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Acquisition of Peroxidase Activity by Rat Alveolar Macrophages During Pulmonary Inflammation

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The authors investigated the ability of rat alveolar macrophages to acquire peroxidase activity in the course of pulmonary inflammation. Granulomatous pulmonary inflammation was induced in bacille Calmette-Guérin (BCG)-immunized rats by intravenous injection of BCG in mineral oil. In contrast to normal alveolar macrophages, which are peroxidase-negative, alveolar macrophages lavaged from the BCG-treated rats showed significant peroxidase activity in large cytoplasmic inclusions compatible with internalized exogenous material. Alveolar macrophage uptake of intact peroxidase-positive neutrophils was also observed. Maximal numbers of peroxidase-positive alveolar macrophages were observed after the initial influx of neutrophils into the lungs, and peroxidase activity could be demonstrated in cell-free lavage fluid during the acute phase of lung injury. Normal alveolar macrophages acquired peroxidase activity after incubation with peritoneal exudate neutrophils, with purified soluble human myeloperoxidase, and with opsonized erythrocytes. It is concluded that alveolar macrophages acquire peroxidase activity from multiple sources during pulmonary inflammation. Internalization of peroxidase by the alveolar macrophage may serve to clear a potentially toxic enzyme(s) from the alveolar space and contribute to the resolution of pulmonary inflammation. (Am J Pathol 1987, 129:567-577)

PEROXIDASES combine with hydrogen peroxide to form enzyme-substrate complexes that can oxidize a variety of compounds. The neutrophil peroxidase myeloperoxidase (MPO) is important in neutrophil microbicidal function.[1,2] Promyelocytes synthesize MPO and package the enzyme into azurophil granules.[3,4] During phagocytosis, MPO is released into the phagocytic vacuole, and in combination with hydrogen peroxide and a halide, powerful oxidants are produced that are toxic for internalized microorganisms.[5] Eosinophils contain a peroxidase that differs structurally from MPO.[6] Erythrocyte hemoglobin has also been shown to have peroxidase activity in some experimental systems.[7]

Phagocytosis, as well as exposure to certain degranulating stimuli, can result in the release of active peroxidase into the extracellular environment.[8,9] Such extracellular peroxidase may cause tissue injury through direct cell lysis[10] or inactivation of inflammatory mediators.[11-13] One potential mechanism for limiting tissue injury from extracellular peroxidase is the uptake of soluble peroxidase by cells other than neutrophils. Mouse peritoneal macrophages, when incubated *in vitro* with a neutrophil granule preparation, acquire the capacity to fix iodine during phagocytosis, suggesting uptake of neutrophil MPO by the macrophages.[14] Recent studies by Dvorak et al have also shown that soluble eosinophil peroxidase can be internalized by guinea pig basophils and mouse mast cells, and that cellular uptake of peroxidase involves vesicular transport and concentration within cytoplasmic granules.[15] Rabbit alveolar macrophages have been previously demonstrated to transport soluble horseradish peroxidase into the cytoplasm,[16] and human alveolar macrophages have the capacity to internalize neutrophil elastase.[17,18] Here we investigate the ability of rat alveolar macrophages to acquire peroxidase activity during the course of pulmonary inflammation.

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Animals

We obtained specific pathogen-free male Fischer 344 rats weighing 150–175 g from Charles River Breeding Laboratories, Kingston, New York. The animals were housed in the animal care facility at the Veterans Administration Medical Center, San Francisco, in laminar flow hoods.

Experimental Lung Injury

Lung injury was induced in 8–10-week-old rats by subcutaneous immunization followed by intravenous injection of bacille Calmette–Guérin (BCG) (Calbiochem, La Jolla, Calif). Rats were immunized subcutaneously with 100 μg of BCG emulsified with complete Freund's adjuvant (DIFCO, Detroit, Mich) at a final concentration of 1 mg BCG/ml (Figure 1). Fourteen days later, immunized rats were given a tail vein injection of 50 μg BCG in mineral oil at 1 mg BCG/ml. Rats were sacrificed for bronchoalveolar lavage at intervals of from 1 to 31 days after intravenous BCG. For controls, we studied untreated rats of similar age.

Isolation of Alveolar Macrophages

Alveolar macrophages were obtained by bronchoalveolar lavage (BAL) as previously reported.[19] We anesthetized the rats with intraperitoneal pentobarbital and lavaged the lungs with calcium and magnesium-free, phosphate-buffered saline (GIBCO, Grand Island, NY) supplemented with 0.6 mM EDTA (Sigma, St. Louis, Mo) and adjusted to rat serum osmolality (328 mosm/l). For each rat, nine 5-ml portions of warmed lavage fluid (37 C) were instilled with a 1–2-minute dwell time and withdrawn. Cytocentrifuge preparations of lavaged cells were examined for differential cell counts (Diff-Quik, American Scientific Products, Sunnyvale, Calif) or for peroxidase activity as described below. Lavaged cell viabilities consistently exceeded 95% by trypan blue exclusion.

Isolation of Peritoneal Neutrophils

Peritoneal exudate neutrophils were obtained after intraperitoneal injection of casein by a modification of the method of Van Epps and Garcia.[20] Normal rats (13–15 weeks old) were given injections of 10 ml of a 50% saturated solution of casein (Sigma). After 15–18 hours, we anesthetized the rats with halothane (Ayerst, New York, NY) and recovered peritoneal cells by lavage with 25 ml of cold (4 C) phosphate-buffered saline. To minimize cell clumping, we washed and centrifuged lavaged cells at 4 C and removed visible cell clumps by adherence to glass pipettes. The washed peritoneal cells were further purified by centrifugation at 940g through Ficoll-Hypaque (1.088 SG, Sigma; Winthrop Laboratories, New York, NY). Purified peritoneal exudate cells were 97.5% ± 1.5% neutrophils by Diff-Quik staining (mean ± standard deviation [SD], n = 9). Nonneutrophil cells included peritoneal macrophages (1.0 ± 0.9%) and eosinophils (1.1% ± 0.6%).

