Pulmonary Antibacterial Defenses with Pure Oxygen Breathing[1] (35756)

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(Introduced by E. P. Radford)

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Exposure to oxygen at higher than atmospheric partial pressures produces toxic effects in all higher animals. Animals kept at approximately 1 atm of pure oxygen develop diffuse pulmonary injury. Pulmonary congestion and edema, hyaline membranes, altered enzyme activity, oxidation of sulfhydryl compounds, and reduced pulmonary surfactant have been demonstrated in oxygen poisoned lungs, (1) but the cellular basis of oxygen toxicity remains unclear (2).

Studies with the electron microscope have shown that the early stages of pulmonary oxygen toxicity are associated with damage to the alveolar capillaries followed by destruction of the type I cells of the alveolar epithelium (3). The type II or large alveolar cells, the bronchial epithelium, and the alveolar macrophages are also exposed to the full brunt of inspired oxygen tensions. The large alveolar cells appear to be resistant to the toxic effects of oxygen and, indeed, these cells are capable of regenerating the alveolar epithelium with continued oxygen exposure (3). The bronchiolar epithelium is morphologically altered in the course of chronic high oxygen exposures (4). A reduced rate of tracheal mucus flow has been demonstrated during acute exposure to 40–100% oxygen at 1 atm (5) but later workers have been unable to confirm this observation (6).

Information concerning the activity of pulmonary antibacterial defenses in toxic oxygen atmospheres has not been available previously. Destruction of finely dispersed inhaled bacteria in the lung is primarily a function of alveolar macrophages (7). A variety of factors has been shown to interfere with bactericidal activity in the lung and presumably to increase the susceptibility of the lung to infection (7, 8). The severity of such infection has been shown to increase with exposure to such oxidizing gases as nitrogen dioxide (9), and ozone (10). In view of these findings, and because of the high incidence of pulmonary bacterial infection in patients receiving high oxygen and respirator therapy, we believe it to be important to evaluate pulmonary defense mechanisms during oxygen exposure.

Methods. A slightly modified version of an apparatus designed by Laurenzi and coworkers (11) was used to expose mice to aerosolized bacteria. The production of an aerosol in which over 90% of the particles were under 3.3 μ in diameter was confirmed using an Andersen sampler (Andersen Samplers, Provo, Utah). The organisms used in different experiments were *Staphylococcus aureus* 502A, *Klebsiella pneumoniae*, type 27, obtained from a clinical isolate (kindly typed by Dr. George T. Hermann of the National Communicable Disease Center) and *K. pneumoniae* type A, strain AD (obtained from Dr. James D. Fenters, IIT Research Institute). Before each experiment, bacteria from stock cultures were grown overnight in trypticase soy broth (Baltimore Biological Laboratories) in shaker bath at 37° and washed twice by centrifugation and suspended in phosphate buffered saline, pH 7.3. Colony counts were performed on the final suspension. Specific-pathogen-free CF No. 1 male mice (Carworth Farms), approximately 25 days old and weighing 9 to 15 g, were exposed to bacterial aerosols in groups of 16.

[1] This investigation was supported by NIH Training Grant No. GM-415 from 7/1/69 to 6/30/70 and by NIH Grant No. 5 PO1 ES 00454.

Immediately after 30 min of exposure, the mice were removed from the aerosol apparatus. Randomly selected groups of equal number were placed in cages in one of two identical Plexiglas chambers of 80-liters capacity. One of these chambers was flushed with compressed air and the other with pure oxygen. The pressure in the chambers was 1 atm and the flow of gas through them was maintained at a rate of approximately 4.3 liters/hr/mouse, a flow calculated to keep the CO_2 concentration below 0.1%. Each time the chambers were opened for removal of animals, the chambers were flushed out with several complete changes of the appropriate gas. Occasional determinations with a Corning Model 16 analyzer of the oxygen concentration of gas exhausted from the chambers showed no detectable difference from that of the gas supplied. Temperature and humidity were not regulated but were 22–25° and 30–50% relative humidity, respectively, and did not differ in the two chambers. Several mice from each experimental group were sacrificed at the end of the aerosol exposure to establish the average number of bacteria inhaled. Additional animals were sacrificed at intervals thereafter for up to 24 hr, to determine the number of viable bacteria remaining in the lungs of air and oxygen breathing animals. The mice were killed by intraperitoneal injection of pentobarbital. The lungs were immediately removed under aseptic conditions, separated from the trachea and proximal mainstem bronchi, and homogenized with 5.0 ml of phosphate buffered saline pH 7.3 in glass and Teflon motorized tissue homogenizers. Tenfold serial dilutions of the homogenates were made in buffer and samples in duplicate were mixed with 10 ml of melted Mueller-Hinton agar (BBL) and poured into petri dishes. Colony counts were determined after 24-hr incubation at 37°. The purity of all cultures was confirmed repeatedly using appropriate selective media. The lungs of additional mice from each experiment were immersed in phosphate buffered formalin and prepared for histologic examination. The lungs of numerous uninfected mice were cultured and examined histologically. These lungs were all sterile and showed no histologic evidence of murine pneumonia or other disease.

