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# Activation of Alveolar Macrophages Exposed to Lavage-Procured Immunoglobulin G Obtained from Normal Rabbit Lungs

MASAYUKI ANDO, MORITAKA SUGA, KIYOSHI SHIMA, MINEHARU SUGIMOTO, AND HARUHIKO TOKUOMI

*The First Department of Internal Medicine, Kumamoto University Medical School, Kumamoto 860, Japan*

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Pulmonary washings from rabbits were freed of cells and added to the monolayers of homologous alveolar macrophages (AM). At 1 h after incubation with the pulmonary washings, many more cells adhered to glass, spread out, and showed enhanced Nitro Blue Tetrazolium reduction. The maximal effect of the pulmonary washings on AM activation was obtained 12 h after incubation. The AM activated by the pulmonary washings showed a higher capacity to inhibit the growth of intracellular BCG, and that capacity was correlated with the intensity of Nitro Blue Tetrazolium reduction by the AM. Gel filtration of the pulmonary washings through Sepharose 4B yielded five fractions. The factor that activated the AM functions was in fraction 4. When the immunoglobulin G in the fraction was removed by an immunoadsorbent column, AM activity was abolished. The effect of the immunoglobulin G was dose dependent, and minimal responses to $10^6$ cells per ml were obtained at a protein concentration of 20 $\mu$g/ml. Lymphokines had no effect on AM activation with respect to the morphological alterations and Nitro Blue Tetrazolium reduction during the 24-h observation time. In summary, AM from normal rabbits were soon activated markedly by lavage-procured immunoglobulin G, but not by lymphokines.

The role of alveolar macrophages (AM) as the major mechanisms of early resistance to bacterial infection has been shown in vivo (11, 17) and in vitro (12, 14). There are some inconsistent reports, however, that AM are unable to kill *Staphylococcus aureus* in vitro (16, 25). LaForce et al. showed that rat AM were unable to kill *S. aureus* in vitro, but could kill the bacilli in vitro when phagocytosis was allowed to take place in the whole animal (16). These results may suggest the possibility that the functions of AM are enhanced by some factors in alveolar lining layers, where AM reside.

The present study evaluates whether there are factors that activate the functions of AM in the pulmonary washings (PW) obtained from normal rabbits. Activation of macrophages in culture was measured by adherence to glass, by morphological alterations, by the amount of Nitro Blue Tetrazolium (NBT) reduction, and by the capacity of inhibition of the intracellular growth of BCG in AM.

The AM activation factor was found to be lavage-procured immunoglobulin G (IgG). Importance of AM activation by IgG in alveolar spaces is discussed with respect to early resistance to bacterial infection.

## MATERIALS AND METHODS

**Animals.** Female New Zealand white rabbits, weighing 2 to 3 kg, were used in all experiments.

**Culture media.** RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 1% L-glutamine (200 mM) and 10% fetal calf serum (FCS), heat inactivated for 30 min at 56°C, was used for all experiments. Penicillin (100 U/ml) was added. In some experiments, 0.2% lactalbumin hydrolysate (Difco Laboratories, Detroit, Mich.) was used instead of FCS.

**Harvest of macrophages.** Animals were exsanguinated by severing the carotid artery with a sharp blade. AM were harvested by the method of Myrvik et al. (22), using heparinized Hanks balanced salt solution (HBSS) and gently flushing the lungs via the trachea. Peritoneal exudate cells (PEC) were obtained with 200 ml of heparinized HBSS 4 days after intraperitoneal injection of 100 ml of 0.4% glycogen in saline, as previously reported (1). The two types of cells were washed three times with HBSS and resuspended in culture medium. Differential counts of AM averaged 98% macrophages, 1% lymphocytes, and 1% polymorphonuclear leukocytes, and those of PEC averaged 88% macrophages, 3% lymphocytes, and 9% polymorphonuclear leukocytes.

**Preparation of macrophage monolayers.** For the culture, cells, at a concentration of $10^6$ per chamber, were dispersed on four tissue culture slides (Lab-

Tek Products, Div. Miles Laboratories, Inc., Westmont, Ill.) as previously reported (1). After 2 h of incubation at 37°C in 5% $CO_2$ and 95% humidified air, the chambers were washed four times with HBSS to remove nonadherent cells, and fresh culture medium was added.

**Preparation of PW.** Normal rabbit lungs were washed five times by bronchial lavage, each with 25 ml of pyrogen-free saline. To determine that the blood in the lavages was not contaminated, two criteria were used, i.e., absence of erythrocytes in the sedimentation of the lavages and absence of hemoglobin assayed by spectrophotometric measurement as oxyhemoglobin (20). The pooled lavages (usually 120 ml) without blood contamination were centrifuged at 400 × *g* for 10 min and passed through a 0.45-$\mu$m membrane filter (Millipore Corp., Bedford, Mass.) to remove mucus, dust, and bacteria. The filtrate was concentrated 20-fold by ultrafiltration on a Diaflo membrane (UM 2, > molecular weight 1,000, Amicon Corp., Lexington, Mass.). After another passage through a 0.45-$\mu$m membrane filter, the solutions were designated as PW. Fresh PW were used in each experiment. Protein was determined by the procedure of Lowry et al. (19), with bovine serum albumin as the standard. Phosphorus was determined by a modification of the method of Beveridge and Johnson (7), and phospholipid content was obtained by multiplying the phosphorus content by 25.

