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EFFECT OF IN VIVO OZONE EXPOSURE ON IN VITRO PULMONARY ALVEOLAR MACROPHAGE MOBILITY

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The effect of in vivo O₃ exposure on the mobility of pulmonary alveolar macrophages (PAM) in vitro was investigated. Eight randomly selected rats were exposed for 4 h. Four rats were exposed to a clean air (sham) atmosphere, and four to an atmosphere containing 1 ppm O₃. PAM were obtained by lung lavage and placed on gold-colloid coated coverslips. The area cleared of gold particles by migrating PAM after 24, 48, and 72 h was used as an indicator of cell mobility. The number of PAM recoverable by lavage was similar for both groups (2 × 10⁵), but the percentage of macrophages that made tracks was significantly smaller with 95% certainty in the O₃ group. For sham-exposed and O₃-exposed groups, the area cleared by PAM increased as the length of incubation increased, with the area cleared by the sham-exposed group being about 50% greater during each time period. When the two groups were compared statistically at each time point, the probability that they differed was, in each case, greater than 95%. It was concluded that the in vitro migrational potential of PAM was most likely decreased by in vivo exposure to O₃.

INTRODUCTION

Pulmonary alveolar macrophages (PAM) are generally considered to be the deep lung's first line of defense against infectious agents and particulate matter (Cohen and Gold, 1975; Green and Kass, 1964; Coffin et al., 1968). Hocking and Golde (1979) reviewed the research on antimicrobial mechanisms of the macrophage and listed several chemicals within these cells that have microbicidal properties. Included were hydrogen peroxide, catalase, superoxide anion, and lysosomal cationic proteins. With respect to nonviable materials deposited in the deep lung, Brain and Corkery...
(1977) suggested that a major role of the macrophage is prevention of incorporation of insoluble materials in tissues of the lung that have low rates of clearance. For example, the alveolar epithelium and alveolar interstitial spaces appear to have quite long turnover times for particulate contaminants. Thus if phagocytosis occurs, potentially toxic particles are prevented from entering a tissue compartment in which they will be in contact with vulnerable cells for protracted periods of time. As resident phagocytes that are relatively abundant in the interstitial connective tissue of the alveolar septa and free in the lumen of the alveolus, PAM are in intimate contact with many inspired substances. Thus they are both an important pulmonary defense mechanism and a potential target for toxic airborne materials.

PAM are believed to migrate to sites of physical, chemical, and microbial challenge, where they phagocytize bacteria, viruses, and other target materials. Stossel (1976) described phagocytosis in seven sequential phases: (1) particle recognition, (2) reception of the message to initiate phagocytosis, (3) transmission of the message from receptor to effector, (4) attachment of the plasma membrane to the particle, (5) assembly of pseudopodia, (6) movement of pseudopodia to engulf the particle, and (7) fusion of pseudopodia. Inhibition of macrophage mobility, or of any phagocytic subprocess, could effectively compromise the net defensive function of PAM and result, for example, in an increased likelihood of respiratory system infections or other disease states.

Another defense mechanism, mucociliary clearance—due to the action of mucus secretion and ciliary motion in the nasopharyngeal and tracheobronchial regions—transports macrophages laden with foreign material out of the lung. However, Green and Kass (1964) showed that the initial rapid clearance of inhaled radioactively labeled bacteria is less dependent on mechanical removal by the mucociliary system than on the in situ phagocytic action of PAM in the terminal lung units.

It is important to understand how environmental air pollutants affect the mucociliary and PAM defense systems. Gaseous pollutants such as O$_3$ and NO$_2$ are poorly removed by the upper airways and therefore can provide significant in situ exposure to PAM. Because O$_3$ is a toxin, many toxicological studies related to air pollution have focused on it. Dowell et al. (1970) noted increased lung macrophage osmotic fragility after in vivo exposure of rabbits, and Hurst et al. (1970) demonstrated a reduction in activity of three lysosomal enzymes in the same cells. Coffin et al. (1968) and Gardner et al. (1969) reported that exposure to O$_3$ inhibits the ability of PAM to inactivate engulfed microorganisms, which might lead to increased susceptibility of the respiratory system to microbial infection.