Histochemical Peroxidase Assay (Diaminobenzidine)

Cytocentrifuge preparations of alveolar macrophages were assayed for peroxidase activity with a modification of the diaminobenzidine (DAB) technique of Graham and Karnovsky.[21] Briefly, cells were fixed for 30 seconds at 4 C in 4% paraformaldehyde, incubated for 30 minutes in 0.5 mg/ml DAB (Sigma), then incubated for 45 minutes in DAB plus 0.01% hydrogen peroxide. The reaction was intensified by incubation in 0.5% cupric nitrate for 1 minute. The cells were Giemsa-counterstained and examined by oil immersion microscopy. Control experiments excluded hydrogen peroxide.

Incubation of Normal Alveolar Macrophages With Purified Rat Peritoneal Neutrophils or Human MPO

Alveolar macrophages were obtained from normal rats by bronchoalveolar lavage, washed, and sus-

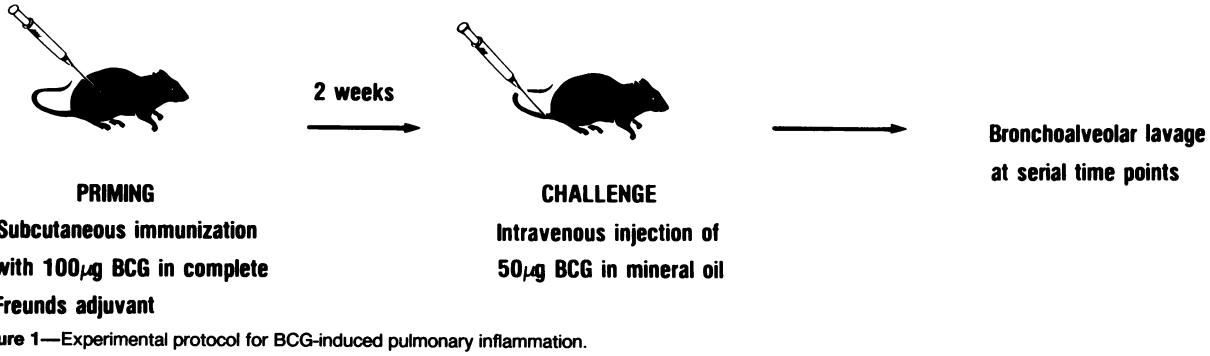

Figure 1—Experimental protocol for BCG-induced pulmonary inflammation.

pended in RPMI 1640 supplemented with 10 mM HEPES buffer, 0.1 mg/ml gentamicin, 200 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. One milliliter of lavaged cells (1×10^6) was added to each well of a four-chamber glass slide (Lab Tek, Miles Labs, Naperville, Ill) and incubated at 37 C in air containing 5% CO_2. After 20 hours, 2×10^6 purified peritoneal neutrophils in 0.1 ml medium were added to each well. The slides were then incubated for various times and assayed for histochemical peroxidase activity.

In separate experiments, purified human MPO (Calbiochem) was added to chamber slides containing normal alveolar macrophages for final MPO concentrations of 0.11 or 0.23 U/ml. The slides containing rat alveolar macrophages and MPO were incubated for 24 hours and assayed for histochemical peroxidase activity (DAB).

Hemoglobin Assay

Hemoglobin was assayed in extracts of lavaged alveolar macrophages as the difference in absorbancy between the oxidized and reduced form of the pyridine hemochromogen by the method of Neufeld et al.[22] This assay is not altered by concomitant peroxidase activity. Lysates of alveolar macrophages were prepared by adding washed alveolar cells to 0.5% hexadecyltrimethyl ammonium bromide (HTAB, Sigma) in 100 mM potassium phosphate buffer, pH 6.0, at a final alveolar cell concentration of 20×10^6 cells/ml. Cells were then sonicated, freeze-thawed three times, sonicated again, and centrifuged at 40,000*g* for 15 minutes at 10 C. One milliliter of supernatant was added to 3 ml of a pyridine-sodium hydroxide mixture (100 ml pyridine [Sigma], 30 ml 1 N NaOH, 170 ml distilled water) and vortexed. Three milliliters of the vortexed sample was placed in a cuvette, and the initial optical density (OD) of the oxidized sample was measured at 558 nm. One hundred microliters of freshly prepared sodium dithionite (155 mg/ml in water, Sigma) was then added, and the OD of the reduced sample was determined after 1–2 minutes. The hemoglobin concentration of the sample was calculated in reference to a standard hemoglobin curve for crystallized rat blood hemoglobin (Sigma) treated in the same way.

Incubation of Normal Alveolar Macrophages With Opsonized Erythrocytes

Rat erythrocytes were obtained from whole blood and opsonized in a 1/1600 dilution of rabbit anti-rat erythrocyte antiserum (Cappel Laboratories, Cochranville, Pa) for 90 minutes at 37 C. The opsonized erythrocytes were then washed, added to normal alveolar macrophages at 25 erythrocytes per alveolar macrophage, and incubated in polypropylene tubes for 16–20 hours at 37 C in 5% CO_2. Uningested erythrocytes were lysed in ammonium chloride, and the alveolar macrophages were examined cytochemically for peroxidase activity.

Assay of Lavage Fluid for Peroxidase Activity (O-Dianisidine)

The first three instilled 5-ml portions of lavage fluid (see *Isolation of Alveolar Macrophages*) were collected, and cell-free supernatant was prepared by centrifugation two times at 350*g* for 10 minutes at 4 C. The supernatant was then diluted with an equal volume of 1.0% HTAB, and peroxidase activity was measured by the o-dianisidine technique.[8] A reaction mix was prepared by adding 1.0 ml of o-dianisidine dihydrochloride (Sigma, 1.67 mg/ml 50 mM potassium phosphate buffer, pH 6.0) to 8.0 ml potassium phosphate buffer and 1.0 ml of 0.005% H_2O_2. Peroxidase activity was assayed as the change in OD at 460 nm within the first 2 minutes for 0.1 ml of supernatant and 2.9 ml of the reaction mixture. Soluble human MPO served as a standard.

