Effects of Oxygen Exposure on In Vitro Function of Pulmonary Alveolar Macrophages

SHEILA A. MURPHEY, JEFFREY S. HYAMS, ARON B. FISHER, and
RICHARD K. ROOT

From the Departments of Medicine and Physiology, University of Pennsylvania
School of Medicine, Philadelphia, Pennsylvania 19174

A B S T R A C T Bacterial infection may complicate pulmonary oxygen (O_2) toxicity, and animals exposed to high O_2 concentrations show depressed in vivo pulmonary bacterial inactivation. Therefore, in vitro studies were undertaken to define the mechanism by which O_2 alters pulmonary antibacterial activity. Normal and BCG pretreated rabbits were exposed to 100% O_2 for 24, 48, and 72-h periods. Pulmonary alveolar macrophages (PAM) were obtained from the experimental animals and from nonoxygen exposed controls by bronchopulmonary lavage. O_2 exposure did not alter cell yield or morphology. PAMs were suspended in 10% serum-buffer, and phagocytosis of [^{14}C]Staphylococcus aureus 502A and [^{14}C]Pseudomonas aeruginosa was measured. Comparison of the percent uptake of the ^{14}C-labeled S. aureus after a 60-min incubation period demonstrated that normal PAMs exposed to O_2 for 48 h showed a statistically significant increase in phagocytosis when compared to their controls (43.5 vs. 29.2%). A similar, but smaller, increase was seen after 24-h O_2 exposures. 48 and 72-h O_2 exposures produced no significant changes in phagocytosis in PAMs from BCG-stimulated rabbits. Normal PAMs also showed an increased phagocytosis of Ps. aeruginosa after 48-h oxygen exposures. No impairment of in vitro bactericidal activity against either S. aureus 502A or Ps. aeruginosa could be demonstrated in PAMs from normal rabbits exposed to O_2 for 48 h. These results indicate that the in vitro phagocytic and bactericidal capacity of the rabbit PAM is relatively resistant to the

toxic effects of oxygen, and that impaired in vivo activity may possibly be mediated by effects other than irreversible metabolic damage to these cells. The mechanism for the observed stimulation of phagocytosis remains to be determined.

INTRODUCTION

The critically ill patient requiring mechanical ventilatory support with high concentrations of oxygen is at significant risk for developing pulmonary oxygen toxicity (1). A common clinical impression is that such patients are also more susceptible to pulmonary bacterial infections (2). Many factors may interact to produce such an effect; however, previous investigations (3–5) have suggested that the pulmonary alveolar macrophage (PAM) [1] may be the major determinant of whole lung bactericidal capability. Inasmuch as Huber, La Force, and Mason (6) have reported that exposure of mice to 100% oxygen for periods of 24–72 h impaired in vivo pulmonary bactericidal activity against aerosols of live Staphylococcus aureus, it is possible that direct toxic effects of oxygen on the alveolar macrophages may result in disturbed function and therefore depressed in vivo bactericidal activity in the intact lung.

Very little data is available on in vitro function of PAMs obtained from oxygen-exposed animals. Since such information would assist in defining the consequences of oxygen toxicity at a cellular level, the present investigations were undertaken to determine the effects of prolonged oxygen exposure at high partial pressures on the in vitro phagocytic and bacteri-

[1] Abbreviations used in this paper: FCS, fetal calf serum; KRB, Krebs-Ringer bicarbonate buffer; PAM, pulmonary alveolar macrophages; TSA, trypticase soy agar; TSB, trypticase soy broth.

cidal activity of alveolar macrophages obtained from rabbits by bronchopulmonary lavage (7). This method has been shown to produce large numbers of viable cells in a relatively pure state; moreover, their morphologic and biochemical characteristics have been well defined by previous investigations (7, 8). To examine the effects of such an exposure on both normal and "activated" macrophages, comparative studies were run using PAM from both normal rabbits and from animals previously inoculated with bacille Calmette-Guerin (BCG) (8). The results obtained indicate that PAM phagocytic and bactericidal capacity, measured under optimal in vitro conditions, show no depression after oxygen exposure. In fact, stimulation of in vitro PAM phagocytosis of bacterial particles after a 48-h oxygen exposure can be demonstrated.

METHODS

Animals. Healthy male New Zealand white rabbits weighing between 2 and 3 kg were used in all studies. They were received from the supplier in lots of 6–12 and housed in single cages. Control and experimental animals in a given experiment usually came from the same lot.

To obtain activated alveolar macrophages for study, some of the rabbits were immunized with BCG. Two 1.0 ml i.v. injections of BCG suspended in 0.85% NaCl and containing approximately 20 mg of BCG per dose were given on 2 consecutive days (9). The animals were sacrificed 12–14 days later.

Serum collection. Whole blood was obtained from normal, healthy rabbits by cardiac puncture and allowed to clot. The clot was centrifuged at 3,000 rpm for 20 min, and the serum was removed. Serum was either used fresh on the day of collection or pooled with other normal sera and stored at $-70°C$ until use.

Pseudomonas hyperimmune serum was prepared by a modification of the procedure of Millican and Rust (10). A laboratory strain of *Pseudomonas aeruginosa* (*vide infra*) was grown overnight in trypticase soy broth (TSB). The organisms were sedimented at 2,200 g for 20 min, washed once with modified Hanks' solution (11), and resuspended in 0.85% NaCl. The concentrations were adjusted to approximately 1×10^9 organism/ml by counting in a Petroff-Hauser chamber. Vol of 0.1, 1.0, 2.0, 2.5, and 5.0 ml of heat-killed organisms were injected sequentially into the marginal ear vein of rabbits every 4 days. The rabbits were bled 12 days after the last injection, and the sera were pooled and stored at $-70°$ until use. ^{14}C labeling of both *S. aureus* and *Ps. aeruginosa* was performed by the techniques of Root, Rosenthal, and Balestra (11).