**Gel filtration.** A column (2.5 by 90 cm) was prepared with Sepharose 4B and Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) by modifying the method of Colacicco et al. (10). Column equilibration and elution were performed with 1.0 M NaCl in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid. Five milliliters of PW (pooled from three rabbits) was passed through a Sepharose 4B column at 4°C at a constant flow rate of 10 ml/h. Fractions of 3 ml were collected, their optical densities were read at 280 nm, and the relevant tubes were pooled into five fractions. The fractions were concentrated to 4 ml and dialyzed against pyrogen-free saline at 4°C for 48 h. Molecular weights were determined essentially by the method of Andrews (2) on a Sepharose 4B and Sephadex G-200 column. Immunoglobulins in the fractions were identified by immunoelectrophoresis and Ouchterlony gel diffusion by using goat anti-rabbit IgG (Hyland Laboratories, Costa Mesa, Calif.) and goat anti-rabbit IgG serum (Medical and Biological Laboratories, Ltd. (MBL), Japan).

**Affinity chromatography.** The purity of the goat anti-rabbit IgG serum (MBL, Japan) was tested by Ouchterlony gel diffusion, and the immunoglobulin fractions from 5 ml of the serum were precipitated with ammonium sulfate at a final concentration of 40% saturation. The precipitates were dissolved in 0.1 M $NaHCO_3$ solution (pH 8.3), and the solutions were passed through a Sephadex G-50 column. The immunoglobulin fractions eluted in the void volume were collected and concentrated by Diaflo ultrafiltration. A total of 84 mg of the immunoglobulin preparation was bound to a bromocyan-activated Sepharose 4B (Pharmacia Fine Chemicals) column (29, 33). After coupling reaction, the gels were washed with a 0.1 M $NaHCO_3$ solution and exposed to 1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.3) for 2 h. The gels were washed sequentially with 0.1 M $NaHCO_3$ solution and 0.1 M acetate buffer (pH 4.0) and then washed with PBS and poured into the column (1 by 15 cm). A total of 3 ml of fraction 4 of the PW eluted from a Sepharose 4B column (protein, 3.3 mg) was slowly applied to the column, and after 10 min the unbound proteins and other components were washed out by slowly pumping 50 ml of PBS through the column. The effluents were concentrated, and the absence of contamination of IgG was checked by Ouchterlony gel diffusion. For the elution of IgG bound to the column, 16 ml of 0.1 M glycine-hydrochloride buffer (pH 2.3) followed by PBS were slowly pumped through the column. The effluents were immediately adjusted to pH 7.0 by the addition of 1 M glycine-NaOH buffer (pH 11.5) in each 2 ml of effluents, collected, and concentrated. Purity of IgG in the solutions was checked by Ouchterlony gel diffusion. A total of 0.7 mg of IgG was obtained. Two fractions were dialyzed against PBS for 48 h and sterilized by filtration.

**Preparation of normal serum IgG.** Rabbit serum IgG was prepared from pooled, normal rabbit serum by chromatography on diethylaminoethyl-cellulose (DE 52, Whatman, England) (28) and by gel filtration with Sephadex G-200 according to molecular size (2). The purity of IgG was determined by Ouchterlony gel diffusion.

**Pyrogen test.** The effluents passed through a Sepharose 4B column were injected into a rabbit intravenously, and the temperature of the rabbit was measured by rectum with a thermometer during the subsequent 4-h period.

**Preparation of LK.** Lymphokines (LK) were prepared from phytohemagglutinin (PHA)-activated lymphocytes, as previously reported (1). In brief, mesenteric lymph node lymphocytes were incubated in the presence of 6 $\mu$l of PHA-P (Difco) per ml for 18 h at 37°C and were washed three times with the culture medium to remove the free PHA-P. After another incubation for 24 h, the culture supernatants were used as a source of LK. The activity of macrophage migration inhibitory factor in the supernatants averaged 40%.

**Cellular adherence and morphological classification.** The number of macrophages remaining adherent around the center of the culture slide in the unit area (0.38 $mm^2$) was counted, and the cells were grouped as rounded or spreading cells by morphological criteria. The former were designated nonactivated macrophages, and the latter were designated activated macrophages, as previously reported (1).

**Qualitative and quantitative NBT reduction by macrophages.** Qualitative NBT (Sigma) reduction by macrophages was observed by the modification of the supravital method of Park et al. (24). Macrophages in the chamber slides were incubated in 0.3% NBT solution at 37°C for 20 min. The cells containing clumped formazan and 15 or more formazan granules were designated positive cells, as previously reported (1). Only 2 to 3% of the AM and PEC were positive in the NBT reduction test when tested immediately after harvesting. Quantitative NBT reduction was per-

formed by the method of Baehner and Nathan (4).

A 0.8-ml portion of culture medium in the chamber slides was gently removed, and 1.0 ml of 0.3% NBT solution was added to the chamber slides. After incubation at 37°C for 30 min, all macrophages, i.e., both attached and detached cells, were collected into the tube containing 1 ml of 1 N HCl. The precipitated formazan was pelleted by centrifugation at 1,000 × *g* for 10 min, resuspended in 2.5 ml of pyridine, and read at an optical density at 515 nm. No reduction of NBT by PW was found when NBT solutions were incubated with PW at 37°C for 30 min.

**Inhibition of the intracellular growth of BCG in the AM activated by PW.** A total of 80 mg of the lyophilized BCG strain (Japan BCG Laboratory, Tokyo) was dissolved in 10 ml of cold saline. The BCG suspension was allowed to stand at 4°C for 20 min and then centrifuged at 35 × *g* for 5 min to remove the larger bacillary clumps. After the opacity of the supernatant was read in a spectrophotometer at 570 nm, it was adjusted to an optical density of 1.52 nm ($2.3 \times 10^7$ viable bacilli/ml) by the addition of saline. The BCG suspension was added to a suspension of AM and incubated in Falcon plastic tubes at 37°C for 1 h. The ratio of bacteria to AM was 10:1. After incubation, the suspensions were collected and centrifuged at 125 × *g* for 5 min. The supernatant was discarded, and the pellets were washed three times with HBSS to remove nonphagocytized bacilli. The BCG-infected AM were resuspended with culture medium and dispersed on four culture slides (Lab-Tek). Without washing the chambers with HBSS, we cultivated the monolayers of infected AM with either PW or saline. The intracellular growth of BCG was evaluated by visually counting the bacilli within the infected macrophages.