Culture of Alveolar Macrophages to Detect Released MPO

To detect release of internalized MPO, normal lavaged alveolar macrophages were cultured *in vitro* with 0.25 units/ml soluble human myeloperoxidase for 4 and 24 hours at 37 C in complete medium. The cells were then washed and incubated for variable times in media containing the following stimuli (all from Sigma): rat serum opsonized zymosan, 5–10 particles per alveolar macrophage; lipopolysaccharide (LPS), 10 μg/ml; phorbol myristic acetate (PMA), 10 μg/ml; or combinations of the different stimuli. Peroxidase activity was assayed in the supernatants of the alveolar macrophage cultures by the o-dianisidine technique.[8]

Preparation of Macrophages for Ultrastructural Localization of Peroxidase

Pelleted cells from lavage fluid or cells adherent to Lab Tek slides were fixed for 10 minutes at 4 C with 1.5% glutaraldehyde (Electron Microscopy Sciences, Warrington, Pa) in a fixation buffer of Gey's balanced salts[23] with 2 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.2), and 2 mM EGTA in a modification of the method of

Pryzwansky.[24] After three washes with fixation buffer, we incubated cells for 30 minutes at room temperature in a medium containing 2 mg/ml DAB in 50 mM Tris-HCl (pH 7.6) with 5% sucrose and 0.03% H_2O_2. For control preparations, we omitted the peroxide substrate. After incubation, cells were washed three times in 0.1 M phosphate buffer (pH 7.2) and post-fixed in ferrocyanide-reduced osmium[25] for 15 minutes at 4 C. Cells were dehydrated[26] and embedded in Epon. Ultrathin sections were stained with lead citrate for 30 minutes and examined at 80 kv with a JEOL 100 CX electron microscope.

Statistics

Data were expressed as mean ± standard deviation. Data were analyzed using the Newman-Keuls multiple range test and paired *t* test.[27]

Results

Lavage Cell Recovery From BCG-Treated Rats

In normal untreated rats, bronchoalveolar lavage recovered $9.1 \pm 1.7 \times 10^6$ cells per rat, of which 99.5% ± 0.7% were alveolar macrophages. In BCG-treated rats, the number of cells recovered by lavage was increased above normal at all time points after intravenous BCG (Figure 2). The maximal number of lavaged cells was $51.7 \pm 9.5 \times 10^6$ per rat on Day 4, but lavaged cell number was significantly increased up to 31 days after BCG ($P < 0.05$). Although the majority of cells were alveolar macrophages at all times after BCG, the percentage of lavaged neutrophils was significantly increased above control levels on Days 2, 3, and 4 after intravenous BCG ($P < 0.05$). Control rats housed for the same time periods as the BCG-treated rats showed normal cell numbers, indicating that the change in cell number was not the result of aging or animal care conditions (data not shown).

Peroxidase Cytochemistry of Alveolar Macrophages From BCG-Treated Rats

In normal rats, only 0.8% ± 0.9% of lavaged alveolar macrophages were positive for cytoplasmic peroxidase (Figure 3A). In contrast, alveolar macrophages from the BCG-treated rats were often peroxidase-positive (Figure 3B). Peroxidase activity was visible as large granular deposits of orange-brown reaction product in the cytoplasm. Alveolar macrophage internalization of peroxidase-positive neutrophils was also observed.

The percentage of peroxidase-positive alveolar macrophages was determined at serial times after the administration of BCG (Figure 4). Peroxidase-posi-