Microorganisms. Laboratory strains of *Staphylococcus aureus* 502A and *Pseudomonas aeruginosa* were used in these studies. All cultures were prepared by overnight growth in TSB at 37°C, centrifuged at 3,000 rpm for 20 min at 4°C, washed once with MHS, and resuspended in Hanks's balanced salt solution. The concentration was adjusted to 5×10^8 organisms per ml, and the suspension was stored at 4°C until use (see above). ^{14}C-labeled *S. aureus* contained 1.5×10^5–4.5×10^5 cpm/ml and *Ps. aeruginosa* 0.8×10^5–2×10^5 cpm/ml.

Oxygen exposure. Normal and BCG-stimulated rabbits were exposed to 100% oxygen in a 250 liter Plexiglas box maintained 5–10 mg above ambient atmospheric pressure to prevent inward leakage of air (12). Oxygen concentrations were monitored by a paramagnetic oxygen analyzer (Beckman model E_2, Beckman Instruments, Inc., Fullerton, Calif.) and carbon dioxide levels by an infrared CO_2 analyzer (Beckman model LB-1). O_2 levels were always greater than 95% of barometric pressure. CO_2 absorbent was added to maintain the P_{CO_2} below 4 mm Hg at all times. The temperature ranged from 25 to 28°C. No direct measurements of humidity were made; however, it was high enough to cause visible condensation on the chamber walls within 48 h. Animals were given water ad lib. but no food. Exposure periods were 24, 48, or 72 h with 2–4 animals exposed at one time. All animals were sacrificed immediately after removal from the chamber by an i.v. air embolus. In most studies control animals were maintained in their cages until sacrifice. Preliminary experiments utilizing control animals placed in the chamber in a room air atmosphere demonstrated that conditions in the chamber itself, apart from 100% O_2, had no effect on survival or on in vitro PAM function.

Technique for obtaining alveolar macrophages. Alveolar macrophages were obtained by the technique of pulmonary lavage as described by Myrvik, Leake, and Fariss (7). Krebs-Ringer bicarbonate buffer (KRB) pH 7.4, containing 5.5 mm glucose and sodium heparin (2u/ml) was used as the lavage medium. The cells obtained by lavage were centrifuged at 1,000 g for 15 min at 4°C, washed once with KRB, and resuspended in KRB–10% heat inactivated fetal calf serum (FCS).

Total cell counts were performed by hemocytometer and differential counts on Wright's stained smears. The percent of viable macrophages in the population was determined by the uptake of 0.1% neutral red dye (13) after a 10-min incubation at 37°C. Morphology was studied with a phase contrast microscope. Cell diameter was estimated with a micrometer eyepiece.

Electron microscopic studies. For electron microscopy, cells from one preparation each of normal control and 24, 48, and 72 h oxygen-exposed animals and from BCG-stimulated control and 48 and 72 h oxygen-exposed animals were fixed for 1 h at 4°C in 3% phosphate buffered gluteraldehyde (pH 7.4, 310 mosmol) with occasional mixing. The cells were centrifuged, rinsed with phosphate buffer, postfixed in 2% osmium tetroxide, dehydrated in ascending grades of alcohol, and embedded in araldite. Thin sections were cut with an LKB ultra microtome (LKB Instruments Inc., Rockville, Md.), stained with uranyl acetate-lead citrate, and examined in a RCA EMU 3-H electron microscope at 50 kV accelerating voltage. Approximately 20–30 cells from each specimen were examined and photographed.

Phagocytic assays. The cell suspensions obtained by pulmonary lavage were adjusted to a concentration of 10×10^6 viable macrophages per ml in KRB-FCS before use. This final suspension was frequently a pool of cells from two or more animals. Phagocytosis of microorganisms was studied by the technique of Root and coworkers (11) using ^{14}C-radiolabeled bacteria. Assays were performed in 12×75 mm glass tubes (RTU disposable culture tubes, Becton-Dickinson & Co., Rutherford, N. J.) containing 5×10^6 macrophages in 10% normal or Pseudomonas hyperimmune serum–KRB. ^{14}C-labeled bacteria were added in a 10:1 bacteria/cell ratio. The final reaction volume was 1.0 ml. The tubes were tumbled end over end (Fisher Roto-Rack, Fisher Scientific Co., Pittsburgh, Pa.) at 37°C. At progressive intervals phagocytosis was halted by the addition of 2.0 ml of cold 0.01 M sodium fluoride (NaF) in KRB-FCS,

and the tubes were then placed in an ice bath. Uningested bacteria were removed by centrifuging the cell pellet at 500 rpm for 5 min at 4°C and washing twice with 4 ml vol of KRB-10% FCS containing 0.01 M NaF. The cell pellet was dried overnight and then digested with 0.5 ml of 0.2 N sodium hydroxide at 56°C for 3 h. The lysate was brought to neutral pH with 0.2 ml of 3% glacial acetic acid, 0.5 ml distilled water was added, and 1.0 ml aliquots were removed to counting vials containing 10.0 ml Aquasol scintillation medium (New England Nuclear, Boston, Mass.) and counted in a Liquid Scintillation Counter (LS-230 Beckman Instruments, Inc.) for sufficient time to assure a counting error of < 1.5%.

The percent uptake by the macrophage pellet of the added radiolabeled bacteria was determined by calculating the ratio of the cell-associated counts to the cell-free standard. All determinations were performed in duplicate, and all experiments were run using paired controls from normal or BCG-stimulated animals not exposed to oxygen. Each experimental and control group usually contained cells pooled from two or more animals.