**Statistical method.** The standard error of the mean and *P* values were calculated by using Student's *t* test (1).

## RESULTS

**Effects of PW on AM activation.** When the PW were added to AM monolayers to a protein concentration of 300 μg per ml of culture medium, many more cells adhered to the chamber slides and spread out and showed increased NBT reduction at 12 h, as compared with the AM in the control cultures to which the same volume (0.1 ml) of saline per ml of culture medium was added (Table 1). Increased NBT reduction by AM exposed to PW was found 1 h after incubation, but its maximal effects were found 12 h after incubation ($P < 0.003$ (Fig. 1). LK that were used as a control stimulant had no effect on AM from normal rabbits, with respect to the morphological alterations and NBT reduction during the 24-h observation time (Fig. 1).

AM exposed to PW inhibited the intracellular growth of BCG. AM in the monolayers incubated with 300 μg of PW per ml showed higher capacity to inhibit the intracellular growth of BCG than did those of control monolayers 3 days after incubation ($P < 0.002$) (Fig. 2 and 3). The amount of NBT reduction by the BCG-infected AM was markedly reduced, as compared with those of noninfected AM in the control monolayers 1 and 3 days after incubation ($P < 0.006$ and $P < 0.017$, respectively) (Fig. 4). However, when the BCG-infected AM were incubated with the PW, the amount of NBT reduction by the AM never reduced. The PW themselves neither inhibited the growth of BCG nor reduced NBT. The findings indicate that the antimicrobial activities of AM exposed to PW were correlated with the intensity of NBT reduction by the AM.

These results may suggest that the factor that activates AM is in the PW obtained from normal rabbit lungs and is different from LK. To find the factor in the PW, the following experiments were carried out with the NBT reduction test as a marker of AM activation.

**Effects of dialysis, temperature, and pH on the activity of the AM activation factor in PW.** The results are summarized in Table 2. The PW were dialyzed against saline at 4°C for 24 h, and the fluid both inside and outside the dialysis sac (Seamless cellulose tubing, Visking Co., Chicago, Ill.) was tested. The activity of the AM activation factor in the PW was not reduced by such dialysis. When the PW were heated at 80°C for 30 min, most of the activity was abolished. Heating at 60°C for 30 min partly reduced the activity.

To observe the effect of pH on the activity of the AM activation factor, the PW were dialyzed

TABLE 1. *Adherence to glass, morphological alterations, and NBT reduction test of AM exposed to PW from normal rabbit lungs*[a]

| Stimulants | Protein (μg/ml) | No. of AM per unit area (0.38 $mm^2$)[b] | *P* values | % Spreading cells[b] | *P* values | % NBT-positive cells[b] | *P* values | Quantitative NBT reduction[c] (% control) |
|---|---|---|---|---|---|---|---|---|
| PW | 300 | 163 ± 16 | $P < 0.05$ | 49.7 ± 4.4 | $P < 0.001$ | 43.0 ± 5.3 | $P < 0.001$ | 580 |
| Control | 0 | 133 ± 3 | | 27.5 ± 2.2 | | 16.4 ± 1.7 | | 100 |

[a] AM were incubated with PW for 12 h at 37°C.
[b] Each value represents the mean of seven experiments with standard error.
[c] Shown are the mean values of duplicated chamber slides of one experiment.

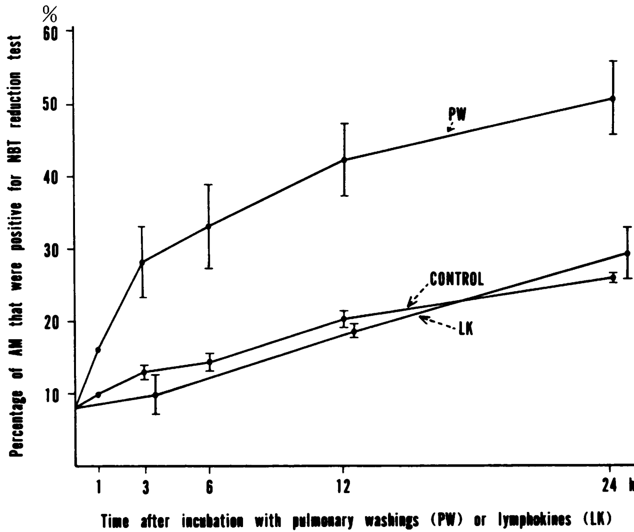


FIG. 1. *Time course of NBT reduction by AM exposed to PW or LK. PW were added to AM monolayers in a protein concentration of 300 μg per ml of culture medium. Culture supernatants of PHA-activated lymphocytes were used as LK and added to AM monolayers instead of culture medium (1). Each point represents the mean of four experiments with standard error. P values (PW versus control) at 3, 6, 12, and 24 h were 0.012, 0.006, 0.003, and 0.003, respectively.*

