SERUM FACTOR REQUIREMENT FOR REACTIVE OXYGEN INTERMEDIATE RELEASE BY RABBIT ALVEOLAR MACROPHAGES

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Reactive oxygen intermediates (ROI) play an essential role in host resistance to microbial infection. ROI produced by phagocytic cells after membrane stimulation (for example, during phagocytosis of bacteria), exert a bactericidal effect through both halogenation and direct oxidation of the ingested microbes. The importance of ROI in this regard is highlighted in patients with genetic defects in neutrophil function, characterized by their inability to produce ROI. In time, these patients develop a chronic granulomatous disease (1). Paradoxically, there is also evidence to show that ROI, when released extracellularly, may cause tissue inflammation and injury (2). For example, neutrophils stimulated by activated complement components or aggregated immunoglobulin release superoxide anion into the extracellular environment (3). At the same time, parenteral administration of superoxide dismutase, a scavenger of superoxide anion, has been shown to have an antiinflammatory effect (4).

The alveolar macrophage (AM), by virtue of its direct exposure to the environment, is generally considered to constitute a “first line of defense” in protecting the host against pulmonary infection by inhaled microorganisms, and is known to produce ROI under certain circumstances (5–9). However, if inhalation of environmental dust capable of activating macrophages caused an inappropriate release of ROI into the alveolar microenvironment, the delicate alveolar tissues might be injured. Clearly, the ability to regulate ROI production and/or release by AM would be highly advantageous to the host.

We now report that resident rabbit AM do not normally release ROI into the extracellular environment when triggered in vitro by membrane-active agents such as phorbol esters (PMA) or concanavalin A (Con A). However, after being conditioned by exposure to serum for a period of 24–48 h, AM readily produce...
and release ROI. This serum-conditioning effect of resident AM is a function of both the concentration of serum used and the duration of treatment, and is reversible and inhibitable by cycloheximide. In addition, NADPH oxidase activity in resident AM is markedly increased as a consequence of serum conditioning. This conditioning effect appears to be due to a discrete serum constituent possessing an estimated molecular weight of 30,000–50,000 (30–50 K); it is not species specific in its action and it is distinct from endotoxin and gamma interferon.

Materials and Methods

**Animals.** Male pathogen-free New Zealand White rabbits weighing 3–5 lbs were purchased from Hazelton Dutchland Inc. (Denver, PA). The rabbits were housed in laminar flow rooms supplied with filtered air and were given rabbit chow and water ad libitum.

**AM Isolation.** Resident AM were harvested by tracheal lavage according to the method of Myrvik (10). Briefly, rabbits were sacrificed by intravenous injection of 75 mg/kg body weight of Ketamine HCl (Parke, Davis & Co., Detroit, MI). The lungs from each rabbit were cannulated via the trachea and lavaged in situ eight times with 50-ml volumes of prewarmed (37°C) Hanks' calcium/magnesium-free balanced salt solution. The cells from individual animals were collected by centrifugation at 40 g for 10 min at 4°C. Any contaminating erythrocytes were lysed by incubating with Tris-buffered NH₄Cl for 5 min at 37°C. The cells were then washed twice with Hanks' balanced salt solution (HBSS) and once with minimum essential medium (MEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cell viability was consistently ~95% and, in general, 5–7 × 10⁷ viable macrophages were obtained from each rabbit. The AM cell concentration was adjusted to 1.0 × 10⁶ cells/ml, and 0.5 ml of this cell suspension was added to each 16-mm-diam well of a Linbro tissue culture plate (Linbro Scientific Co., Hamden, CT), resulting in 5.0 × 10⁵ cells/well at a density of 2.5 × 10⁵ cells/cm². The plates were incubated at 37°C in 5% CO₂ to allow adherence of macrophages. After 2 h of incubation, the monolayers, which were >99% macrophages as determined by differential staining, were washed with HBSS and reincubated with MEM (with or without serum as a conditioning agent) for the desired length of time before assaying ROI activity. In lipopolysaccharide (LPS) priming experiments, AM were incubated for up to 72 h with MEM containing 0.001–1.0 μg/ml LPS before assaying ROI activity. Growth factors (Gibco Laboratories, Grand Island, NY) used for conditioning AM were insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (100 ng/ml), fibroblast growth factor (100 ng/ml), hydrocortisone (10⁻¹⁰ M), T₃ (10⁻¹⁰ M), sodium selenite (10⁻⁶ M), putrescine (5 μg/ml), fibronectin, and a trace element mix. The conditioning activity of these factors was assayed after 48 h of incubation with AM monolayers.

**Hydrogen Peroxide Assay.** Production of H₂O₂ by AM was quantitated by a method described by Pick and Keisari (11), based on the H₂O₂-mediated and horseradish peroxidase (HRP)-dependent oxidation of phenol red, yielding a reaction product that absorbs maximally at 610 nm. The phenol red assay solution (PRS) contains 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.28 mM phenol red (phenolsulfonphthalein, sodium salt; Sigma Chemical Co., St. Louis, MO), and 8.5 U/ml HRP (type II, 170 purogallin U/mg solid; Sigma Chemical Co.). To assay H₂O₂ production of the total AM population, including those viable AM that became detached from the monolayers during incubation, the culture medium was collected from each well, centrifuged, and any pelleted cells resuspended in 1 ml PRS. A 10 μl vol of the appropriate triggering agent (e.g., Con A) was then added to the PRS containing the nonadherent AM, at which point the mixtures were added back to the appropriate wells containing the adherent AM, and incubated at 37°C with 5% CO₂ for 30 or 90 min. Controls included wells with PRS but no triggering agent and wells with PRS but no cells. At the completion
of incubation, the culture fluids were collected, centrifuged, and the supernatant fluids transferred to clean test tubes. The cell-free fluids were made alkaline by the addition of 10 μl of 1 N NaOH per tube; the absorbance (610 nm) of each sample was then determined against a blank of PRS to which 10 μl of 1 N NaOH was added. Standard curves were made, using the same preparation of PRS, with 1–30 μM H₂O₂ solutions. The standards were treated in the same manner as the experimental cultures. All stimulants and controls were tested in triplicate for each rabbit. In some instances, 1,250 U/ml catalase (purified powder from bovine liver, 1,860 U/mg protein; Sigma Chemical Co.) was used to specifically inhibit H₂O₂ accumulation.