Our study was designed to examine the effect of in vivo O$_3$ exposure on one aspect of phagocytic activity—PAM mobility. We obtained PAM by lavage from the lungs of both 1 ppm O$_3$-exposed rats and clean air (sham)-exposed rats. Cell mobility was analyzed by measuring the surface
area cleared by PAM as they moved across gold-colloid coated glass coverslips. Surface areas cleared by PAM from the two groups of rats were measured and compared by appropriate statistical tests. It was found that in vivo O₃ exposure significantly reduced the mobility of PAM in vitro.

MATERIALS AND METHODS

Gold-colloid coated coverslips were prepared as described by Albrecht-Buehler (1977). Sterile glass coverslips, 22 X 22 mm (Corning Glass Works, Science Products Division, Corning, N.Y.; coverslip thickness no. 1), were dipped into a 1% solution of filtered serum albumin (ICN Division of K & K Laboratories, Irvine, Calif.) for 5 s. A 0.2-µm membrane filter (Nalgene Labware Department, Rochester, N.Y.) was used to filter the albumin. A coverslip was then drained by (1) touching its edge to a paper towel, (2) dipping it in absolute ethanol for 3 s, and (3) again touching its edge to a paper towel. The coverslip was placed in a 35 X 10 mm petri dish (Falcon Plastics Division of BioQuest, Oxnard, Calif.) on top of a warming plate held at 60°C, and then allowed to dry.

A colloidal gold suspension was prepared by combining 1.8 ml 14.0 mM AuCl₄⁻ solution (Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, N.J.) and 6 ml 36.5 mM Na₂CO₃ solution with 11 ml distilled H₂O. The mixture was heated to boiling in a 100-ml glass beaker on a hot plate. Then 1.8 ml 4.1% formaldehyde was added, initiating colloidal gold precipitation. Approximately 2 ml of the gold particle suspension was poured into three petri dishes, each containing a coverslip, and allowed to cool at room temperature for 3 h. Excess solution was removed by repeated gentle washing with distilled water, and the coverslips were stored under distilled water in a petri dish until used.

Before being seeded with macrophages, the coverslips were sequentially washed in three 60 X 15 mm petri dishes (Falcon Plastics) containing (1) cell culture growth medium (Dulbecco’s minimum essential medium; Grand Island Biological Co., Grand Island, N.Y.), (2) 10% fetal calf serum (Grand Island Biological Co.), and (3) Gentamycin (100 µg/ml). They were then transferred to sterile 35 X 10 mm petri dishes (Falcon Plastics).

The study was performed with eight white, male, specific pathogen free Sprague-Dawley rats (Hilltop Lab Animals, Inc., Chatsworth, Calif.) ranging in weight from 348 to 430 g. Four randomly selected rats exposed to clean air (sham) served as controls, while the other four were simultaneously exposed to 1 ppm O₃ for 4 h. O₃ was generated by passing medical grade O₂ through a Sander Ozonizer (type III; Sander, Osterberg, West Germany). The rats were coded so that the experimenters did not know whether a particular rat was O₃-exposed or sham-exposed.