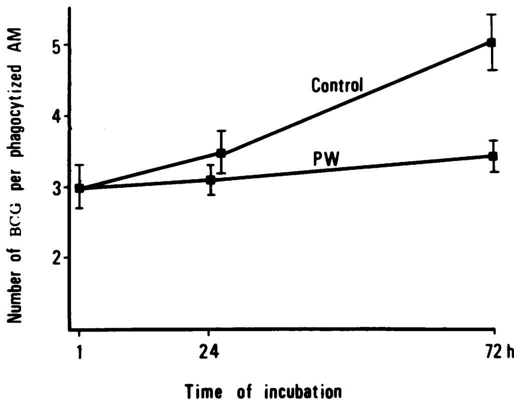


FIG. 2. *Inhibition of the intracellular growth of BCG in the AM exposed to PW. Each value represents the mean of five experiments with standard error. The average number of BCG per phagocytized AM in each experiment was obtained by counting 100 phagocytized AM around the center of the chamber slides. P value for 3 days: $P < 0.002$. The average number of BCG per phagocytized AM of 500 cells (five experiments with 100 cells in each experiment) was: 1 day, PW = 3.1 ± 0.1, control = 3.5 ± 0.1; 3 days, PW = 3.2 ± 0.1, control = 5.0 ± 0.2, (mean ± standard error).*

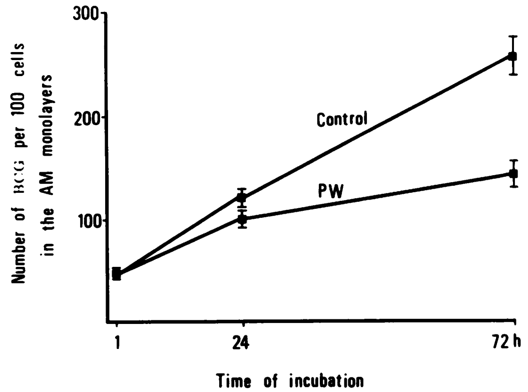


FIG. 3. *Number of BCG per 100 cells in the monolayers of BCG-infected AM that were incubated in the presence or absence of PW. Each value represents the mean of five experiments with standard error. In each experiment, 300 to 500 cells of both phagocytized and nonphagocytized AM were counted. The P value for 3 days: $P < 0.001$.*

against Britton-Robinson buffer (9) in a wide pH range from 24 h and readjusted to neutrality. After dialysis against saline for 48 h, the PW were passed through a 0.45-μm membrane filter to remove the fine precipitate. The activity of the AM activation factor in PW remained unchanged in the pH range between 4 and 9 (Table 2). However, when the PW were treated with pH 2, the activity of the factor was abolished. Treatment at pH 11 partly reduced the activity. These results indicated that the factor in PW was nondialyzable and unstable to both heat and extremes of pH.

**Activities of the AM activation factor in fraction 4 of the PW eluted from a Sepha-**

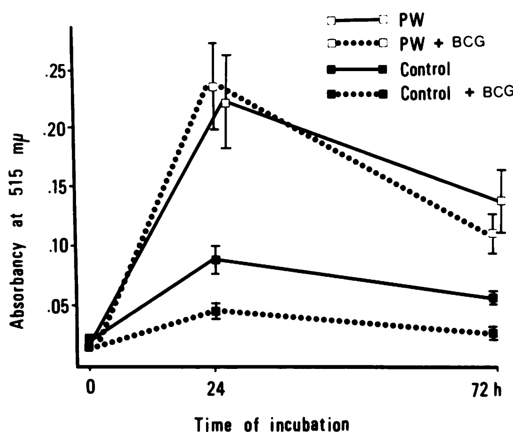


FIG. 4. *Effect of BCG infection on the NBT reduction by AM. Each of BCG-infected (····) and non-infected (——) AM was incubated in the presence (□) or absence (■) of PW. Each value represents the mean of five experiments with standard error. P values for 24 h: PW versus control = $P < 0.006$, PW + BCG versus control + BCG = $P < 0.001$, PW versus PW + BCG = $P > 0.40$, control versus control + BCG = $P < 0.006$; and for 72 h: PW versus control = $P < 0.006$, PW + BCG versus control + BCG = $P < 0.002$, PW versus PW + BCG = $P > 0.20$, control versus control + BCG = $P < 0.005$.*

TABLE 2. *Effect of dialysis, heating, and pH on the stability of the factor in PW that activates AM*

| Treatment | PW[a] | % Spreading AM[b] | % NBT test positive AM[b] |
|---|---|---|---|
| | None | 18 | 11 |
| | 300 | 34 | 33 |
| Dialysand | 300 | 34 | 31 |
| Dialysate | 300 | 19 | 12 |
| Heating at 60°C for 30 min | 300 | 30 | 25 |
| Heating at 80°C for 30 min | 300 | 19 | 12 |
| pH 2 for 24 h | 300 | 22 | 16 |
| pH 4 for 24 h | 300 | 34 | 33 |
| pH 9 for 24 h | 300 | 33 | 33 |
| pH 11 for 24 h | 300 | 34 | 28 |

[a] Protein concentration (μg/ml) of PW.

[b] AM were incubated with PW for 6 h at 37°C. Each value represents the mean of duplicated chamber slides of one experiment. Variations between duplicates ranged within 3% in each value.

**rose 4B.** To find the AM activation factor, the PW were applied to a Sepharose 4B column and the column effluents were pooled into five fractions: Fr-I, Fr-II, Fr-III, Fr-IV, and Fr-V (Fig. 5). Each fraction was concentrated to 4 ml and tested for the effects on AM activation. When fraction 4 (Fr-IV, molecular weight, 50,000 to 200,000) was added to AM monolayers, many more cells adhered to the chamber slides and spread out and showed increased NBT reduction 12 h after incubation ($P < 0.004$, $P < 0.001$, and $P < 0.004$, respectively) (Table 3). NBT reduction by AM was correlated with the spreading of the cells, but the rounded cells, as well as the spreading cells, in the culture slides stimulated by Fr-IV showed enhanced NBT reduction as compared with those in the controls. Other fractions had no effects on AM activation when judged by these criteria of AM activation.