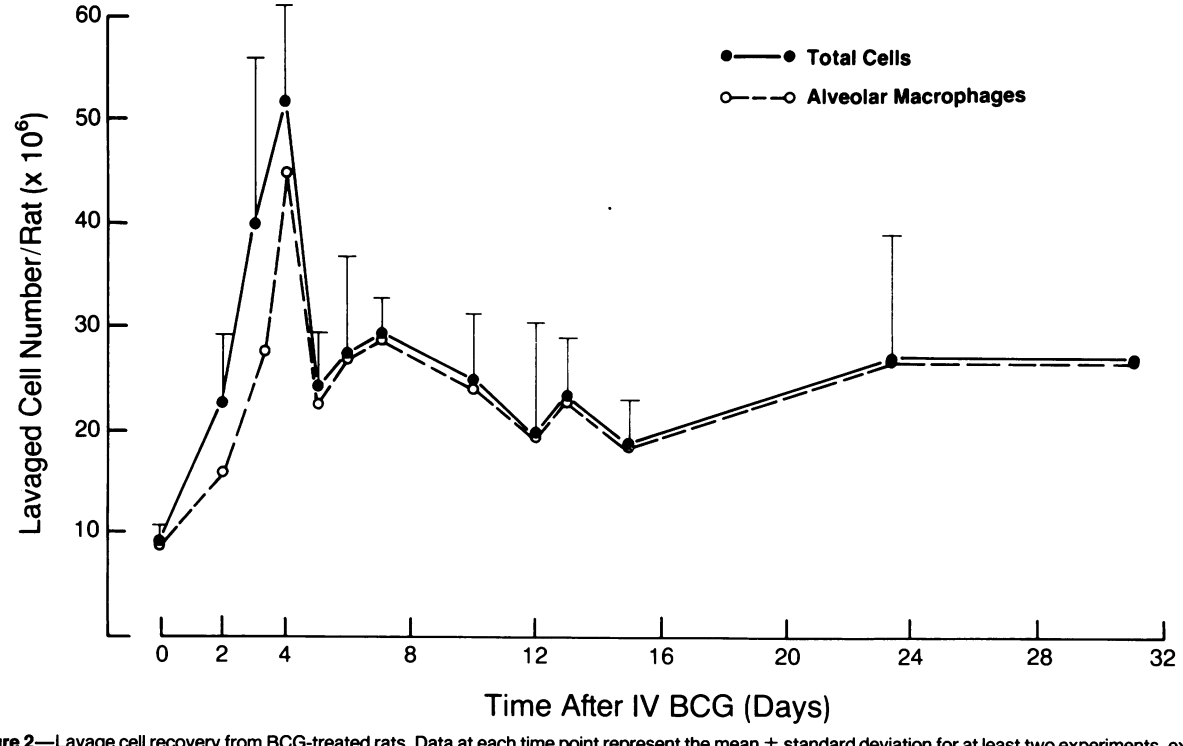

Figure 2—Lavage cell recovery from BCG-treated rats. Data at each time point represent the mean ± standard deviation for at least two experiments, except Day 31.

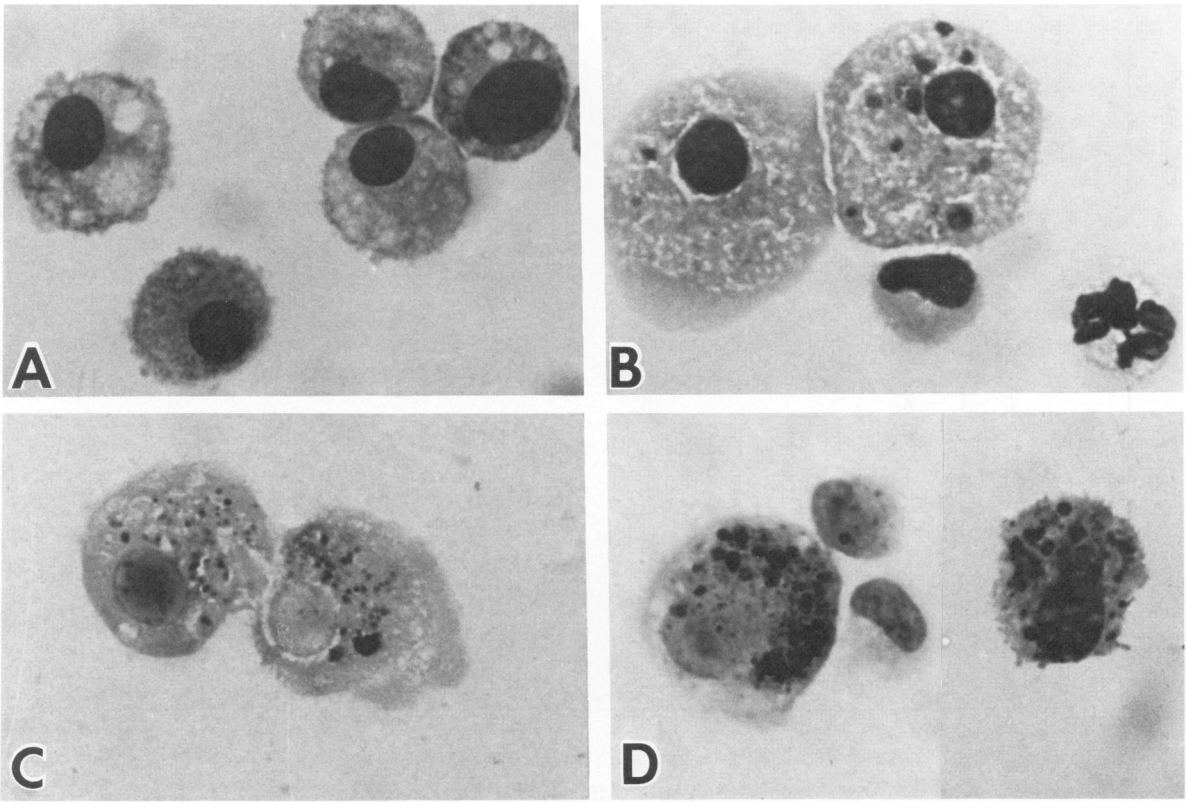

Figure 3—Peroxidase cytochemistry of lavaged alveolar macrophages. **A**—Normal alveolar macrophages. **B**—Alveolar macrophages lavaged from immunized rats 4 days after intravenous BCG. (Note the degranulated neutrophil in lower right corner.) **C**—Normal alveolar macrophages after a 24-hour incubation with peritoneal neutrophils. **D**—Normal alveolar macrophages after a 24-hour incubation in 0.23 U/ml soluble MPO. Peroxidase activity was not visible in the cytoplasm of normal alveolar macrophages immediately after lavage (**A**) or after *in vitro* culture for up to 48 hours. Peroxidase-positive cytoplasmic deposits were visible in alveolar macrophages lavaged from the BCG-treated animals (**B**) and in normal alveolar macrophages after *in vitro* culture with peritoneal neutrophils (**C**) or soluble MPO (**D**). (DAB, Diff-Quik, ×900)

tive alveolar macrophages increased to 50.5% ± 4.7% of lavaged alveolar macrophages on Day 6 and remained significantly increased above normal up to 31 days after BCG ($P < 0.05$). Figure 4 shows that maximal numbers of peroxidase-positive alveolar macrophages were observed after the influx of neutrophils into the lungs, during the acute phase of BCG injury.