In quantitative bacterial clearance studies, the mean viable count at any time after infection was expressed as a percentage of the initial count determined in animals sacrificed immediately following the inhalation period. This technique permitted comparison of the results of experiments in which widely differing numbers of bacteria were inhaled. As has been demonstrated previously (11), the clearance rate of nonpathogenic bacteria from the lung did not vary with the inhaled dose, within the range used. Clearance is defined here as the proportion of originally inhaled organisms which have been rendered nonviable in the lungs at any time after inhalation.

In mortality studies with inhalation of virulent bacteria, mice were maintained in the oxygen and air chambers for 24 hr after inhalation of bacteria, then all were removed to the same animal room environment and the number dying was checked at 12 hr intervals for 2 weeks.

To determine the sensitivity of the mice used to factors previously reported to interfere with alveolar macrophage function (7), 4 mice were given an intraperitoneal injection of 1.0 mg/25 g of body weight of 19% ethanol in sterile water immediately after exposure to *S. aureus* 502A and their clearance of these bacteria after 6 hr was compared with that of untreated mice.

In vitro bacterial growth curves were determined in broth equilibrated with gas mixtures chosen to approximate the alveolar gases of animals breathing 100% oxygen and air, respectively. The gas mixtures used were, before humidification, 95% oxygen with 5% CO_2 and 95% air with 5% CO_2. The gases were filtered (style 272R canister, Acme Protection Equipment Co.) and passed through fritted gas washing discs (A. H. Thomas and Co.) into 1-liter graduated cylinders filled with sterile distilled water. The humidified gas was then bubbled through another set of fritted discs at a rate of 8.5 liter/hr into 500-ml graduated cylinders containing 400 ml of nutrient broth (Fisher), 0.2 ml of Antifoam FD-82 (Hodag Chemical Corp., Skokie, Ill.), and an inoculum of the appropriate

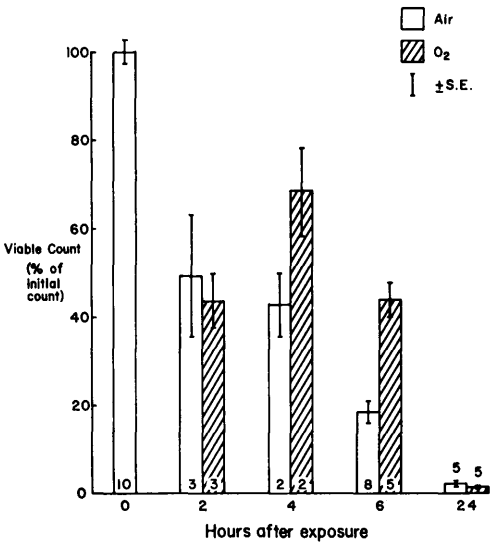

FIG. 1. Effect of pure oxygen breathing on the destruction of *S. aureus* 502A in the lung. Numbers in bars are the number of mice in each group.

bacterial strain. The cylinders were kept in a water bath at 37°. The bacteria used were *S. aureus* 502 A and *K. pneumoniae* type 27. Growth rates were determined through the logarithmic growth phase using viable counts made by the tube dilution and pour plate method. All counts were performed in duplicate and the growth curve for each strain and gas mixture was determined three times.