Effect of increased oxygen tension on phagocytosis. Most of the phagocytic assays were performed in solutions equilibrated with room air. To study the effect of an enriched oxygen atmosphere on the in vitro assay, the lavage returns were gassed with a 95% oxygen–5% CO_2 mixture for 2 min reaching a Po_2 of approximately 700 mm Hg as measured with the use of a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Phagocytic assays were performed in 13 × 75-mm glass tubes with rubber stoppers (Vacutainer, Becton-Dickinson Corp., Rutherford, N. J.) which were gassed with 95% O_2–5% CO_2 for 30 s before the ^{14}C-labeled bacteria were added.

Bactericidal assays. Bactericidal assays were performed by a modification of the technique of Hirsch and Strauss (14) as detailed by Root et al. (11). 5×10^6 macrophages in KRB-10% serum (normal, or Pseudomonas immune for Pseudomonas studies) were added to 12 × 75-mm glass tubes (Becton-Dickinson & Co.). These were warmed for 10 min at 37°C before the addition of 0.1 ml of bacterial suspension providing a final ratio of bacteria to cells of approximately 10:1. The final volume was 1.0 ml. The tubes were rotated end over end at 37°C. Aliquots of 0.01

or 0.001 ml were removed at intervals with calibrated platinum loops and added to 10 ml distilled water. After a 10-min interval to permit cell lysis, 1.0 ml and 0.1 ml vol were added to trypticase soy agar (TSA) pour plates and incubated overnight at 37°C. The number of colonies per plate provide a measurement of the total viable bacterial count. All experiments were performed in duplicate and included cell-free control suspensions treated in the same manner.

A modification of the method of Holmes, Quie, Windhorst, and Good (15) was used to determine the numbers of viable intracellular bacteria remaining. After 30 min of incubation to permit adequate phagocytosis, 10 U of lysostaphin (16) for assays with *S. aureus* or 40 μg of gentamicin for assays with *Ps. aeruginosa* were added to the tubes to kill any extracellular bacteria still present. At intervals, aliquots were removed as described above, and serial dilutions were used to prepare TSA pour plates.

The amount of phagocytosis occurring at 30 min was estimated from the simultaneous uptake of ^{14}C-labeled bacteria (*vide supra*) and was used to determine the total number of intracellular bacteria killed by the cells progressively with time.

RESULTS

Macrophage characteristics. The characteristics of the alveolar macrophages obtained by pulmonary lavage from the various groups of experimental animals are shown in Table I. The mean cell yields from the normal and the BCG-stimulated rabbits are comparable to those in the literature (7, 8, 17–19). PAMs from normal animals exposed to 100% oxygen for varying periods (24–72 h) showed no statistically significant differences in mean cell yield per animal, viability of cells, or morphology from the control animals. However, after 72 h of oxygen exposure, a marked heterophile response was seen in the lavage returns from normal rabbits. Correction for this factor revealed a

TABLE I

Macrophage Characteristics

| Animals		No. experiments	Cell yield ×10⁷*	Viability*	Macrophages*	Size Mean	Size Range
				%	%	μm	μm
Normal control		27	7.89 × 10⁷ ±1.45	94.3±0.55	96.7±0.33	6	5–10
O₂ Exposure	24 h	3	5.58 × 10⁷ ±1.17	93.3±0.33	92.0±1.52		
	48 h	21	8.79 × 10⁷ ±1.35	94.8±0.42	94.4±0.74	6.5	5–10
	72 h	3	4.90 × 10⁷ ±0.80	91.5±1.5	66.5±3.50		
BCG stimulated control		11	32.00±13.1	93.9±1.7	87.5±3.0	10	8–12
O₂ Exposure	48 h	4	26.26	94.7±2.7	95.0±0.5	11	8–12
	72 h	3	30.0	95.3±4.7	91.6±4.1	10	8–12