Superoxide Anion Assay. O₂⁻ was measured by the reduction of ferricytochrome c (12). Both the adherent and any nonadherent AM from individual wells were assayed as previously described for the H₂O₂ assay. Briefly, the nonadherent cells from each well were harvested in test tubes and resuspended in 1 ml of an 80 μM solution of ferricytochrome c (type III; Sigma Chemical Co.) in HBSS lacking phenol red. The triggering agents (in 10 μl volume) were added to the tubes, at which time the assay medium was added back to the corresponding wells containing adherent cells and the cultures incubated for 90 min at 37°C in 5% CO₂. Controls included AM in cytochrome c solution containing HBSS but no added triggering agent, and cultures containing cytochrome c solution and triggering agent but no macrophages. Each concentration was tested in triplicate for each rabbit. After incubation, the supernatant fluids from each culture were harvested by centrifugation for 10 min at 1,500 rpm at 4°C, and the absorbance measured at 550 nm against blanks consisting of cytochrome c solution (also incubated for 90 min at 37°C). The amount of cytochrome c reduced was determined by the formula, ΔA₅₅₀ = 2.1 × 10⁴ M⁻¹ cm⁻¹ (12). The specificity of cytochrome c reduction was assessed by the addition of 300 U/ml superoxide dismutase (SOD) (type I from bovine blood, 3,000 U/mg protein; Sigma Chemical Co.) to some samples.

pH Stability of Macrophage-conditioning Activity. To examine whether the factor(s) in serum that conditions AM for ROI production was acid labile, a 5% solution of fetal bovine serum (FBS) in MEM was adjusted to pH 2.0 with concentrated HCl. The pH-adjusted FBS solution and control FBS solution (pH 7.4) were maintained at 4°C for 2 h. The pH of the acid-treated FBS solution was then readjusted to 7.4 with 40% saturated NaOH and the medium sterilized by passage through a 0.45 μm filter. AM monolayers were conditioned with either control or acid-treated medium for 48 h, at which time the cells were assayed for their ability to produce H₂O₂ by triggering the cells with 10 μg/ml Con A.

NADPH Oxidase Assay. NADPH oxidase activity was assessed by measuring O₂⁻ production in deoxycholate (DOC)-treated AM in the presence of exogenous NADPH and required the use of acetylated ferricytochrome c to avoid detection of cytochrome reductase activity (13). Ferricytochrome c was acetylated by the procedure of Tsunawaki and Nathan (13), based on the method described by Kakinuma and Minakami (14). Briefly, 500 mg of ferricytochrome c in 10 ml of half-saturated sodium acetate was slowly mixed with acetic anhydride at 100 mol/mol of ferricytochrome c for 40 min at 0°C, followed by 48-h dialysis against distilled water. The efficiency of the acetylated ferricytochrome c at detecting O₂⁻ was ~50% of native ferricytochrome c. The rate of NADPH oxidase-induced O₂⁻ production was assayed as described by Tsunawaki and Nathan (13), with modifications. Freshly harvested AM, or AM that had been cultured 48 h with or without serum containing medium, were incubated 45 min with 10 mM diethyldithiocarbamic acid (DDC) to inhibit endogenous SOD activity. Cells were harvested with a rubber policeman, washed with HBSS, and suspended in 1 ml HBSS (pH 7.5) with 30 μM acetylated ferricytochrome c and 2 mM NaN₃ (to inhibit cytochrome oxidase), at a cell concentration of 1–2 × 10⁶ cells/ml. The suspension was made in a 1 cm light path cuvette in the thermostatted (37°C) chamber of a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) set to continuously read absorbance at 550 nm. To initiate O₂⁻ production, the intact cells were triggered with 1 μg/ml PMA. When the rate of O₂⁻ formation reached its maximum, the cells were lysed by addition of 0.07% (wt/vol) DOC, and the NADPH oxidase activity was determined by measuring the rate of ferricytochrome
c reduction after immediate addition of NADPH (type X; Sigma Chemical Co.) at a saturation concentration of 1.2 mM. The addition of 300 U/ml SOD to either the intact cells or lysed cells completely abolished O₂ ferricytochrome c reduction. Activity was expressed as nanomoles of acetylated ferricytochrome c reduced per minute per 10⁶ cells.

Ammonium Sulfate Precipitation. As an initial step in isolating the homologous serum factor(s) responsible for AM conditioning, pooled rabbit serum (175 ml) was separated into two fractions by ammonium sulfate precipitation. A 40% saturation of ammonium sulfate was achieved by addition of powdered salt to pooled rabbit serum at 4°C. After 4 h the precipitated protein was removed by centrifugation and sufficient ammonium sulfate added to the supernatant fluid to reach 80% saturation. The precipitable material obtained after both steps was redisolved in distilled H₂O and then dialyzed against 0.05 M Tris/0.15 M NaCl, pH 8.0. A small portion of each ammonium sulfate cut was also dialyzed against MEM and assayed for ROI-conditioning activity.

Affinity Chromatography. After dialysis, the protein fraction that precipitated at 40–80% salt saturation was applied to a 2.5 × 90 cm Cibacron Blue affinity column (Affigel-blue; Bio-Rad Laboratories, Richmond, CA), equilibrated with 0.05 M Tris/0.15 M NaCl, pH 8.0, at a flow rate of ~148 ml/h. Nonbinding proteins were eluted with 0.05 M Tris/0.15 M NaCl, pH 8.0, and binding proteins eluted with 2 M NaSCN. Aliquots of the various fractions (12 ml/tube) were dialyzed against MEM and assayed for their ROI-conditioning activity. Fractions containing activity were pooled and concentrated by lyophilization.

Gel Filtration. The active material from the affinity chromatography step was applied to a 2.5 × 60 cm Sephacryl S-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with PBS, pH 7.2, at a flow rate of ~48 ml/h. The column was calibrated with bovine serum albumin (66 K mol wt), ovalbumin (45 K), and alpha chymotrypsinogen A (25 K). The exclusion volume was determined by using Blue Dextran 2000. Eluted 3-ml fractions were dialyzed against MEM and tested for their ROI-conditioning activity.