**Effect of the IgG in fraction 4 on AM activation.** From the results of immunoelectrophoresis (Fig. 6) and the molecular size of fraction 4 (Fig. 5), it was hypothesized that IgG in the PW was the AM activation factor. When IgG in this fraction was removed by the immunoadsorbent column-bound anti-rabbit IgG, the residual solution, i.e., Fr-IV (IgG free), had no effect. IgG in fraction 4 had effects on AM activation similar to those of Fr-IV of the PW (Table 4). The effects of lavage-procured IgG on AM activation were dose dependent, and its minimal responses to $10^6$ cells per ml were obtained at a protein concentration of 20 μg per ml of culture medium (Fig. 7). The molecular weight of lavage-procured IgG was 160,000 when estimated by the method of Andrews with Sephadex G-200. IgG in normal rabbit serum had effects on AM activation similar to those of IgG in fraction 4 of the PW. In fraction 3 (Fr-III, molecular weight, 200,000 to 700,000) of the PW, there was immunoglobulin, probably secretory

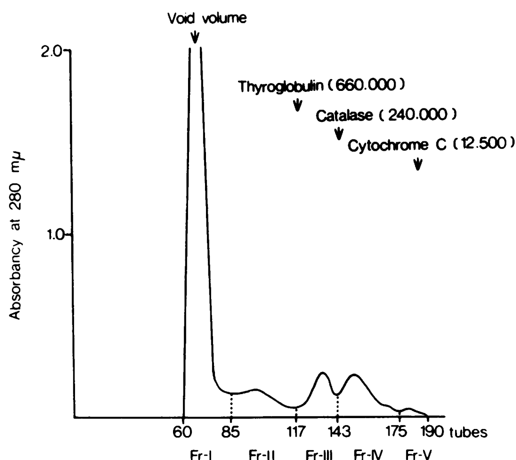


FIG. 5. *Fractionation of PW on Sepharose 4B. PW (4 ml) were applied on a column (2.5 by 90 cm). Effluent was collected in 3-ml fractions. Effluents were pooled as indicated in the figure.*

TABLE 3. *Effects of the fractions of PW eluted from Sepharose 4B column on the adherence to glass, spreading, and NBT reduction of AM*[a]

| Stimulant[b] | Protein (μg/ml)[c] | Phospholipid (μg/ml)[c] | No. of AM per unit area (0.38 mm²) | P values | % Spreading cells | P values | % NBT-positive cells | P values | Quantitative NBT reduction (% control) | P values |
|---|---|---|---|---|---|---|---|---|---|---|
| Fr-I | 62 | 560 | 131 ± 6 | | 21 ± 1 | | 12 ± 2 | | 90 ± 10 | |
| Fr-II | 54 | 320 | 125 ± 3 | | 21 ± 2 | | 11 ± 1 | | 90 ± 10 | |
| Fr-III | 100 | 552 | 127 ± 14 | | 31 ± 3 | $P < 0.012$ | 14 ± 2 | | 90 ± 20 | |
| Fr-IV | 100 | 270 | 163 ± 7 | $P < 0.004$ | 52 ± 5 | $P < 0.001$ | 42 ± 8 | $P < 0.004$ | 400 ± 80 | $P < 0.008$ |
| Fr-V | 50 | 266 | 128 ± 14 | | 22 ± 2 | | 12 ± 1 | | 120 ± 20 | |
| Control | 0 | 0 | 133 ± 3 | | 20 ± 2 | | 10 ± 1 | | 100 | |

[a] AM were incubated with the fractions of PW for 12 h at 37°C.

[b] Each fraction was concentrated to the same volume of 4 ml, and 0.1 ml of each fraction was added to the chamber slides except for 0.2 ml of Fr-V.

[c] Protein and phospholipid concentrations of the stimulant per ml of culture medium are shown. *P* values are at the value of control. Each value represents the mean of four experiments with standard error.

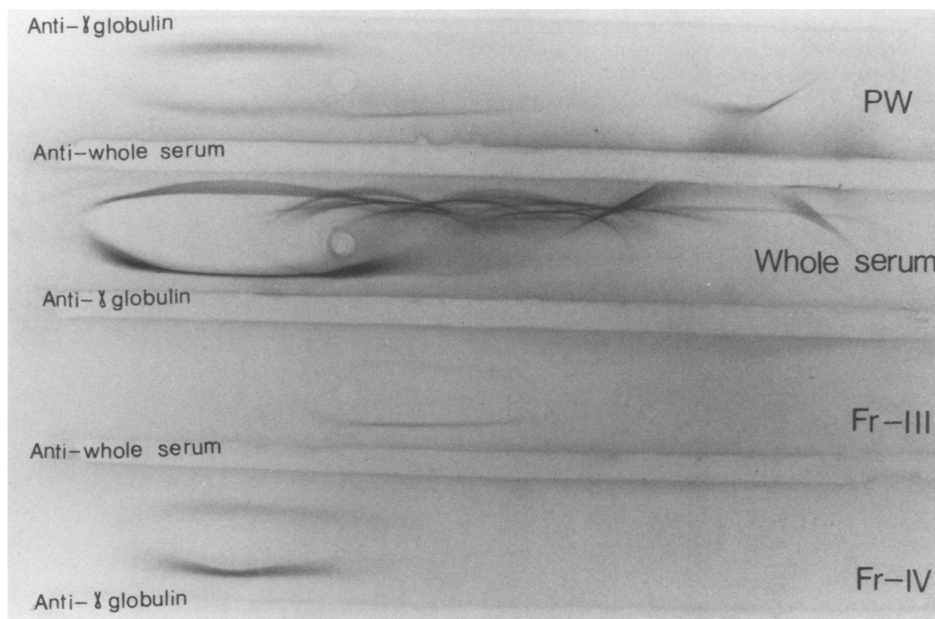


FIG. 6. *Immunoelectrophoresis of the fractions obtained by gel filtration on Sepharose 4B. Protein concentration: PW, 3 mg/ml; Fr-III, 1 mg/ml; Fr-IV, 1 mg/ml.*

IgA as estimated by the molecular size and by immunoelectrophoresis (Fig. 5 and 6), but the fraction (Fr-III) had no effects on the AM (Table 2). FCS used in culture medium was aggammaglobulinemic, and its effect on AM activation was similar to that of lactalbumin hydrolysate, which was used in culture medium instead of FCS.