Incubation of Normal Alveolar Macrophages With Casein-Elicited Neutrophils

To see whether normal alveolar macrophages could acquire peroxidase activity from inflammatory neutrophils, we incubated alveolar macrophages lavaged from normal rats with a neutrophil-rich preparation of casein-elicited peritoneal cells (97.5% ± 1.4% neutrophils) at a ratio of two neutrophils for each alveolar macrophage. After incubation with neutrophils, peroxidase activity was visible in the cytoplasm of the alveolar macrophages (Figure 3C). This activity was present in a granular pattern similar to the peroxidase-positive alveolar macrophages from the BCG-treated rats. When the alveolar macrophage-neutrophil cultures were assayed at serial incubation times, we observed a time-dependent increase in alveolar macrophage peroxidase activity (Figure 5). The percentage of peroxidase-positive alveolar macrophages increased from a resting value of 2.2% ± 1.7% positive to 69.6% ± 11.3% positive after a 24-hour incubation with neutrophils ($P < 0.001$ for all time points in comparison with control). Peroxidase-positive control cells were attributed to alveolar macrophage phagocytosis of contaminating neutrophils or erythrocytes, the latter known to react with DAB.[26]

Incubation of Normal Alveolar Macrophages With Soluble Human MPO

To see whether normal alveolar macrophages could internalize soluble myeloperoxidase, we incubated alveolar macrophages lavaged from normal rats with purified soluble human MPO. After 24 hours in cul-

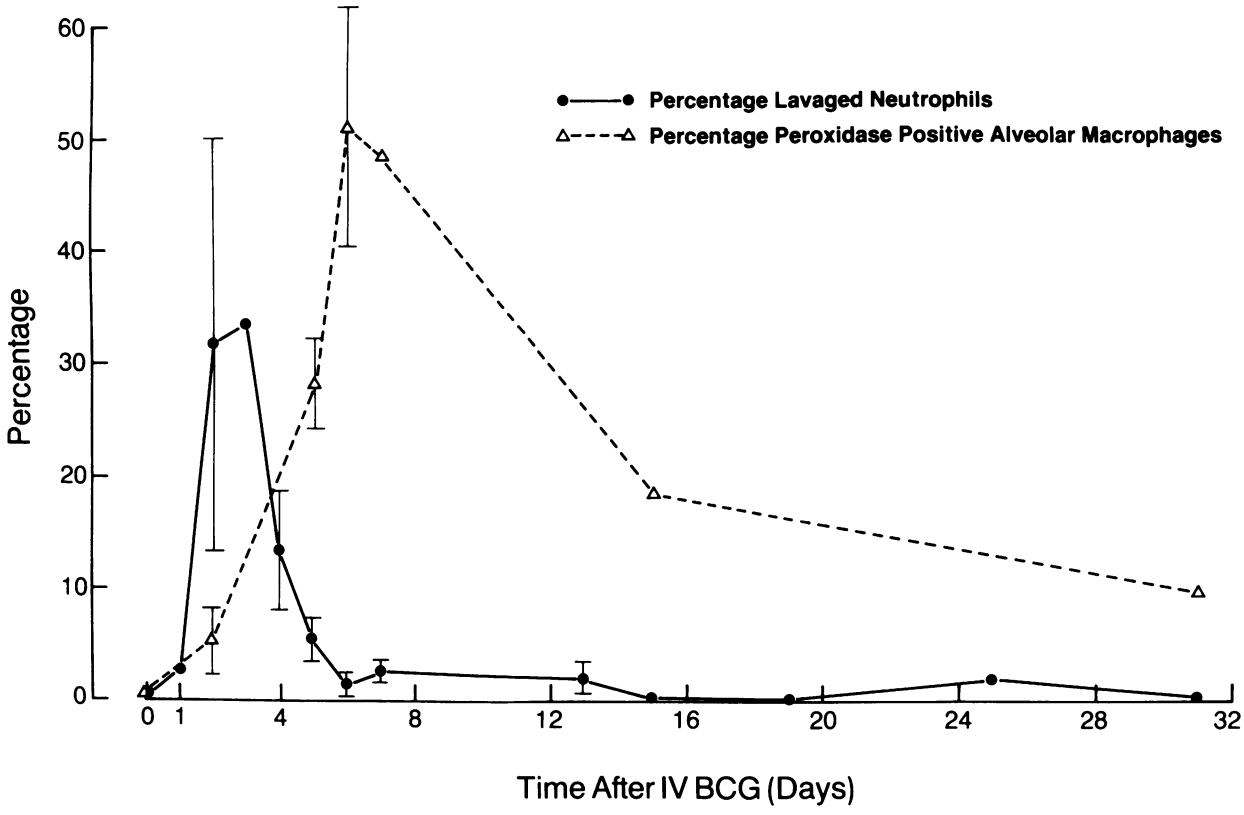

Figure 4—Percentages of lavaged neutrophils and peroxidase-positive alveolar macrophages from BCG-treated rats. Data at each time point represent the mean ± SD for at least two experiments. Data points without error bars represent single experiments using 2 or more rats.

ture, alveolar macrophages incubated in 0.23 U/ml of MPO had peroxidase activity in 76.3% ± 12.5% of cells (n = 2), whereas alveolar macrophages cultured alone had activity in only 2.2% ± 1.7% ($P < 0.001$). The increase in alveolar macrophage peroxidase activity was dose-dependent, with proportionally less activity observed (52.3% of cells peroxidase-positive) after a 24-hour incubation in a lower concentration of MPO (0.11 U/ml). The light-microscopic pattern of cytoplasmic peroxidase after incubation with MPO was similar to that observed in alveolar macrophages from the BCG-treated rats (Figure 3D).