Chemicals. The tissue culture reagents MEM, FBS, HBSS (10×), streptomycin, penicillin, l-glutamine, and sodium pyruvate were purchased from Gibco Laboratories. NU serum, which contains FBS and specific growth factors, was obtained from Collaborative Research Inc., Lexington, MA. H₂O₂ was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ, as a 30% solution. Standards were made by initially diluting 1.0 ml of a 30% solution of H₂O₂ in 1,000 ml H₂O. The actual concentration was determined from its absorption at 230 nm, using an extinction coefficient of 81 M⁻¹ cm⁻¹ (11). PMA was obtained from Sigma Chemical Co. and was diluted in dimethylsulfoxide at 5 mg/ml, aliquoted, and stored at −70°C. Con A (Calbiochem-Behring Corp., La Jolla, CA) was obtained at a concentration of 25 mg/ml in 5% sterile glucose, and stored at 4°C. LPS (from Escherichia coli 0128-B12; Difco Laboratories Inc., Detroit, MI) was dissolved in H₂O at a concentration of 10 mg/ml and stored at 4°C. Cycloheximide (Sigma Chemical Co.) was dissolved in distilled H₂O at a concentration of 1 mg/ml. DDC (Sigma Chemical Co.) was dissolved in H₂O at a concentration of 1 M. Recombinant human gamma interferon (rIFN-γ), from an E. coli host recombinant DNA procedure (AMGen Biologicals, Thousand Oaks, CA) was diluted in 40 mM Tris-HCl, pH 7.5, to a concentration of 10⁶ U/ml. The activity of this rIFN-γ preparation was determined by AMGen Biologicals by assaying its ability to inhibit the cytopathic effect in HeLa cells infected with encephalomyocarditis virus.

Results

In our initial experiments we found that addition of membrane-active agents, such as Con A or PMA, to freshly harvested rabbit AM resulted in little if any incremental release of ROI. However, after these cells had been cultured in vitro for 24–48 h in the presence of FBS, subsequent addition of Con A or PMA readily triggered ROI release. The effectiveness of such serum conditioning depended upon the concentration of serum used, with maximal ROI production after conditioning with an FBS concentration of 7.5% or greater (Fig. 1).
We next examined the time required to condition rabbit AM for ROI production, using a fixed concentration of FBS (5%). No significant H₂O₂ or O₂⁻ release was observed unless the cells had been conditioned in serum-containing medium for at least 24 h, with 48 h required in most instances (Figs. 2 and 3). This experiment also demonstrated that whereas both Con A and PMA readily stimulated ROI release from serum-conditioned AM, LPS stimulated poorly or not at all. Using AM conditioned for 48 h in 5% FBS, we determined the optimal Con A concentration for triggering ROI release. Maximal H₂O₂ accumulation was triggered by 10 μg Con A/ml, whereas maximal O₂⁻ accumulation was achieved using 50–250 μg Con A/ml (Fig. 4). Similar dose-response studies using LPS as a triggering agent (at 1.0 ng/ml to 100 μg/ml) confirmed the inability of this agent to trigger ROI release from serum-conditioned AM (data not shown).

Next, we compared FBS, autologous rabbit serum, pooled human serum, and
FIGURE 3. O$_2$ release by AM triggered after 0, 24, 48, and 72 h of incubation in medium containing 5% FBS. Each point represents the mean of nine values from three rabbits assayed in triplicate (bars indicate SEM). (●) 5.0 µg/ml PMA; (△) 50 µg/ml Con A; (●) 250 µg/ml Con A; (□) 10 µg/ml LPS; and (○) 100 µg/ml LPS.

FIGURE 4. Con A triggering of O$_2$ and H$_2$O$_2$ release by AM. Cells were conditioned in medium containing 5% FBS for 48 h, at which time the cells were triggered with Con A. Each point represents the mean of six values from two rabbits assayed in triplicate (bars indicate SEM). (○) H$_2$O$_2$ values; and (□) O$_2$ values.

NU serum (a semidefined serum substitute containing FBS) for their ability to condition AM for subsequent ROI production. Each was used at a final concentration of 10%, which preliminary experiments had shown to be optimal. All serum preparations conditioned AM for subsequent ROI production upon triggering by Con A, with FBS the most effective (Table I). In contrast, neither bovine, rabbit, nor human serum albumin proved capable of conditioning AM, thus arguing against a simple protein nutrient effect (Table I). Another possible explanation for this serum effect would be that serum supplies the necessary
TABLE I

Effect of Pretreatment of AM With Serum or Albumin on H₂O₂ Release

| Exp. | Conditioning agent          | H₂O₂/30 min per 5 x 10⁵ cells | nmol   |
|------|-----------------------------|------------------------------|--------|
| 1    | None                        | 2.1 ± 0.4                    |        |
|      | 10% Autologous serum        | 9.5 ± 1.6                    |        |
|      | 10% NU serum                | 17.0 ± 2.1                   |        |
|      | 10% FBS                     | 24.2 ± 0.9                   |        |
| 2    | None                        | 4.2 ± 1.3                    |        |
|      | 5% FBS                      | 22.5 ± 0.1                   |        |
|      | 10% FBS                     | 21.5 ± 0.1                   |        |
|      | 4.0 mg/ml Human albumin     | 4.0 ± 1.5                    |        |
|      | 4.0 mg/ml Rabbit albumin    | 3.1 ± 0.7                    |        |
|      | 4.0 mg/ml Bovine albumin    | 3.7 ± 0.6                    |        |
| 3    | None                        | <0.5                         |        |
|      | 10% FBS                     | 16.1 ± 0.6                   |        |
|      | 10% Human serum             | 9.1 ± 0.9                    |        |

AM were cultured 48 h with the indicated conditioning agent, then assayed for Con A (10 μg/ml)-stimulated H₂O₂ release. Data represent the mean of nine values from three rabbits assayed in triplicate ± SEM.