These results clearly show that the factor in PW which increased adherence of AM to glass, spread out AM, and enhanced NBT reduction by AM was lavage-procured IgG.

**Effect of PW on PEC activation.** NBT reduction by PEC was enhanced by both Fr-III and Fr-IV 24 h after incubation. To investigate the discrepancy of the activation between PEC and AM, PW were eluted through a Sephadex G-200 column, and the resulting fractions were tested on the PEC monolayers. Both fraction 1 (molecular weight, >200,000, IgG free) eluted in the void volume and fraction 2, which contained IgG, had similar effects on the NBT reduction by PEC (Fig. 8). In other words, PEC activation was induced by various stimulants, not only by lavage-procured IgG, but also by other unknown factors in the PW or LK (1).

**Observations on the pyrogenicity of the AM activation factor in the PW.** After 5 ml (protein, 1 mg) of either Fr-III or Fr-IV was

TABLE 4. *Morphological alterations and NBT reduction of AM exposed to IgG in fraction 4 of the PW and IgG in serum*[a]

| Stimulant | Protein (μg/ml) | % Spreading cells | P values[b] | % NBT-positive cells | P values[b] | Quantitative NBT reduction (% control) | P values[b] |
|---|---|---|---|---|---|---|---|
| Fr-IV of PW[c] | | | | | | | |
| Whole Fr-IV | 100 | 52 ± 5 | $P < 0.005$ | 42 ± 8 | $P < 0.006$ | 400 ± 80 | $P < 0.006$ |
| Fr-IV (IgG free) | 40 | 21 ± 1 | | 15 ± 2 | | 110 ± 10 | |
| IgG in Fr-IV | 40 | 48 ± 2 | $P < 0.004$ | 34 ± 2 | $P < 0.001$ | 220 ± 20 | $P < 0.001$ |
| | 10 | | | | | 140 ± 10 | $P < 0.015$ |
| Rabbit serum[d] | | | | | | | |
| Serum (IgG free) | 340 | | | | | 180 | |
| IgG in serum | 300 | | | | | 830 | |
| | 150 | | | | | 700 | |
| Control | 0 | 23 ± 6 | | 12 ± 2 | | 100 | |
| LAH[e] | | | | | | 100 | |

[a] AM were incubated with the stimulants for 12 h at 37°C.
[b] P values are at the value of control.
[c] Each value represents the mean of four experiments with standard error.
[d] Shown are the mean of duplicated chamber slides of one experiment.
[e] Lactalbumin hydrolysate (LAH, 0.2%) was used in the culture medium instead of 10% FCS. The value represents the mean of duplicated chamber slides of one experiment.

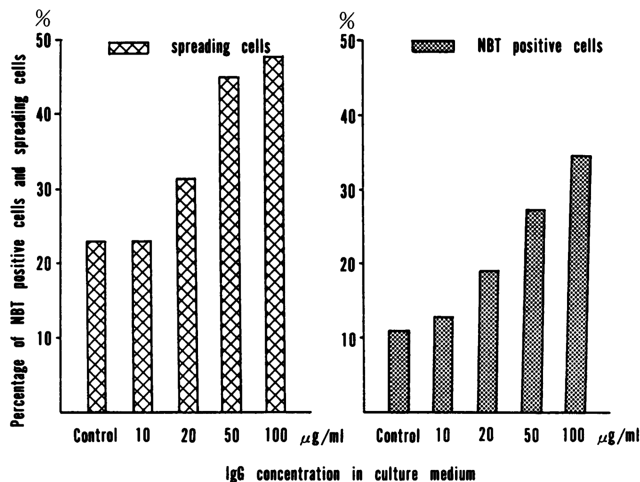


FIG. 7. *Correlation of the dose of lavage-procured IgG to morphological alterations and NBT reduction of AM. AM were incubated with lavage-procured IgG for 12 h at 37°C. Each value represents the mean of duplicated chamber slides of one experiment. Variations between duplicates ranged within 3% in each value.*

injected into a rabbit intravenously, the temperature taken by rectum did not fluctuate more than 0.3°C, similar to that of nontreated rabbits, during a 4-h observation time. Thus, the factor had no pyrogenic effects.

## DISCUSSION

The results presented in this report clearly show that the factor that activated AM with respect to adherence to glass, morphological alterations, NBT reduction, and antimicrobial activity was in the PW obtained from normal rabbit lungs. The factor was found to be IgG in fraction 4 of the PW eluted from Sepharose 4B when judged by the intensity of NBT reduction by the AM as a marker of AM activation. When the IgG in the fraction was removed by the immunoadsorbent column-bound anti-rabbit IgG, the activity in the fraction was abolished. The IgG obtained from the fraction had similar