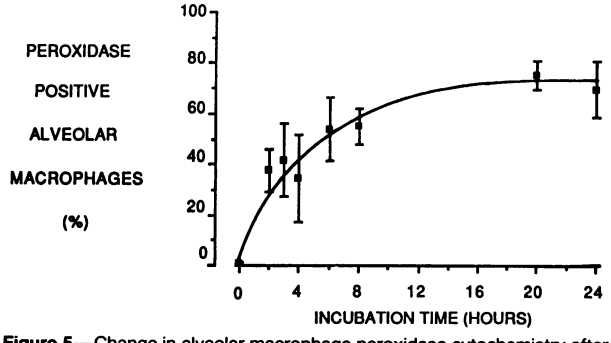

Figure 5—Change in alveolar macrophage peroxidase cytochemistry after incubation with peritoneal neutrophils. Normal alveolar macrophages were incubated for 2–24 hours with peritoneal neutrophils at a ratio of two neutrophils for each alveolar macrophage. Data at each time point represent the mean ± standard deviation for at least three experiments. The curve connecting the data points was hand-fit. Control alveolar macrophages were 0.8% ± 0.9% peroxidase-positive immediately after lavage and 2.2% ± 1.7% positive after a 24-hour culture without added neutrophils.

Concentration of Hemoglobin in Alveolar Macrophage Cell Lysates

Because hemoglobin may have peroxidase activity,[7] we considered the possibility that some of the peroxidase visible in the alveolar macrophage cytoplasm may have resulted from ingested red cells or hemoglobin. Lavaged alveolar macrophages were recovered from BCG-treated rats at serial times and solubilized in 0.5% HTAB. No hemoglobin was detectable in alveolar macrophage lysates from normal animals. In alveolar macrophages from injured rats, hemoglobin was detectable on Day 4 (90 μg for 20 × 10^6 alveolar cells), but not on Days 5, 7, or 13 after intravenous BCG (less than 2 μg/20 × 10^6 cells).

Incubation of Normal Alveolar Macrophages With Opsonized Erythrocytes

When normal alveolar macrophages were incubated with opsonized rat erythrocytes, internalized

erythrocytes were visible as clumps of peroxidase-positive cytoplasmic material. The light-microscopic pattern of peroxidase activity in the alveolar macrophage cytoplasm was similar to that observed in the BCG alveolar macrophages. In the absence of an erythrocyte-specific opsonizing antibody, alveolar macrophages failed to take up the red cells.

Peroxidase Activity in Lavage Fluid From Normal and BCG-Treated Rats

Using the o-dianisidine technique, we assayed cell-free lavage fluid from normal and BCG-treated rats for peroxidase activity (Table 1). No activity was detectable in the lavage fluid from normal rats. In the BCG-treated rats, peroxidase activity was detectable on Days 1–3 after intravenous BCG but not subsequently. A comparison of Table 1 with Figure 4 shows that peroxidase activity in lavage fluid immediately preceded the appearance of peroxidase-positive cytoplasmic deposits in lavaged alveolar macrophages. Lavage fluid peroxidase activity could not be attributed to contaminating erythrocytes, because control experiments showed that solubilization of lavage fluid in HTAB eliminated reactivity of rat hemoglobin in the o-dianisidine reaction.

Alveolar Macrophage Release of Internalized Human MPO

To see whether alveolar macrophages containing MPO could be stimulated to release the enzyme, we incubated normal alveolar macrophages in 0.25 U/ml of soluble human MPO as described above. After 20 hours' incubation, the macrophages were washed and further incubated with a variety of *in vitro* stimuli. Soluble peroxidase activity was assayed in the alveolar macrophage supernatants with the dianisidine technique. Alveolar macrophages incubated with MPO could not be shown to release active enzyme either spontaneously or after incubation with opsonized zymosan (0.5, 2, 11, 16, and 24 hours incubation), 10 μg/ml LPS (24 hours' incubation), or 10 μg/ml PMA (24 hours' incubation).

Table 1—Peroxidase Activity in Lavage Fluid From Control and BCG-Treated Rats

Time after BCG (days)	Peroxidase activity (U/ml ± SD)*		
0 (control)	<0.002		n = 2
1	0.020	±0.021	n = 2
2	0.128	±0.096	n = 2
3	0.050	±0.031	n = 2
4	<0.002		n = 2
5	<0.002	—	n = 1
6	<0.002	—	n = 1
7	<0.002	—	n = 1
10	<0.002	—	n = 1
21	<0.002	—	n = 1

*Assayed by o-dianisidine technique in recovered fluid from the first three 5-ml lavage aliquots. One unit of peroxidase activity was defined as the amount of enzyme that would decompose 1.0 μmol of hydrogen peroxide/minute at 25 C, pH 6.0.

Ultrastructural Localization of Peroxidase Activity in Alveolar Macrophages

Alveolar macrophages freshly lavaged from normal rats or maintained for 2 days in culture did not contain peroxidase-positive cytoplasmic deposits (Figure 6A). However, the majority of alveolar macrophages lavaged from animals on the fourth day after intravenous BCG contained peroxidase-positive vacuoles corresponding to the cytoplasmic deposits seen by light microscopy (Figure 6B). In addition, ingested neutrophils could sometimes be recognized. Although many peroxidase-positive inclusions were homogeneous, some contained flocculent or granular reactivity. The homogeneous peroxidase-positive inclusions, varying from 80 to 800 nm, were round, ovoid, dumbbell-shaped, or wormlike. Sometimes reactivity was found in larger inclusions outlining myelin figures in secondary lysosomes, in endocytic vacuoles with a peripheral rim of reactivity, or in ingested cells. Because of the reactivity of hemeprotein with DAB,[7] some large inclusions could not be distinguished from ingested erythrocytes. At no time was reactivity observed in the Golgi apparatus of the cell.

Alveolar macrophages incubated with neutrophils displayed large numbers of pleomorphic and heterophagic vacuoles containing peroxidase-positive flocculent, granular, or homogeneous reaction product (Figure 6C). Remnants of ingested cells were also present, and some of these still retained peroxidase-positive neutrophil granules (Figure 6C). Although homogeneous, dark granules the size of PMN azurophil granules occurred alone and in some of the vacuoles, we could not determine whether they represented ingestion of intact neutrophil granules or ingestion of soluble MPO and subsequent condensation. Alveolar macrophages incubated with soluble MPO showed the same types of vacuoles: those with flocculent peroxidase-positive product (Figure 6D) and those with granular or condensed reaction product.