Results. S. aureus 502A was killed rapidly following inhalation by mice (Fig. 1). Only 1–2% of the originally inhaled dose (Table I) remained viable in the lungs of normal animals 24 hr after exposure. Inhibition of clearance in oxygen was observed at 4 and 6 hr following exposure to bacteria. At 6 hr, 18.6 ± 2.4% of the original dose could be recovered from the lungs of air breathing mice, as compared to 44.0 ± 3.7% recovered in oxygen breathing animals. This difference is significant, with $p<.01$. By 24 hr, all animals had cleared over 96% of the original dose and there were no significant differences between the two groups. For comparison, the proportion remaining in mice given alcohol was 47.2 ± 12.6% at 6 hr, a degree of inhibition of clearance comparable both to that induced by oxygen breathing and to that previously reported with alcohol (7). The lungs of animals examined histologically at various times after exposure to this organism were always completely normal, being indistinguishable from uninfected controls and showing no sign of any inflammatory response.

K. pneumoniae type 27 was also avirulent for these mice. With this organism, the mean viable counts at 6 hr were 70.6 ± 11.3 and 31.5 ± 3.8% of the original dose, for air and oxygen breathing animals, respectively, (Fig. 2). This difference is significant, with $p<.01$. Oxygen breathing thus promotes early inactivation of *K. pneumoniae*. By 24 hr all animals had killed more than 98% of the inhaled bacteria and the air and oxygen groups did not differ significantly. No histologic lesion could be detected in the lungs of animals inhaling this organism.

The mouse-adapted *K. pneumoniae* type A strain AD proved highly pathogenic. An inhaled dose of 1.15×10^3 organisms proved lethal to over 90% of infected mice in 1 week, while as few as 25 organisms were capable of inducing a small incidence of progressive infection. A diffuse bronchopneumonia was induced within 24 hr with adequate doses, and animals dying of the infection had involvement of a large proportion of their pulmonary parenchyma but not of other organs. As has been reported by Ehrlich (9), the clear-

TABLE I. Mean Number of Viable Bacteria Present in the Lungs of Mice Immediately Following Aerosol Exposure.

Each experiment represents a separate run. Means are based on counts from 2–4 mice.

Bacterial Strain	Expt. no.	Mean initial viable count
S. aureus 502A	1	3.38×10^4
	2	1.39×10^5
	3	5.83×10^5
	4	1.51×10^5
K. pneumoniae type 27	1	8.33×10^3
	2	1.52×10^4
	3	1.95×10^5
K. pneumoniae type A strain AD	1	1.78×10^3
	2	43
	3	1.35×10^3
	4	360
	5	25
	6	1.15×10^3

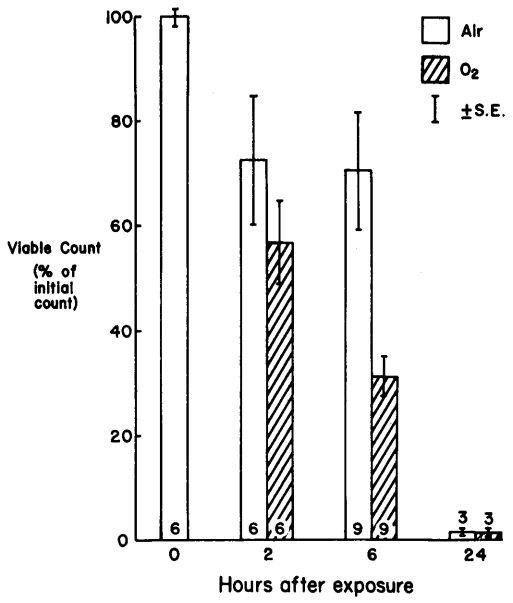

FIG. 2. Effect of pure oxygen breathing on the destruction of *K. pneumoniae* type 27 in the lung.

ance of this organism for several hours after inhalation follows a course similar to that of avirulent bacteria (Fig. 3). At 6 hr, the proportion of bacteria remaining viable in air and oxygen breathing mice was 40.7 ± 11.1 and 15.8 ± 5.2%, respectively. The difference is not significant ($p>0.10$) but the direction of the effect produced by oxygen is the same as was shown with the avirulent *K. pneumoniae* strain. After 6 hr, however, the ability of the host to limit bacterial growth was overwhelmed, and bacterial counts increased rapidly. The variability of bacterial growth rate in individual animals was very high, and continued oxygen exposure for 24 hr produced no statistically demonstrable effect.