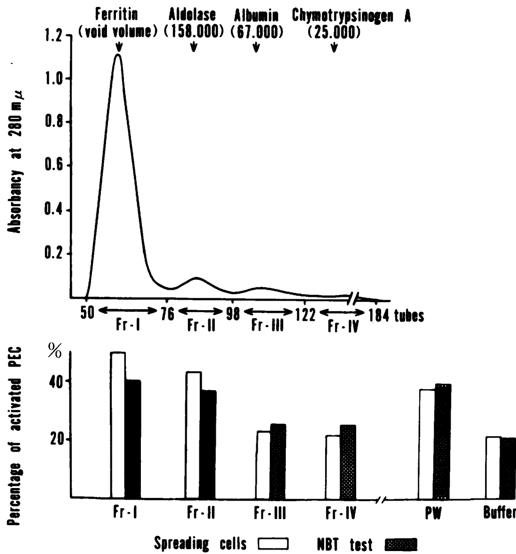


FIG. 8. *Activation of PEC exposed to the fractions of PW passed through a Sephadex G-200 column. Histochemical assay for the qualitative NBT reduction test was carried out to observe the morphological alterations of PEC. PEC were incubated with the stimulants for 48 h at 37°C. Protein concentrations (μg/ml) of the stimulants were: Fr-I, 30; Fr-II, 45: Fr-III, 85; Fr-IV, 85; and PW, 365. Each value represents the mean of two experiments.*

effects on AM activation as the PW. It is unlikely that the lavage-procured IgG was aggregated or denatured because the molecular weight of the IgG was 160,000, and rabbit serum IgG obtained by ion-exchange chromatography and by gel filtration had an effect on the AM similar to that of lavage-procured IgG. The immunoglobulin in fraction 3 (molecular weight, 200,000 to 700,000), probably secretory IgA as estimated by the molecular size and by immunoelectrophoresis, had no effect on AM activation. These findings may suggest that the Fc receptor on macrophages is critical for the activation by IgG. Macrophage receptors for immunoglobulins are specific for the Fc fragments of IgG and IgM, but not for those of IgA (3, 30).

NBT reduction by AM may reflect the enhanced oxidative metabolism of macrophages. This is consistent with our data showing that spreading cells were more positive for the NBT reduction test than were rounded cells and that PEC activated by either activated lymphocytes or LK also show an enhanced NBT reduction (1). There is evidence that PEC activated by LK show enhanced oxidative metabolism (23). Moreover, it is well known that NBT reduction in neutrophils is associated with oxidative metabolism characterized by increases in oxygen consumption, hexose monophosphate shunt activity, and producton of hydrogen peroxide (4, 26). Baehner et al. have shown that the majority of NBT reduction by leukocytes is due to superoxide anion (5). If so in AM, the results presented here suggest that IgG may directly act on the oxidative metabolism of AM. Subsequent changes in macrophage function may be important on the defense mechanism against bacterial infection in lungs, probably by the $H_2O_2$-generating microbicidal system (14). In fact, the AM activated by PW showed a higher capacity to inhibit the intracellular growth of BCG in the AM, and the antimicrobial activity of the activated AM was correlated with the intensity of NBT reduction by the AM. Further experiments on the mechanisms of the antimicrobial activity of the AM activated by lavage-procured IgG are being studied in our laboratory with respect to oxidative metabolisms.

Recently, LaForce et al. (15, 16) and Juers et al. (13) reported that the *S. aureus* incubated for 1 h with alveolar lining material were killed more readily after ingestion by AM than *S. aureus* that were similarly incubated in a balanced salt solution and postulated that alveolar lining material stimulated the bactericidal activity of AM. They used the sediment of cell-free lung lavage fluids as alveolar lining material and suggested that the AM activation factor may be lipids in the lining material, but not proteins. If so, the factor they reported may be different from the lavage-procured IgG reported here. Fraction 1 (Fr-I) eluted in the void volume contained high concentrations of phospholipids, but had no effect on AM activation when judged by our criteria.

The findings that AM from normal rabbit lungs were soon activated markedly by both lavage-procured IgG and serum IgG, but not by LK, and that the AM activation by lavage-procured IgG was dose dependent may be important with respect to AM function in normal alveolar spaces or in the early resistance to bacterial infection. In normal alveolar spaces, moderately activated AM by normal concentrations of IgG would ingest more readily inhaled particles or kill low-virulent bacilli. IgG is present in relatively high concentrations in normal alveolar spaces (8, 27). On the other hand, in the early stage of bacterial infection, AM markedly activated by high concentrations of serum IgG transuded from blood into alveolar spaces may play important roles in the early resistance to bacterial infection. Serum transudation occurs immediately after inflammation (21), in contrast with the production of LK by sensitized lymphocytes, which may take 6 h after contact with corresponding antigens (6).

Different responses of AM and PEC to lavage-

procured IgG or LK may depend on the differences of the membrane receptors or the metabolic pathways of these cells. Our results that LK did not have an effect on AM activation but did on PEC (1) are consistent with the evidence that AM from inflammatory lesions respond to migration inhibitory factor (12), but AM from normal lungs do not respond (18). Both AM in inflammatory lesions and PEC are newly derived macrophages from blood monocytes (32, 31). Further experiments are needed to clarify the mechanisms of the discrepancy in response.

From the results reported here, it seems reasonable to conclude that the function of AM in normal or inflammed lungs is regulated by the concentration of IgG in the alveolar spaces.