TABLE II

pH Stability of Serum-conditioning Activity

| Conditioning agent | Triggering agent   | H₂O₂/30 min per 5 x 10⁵ cells | nmol |
|--------------------|--------------------|------------------------------|------|
| 5% FBS             | 10 μg/ml Con A     | 17.1                         |      |
| 5% Acid-treated FBS| 10 μg/ml Con A     | 11.5                         |      |
| None               | 10 μg/ml Con A     | 1.4                          |      |

AM were cultured for 48 h with MEM containing either 5% FBS or 5% acid-treated FBS, then triggered with 10 μg/ml Con A, and assayed for H₂O₂ release.

growth factor(s) and/or nutrient(s) essential for ROI production. We tested the ability of AM to release ROI after preincubation for 48 h with insulin, transferrin, epidermal growth factor, fibroblast growth factor, hydrocortisone, progesterone, fibronectin, sodium selenite, putrescine-HCl, and a trace element mixture, either alone or in a limited number of combinations. In no case did such pretreatment condition AM for subsequent ROI production upon stimulation with Con A.

The ability of monocyte-derived macrophages to release ROI is known to be enhanced by IFN-γ, a 50 K mol wt glycoprotein lymphokine that is 90% inactivated by acid treatment at pH 2 for 2 h (15). As one way to determine whether our serum factor could be IFN-γ, we adjusted the pH of a 5% solution of FBS in MEM to 2.0, held the pH at 2.0 for 2 h at 4°C, then readjusted it to 7.4. When compared with control serum solutions maintained at pH 7.4, the acid-treated preparation was still active, retaining 67% of its activity (Table II).
Next, we tested human rIFN-γ for conditioning activity of rabbit AM, since we had found that human serum was fully capable of conditioning rabbit AM for ROI release (Table I). No activity was detected after incubation of rIFN-γ with rabbit AM for 48 h at 0.01–1,000 U/ml (data not shown).

While LPS does not trigger ROI production by peritoneal macrophages (16, 17), it has been reported (18) to "prime" such cells, causing them to release ROI in enhanced quantities upon subsequent triggering with other membrane-active agents. Thus, it seemed possible that the conditioning effect we were observing with serum resulted from priming by contaminating endotoxin. Accordingly, we precultured AM in medium containing LPS at 1.0 ng/ml to 1.0 µg/ml, but in the absence of serum; as positive and negative controls, we cultured other AM in the presence of 5% FBS or with serum-free medium, respectively. After 24, 48, and 72 h of culture, the cells were triggered with Con A and assessed for ROI production. As expected, cells incubated for 48–72 h in the presence of 5% FBS readily released H₂O₂, while cells incubated in medium alone produced little if any H₂O₂. Cells incubated in the presence of LPS alone showed no increase in H₂O₂ release over that by media controls (Table III). In related experiments, we further observed that addition of LPS to FBS failed to augment the ability of FBS to condition AM for ROI release (data not shown).

The reversibility of this serum effect was assessed by incubating aliquots of AM for 48 h in the presence and absence of serum, respectively. At this point, cells that had been initially cultured in serum-containing medium were washed and recultured for an additional 48 h in serum-free medium, while cells initially cultured in the absence of serum were washed and recultured in serum-containing medium. Control cultures containing MEM or MEM supplemented with 5% FBS were also cultured for 48 h, at which time the monolayers were washed and recultured in the same medium. The ability of these AM to release H₂O₂ upon triggering with Con A was assessed throughout the culture period. As expected (Fig. 5), cells initially cultured in serum-containing medium acquired the ability to release ROI; cells cultured in serum-free medium did not. However, upon transfer to serum-free medium, the serum-conditioned cells rapidly lost the

### Table III

| Conditioning agent | Triggering agent | H₂O₂/30 min per 5 × 10⁵ cells |
|--------------------|-----------------|-----------------------------|
|                    |                 | 24 h | 48 h | 72 h |
| None               | 10 µg/ml Con A  | 0.8 ± 0.3 | 6.1 ± 2.1 | 4.6 ± 1.9 |
| 5% FBS             | 10 µg/ml Con A  | 7.9 ± 1.9 | 24.3 ± 4.1 | 25.0 ± 1.7 |
| 0.001 µg/ml LPS    | 10 µg/ml Con A  | 0.8 ± 0.2 | 6.1 ± 2.2 | 7.6 ± 3.5 |
| 0.01 µg/ml LPS     | 10 µg/ml Con A  | 0.9 ± 0.4 | 5.5 ± 2.1 | 7.6 ± 3.0 |
| 0.1 µg/ml LPS      | 10 µg/ml Con A  | 0.9 ± 0.3 | 6.7 ± 2.5 | 7.2 ± 2.8 |
| 1.0 µg/ml LPS      | 10 µg/ml Con A  | 0.9 ± 0.3 | 5.5 ± 2.3 | 7.9 ± 3.5 |
| None               | None            | 0.0 ± 0.0 | 4.8 ± 1.7 | 1.9 ± 1.0 |
| 5% FBS             | None            | 0.5 ± 0.2 | 13.1 ± 1.2 | 11.3 ± 2.3 |

AM were conditioned for 24–72 h with the appropriate conditioning agents, triggered with or without 10 µg/ml Con A, and assayed for H₂O₂ release. Data represents the mean of nine values from three rabbits assayed in triplicate ± SEM.
ability to release $H_2O_2$, whereas cells transferred from serum-free medium to serum-containing medium showed a progressive increase in $H_2O_2$-releasing capacity (Fig. 5).