Because of this variability in individual response, the time of death was determined in 53 mice which inhaled a mean dose of 1.15 × 10^3 organisms (Fig. 4). Half of this group were kept in pure oxygen for 24 hr following infection. After 5 days, mortality was 86.2% for the mice kept in air and 89.6% for those in oxygen. The remaining mice died of the infection with 2 weeks. The mean survival time of those dying within 5 days was 59.5 ± 3.5 hr in air and 74.8 ± 4.1 hr in animals kept in oxygen for 24 hr. This difference is significant, with $p<.01$. The accelerated early clearance of *K. pneumoniae* by pure oxygen breathing animals thus is associated with a prolonged survival with pneumonia induced by the same bacterial species. It should be noted that all deaths occurred more than 1 day after removal from the oxygen chamber.

Normal mice maintained in pure oxygen for 24 hr showed no consistent increase in the ratio of lung weight:body weight, nor did they have histologic evidence of oxygen toxicity.

In the *in vitro* growth experiments, the logarithmic phase of *S. aureus* was inhibited by high oxygen tension (Fig. 5). The growth rate of *K. pneumoniae* was the same at both oxygen concentrations in all experiments. No effect was observed on the lag phase of growth. Oxygen tensions used were approximately 140 and 680 mm Hg. The results of 3 experiments with each organism did not differ from those shown.

Discussion. These studies demonstrate that pure oxygen breathing produces an inhibition of the early phase of intrapulmonary

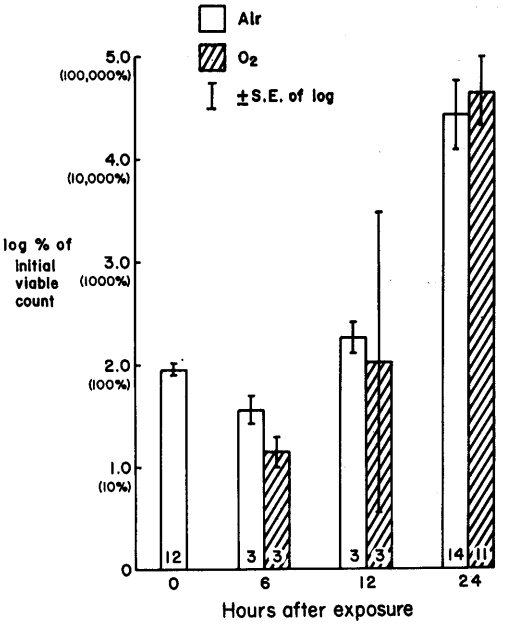

FIG. 3. Effect of pure oxygen breathing on the growth of virulent *K. pneumoniae* type A strain AD in the lung. The number of animals in each group is shown in bars.

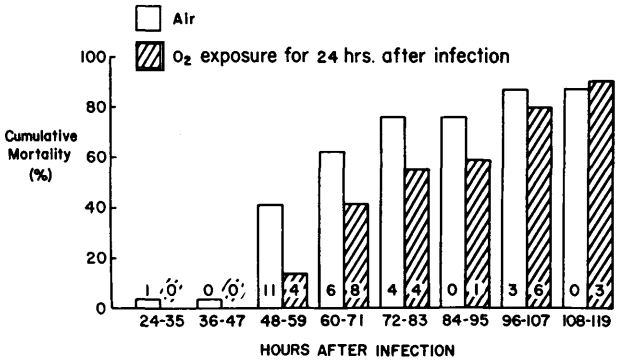

FIG. 4. Cumulative mortality from *K. pneumoniae* type A, strain AD pneumonia in oxygen and air breathing mice. The number of deaths occurring in each 12-hr time period are shown in bars.

destruction of *S. aureus* while clearance of *K. pneumoniae* is promoted. Oxygen also confers protection against the lethal effects of *K. pneumoniae* inhalation, as measured by prolonged survival time following lethal infection. The importance to these effects of altered pH and CO_2 tension has not been studied.

The destruction in the lung of finely dispersed inhaled bacteria has been shown by Kass *et al.* (7) to be due predominantly to the bactericidal activity of alveolar macrophages. A number of factors, including hypoxia, starvation, alcohol administration, cortisone administration, tobacco smoke, viral infection, and renal failure have been shown to inhibit the intrapulmonary clearance of bacteria and, at least by implication, to enhance susceptibility to infection (7). Different clearance rates are seen with different bacteria, reflecting their specific sensitivity to killing by macrophages. In addition, specific adverse influences have been shown to have somewhat disparate effects on the destruction of various bacterial species (7). A paradoxical effect, as has been shown for the early clearance of *S. aureus* and *K. pneumoniae* in pure oxygen atmospheres, has not been demonstrated under any other conditions.