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## LITERATURE CITED

1. **Ando, M., M. Suga, K. Shima, M. Sugimoto, S. Higuchi, T. Tsuda, and H. Tokuomi.** 1976. Different effects of phytohemagglutinin-activated lymphocytes and their culture supernatants on macrophage function. Infect. Immun. **13**:1442-1448.
2. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. **91**:222-233.
3. **Arend, W. P., and M. Mannik.** 1975. Quantitative studies on IgG receptors on monocytes, p. 303-314. *In* R. Van-Furth (ed.), Mononuclear phagocytes in immunity, infection and pathology. Blackwell, Oxford.
4. **Baehner, R. L., and D. G. Nathan.** 1968. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. N. Engl. J. Med. **278**:971-976.
5. **Baehner, R. L., S. K. Murrman, J. Davis, and R. B. Johnston.** 1975. The role of superoxide anion and hydrogen peroxide in phagocytosis-associated oxidative metabolic reactions. J. Clin. Invest. **56**:571-576.
6. **Bennett, B., and B. R. Bloom.** 1967. Studies on the migration inhibitory factor associated with delayed hypersensitivity: cytodynamics and specificity. Transplantation **5**:996-1000.
7. **Beveridge, J. M. R., and S. E. Johnson.** 1949. Determination of phospholipid phosphorus. Can. J. Res. Sect. E **27**:159-163.
8. **Bignon, J., M. C. Jaurand, M. C. Piinchon, C. Sapin, and J. M. Warnet.** 1976. Immunoelectron microscopic and immunochemical demonstrations of serum proteins in the alveolar lining material of the rat lung. Am. Rev. Respir. Dis. **113**:109-120.
9. **Britton, H. T. S., and R. A. Robinson.** 1931. Universal buffer solutions and the dissociation constant of veronal. J. Chem. Soc. **134-1**:1456-1462.
10. **Colacicco, G., A. B. Buckelew, and E. M. Scarpelli.** 1973. Protein and lipid-protein fractions of lung washings: chemical characterization. J. Appl. Physiol. **34**:743-749.
11. **Green, G. M., and E. H. Kass.** 1964. The role of the alveolar macrophages in the clearance of bacteria from the lung. J. Exp. Med. **119**:167-175.
12. **Johnson, J. D., W. L. Hand, N. L. King, and C. G. Hughes.** 1975. Activation of alveolar macrophages after lower respiratory tract infection. J. Immunol. **115**:80-84.
13. **Juers, J. A., R. M. Rogers, J. B. McCurdy, and W. W. Cook.** 1976. Enhancement of bactericidal capacity of alveolar macrophages by human alveolar lining material. J. Clin. Invest. **58**:271-275.
14. **Klebanoff, S. J., and C. B. Hamon.** 1975. Antimicrobial systems of mononuclear phagocytes, p. 507-529. *In* R. Van-Furth (ed.), Mononuclear phagoctes in immunity, infection and pathology. Blackwell, Oxford.
15. **LaForce, F. M.** 1976. Effect of alveolar lining material on phagocytic and bactericidal activity of lung macrophages against Staphylococcus aureus. J. Lab. Clin. Med. **88**:691-699.
16. **LaForce, F. M., W. J. Kelly, and G. L. Huber.** 1973. Inactivation of Staphylococci by alveolar macrophages with preliminary observations on the importance of alveolar lining material. Am. Rev. Respir. Dis. **108**:784-790.
17. **Laurenzi, G. A., L. Berman, M. First, and E. H. Kass.** 1964. A quantitative study of the deposition and clearance of bacteria in the murine lung. J. Clin. Invest. **43**:759-768.
18. **Leu, R. W., A. L. W. F. Eddleston, J. W. Hadden, and R. A. Good.** 1972. Mechanism of action of migration inhibitory factor (MIF). I. Evidence for a receptor for MIF present on the peritoneal macrophage but not on the alveolar macrophage. J. Exp. Med. **136**:589-603.
19. **Lowry, O. H., N. J. Rosebrough,. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
20. **Miale, J. B.** 1967. Laboratory medicine: hematology, 3rd ed., p. 532-534. C. V. Mosby Co., St. Louis.
21. **Movat, J. B.** 1971. The acute inflammatory reaction, p. 1-129. *In* H. Z. Movat (ed.), Inflammation, immunity and hypersensitivity. Harper & Row, Publishers, New York.
22. **Myrvik, Q. N., E. S. Leake, and B. Fariss.** 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. J. Immunol. **86**:128-132.
23. **Nathan, C. F., H. G. Remold, and J. R. David.** 1973. Characterization of a lymphocyte factor which alters macrophage functions. J. Exp. Med. **137**:275-290.
24. **Park, B. H., S. M. Fikrig, and E. M. Smithwick.** 1968. Infection and nitroblue tetrazolium reduction by neutrophils. Lancet **ii**:532-534.
25. **Pavillard, E. R. J., and D. Rowley.** 1963. *In vitro* phagocytic and bactericidal ability of alveolar and peritoneal macrophages of normal rats. Aus. J. Exp. Biol. **41**:265-274.
26. **Reed, P. W.** 1969. Glutathione and the hexose monophosphate shunt in phagocytizing and hydrogen peroxide-treated rat leukocytes. J. Biol. Chem. **244**:2459-2464.
27. **Reynolds, H. Y., and H. H. Newball.** 1974. Analysis of proteins and respiratory cells obtained from human lungs by bronchial lavage. J. Lab. Clin. Med. **84**:559-573.
28. **Sober, H. A., and E. A. Peterson.** 1958. Protein chromatography on ion exchange cellulose. Fed. Proc. **17**:1116-1126.
29. **Tamerius, J., J. Nepom. I. Hellström, and K. E. Hellström.** 1976. Tumor-associated blocking factors: isolation from sera of tumor-bearing mice. J. Immunol. **116**:724-730.
30. **Tizard, I. R.** 1971. Macrophage-cytophilic antibodies and the functions of macrophage-bound immunoglobulins. Bact. Rev. **35**:365-378.
31. **Van-Furth, R., and Z. A. Cohn.** 1968. The origin and kinetics of mononuclear phagocytes. J. Exp. Med. **128**:415-433.
32. **Velo, G. P., and W. G. Spector.** 1975. Alveolar macrophages and granuloma formation, p. 951-957. *In* R. Van-Furth (ed.), Mononuclear phagocytes in immunity, infection and pathology. Blackwell, Oxford.
33. **Wofsy, L., and B. Burr.** 1969. The use of affinity chromatography for the specific purification of antibodies and antigens. J. Immunol. **103**:380-382.