* Mean±SE.
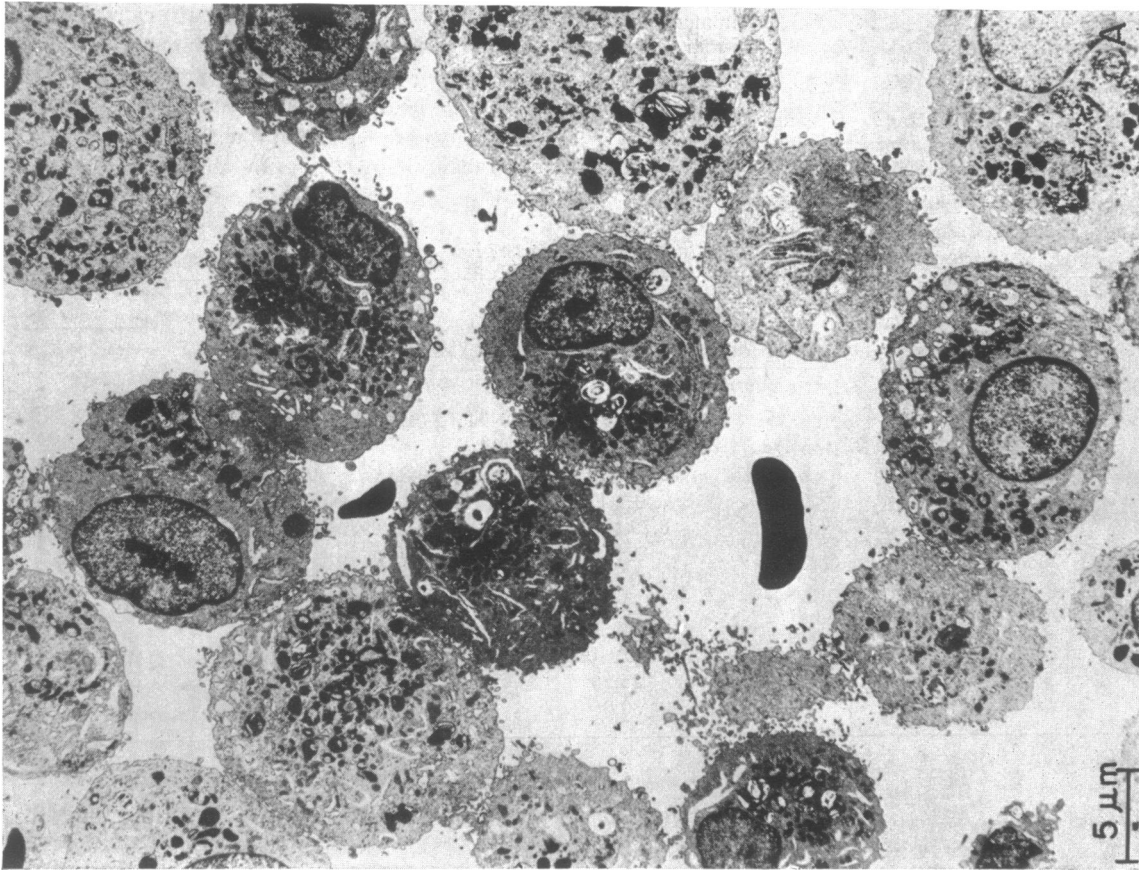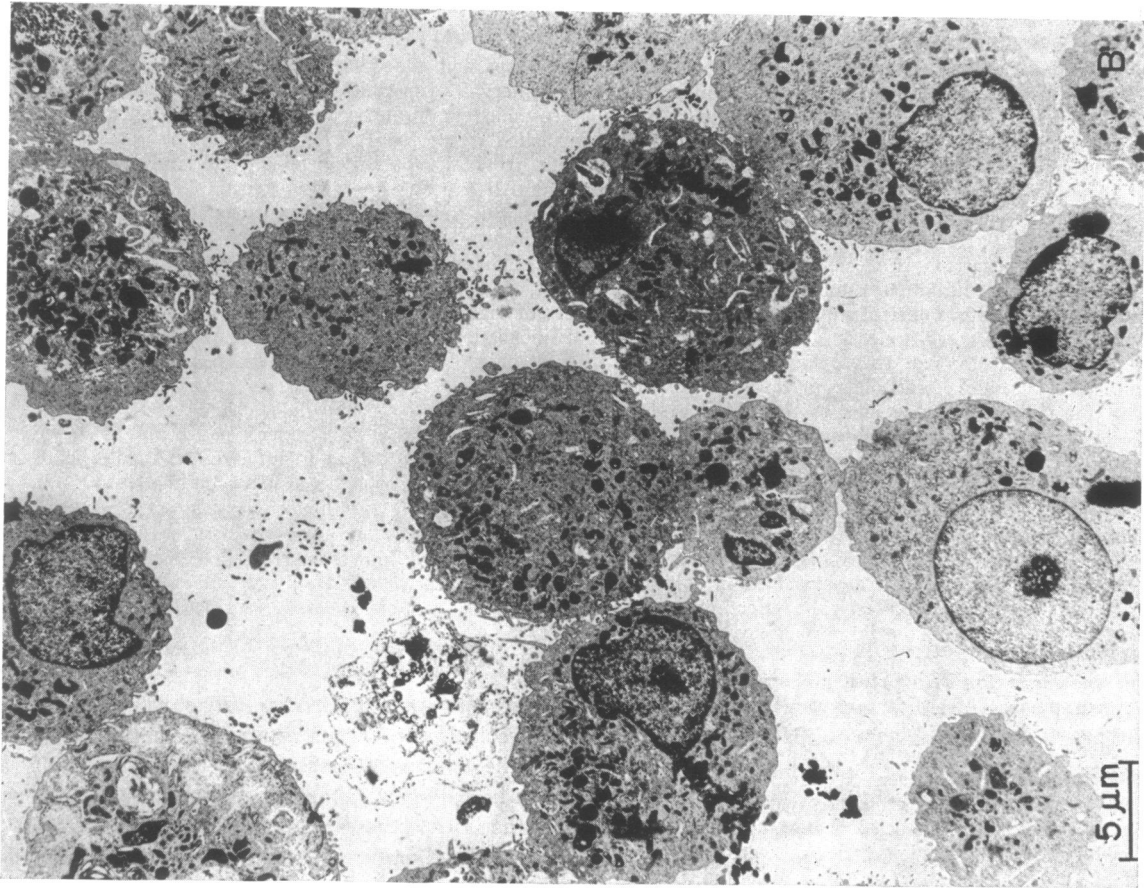
FIGURE 1 Electron photomicrographs of pulmonary alveolar macrophages from normal (A) and BCG-treated (B) rabbits exposed to 100% oxygen for 72 h. Magnification ×2,800.
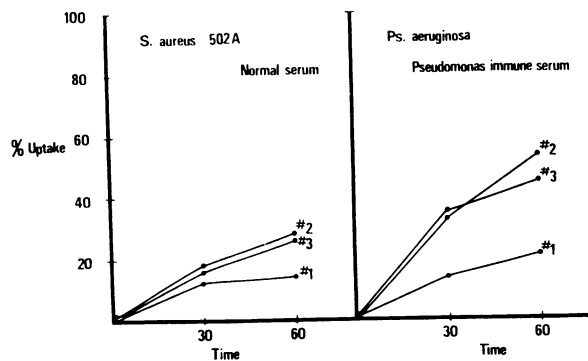
FIGURE 2 Variation in phagocytosis of ¹⁴C-radiolabeled *S. aureus* 502A and *Ps. aeruginosa* by normal pulmonary alveolar macrophages from different animals.

decrease in the number of alveolar macrophages obtainable by lavage at this time period. The mean survival time for adult rabbits exposed to 100% oxygen has been reported to be 76.8 h (20). The mortality rate in our normal experimental animals exposed for 72 h was 50%. All of the survivors were cyanotic and tachypneic; their lungs were edematous and hemorrhagic with focal areas of consolidation. No mortality was observed with 24 and 48-h oxygen exposures; the animals appeared healthy, and no gross pulmonary pathology was observed.