Discussion

The major observation of this study is that alveolar macrophages from rats with BCG-induced pulmonary inflammation develop increased cytoplasmic staining for peroxidase. The presence of peroxidase-

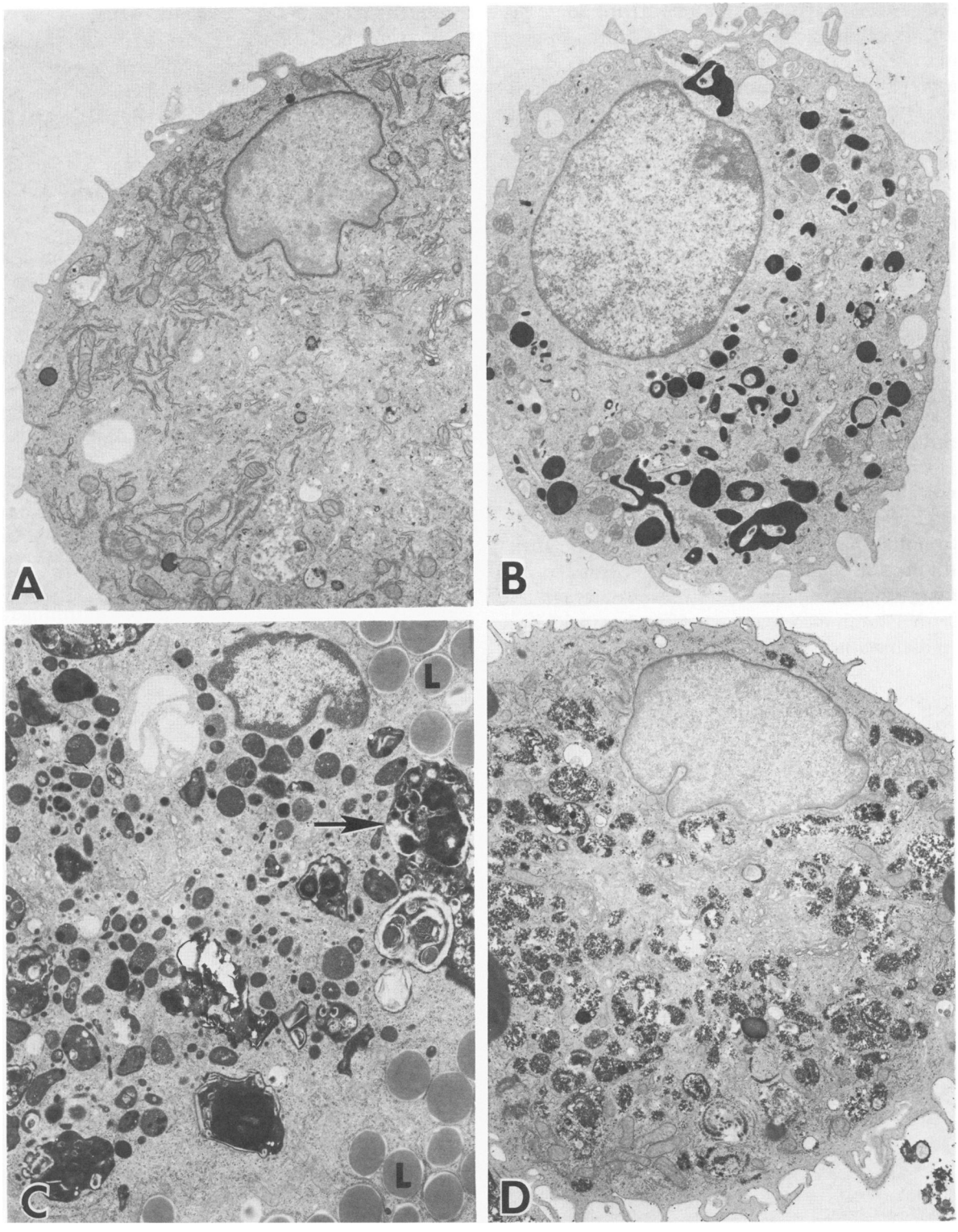

Figure 6—Ultrastructural peroxidase cytochemistry of lavaged alveolar macrophages. **A**—Normal alveolar macrophage after 48-hour *in vitro* culture. **B**—Alveolar macrophage lavaged from an immunized rat 4 days after intravenous BCG. **C**—Normal alveolar macrophage after 24-hour *in vitro* culture with peritoneal neutrophils. **D**—Normal alveolar macrophage after 24-hour *in vitro* culture in 0.11 U/ml MPO. Peroxidase activity was not visible in the cytoplasm of normal alveolar macrophages (**A**). Cytoplasmic reaction product was present in alveolar macrophages from BCG-treated rats (**B**) and in alveolar macrophages after incubation with neutrophils (**C**) or soluble MPO (**D**). The *arrow* marks the ingested neutrophil. *L*, lipid. (DAB, lead citrate, **A**, ×7600; **B**, **C**, **D**, ×6500)

positive alveolar macrophages accompanied the influx of neutrophils during acute inflammation, but the number of peroxidase-positive alveolar macrophages remained increased even at later stages of pulmonary inflammation, when the absolute number of lavaged neutrophils had returned to baseline levels. Such changes in alveolar macrophage peroxidase activity have not been previously described in animals with BCG-induced lung injury.