In preliminary studies to assess a possible mechanism by which serum enhances the amount of ROI released by AM, we examined the NADPH oxidase activity of freshly harvested cells as well as cells cultured 48 h in serum-containing or serum-free media. Pretreatment of all cells with 10 mM DDC to inhibit endogenous SOD activity contributed to the sensitivity of the assay. Initial experiments with freshly harvested AM showed that PMA-triggered intact cells were routinely unable to generate $O_2^-$. Also, lysis of these freshly isolated cells with DOC, followed by addition of NADPH, resulted in no detectable NADPH oxidase activity (Table IV). Intact cells incubated with 10% FBS for 48 h, however, were capable of producing $O_2^-$ when triggered with PMA. When these triggered cells were lysed with DOC and assayed for NADPH oxidase activity by adding a saturating concentration of NADPH, they continued to produce $O_2^-$. In contrast, the rate of $O_2^-$ production by intact AM incubated in serum-free medium for 48 h was <20% of that by conditioned AM and, again, no NADPH oxidase activity could be detected in lysed cell preparations. The detection of any $O_2^-$ by intact as well as lysed cells in these experiments could be totally inhibited by the
TABLE IV

NADPH Oxidase Activity of AM

| Conditioning agent | Time (h) | Intact cells | DOC-treated cells plus 1.2 mM NADPH |
|--------------------|----------|--------------|-------------------------------------|
| None               | 0        | <0.1         | <0.1                                |
| None               | 48       | 0.26 ± 0.07  | <0.1                                |
| 10% FBS            | 48       | 1.27 ± 0.18  | 0.80 ± 0.22                         |

Freshly harvested AM, and AM cultured 48 h with or without serum, were assayed for O$_2^-$ production after triggering the intact cells with 1 µg/ml PMA. To assay NADPH oxidase activity we lysed the intact cells with DOC and measured the rate of O$_2^-$ production after addition of 1.2 mM NADPH as the substrate. Data represent the mean of three experiments ± SEM.

TABLE V

Cycloheximide Inhibition of Serum-conditioning Activity

| Conditioning agent | Time of cycloheximide addition (h) | H$_2$O$_2$/30 min per 5 × 10$^6$ cells (nmol) |
|--------------------|-----------------------------------|---------------------------------------------|
| None               | —                                 | 1.6 ± 0.5                                   |
| 10% FBS            | —                                 | 19.0 ± 1.0                                  |
| 10% FBS + 0.5 µg/ml CyH | 18                                      | 13.1 ± 1.4                                  |
| 10% FBS + 1.0 µg/ml CyH | 18                                      | 5.2 ± 0.6                                   |
| 10% FBS + 0.5 µg/ml CyH | 24                                      | 19.6 ± 0.8                                  |
| 10% FBS + 1.0 µg/ml CyH | 24                                      | 20.2 ± 0.6                                  |

AM were cultured for 24 h in medium containing 10% FBS. Cycloheximide (CyH) was added to some cultures at a final concentration of 0.5 or 1.0 µg/ml at 18 or 24 h. All cultures were then triggered with Con A and assayed for H$_2$O$_2$ release. Data represent the mean of six values from two rabbits assayed in triplicate ± SEM.

addition of 300 U/ml SOD. In a single experiment using AM from three rabbits, we determined the $V_{max}$ and $K_m$ of NADPH oxidase for serum-conditioned cells to be 2.7 nmol/min per 10$^6$ cells and 0.57 mM NADPH, respectively. Due to the low activity of freshly isolated AM or AM cultured 48 h in serum-free medium, we were unable to determine a $V_{max}$ or $K_m$ for these cells.

In a separate study, we have shown that the protein synthesis inhibitor, cycloheximide, inhibits serum conditioning of rabbit AM for subsequent triggered ROI release (Table V). In these experiments, AM cultures were incubated for 24 h with 10% FBS. In addition, some cultures received 0.5 or 1.0 µg/ml cycloheximide, either at 18 h after culture initiation or just before triggering at 24 h. These concentrations of cycloheximide have been previously shown (19) to inhibit protein synthesis of mouse peritoneal macrophages. All cultures were then washed and assayed for their ability to release H$_2$O$_2$ upon Con A triggering. The results clearly show that serum-conditioned cells incubated for the final 6 h with 0.5 or 1.0 µg/ml cycloheximide released significantly reduced amounts of H$_2$O$_2$ (Table V). Addition of cycloheximide just before triggering at 24 h had
Figure 6. Gel filtration of AM-conditioning activity on Sephacryl S-200. Dialyzed fractions (1:2 dilution) were incubated with AM for 48 h before assessment of Con A (10 μg/ml)-triggered H$_2$O$_2$ release. The H$_2$O$_2$ generating activity (bars) and A$_{280}$ (---) are indicated as well as the position of protein markers used in column calibration (†).

Lastly, we undertook initial studies to characterize the component(s) in serum responsible for this conditioning effect. Pooled rabbit serum was fractionated as outlined in Materials and Methods. The conditioning activity precipitated at 40–80% saturated (NH$_4$)$_2$SO$_4$, and did not bind to a Cibacron Blue affinity column. Subsequent gel filtration analysis on Sephacryl S-200 revealed a major peak of activity with an estimated molecular weight of 30–50 K (Fig. 6).

Discussion

The issue of whether AM produce and release ROI is of particular importance since these cells are present within the air spaces in large numbers, and are likely to come into direct contact with leukocyte-activating agents inhaled from the environment (20). While numerous laboratories (12, 21–23) have shown that stimulated neutrophils and peritoneal macrophages produce and release ROI into the extracellular environment, experiments with AM have yielded inconsistent results. For example, while rat and guinea pig AM stimulated by PMA or heat-killed E. coli will produce ROI (5–7), freshly isolated rabbit AM release little or no ROI when stimulated with zymosan, opsonized zymosan, heat-killed bacteria, F-Met-Leu-Phe, or Con A (8, 24–28). Similarly, human AM produce relatively low levels of ROI relative to that produced by human neutrophils (29, 30), leading Papermaster-Bender et al. (30) to conclude that an inability of the no effect on H$_2$O$_2$ release. Moreover, incubation of cells with these concentrations of cycloheximide for 6 h did not affect the cell number or cell viability as determined by dye exclusion.
AM to produce ROI might protect the lung against inflammatory damage. The reported inability of rabbit AM to release ROI has not been absolute, however, since they have been shown (8) to release ROI when triggered by Con A or heat-killed organisms in the presence of cytochalasin B. Sugimoto et al. (9) showed that rabbit AM cultured for 24 h in the presence of 10% FBS released ROI after addition of cytochalasin B alone. However, the mechanism of action of cytochalasin B in these studies is not clear; it may act by inhibiting internalization of membrane segments (31), by increasing the activation of NADPH oxidase (32), or by direct stimulation of the cell membrane (9).