BCG immunization 2 wk before sacrifice produced a 5–10-fold increase in cell yield. As expected from previous reports (8, 12), the macrophages obtained were larger and contained more intracytoplasmic organelles. The total lavage return was somewhat more heterogenous than that from the normal rabbits, containing slightly higher numbers of heterophiles and small mononuclear cells. Oxygen exposures of 48 and 72-h duration produced no significant changes in cell yields, viability, or differential counts. The mortality for BCG-stimulated rabbits exposed to 100% oxygen
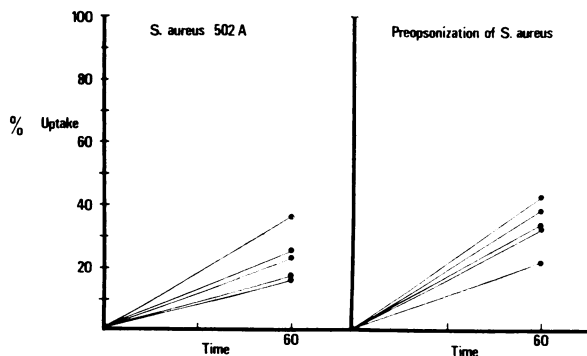

FIGURE 3 Variation in phagocytosis of ¹⁴C-labeled *S. aureus* by a single pool of normal PAMs using different normal rabbit sera as opsonin sources.
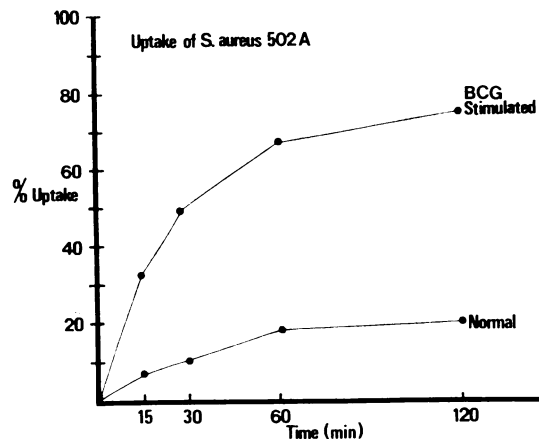

FIGURE 4 Phagocytosis of ¹⁴C-labeled *S. aureus* 502A by normal and by BCG-stimulated PAMs.

was only 20% after 72 h oxygen exposure; the survivors were tachypneic but not visibly cyanotic, and focal hemorrhages and consolidation were not as pronounced as that seen in similarly exposed normal animals.

Electron photomicrographs showed no differences between the control and the oxygen-exposed PAMs at any time interval in either the normal or the BCG-treated rabbits (Fig. 1). Nuclear structure and cytoplasm were studied specifically for vacuolization, abnormal inclusions, or evidence of increased phagocytic activity. Ribosomal morphology appear normal. The mitochondrial cristae and outer membranes were intact with no evidence of swelling. On gross inspection, the number of lysosomes per cell appeared similar in control and oxygen-exposed cells.

Phagocytic studies. In most experiments, the kinetics of phagocytosis were studied using [¹⁴C]*S. aureus* 502A as the particle and normal rabbit serum as the opsonin source. Under the experimental conditions described, the percent uptake of bacteria was almost linear for the first 60 min; beyond this point the rate decreased. A moderate range of variability in the absolute values for phagocytosis was noted in our normal rabbit population. Using the percent uptake of *S. aureus* 502A at 60 min as a reference point, it was possible to demonstrate that variability was due to both humoral and cellular factors. The percent uptake of *S. aureus* by PAMs from three different normal rabbits studied on 1 day using the same serum pool as an opsonin source, ranged between 14 and 28% uptake at 60 min incubation (see Fig. 2). A pool of normal cells studied with five different sera, both fresh and frozen (stored at − 70°C), gave percent uptakes ranging from 16 to 36% at 60 min (see Fig. 3). Prepsonization of the [¹⁴C]*S. aureus* with normal sera for 30 min before use did not decrease the degree of variability (see Fig. 3). Since both cellular and humoral factors appeared to
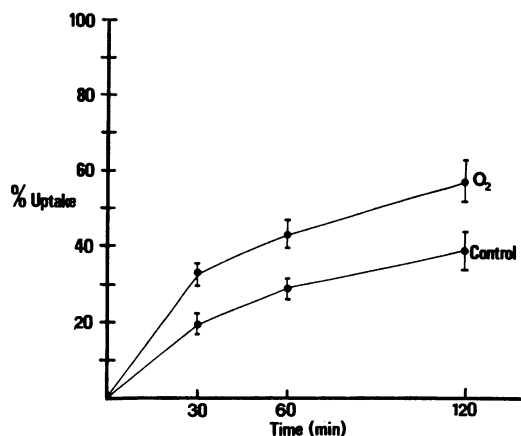
Effects of Oxygen on Pulmonary Alveolar Macrophages 507

FIGURE 5 Phagocytosis of ^{14}C-labeled *S. aureus* 502A by PAMs from normal rabbits and by rabbits exposed to 100% O₂ for 48 h.