Each rat, placed in an individual compartment of a wire cage, was exposed in a stainless steel Rochester-type (Leach et al., 1959) 1-m³ exposure chamber, where O₃ or clean air was delivered. The O₃ was
measured in the breathing zone of the animals with a calibrated ultraviolet absorption monitor (Dasibi Environmental Corp., Glendale, Calif.). Each rat was removed immediately after exposure, injected ip with 50 mg/kg sodium pentobarbital (Western Medical Supply, Inc., Arcadia, Calif.), and lung-lavaged as described by Myrvik et al. (1971). The anesthetized rat was positioned on its back and its trachea exposed and partially transected. Polyethylene tubing (1.67 mm ID, 2.42 mm OD) (Clay Adams Division of Becton, Dickinson, and Co., Orangeburg, N.Y.) was inserted into the tracheal opening and secured with an annular ligature. The tubing was attached to an 18-gauge needle, which was connected to a 12-cm³ plastic syringe via a three-way valve. Lungs were collapsed by drawing air into the syringe, and the valve was closed immediately to prevent reinflation. Hanks balanced salt solution (Grand Island Biological Co.) with Gentamycin was injected (8 ml) into the lungs through the cannula, withdrawn, and placed into a centrifuge tube. Eight successive washes were performed; the resulting PAM suspensions were centrifuged at 1500 rpm for 5 min. The supernatant solutions were discarded and the pellets pooled and resuspended in 8 ml of the cell culture growth medium described earlier. The new suspension was centrifuged at 1500 rpm for 5 min. Again, the supernatant was discarded and the pellet suspended in 2 ml growth medium. A hemocytometer was used to measure cell concentration and the final PAM suspension was diluted to a density of 1 × 10⁵ cells/ml. Portions (~2 ml) of this final cell suspension were pipetted into separate petri dishes containing a single gold-colloid coated coverslip. The petri dishes were then placed in an incubator at 37°C in an atmosphere of 5% CO₂ and 95% air. The growth medium was changed after 24 h. Phagokinetic (Albrecht-Buehler, 1977) tracks were photographed after 24, 48, and 72 h with a Zeiss inverted microscope with Polaroid attachment, using phase optics in light-field illumination (Fig. 1). A 20X objective lens was used.

Each photograph was analyzed “blind” (origin of PAM unknown to experimenter) by placing a 1-mm² grid over it and counting the number of squares infringed on by each cell’s cleared area. Blind counts of the area cleared on photographs were made by two independent observers. The total number of tracks observed was 489. Percent viability was not measured; the presence of cleared tracks indicated that most identifiable cells were viable at all measurement times.

RESULTS

The number of PAM recovered by lavage was similar (2 × 10⁵) for both the O₃- and sham-exposed groups. However, the percent macrophages that actually made tracks was significantly smaller in the O₃-exposed group (p < 0.05) (Fig. 2). For control and O₃-exposed groups, the area cleared by the
FIGURE 1. Track made on a glass surface coated with gold particles by a cultured, sham-exposed PAM after 48 h incubation. It is not clear whether the gold was ingested by the macrophage or merely adhered to its outer surface. Measured track area is approximately $4.1 \times 10^{-3} \text{ mm}^2$.

FIGURE 2. Respective fractions of PAM that made tracks for the $\text{O}_3$-exposed and sham-exposed groups were determined and compared. A two-tailed $t$-test of the mean fraction values of the sham and $\text{O}_3$ groups indicated that at each sample time the fraction of macrophages that made tracks was significantly greater in the sham group with 95% certainty. Standard errors ranged from 0.009 to 0.029 and are too small to be represented graphically. In each case, the number of samples exceeded 300.
macrophages increased as the lengths of incubation increased (Fig. 3). At each sample time the area cleared by the cells exposed to O$_3$ was about half that of the control cells. When the two groups were compared statistically at each point on the curves (Fig. 3), the probability that they differed was, in each case, greater than 0.95. We concluded that macrophage mobility and ability to clear colloidal gold from the coverslip surface were significantly affected by the previous in vivo exposure to 1 ppm O$_3$.

The standard error of the data points was approximately 12% of the mean value for the sham group and about 6% for the O$_3$ group. The reason for this difference (if real) is not known, but it may be related to differences in homogeneity between the cell populations recovered by lavage from the two groups.

The slope of the curve of area cleared versus time was greatest for both groups from 48 to 72 h, which is indicative of increasing macrophage mobility. This might be caused by recovery from the stresses related to the lavage and placement in the in vitro environment.