In this paper we show that normal resident rabbit AM are indeed fully capable of releasing ROI after stimulation by membrane-active agents after AM have been conditioned by culture in the presence of 5–10% serum for 24–48 h. Moreover, this effect appears to be due to a discrete macromolecular serum constituent that precipitates at 40–80% saturated ammonium sulfate, does not bind Cibacron Blue affinity columns, and has an apparent molecular weight of approximately 30–50 K. These findings, together with the inability of serum albumin to condition AM, suggest that such conditioning of AM for ROI release is not simply due to a general protein effect. Moreover, our inability to condition AM with LPS in the absence of serum further indicates that this serum factor is also not due to contaminating endotoxin.

To ascertain whether such a serum-conditioning requirement was unique for normal rabbit alveolar macrophage ROI production, we have also recently studied alveolar macrophages from germ-free rats that were confirmed to have no pulmonary infections. Like normal rabbit AM, O₂⁻ release was demonstrated only after these rat AM had been precultured for 48–72 h in 5% FBS (manuscript in preparation).

Evidence exists for a conditioning action by serum proteins on macrophage function. Musson (33) found that human serum induces maturation of human monocytes in culture, as assessed by changes in cytolytic activity, intracellular lysosomal enzymes, and nonspecific esterase activity. Bianco et al. (34) described two distinct serum factors (one of which appears to be a low molecular weight, complement-derived protein) that cause peritoneal macrophages to spread and increase their surface area. Leonard et al. (35) reported that serum albumin caused both resident and mouse peritoneal exudate macrophages to become responsive to chemotactic stimuli. They also described another serum protein, which they termed "macrophage-stimulating protein," which enhanced the responsiveness of resident peritoneal macrophages to chemotactic stimuli, with a molecular weight of 100 K and a pI of 7.0 (35). Hsueh et al. (36) recently reported that both FBS and rabbit serum, but not albumin, stimulate cultured rabbit AM to synthesize prostaglandins and release lysosomal enzymes. They also observed that, after gel filtration chromatography, the major peak of activity was associated with an apparent molecular weight of 150 K. Chapman and Hibbs (37) described a human serum factor that enhances the tumoricidal capacity of peritoneal macrophages, and was found in the last of three protein peaks obtained after passage of whole serum through a Sephadex G-200 column. Finally, data published by Johnston et al. (38) suggest that resident mouse peritoneal macro-
phages may show enhanced ROI release if they are preincubated in serum-supplemented medium (20%) before stimulation.

The question arises whether our serum factor is providing one of the two signals reportedly required for macrophage ROI production (18, 22). For example, muramyl dipeptide (MDP) and LPS have both been shown (18, 22, 39) to deliver a first signal that "primes" peritoneal macrophages, enhancing their production of ROI upon triggering by the second signal, PMA or opsonized zymosan. Whereas Pabst and Johnston (18) reported that such LPS priming was evident within 30 min, in our systems the effect produced by serum required 24-48 h. Moreover, we were unable to demonstrate any enhancing action by LPS on either resident or serum-conditioned alveolar macrophages, even after 72 h of incubation. It is noteworthy that previous studies of macrophage priming have been carried out, for the most part, in the presence of 10-20% FBS. Thus, the priming action of LPS and MDP seems to be distinct from the serum effect that we describe; the possibility that MDP replaces or augments the serum effect remains untested.

Of particular relevance is the potential relationship of this serum factor to IFN-γ, a 50 K mol wt glycoprotein produced by activated lymphocytes. Both human monocyte-derived macrophages and mouse peritoneal macrophages have been shown (40, 41) to be activated by human or mouse IFN-γ, respectively, enhancing their ability to produce ROI upon stimulation with PMA. We feel it unlikely that our serum factor is IFN-γ for the following reasons: (a) it seems unlikely that normal rabbit serum, pooled human serum, and, in particular, FBS, all would contain biologically active concentrations of IFN-γ; (b) demonstrations of lymphocyte-derived IFN-γ potentiation of ROI production have been performed in the presence of 10-20% serum (40, 41), concentrations that, by themselves, are maximally effective in our assay; (c) our factor retained 67% of its activity when held at pH 2.0 for 2 h, whereas IFN-γ is inactivated under these conditions (15); (d) while human serum readily conditioned rabbit AM for ROI release, human rIFN-γ did not at concentrations of 0.01-1000 U/ml.

Recently, Tsunawaki and Nathan (13) reported that increased superoxide-generating capacity of activated (e.g., NaIO₄ elicited) vs. resident mouse peritoneal macrophages can be attributed to an increase in the affinity of their NADPH oxidase for its substrate. These investigators found that the $K_m$ of NADPH oxidase was considerably lower for activated cells, whereas the $V_{max}$ of the enzyme from activated cells was comparable to that of resident macrophages. In a similar study, Sasada et al. (42) reported that LPS-elicited mouse peritoneal macrophages contained higher cellular concentrations of NADPH, while their NADPH oxidase displayed a higher $V_{max}$ and a lower $K_m$ for NADPH than did the oxidase from normal cells. Since both groups of investigators suggested that the enhanced oxidative response of activated (or conditioned) macrophages is due, in part, to modification of this enzyme responsible for the production of $O_2^-$, we looked for similar differences between serum-conditioned AM and freshly harvested AM or AM cultured in the absence of serum. We readily detected NADPH oxidase activity in rabbit AM conditioned with serum-containing medium for 48 h, and determined $V_{max}$ and $K_m$ values of 2.7 nmol/min per $10^6$ cells and 0.57 mM NADPH, respectively. On the other hand, cells incubated 48 h in serum-free
medium or freshly harvested AM showed no detectable NADPH oxidase activity. Therefore, it was impossible to determine $V_{\text{max}}$ and $K_m$ values using these cells. The observed $K_m$ for serum-conditioned rabbit AM was comparable to that reported for resident (but ROI producing) mouse peritoneal macrophages (13), but the observed $V_{\text{max}}$ of our cells was notably greater than that of resident activated mouse peritoneal cells.

Our finding that the presence of cycloheximide significantly reduces $H_2O_2$ release by serum-treated cells suggests that protein synthesis is required for the serum-conditioning effect. Such protein synthesis is presumably involved in the generation of NADPH oxidase activity, and conceivably includes the enzyme itself. Moreover, there appears to be a requirement for ongoing protein synthesis, since our reversibility experiment shows that serum-conditioned cells transferred to serum-free media lose their ability to generate ROI.