FIGURE 7 Phagocytosis of ^{14}C-labeled *S. aureus* 502A by BCG-stimulated PAMs from control rabbits and from rabbits exposed to 100% O₂ for 48- and 72-h periods.

contribute to variability in phagocytosis among "normal" animals, several measures were employed to attempt to reduce this to a minimum. In each experiment, PAMs from control or BCG-immunized animals maintained under atmospheric conditions were studied simultaneously with the oxygen-exposed cells. In over half of the experiments, cells were pooled from two or more animals to obtain sufficient quantities of cells and to reduce the effect of individual variations in macrophage activity. In all experiments the same serum was used as the opsonin source for both control and experimental cells. However, use of a single serum pool over a 3-mo period did not significantly change the amount of variability when compared to a period in which fresh serum from the normal control was used for each experiment.
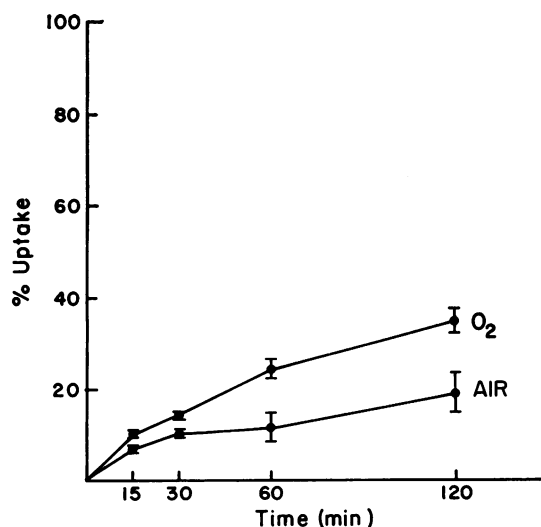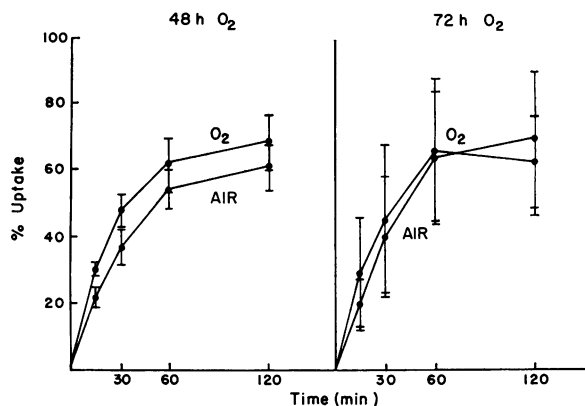

FIGURE 6 Phagocytosis of ^{14}C-labeled *S. aureus* 502A by normal PAMs and by PAMs from rabbits exposed to 100% O₂ for 24 h.

BCG immunization produced a significant increase in the rate and total amount of phagocytosis of *S. aureus* when compared to normal, unstimulated PAMs. A representative experiment is shown in Fig. 4. BCG-stimulated cells, however, showed a daily variability in phagocytosis similar to that observed in normal PAMs.

Effect of oxygen on phagocytosis. The majority of our experiments were performed after oxygen exposures of 48-h duration, a time at which a functional effect could be detected, but at which no gross morphologic changes in the lungs nor alteration in survival by oxygen toxicity could be observed. PAMs from normal rabbits exposed to 100% oxygen for 48 h showed a significant increase in phagocytosis of *S. aureus* when compared to controls ($P < 0.01$ at 30 and 60 min incubation) by the *t* test (21) (see Fig. 5). The increase in phagocytic activity of 48-h oxygen-exposed PAMs was similar to that seen in cells obtained from nonoxygen-exposed animals after BCG immunization. A similar increase in phagocytosis of *S. aureus*, though of lesser magnitude, was seen after 24-h oxygen exposures (see Fig. 6).

Because of the marked heterophile contamination in the cell preparation obtained from normal rabbits after 72-h oxygen exposures, macrophage function could not be properly evaluated. Addition of heterophiles obtained from peripheral blood by dextran sedimentation to normal macrophages in numbers comparable to those in the 72-h lavage preparations showed that the more rapid phagocytic activity of these cells obscured the slower rates of phagocytosis by the PAMs.

Oxygen exposure of BCG-stimulated rabbits of 48 and 72 h duration produced small but not statistically significant increases in in vitro phagocytosis of *S. aureus* when compared to control PAMs (see Fig. 7).

508   S. A. Murphey, J. S. Hyams, A. B. Fisher, and R. K. Root

TABLE II

In Vitro Bactericidal Activity against S. aureus 502A

		24 h O₂ exposure*		48 h O₂ exposure‡	
		Control	O₂	Control	O₂
% phagocytized at 30 min (Mean ±SE)		10.55±0.78	15.41±2.21	18.63±3.78	24.25±5.54
% Intracellular killing (Mean±SE)	40′	81.71±88	84.61±8.48	75.11±8.18	85.44±3.93
	60′	89.51±2.09	91.15±3.95	82.02±6.36	88.89±1.55
	120′	94.00±1.68	90.95±3.44	91.58±3.01	92.73±2.70

* Two experiments.
‡ Four experiments.

The increase in phagocytic activity of PAMs from normal rabbits exposed to 100% O₂ was also demonstrated for *Ps. aeruginosa*. Using 10% hyperimmune serum as the opsonin source, the percent uptake of Pseudomonas was increased in PAMs from normal rabbits exposed to oxygen for 48 h when compared to controls, although statistical significance was not achieved in the small number of studies done (see Fig. 8).

Phagocytosis in an enriched oxygen environment. The above studies were all performed in room air. To

FIGURE 8 Phagocytosis of ¹⁴C-labeled *Ps. aeruginosa* by PAMs from normal rabbits and from rabbits exposed to 100% O₂ for 48 h.

more closely approximate conditions in the intact lung during oxygen exposure, phagocytic assays were performed in a closed system achieving an O₂ saturation of approximately 90%. Both *S. aureus* and *Ps. aeruginosa* were used as particles. No consistent stimulation or depression of phagocytes could be demonstrated in this system in PAMs from other normal control or 48 h oxygen-exposed animals. The increase in phagocytic activity of the PAMs from oxygen-exposed animals when compared to normal controls remained unchanged.

Bactericidal assays. Table II shows the mean intracellular killing of *S. aureus* 502A at 40, 60, and 120 min after ingestion. The macrophages were allowed a 30-min period for phagocytosis, after which lysostaphin was added to remove extracellular bacteria. The percent of intracellular organisms killed were calculated using as the base line the number of organisms phagocytized in 30 min. There was no significant difference in the intracellular killing rates between the control PAMs and those exposed to oxygen for 24 or 48 h.