Our first impression was that peroxidase-positive alveolar macrophages in the BCG-treated animals represented monocyte influx into the alveolar space. The absence of peroxidase activity in the alveolar macrophage Golgi apparatus seemed to rule out induction of endogenous enzymatic activity. Blood monocytes contain peroxidase-positive primary granules, which are lost as the cell matures into a macrophage.[28] Mature tissue macrophages, including alveolar macrophages, do not contain myeloperoxidase in cytoplasmic granules.[29-31] Electron microscopy of alveolar macrophages from the injured animals, however, demonstrated cytoplasmic peroxidase activity within vacuoles much larger and more numerous than primary granules. These vacuoles were compatible with internalized exogenous material. The most obvious exogenous source of peroxidase was the inflammatory neutrophil, and the temporal relationship between lavaged neutrophils and peroxidase-positive alveolar macrophages suggested that alveolar macrophages acquired peroxidase activity from neutrophils. In further support of this hypothesis, degranulated neutrophils with no detectable peroxidase activity were frequently observed among lavaged cells in the acute phase of BCG-induced inflammation.

We postulated that alveolar macrophages in the BCG-treated animals acquired MPO activity from inflammatory neutrophils. To test this hypothesis, normal rat alveolar macrophages were incubated *in vitro* with a neutrophil-rich preparation taken from rats injected with casein. After incubation with neutrophils, the normal alveolar macrophages acquired cytoplasmic deposits with peroxidase activity in a time-dependent manner. Significant cytoplasmic peroxidase was detectable in alveolar macrophages after as little as 60 minutes of incubation with neutrophils. Electron microscopy demonstrated peroxidase-positive cytoplasmic vacuoles similar to those seen in alveolar macrophages from the BCG-treated rats. The results of these *in vitro* studies supported our hypothesis and suggested that the peroxidase-positive alveolar macrophages observed in the BCG-treated animals resulted from an interaction with inflammatory neutrophils.

Hemeprotein has been shown to have peroxidase activity in the DAB assay,[7] and red blood cells were present in cells lavaged from animals during the acute phase of BCG-induced injury. Using a hemoglobin assay unaffected by peroxidase, we were able to detect hemoglobin in extracts of alveolar macrophages during the acute phase of BCG lung injury. In addition, normal alveolar macrophages acquired cytoplasmic peroxidase activity after *in vitro* phagocytosis of opsonized erythrocytes. This information suggested that some of the peroxidase activity observed in the BCG alveolar macrophages may have been derived from ingested erythrocytes.

There are two possible mechanisms for alveolar macrophage acquisition of exogenous peroxidase activity: vesicular transport of soluble peroxidase or phagocytosis of peroxidase-positive cells by the alveolar macrophage. When purified human MPO was added to normal rat alveolar macrophages, the number of peroxidase-positive cytoplasmic granules markedly increased, indicating that rat alveolar macrophages have the capacity to internalize soluble MPO. A similar capacity to internalize extracellular enzymatic material has been demonstrated for rabbit alveolar macrophages and horseradish peroxidase[16] and for mouse mast cells and soluble eosinophil peroxidase.[15] Furthermore, we were able to demonstrate peroxidase activity in cell-free lavage fluid during the acute phase of BCG lung injury, immediately prior to the appearance of histochemical peroxidase in the alveolar macrophage cytoplasm. Peroxidase activity has been previously reported in lavage fluid from humans with the adult respiratory distress syndrome.[32] With regard to alveolar macrophage phagocytosis of peroxidase-positive cells, internalized neutrophils were observed in alveolar macrophages from BCG-treated animals as well as in normal alveolar macrophages after incubation with neutrophils. Newman *et al* have previously demonstrated that inflammatory, but not normal, rabbit alveolar macrophages can phagocytize senescent neutrophils and acquire peroxidase activity,[33] whereas Atwal showed the engulfment of neutrophils by rat peritoneal exudate macrophages.[34] Alveolar macrophages also may phagocytize erythrocytes, and our data indicate that this may also result in cytoplasmic peroxidase activity. Thus, our *in vitro* data indicate that rat alveolar macrophages have the capacity both to internalize soluble myeloperoxidase and to ingest peroxidase-positive neutrophils and erythrocytes. It is possible that both mechanisms are responsible for the alveolar macrophage peroxidase activity observed *in vivo* during BCG-induced pulmonary inflammation.

Mononuclear cells with cytoplasmic deposits of

peroxidase activity have been interpreted by other investigators as blood monocytes migrating to sites of tissue inflammation.[29,35-38] Our demonstration of small peroxidase-positive cytoplasmic deposits in alveolar macrophages incubated with soluble MPO, neutrophils, and erythrocytes suggests that this interpretation should be reevaluated. Neutrophils and erythrocytes are typically present during acute inflammation and may contribute peroxidase activity to resident tissue macrophages as well as to monocytes arriving from the blood.

The functional significance of alveolar macrophage uptake of neutrophil MPO is of considerable interest. The uptake of neutrophil MPO may reflect the scavenger role of the alveolar macrophage and serve to remove a potentially injurious substance from the alveolar space. In this regard, MPO has been shown to be directly toxic to mammalian cells,[10,39] to inactivate chemotactic factors,[11] and to degrade prostaglandins[13] and leukotrienes.[40] Once taken up by the alveolar macrophage, neutrophil MPO appears to be retained within the cell. In contrast to neutrophil elastase, which can be released from the alveolar macrophage under certain conditions,[18] we were unable to demonstrate spontaneous or stimulated release of internalized MPO. It is also theoretically possible that the alveolar macrophage could utilize internalized MPO to enhance its own microbicidal or tumoricidal function. MPO in combination with hydrogen peroxide and a halide has potent microbicidal[1,2,41] and tumoricidal activity,[42,43] and limited evidence suggests that macrophages can utilize internalized peroxidase to enhance the killing of ingested microorganisms.[14,44]

In conclusion, the results of these studies indicate that rat alveolar macrophages acquire cytoplasmic peroxidase activity during the course of BCG-induced pulmonary inflammation. Cytoplasmic peroxidase activity is probably derived from several sources, including uptake of soluble neutrophil MPO and/or phagocytosis and digestion of intact neutrophils and erythrocytes. Such internalization of peroxidase by the alveolar macrophage may serve to remove a toxic enzyme from the alveolar space and contribute to the resolution of pulmonary inflammation.

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