**DISCUSSION**

The finding that larger areas were cleared by macrophages between 48 and 72 h than between 0 and 24 or 24 and 48 h led to the following
question. Assuming that all of the cleared gold was ingested, what do the data imply about the capacity of the macrophage for phagocytosis of inert particles? The assumption that all cleared particles were ingested may not be valid, as some material cleared from the coverslip may have been carried by the cells on their outer surfaces. The average amount of gold, $X$, cleared from the glass surface after 72 h by control and O$_3$-exposed macrophages can be calculated as

$$X = \frac{M m v s}{S}$$

where $M =$ atomic weight of Au = 197 g/mol
$m =$ molarity of original AuCl$_4$H solution = $14.0 \times 10^{-3}$ mol/l
$v =$ volume of AuCl$_4$H solution used to prepare the coverslip coating = $1.8 \times 10^{-3}$ l
$S =$ average surface area cleared by a macrophage = 0.0051 mm$^2$ (control) and 0.0022 mm$^2$ (O$_3$-exposed)
$s =$ surface area of petri dishes and coverslips on which gold precipitate was deposited = 2890 mm$^2$ for 3 petri dishes

Substituting in numerical values, we compute:

$$X \text{ (control)} = 88 \times 10^{-10} \text{ g Au}$$
$$X \text{ (O$_3$-exposed)} = 38 \times 10^{-10} \text{ g Au}$$

These values for the mass of gold accumulated per macrophage can be compared with the computed mass of a macrophage. From scanning electron micrographs (Waters et al., 1975) the PAM appears to be roughly a hemisphere with base diameter about 14 $\mu$m. Therefore the volume of a macrophage is about $7.2 \times 10^{-10}$ cm$^3$ and its mass about $7 \times 10^{-10}$ g.

Thus at 72 h the average control macrophage had cleared about 13 times its weight in gold and the O$_3$-exposed macrophage about 8 times its weight. Also, at 72 h there was no evidence that macrophage movement was slowing down. These calculations suggest that PAM may have an enormous capacity for engulfing foreign material that is nontoxic and small enough to ingest.

In previous studies we found that a single 4-h exposure of rats to 1 ppm O$_3$ in air produced two kinds of lesions in the alveolar spaces (Crocker et al., 1978; Phalen et al., 1980). At 48 h after exposure, free cells, some of which were macrophages, were seen in greatly increased numbers in the air spaces. Focal lesions, characterized by thickened alveolar septae, were abundant and widely distributed throughout the lung volume. Measurements of clearance rates of inert particles in O$_3$-exposed rats showed that the early clearance phase (0-50 h after exposure) was significantly but transiently depressed. However, measurements of long-term particle clearance (50-400 h) indicated that stimulation or accelera-
tion of clearance had occurred more than 2 d after O$_3$ exposure. The stimulation was statistically significant and appeared to be due to increased numbers of free cells participating in particle clearance. These observations suggest that net deep-lung clearance in the whole animal, which is in part due to macrophage action, can be accelerated by O$_3$ exposure, even though individual cell mobility may be temporarily decreased. The increased number of mobile cells \textit{in vivo} appears to overcome the transient depressant effect of O$_3$ on individual cell mobility.

The results reported here provide evidence of an \textit{in vivo} effect of O$_3$ on pulmonary alveolar macrophage function. The findings are consistent with the hypothesis that the O$_3$-induced damage may be partly reversible, since the rate of area clearance increased with time. The fraction of macrophages making tracks did not change significantly, during the \textit{in vitro} incubation, from that observed 24 h after coverslip seeding (Fig. 2). However, at each sampling time the fraction of active cells from O$_3$-exposed rats was lower than that from sham-exposed rats. Further, it does not appear that cells in the O$_3$-exposed population which were inactive 24 h after exposure, regained the ability to move during the subsequent incubation period. Overall, the results suggest that O$_3$ damage that completely inactivates macrophages is irreversible, while sublethal damage that only reduces mobility may be subject to repair. The nature of these kinds of damage remains to be determined.

In this study the mechanism of action of O$_3$ may have involved direct effects on the cells \textit{in vivo} or indirect effects. For example, O$_3$ may react in the lung, giving products that alter macrophage function. Alternatively, O$_3$ exposure may have led to recovery by lavage of greater numbers of less mobile cells than of more mobile cells. Until this is resolved, the conclusion that O$_3$ acted directly on the cells \textit{in vivo} is tentative.

\section*{REFERENCES}


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