Killing of *Ps. aeruginosa* by PAMs from rabbits exposed to 100% O₂ for 48 h was studied in the same manner. No differences in in vitro killing between the control and the O₂ exposed cells could be demonstrated.

DISCUSSION

Alveolar macrophages may be obtained from rabbits by pulmonary lavage in quantities sufficient to permit a variety of in vitro studies. As shown by Myrvik et al. (7), these cell preparations are quite viable and relatively free from contamination by other cell types such as erythrocytes, heterophiles, and lymphocytes. BCG immunization, as demonstrated by Myrvik et al. (8), produces a much greater cell yield of "activated" macrophages which are larger, more adherent to glass, and more avidly phagocytic than normal PAMs.

Our "normal" rabbit PAMs demonstrated a moderate degree of individual variation in in vitro phagocytosis which had to be taken into account in evaluating our results. Differences in the opsonic capacity of in-
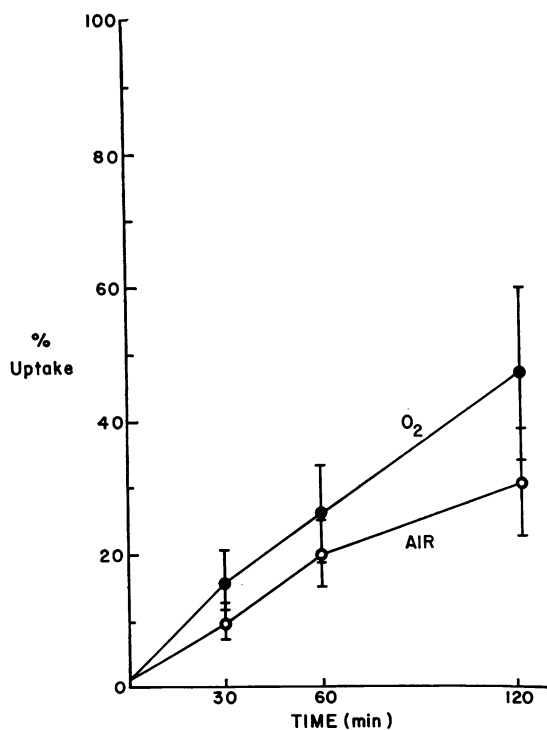
dividual normal rabbit sera were demonstrated and could be eliminated by the use of pooled control sera. Nevertheless, there was an inherent variability in cells from different animals studied under the same conditions which could not be entirely eliminated from the experimental design. BCG "activation" did not remove this individual variation. Accordingly, paired normal or BCG-treated controls housed in room air were used in all experiments to allow comparison with oxygen-exposed animals.

Our in vitro studies cover both the early and late phases of pulmonary oxygen toxicity. Although there were no differences in the morphology or numbers of PAMs obtained from normal control and experimental rabbits after 48 h of oxygen exposure, the cells from oxygen-exposed animals showed a statistically significant increase in the in vitro phagocytosis of S. aureus when compared to the normal controls. The degree of stimulation closely parallels that seen after BCG immunization of normal rabbits. The same effect, though less pronounced, is seen after 24-h oxygen exposures. The phagocytosis of Ps. aeruginosa was also increased after 48 h oxygen exposure of normal rabbits. No statistically significant increase in phagocytosis could be demonstrated in PAMs from BCG-immunized rabbits after 48 or 72 h exposure to 100% oxygen, possibly because they are already maximally stimulated. No change in intracellular bactericidal activity of PAMs from normal rabbits against either S. aureus 502A or Ps. aeruginosa could be found after 24 or 48-h exposures. Our results, therefore, suggest that the PAMs obtained from rabbits after a moderate period of oxygen exposure are not functionally impaired with respect to the in vitro parameters studied. Indeed, in vitro phagocytosis in PAMs from normal rabbits seems to be increased after oxygen exposure.

Since previous in vivo studies in the mouse have demonstrated depression of whole lung phagocytic and bactericidal capacity after oxygen exposure (2, 6, 22, 23), the possible reasons for discrepancy between in vitro and in vivo data must be further explored.

Our in vitro phagocytic and bactericidal assays are performed under optimal conditions for bacterial opsonization and phagocytosis. Preliminary studies of phagocytosis using cell monolayers vs. cell suspensions indicated that loss of cells from monolayers after phagocytosis would make quantitation of our results less accurate; therefore, our experiments were performed with cell suspensions. Whether suspension or monolayer assays more closely approximate normal physiologic conditions for the PAM is a moot point, however, since it seems likely that any major defect in phagocytosis should be apparent in either system.

Another possible reason for differences with previously reported work is that of species variability. Most

studies of in vivo pulmonary bactericidal activity have been carried out in the mouse and rat. Comparable in vivo data does not exist for a rabbit model. There may be species differences in sensitivity to oxygen toxicity; however, the mean survival time in 100% oxygen at one atmosphere for mice varies between 77 and 146 h, depending on the age and strain studied, while that of rabbits ranges from 70 to 84 h (20). The significance of species differences in sensitivity to pulmonary oxygen toxicity remains uncertain.

Since pulmonary oxygen toxicity is known to produce a variety of physiologic changes in the lung, including intra-alveolar exudation, focal atelectasis, alterations in tracheal mucous flow, and decreased production of surfactant (20), it is also quite possible that these alterations in its environment may affect macrophage function and thereby alter whole lung bactericidal activity. Indeed, LaForce, Kelley, and Huber (24) have shown that alveolar lining material obtained from rats by bronchopulmonary lavage is capable of stimulating the bactericidal activity of the rat PAM both in a base-line state and after oxygen exposure (25). Since the rabbit PAM demonstrate a much greater in vitro phagocytic and bactericidal capacity than that reported for the rat PAM in the absence of accessory factors, the interspecies significance of this finding remains to be determined. Furthermore, the question of the relationship between animal observations in various species and clinical applicability to man cannot be answered at this time.