In addition to observing that serum conditions AM for enhanced ROI generation upon stimulation, we have confirmed the differential ability of Con A and LPS to trigger ROI release. This is of particular interest to us because previous studies in our laboratory (43, 44) revealed that Con A, when inhaled repeatedly, initiates immune complex formation in the lung, whereas LPS does not, even though both are capable of initiating pulmonary interstitial inflammation. The mechanism by which Con A initiates immune complex formation is not known; it might be mediated in part by stimulation of conditioned AM to release ROI. The inability of LPS to trigger ROI production by AM parallels findings by others using peritoneal macrophages (16, 17). This contrasts with the highly effective triggering by LPS of interleukin 1 release from AM (45), illustrating the differential responsiveness of macrophages to diverse stimuli (22).

In conclusion, normal resident rabbit AM are notable in that (a) they require conditioning by a plasma factor(s) before they can be triggered to release ROI, and (b) they do not appear to be primed or triggered by LPS for subsequent ROI production. We propose that these properties constitute an inherent protective mechanism of the lung against AM-mediated tissue injury, in accord with the concept proposed by Papermaster-Bender (30). However, it is likely that pulmonary inflammatory reactions, initiated by other mediator systems, may cause increases in vascular permeability, with transudation of plasma proteins into alveolar spaces, thereby causing AM to become conditioned in situ for enhanced generation of ROI.

Summary

Alveolar macrophages (AM) from pathogen-free rabbits were unable to release reactive oxygen intermediates (ROI) unless they were conditioned in serum for 24–48 h before triggering with membrane-active agents. The degree of serum conditioning of AM depended upon the concentration of serum used; optimal ROI release was obtained at or above 7.5% fetal bovine serum (FBS). FBS, autologous rabbit serum, pooled rabbit serum, and pooled human serum were each capable of conditioning AM for release of ROI. Serum conditioning of AM requires synthesis of new protein(s); and the enzyme required for ROI production, NADPH oxidase, was only detectable in serum-conditioned cells. Moreover, serum-conditioned cells lost their ability to release ROI after transfer to serum-
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free medium, while cells maintained in serum-free medium acquired the capacity to release ROI after their transfer to serum-containing medium, demonstrating the reversibility of the phenomenon. Initial purification data indicate that conditioning is mediated by a discrete serum constituent, which precipitates 40–80% saturated ammonium sulfate, does not bind to Cibacron Blue columns, and has a molecular weight of 30,000 to 50,000, as determined by molecular exclusion chromatography. Unlike gamma interferon, which also enhances ROI release by macrophages, our serum-conditioning factor is not acid labile, retaining 67% of its activity after 120 min incubation at pH 2.0. Moreover, it does not appear to be a contaminating endotoxin, since LPS neither conditioned AM for ROI production, nor triggered ROI production by serum-conditioned AM. We propose that such a conditioning requirement may normally protect the lung against ROI-mediated tissue injury. However, during a pulmonary inflammatory reaction initiated by other mediator systems, the resulting transudation of plasma proteins into the alveolar spaces may condition AM in situ for ROI production.