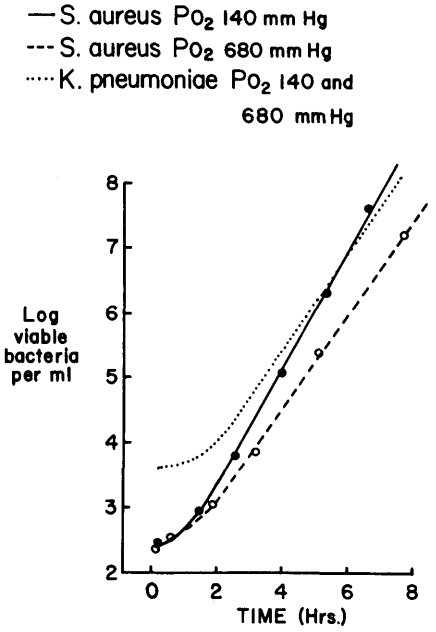

FIG. 5. Effect of oxygen tension on the *in vitro* growth of *S. aureus* and *K. pneumoniae* type 72 in nutrient broth.

The differing effect of oxygen breathing on early intrapulmonary clearance of *S. aureus* and *K. pneumoniae* could be explained by differing mechanisms of destruction of the two organisms. However, previous workers have demonstrated that both gram-positive and gram-negative organisms are subject to destruction by alveolar macrophages (12). The similar overall clearance of *S. aureus* and *K. pneumoniae* shown in the present work also argues for involvement of the same mechanism. The dependence of alveolar macrophages on oxidative metabolism is well known (12). It has been suggested, however, that aerobic mechanisms may be more important for staphylococci than for *Proteus* (7). A similar metabolic explanation for the present results seems reasonable.

The *in vitro* bacterial growth rates obtained do not explain the phenomenon observed *in vivo*. Much previous work has shown that oxygen at hyperbaric pressures has a bacteriostatic effect on aerobic organisms (13). Within the range of oxygen pressures used in the present study, Moore and Williams (14) obtained inhibition of growth of staphylococci and of *P. pestis* and *M. tuberculosis*, but little or no effect on a variety of gram-negative enteric organisms. Other investigators have reported inhibition of staphylococci, streptococci, and a variety of gram-negative bacteria at oxygen pressures approaching 1 atm (13, 15). Reversal of bacterial oxygen toxicity by enrichment of the growth medium has been reported by Gottlieb (16) and Young (17). The latter author has, in addition, shown that oxygen inhibits uptake of nutrients by bacterial cells. It may be that this interaction of oxygen tension and bacterial nutrition, while not observable in the present *in vitro* experiments, did influence bacteria in the intrapulmonary milieu.

Some experimental infections due to aerobic pyogenic bacteria have been suppressed with oxygen at hyperbaric pressures (18), but not at the pressures used in this study. Using intraperitoneal infection with *S. aureus*, Barnwell *et al.* (19) could demonstrate no effect on mortality of mice, with pure oxygen at 1 atm.

It may be postulated that effects of the type demonstrated are of significance in the selection of infecting bacteria in persons receiving oxygen therapy. Infections associated with oxygen and respirator therapy are frequently caused by nosocomial bacteria, and the mechanism by which specific organisms are selected is unclear. Oxygen poisoning is associated with pulmonary edema, a condition known to increase markedly susceptibility to bacterial infection (20). This association has recently been shown in oxygen toxicity as well (21).

These studies show that oxygen breathing does not have a major effect on the intrapulmonary destruction of inhaled bacteria. The practical significance of the transient effects demonstrated is unclear. Similar differences in short-term clearance rates have been considered by others as major determinants of susceptibility (7), and have been associated with depressed mobilization of alveolar macrophages (22).

Summary. After exposure to bacterial aerosols, mice were placed in air and in pure oxygen at 1 atm total pressure. Animals breathing pure oxygen showed an inhibition of early intrapulmonary clearance of *Staphylococcus aureus* and enhanced early clearance of *Klebsiella pneumoniae*. Furthermore, oxygen breathing for 24 hr after infection produced a delay of subsequent mortality due to *K. pneumoniae*.

In vitro bacterial growth studies showed inhibition of growth of *S. aureus* by high oxygen and no effect on the growth of *K. pneumoniae*.

Exposure to pure oxygen for 24 hr does not appear to have any major effect on the ability of the lung to inactivate inhaled bacteria.

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Received Sept. 24, 1970. P.S.E.B.M., 1971. Vol. 137.