Our studies were not designed to investigate these additional factors, but instead to obtain a more precise evaluation of the phagocytic and bactericidal capabilities of the alveolar macrophage than is possible in investigations of the intact lung. Our results suggest that the rabbit PAM itself is relatively resistant to the toxic effects of prolonged oxygen exposure with respect to in vitro phagocytic and bactericidal function. It is possible that depression of in vivo whole lung bactericidal activity during oxygen toxicity may then be due to factors other than PAM cellular dysfunction. The mechanism of the stimulation of in vitro phagocytosis observed in normal PAMs from oxygen-exposed rabbits and from BCG-immunized animals remains to be determined. Perhaps this in vitro model will be able to provide some of the answers to this question.

ACKNOWLEDGMENTS

REFERENCES

1. Winter, P. M., and G. Smith. 1972. The toxicity of oxygen. *Anesthes.* **37**: 210–241.
2. Huber, G. L., and F. M. LaForce. 1970. Progressive impairment of pulmonary antibacterial defense mechanisms associated with prolonged oxygen administration. *Ann. Intern. Med.* **72**: 808a. (Abstr.)
3. Green, G. M., and E. H. Kass. 1964. The role of the alveolar macrophage in the clearance of bacteria from the lung. *J. Exp. Med.* **119**: 167–175.
4. Kass, E. H., G. M. Green, and E. Goldstein. 1966. Mechanisms of antibacterial action in the respiratory system. *Bacteriol. Rev.* **30**: 488–497.
5. Green, G. M. 1970. In defense of the lung. *Am. Rev. Respir. Dis.* **102**: 691–703.
6. Huber, G., M. La Force, and R. Mason. 1970. Impairment and recovery of pulmonary antibacterial defense mechanisms after oxygen administration. *J. Clin. Invest.* **49**: 47a.
7. Myrvik, Q. N.. E. S. Leake, and B. Fariss. 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. *J. Immunol.* **86**: 128–132.
8. Myrvik, Q. N., E. S. Leake, and S. Oshima. 1962. A study of macrophages and epithelioid-like cells from granulomatous (BCG-induced) lungs of rabbits. *J. Immunol.* **89**: 745–751.
9. Massaro, D. 1968. Alveolar cells: incorporation of carbohydrate into protein and evidence for intracellular protein transport. *J. Clin. Invest.* **47**: 366–374.
10. Millican, R. C., and J. D. Rust. 1960. Efficacy of rabbit pseudomonas antiserum in experimental *pseudomonas aeruginosa* infection. *J. Infect. Dis.* **107**: 389–394.
11. Root, R. K., A. S. Rosenthal, and D. J. Balestra. 1972. Abnormal bactericidal, metabolic, and lysosomal functions of Chediak-Higashi syndrome leukocytes. *J. Clin. Invest.* **51**: 649–665.
12. Fisher, A. B., S. Diamond, and S. Mellen. 1974. Effect of O_2 exposure on metabolism of the rabbit alveolar macrophage. *J. Appl. Physiol.* **37**: 341–345.
13. Hanks, J. H., and J. H. Wallace. 1958. Determination of cell viability. *Proc. Exp. Biol. Med.* **98**: 188–192.
14. Hirsch, J. G., and B. Strauss. 1964. Studies on heat-labile opsonin in rabbit serum. *J. Immunol.* **92**: 145–154.
15. Holmes, B., P. G. Quie, D. B. Windhorst, and R. A. Good. 1966. Fatal granulomatous disease of childhood. An inborn abnormality of phagocytic function. *Lancet.* **1**: 1225–1228.
16. Tan, J. S., C. Watanankunakorn, and J. P. Phair. 1971. A modified assay of neutrophil function: use of lysostaphin to differentiate defective phagocytosis from impaired intracellular killing. *J. Lab. Clin. Med.* **78**: 316–322.
17. Gee, J. B. L., C. L. Vassallo, P. Bell, J. Kaskin, R. E. Basford, and J. B. Field. 1970. Catalase-dependent peroxidative metabolism in the alveolar macrophage during phagocytosis *J. Clin. Invest.* **49**: 1280–1287.
18. Ouchi, E., R. J. Selvaraj, and A. J. Sbarra. 1965. The biochemical activities of rabbit alveolar macrophages during phagocytosis. *Exp. Cell. Res.* **40**: 456–468.
19. Heise, E. R., Q. N. Myrvik, and E. S. Leake. 1965. Effect of bacillus Calmette-Guerin on the levels of acid phosphatase, lysozyme, and cathepsin in rabbit alveolar macrophages. *J. Immunol.* **95**: 125–130.
20. Clark, J. M., and C. J. Lambertsen. 1971. Pulmonary oxygen toxicity: a review. *Pharmacol. Rev.* **23**: 37–133.
21. Lewis, A. 1966. Biostatistics. Reinhold Publishing Corp., New York. 227–230.
22. Shurin, P. A., S. Permutt, and R. L. Riley. 1971. Pulmonary antibacterial defenses with pure oxygen breathing. *Proc. Soc. Exp. Biol. Med.* **137**: 1202–1208.
23. Goldstein, E., W. Lippert, and D. Warshauear. 1974. Pulmonary alveolar macrophage. Defenter against bacterial infection in the lung. *J. Clin. Invest.* **54**: 519–528.
24. La Force, F. M., W. J. Kelley, and G. L. Huber. 1973. Inactivation of staphylococci by alveolar macrophages with preliminary observations on the importance of alveolar lining material. *Am. Rev. Respir. Dis.* **108**: 784–790.
25. Huber, G., S. Goodenough, and D. O'Connell. 1974. Oxygen toxicity and bacterial infection in the lung: impairment of alveolar lining material versus alveolar macrophage bactericidal activity. *Clin. Res.* **22**: 506a. (Abstr.)