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References
1. Holmes, B., A. R. Page, and R. A. Good. 1967. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. J. Clin. Invest. 46:1422.
2. Crapo, J. O., and B. A. Freeman. 1982. Biology of disease. Free radicals and tissue injury. Lab. Invest. 47:412.
3. Goldstein, I. A., D. Roos, H. B. Kaplan, and G. Weissman. 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. J. Clin. Invest. 56:1155.
4. Pertone, W. F., D. K. English, K. Wong, and J. M. McCord. 1980. Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma. Proc. Natl. Acad. Sci. USA. 77:1159.
5. Kaneda, M., K. Kakinuma, T. Yamaguchi, and K. Shimada. 1980. Comparative studies on alveolar macrophages and polymorphonuclear leukocytes. II. The ability of guinea pig alveolar macrophages to produce H$_2$O$_2$. J. Biochem. 88:1159.
6. Sweeney, T. D., V. Castranova, L. Bowman, and P. R. Miles. 1981. Factors which affect superoxide anion release from rat alveolar macrophages. Exp. Lung Res. 2:85.
7. Ward, P. A., R. E. Duque, M. C. Sulavik, and K. J. Johnson. 1983. In vitro and in vivo stimulation of rat neutrophils and alveolar macrophages by immune complexes. Production of O$_2^-$ and H$_2$O$_2$. Am. J. Pathol. 110:297.
8. Rossi, F., B. Ballavite, A. Dobrina, P. Dri, and G. Zabucchi. 1978. Oxidative metabolism of mononuclear phagocytes. In Mononuclear Phagocytes. R. van Furth, editor. Blackwell Scientific Publications, Oxford. 1187–1217.
9. Sugimoto, M., S. Higuchi, M. Ando, S. Horio, and H. Tokuomi. 1982. The effect of cytochalasin B on the superoxide production by alveolar macrophages obtained from normal rabbit lungs. J. Reticuloendothel. Soc. 51:117.
10. Myrvik, Q. N. 1961. Studies on pulmonary alveolar macrophages from normal rabbit: a technique to procure them in a high state of purity. J. Immunol. 86:126.
11. Pick, E., and Y. Keisari. 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Methods. 38:161.
12. Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1973. Biological defence mechanisms: the production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 52:741.
13. Tsunawaki, S., and C. F. Nathan. 1984. Enzymatic basis of macrophage activation. Kinetic analysis of superoxide production in lysates of resident and activated mouse peritoneal macrophages and granulocytes. J. Biol. Chem. 259:4305.
14. Kakinuma, K., and S. Minakami. 1978. Effects of fatty acids on superoxide radical generation in leukocytes. Biochim. Biophys. Acta. 538:50.
15. Kleinschmidt, W. J., and R. M. Schultz. 1982. Similarities of murine gamma interferon and the lymphokine that renders macrophages cytotoxic. J. Interferon Res. 2:291.
16. Kaku, M., K. Yagawa, S. Nagao, and A. Tanaka. 1983. Enhanced superoxide anion release from phagocytes by muramyl dipeptide or lipopolysaccharide. Infect. Immunol. 39:559.
17. Nathan, C. F., L. H. Brukner, S. C. Silverstein, and Z. A. Cohn. 1979. Extracellular cytosis by activated macrophages and granulocytes. I. Pharmacologic triggering of effector cells and the release of hydrogen peroxide. J. Exp. Med. 149:84.
18. Pabst, M. J., and R. B. Johnston. 1982. Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. J. Exp. Med. 151:101.
19. Blasi, E., R. B. Herberman, and L. Varesio. 1984. Requirement for protein synthesis for induction of macrophage tumoricidal activity by IFN-α and IFN-β but not IFN-γ. J. Immunol. 132:3226.
20. Willoughby, W. F., and J. B. Willoughby. 1984. Antigen handling. In Immunology of the Lung and Upper Respiratory Tract. John Bienenstock, editor. McGraw-Hill Book Co., New York. 174–190.
21. Klebanoff, S. J., and R. A. Clark. 1978. The Neutrophil and Clinical Disorders, Elsevier/North-Holland Biomedical Press, Amsterdam. 283–488.
22. Nathan, C. F., and R. K. Root. 1977. Hydrogen peroxide release from mouse peritoneal macrophages. Dependence on sequential activation and triggering. J. Exp. Med. 146:1648.
23. Pick, E., and Y. Keisari. 1981. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages, induction by multiple nonphagocytic stimuli. Cell. Immunol. 59:301.
24. De Chatelet, L. R., D. Mullikin, and C. E. McCall. 1975. The generation of superoxide anion by various types of phagocytes. J. Infect. Dis. 131:433.
25. Tsan, M. F. 1977. Stimulation of the hexose monophosphate shunt independent of hydrogen peroxide and superoxide production in rabbit alveolar macrophages during phagocytosis. Blood. 50:935.
26. Hatch, G. E., D. E. Gardner, and D. B. Menzel. 1978. Chemiluminescence of phagocytic cells caused by N-formylmethionyl peptides. J. Exp. Med. 147:182.
27. Miles, P. R., V. Castronova, and P. Lee. 1978. Reactive forms of oxygen and chemiluminescence in phagocytizing rabbit alveolar macrophages. Am. J. Physiol. 235:C103.
28. Yamaguchi, T., K. Kakinuma, M. Kaneda, and K. Shimada. 1980. Comparative studies on alveolar macrophages and polymorphonuclear leukocytes. I. H₂O₂ generation by rabbit alveolar macrophages. J. Biochem. 87:1449.
29. Hoidal, J. R., J. E. Repine, G. D. Beall, F. L. Rasp, and J. G. White. 1978. The effect of phorbol myristate acetate on the metabolism and ultrastructure of human alveolar macrophages. Am. J. Pathol. 91:469.
30. Papermaster-Bender, G., M. E. Whitcomb, A. L. Sagone. 1980. Characterization of
the metabolic responses of the human pulmonary alveolar macrophages. *J. Reticuloendothel. Soc.* 28:129.

31. Gee, J. B. L., and A. S. Khandwala. 1976. Oxygen metabolism in the alveolar macrophage: friend or foe? *J. Reticuloendothel. Soc.* 19:229.

32. Zabucchi, G., G. Berton, and M. R. Soranzo. 1981. Mechanism of the potentiating effect of cytochalasin B on the respiratory burst induced by concavalin A in leukocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 125:165.

33. Musson, R. A. 1983. Human serum induces maturation of human monocytes in vitro. Changes in cytolytic activity, intracellular lysosomal enzymes, and nonspecific esterase activity. *Am. J. Pathol.* 111:331.

34. Bianco, C., A. Eden, and Z. A. Cohn. 1976. The induction of macrophage spreading: role of coagulation factors and the complement system. *J. Exp. Med.* 144:1531.

35. Leonard, E. J., and A. Skeel. 1980. Functional differences between resident and exudate peritoneal mouse macrophages: specific serum protein requirements for responsiveness to chemotoxins. *J. Reticuloendothel. Soc.* 28:437.

36. Hsueh, W., R. L. Jordon, H. H. Harrison, and M. A. Cobb. 1983. Serum and plasma stimulate prostaglandin production by alveolar macrophages. *Prostaglandins.* 25:793.

37. Chapman, H. A., and J. B. Hibbs. 1977. Modulation of macrophage tumoricidal capability by components of normal serum: a central role for lipid. *Science (Wash. DC)* 197:282.

38. Johnston, R. B., C. A. Godzik, and Z. A. Cohn. 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. Exp. Med.* 148:115.

39. Metzger, Z., J. T. Hoffeld, and J. J. Oppenheim. 1981. Regulation by PGE2 of the production of oxygen intermediates by LPS-activated macrophages. *J. Immunol.* 127:1109.

40. Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin. 1983. Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* 158:670.

41. Pace, J. L., S. W. Russell, R. D. Schreiber, A. Altman, and D. H. Katz. 1983. Macrophage activation: priming activity from a T-cell hybridoma is attributable to interferon-γ. *Proc. Natl. Acad. Sci. USA.* 80:3782.

42. Sasada, M., M. J. Pabst, and R. B. Johnston. 1983. Activation of mouse peritoneal macrophages by lipopolysaccharide alters the kinetic parameters of the superoxide-producing NADPH oxidase. *J. Biol. Chem.* 258:9631.

43. Willoughby, W. F., and J. B. Willoughby. 1984. Immunologic mechanisms of parenchymal lung injury. *Environ. Health Perspect.* 55:239.

44. Willoughby, W. F., J. B. Willoughby, B. B. Cantrell, and R. Wheelis. 1979. In vivo responses to inhaled proteins. II. Induction of interstitial pneumonitis and enhancement of immune complex-mediated alveolitis by inhaled concavalin A. *Lab. Invest.* 40:399.

45. Simon, P. L., and W. F. Willoughby. 1981. The role of subcellular factors in pulmonary immune function: physiological characteristics of two distinct species of lymphocyte-activating factor produced by rabbit alveolar macrophages. *J. Immunol.* 126:1534.