indicate that different species have different rates of clearance and different susceptibilities to agents such as alcohol and hypoxia.

The bacterial clearance curve in normal animals is logarithmic in nature resembling, in this respect, the removal of bacteria from the blood stream (8). Blood clearance, however, proceeds at a considerably more rapid rate. Removal of bacteria from the lung, as measured by viability, is more rapid than removal of inhaled inanimate particles. Studies of dust clearance show that the half life is measured in hours (9) to months (10) rather than in minutes as in these bacterial stud-

ies. The distinction is that factors affecting viability may not necessarily affect the physical presence or transport of the bacterial particle. In short, bacteria may be killed in the lung, but their products may remain in a nonviable state with consequences that remain to be defined. Since viability is necessary for multiplication and disease production, it is an essential parameter to measure in bacterial studies and distinguishes problems of infectious diseases from problems of pneumoconioses.

The clinical association between alcoholic intoxication and pneumonia is long established. Numerous experimental studies in animals (11-13) have shown that intoxicated animals have a higher than normal incidence of morbidity and mortality in bacterial infection of the lungs and elsewhere. Nungester and Klepser (12) attributed this susceptibility to the depressed glottal function and increased aspiration of mucus caused by alcoholic intoxication. Interest in the mechanism of alcohol-induced susceptibility is summarized by Pickrell (13). Neither leukocytic phagocytosis (14) nor ciliary action (15) is inhibited by alcohol at tissue levels comparable to those obtained during *in vivo* intoxication. Pickrell (13), however, and later Lushbaugh (16) studied the inflammatory response to intradermally injected pneumococci in mice and in rabbits and found that although phagocytosis per se is not affected by alcoholic intoxication *in vivo*, organisms multiply and overwhelm the host because a depression of capillary permeability prevents exudation of edema fluid and leukocytes into the inflammatory area. This formulation does not explain the mechanism in the lung, since exudation and polymorphonuclear leukocytic infiltration were not seen histologically as part of the bacterial clearance process in normal animals. The studies presented show a dose-related depressant effect of alcohol on the defense mechanisms of the bronchopulmonary tree. The effect occurred with mildly intoxicating doses. It appears from the oxygen studies that this effect is not related to hypoxia induced by hypoventilation.

It is well established experimentally that corticosteroids increase host susceptibility to infection (17). Corticosteroids depress the inflammatory and specific immune responses. The action of corticosteroids on phagocytosis differs with

dose level (18). The massive doses used in these experiments resulted in a minimal but definite slowing of bacterial clearance from the lung. Lurie (19) has shown in rabbits that hydrocortisone brings about diminished killing of inhaled tubercle bacilli by the mononuclear macrophages of the lung and slows removal of the organisms to the lymphatics and lymph nodes.

The inhibitory action of starvation seemed to be roughly proportional to the extent of weight loss. A variety of specific nutritional deficiencies inhibits phagocytosis (20), although Kuna, Blattberg, and Reiman (21) found that acute starvation enhanced that process. Starvation also may reduce mucus secretions in the tracheobronchial tree and thus perhaps the efficacy of the mucociliary stream. Obviously, more extensive studies are needed to work out these interactions more precisely.

The depression of bacterial clearing caused by hypoxia may be of more general significance in the pathogenesis of pulmonary bacterial infection. There are a great number of diseases that are associated both with hypoxia and increased susceptibility to infection. These pathologic conditions that interfere with ventilation of the lung locally or diffusely would be expected to produce the greatest degree of hypoxia in local areas of the lung itself. By contrast, hypoxia caused by defects distal to the alveolus (e.g., alveolar-capillary block) would not lead to depressed clearance so long as ventilation of the lung was preserved. Maintenance of adequate oxygenation appears to be essential not only to the function of distant tissues but also for the function and defense of the lung itself.

Animal studies by Berry and Mitchell (22) and Ehrlich and Mieszkuc (23) demonstrated an increase in susceptibility to peritoneal infection with *Staphylococcus typhimurium* and to respiratory infection with *Klebsiella pneumoniae* in mice exposed to simulated high altitudes of 18,000 and 20,000 feet respectively, but no specific defense mechanism was implicated. In Ehrlich's studies, exposure to high altitude before infection seemed to influence susceptibility more than exposure after infection, although Berry and Mitchell noted a significant increase in susceptibility in animals kept at high altitude after infection as against those kept at atmospheric pressure.

The mechanism by which hypoxia acts to depress clearing activity is uncertain, but presumably hypoxia alters the metabolism of the cells responsible for removing bacteria. Berry (24) noted decreased citric acid levels in animals kept at 18,000 feet and postulated an effect on energy metabolism.

Several observations point toward the alveolar phagocyte as the cell whose activity is impaired by hypoxia. Krueger and Smith (25) found no change in the rate of ciliary beat in the rabbit trachea placed in atmospheres of air, nitrogen, or carbon dioxide, suggesting that the mucociliary stream is not slowed by hypoxia. Oren and co-workers (26), however, have recently demonstrated *in vitro* that the phagocytic activity of alveolar macrophages is markedly depressed by oxygen deprivation and by cyanide. Dannenberg, Walter, and Kapral (27) found these cells to have a high resting level of oxygen uptake, a level little influenced by phagocytic activity. These observations suggest that alveolar macrophages depend on oxidative metabolism for the energy for phagocytic activity and are unsuited metabolically to carry out this function under conditions of reduced oxygen tension. A morphologic basis for the impairment of these cells by the hypoxia of high altitude may be found in the electron microscopic findings of Schulz (28), who has demonstrated mitochondrial swelling and vacuolar transformation in alveolar cells of rat lung subjected to conditions of high altitude.

Summary

1) Intoxication of mice with ethanol depressed significantly the rate at which inhaled staphylococci were cleared by the lungs. The depressant effect was dose related and was seen with doses small enough to produce only ataxia in the animals. The effect appeared unrelated to ethanol-induced respiratory depression.

2) Hypoxia had a similar depressant effect on bacterial clearing. The effect was produced acutely in animals exposed to simulated high altitude immediately after infection. There was no adaptive effect seen in animals kept at high altitude up to 48 hours before infection.

3) Acute starvation caused depression of bacterial clearing apparently related in linear fashion

to the percentage of body weight loss. Corticosteroid injection had a mildly depressant effect.

4) Inhibition of bacterial clearance may render the lung more susceptible to bacterial infection. Inferential evidence suggests that the alveolar macrophage plays an important role in the resistance of the bronchopulmonary tree to bacterial infection.

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