# HUMAN PULMONARY MACROPHAGE-DERIVED MUCUS SECRETAGOGUE

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Hypersecretion of mucus accompanies pulmonary diseases such as asthma, bronchitis, and pneumonia, among others. Neither the role of mucus nor the mechanisms responsible for controlling its secretion in these diseases is clear although recent studies have uncovered a number of putative secretagogues including neurohormones (1–5), mast cell-derived mediators of allergy (4, 6–8), and oxidative derivatives of arachidonic acid (6–9) able to stimulate mucous glycoprotein release in vitro.

The pulmonary macrophage (PM)[1] is the resident phagocytic cell in the lung and functions as the first line of defense against inhaled particulates. Because of the nature of its motility, phagocytic capability, and secretion of multiple products, the PM is thought to participate in lung inflammation both as a primary cell in host defense and to recruit other cell types to the lung (10, 11). It is likely that PM participate in some manner in each pulmonary condition in which increased mucus secretion is associated, although no relationship between PM and mucus secretion has previously been reported. We undertook this study to investigate whether PM could elaborate factors able to stimulate mucus secretion and thus participate in this lung function as well.

## Materials and Methods

*Preparation of Human Airways for Culture.* Human lungs were obtained at surgery primarily from tumor resection. All of the subjects from whom lung tissue was obtained were former or current smokers, and no attempt to correlate their pulmonary function with mucus secretion was made. Normal-appearing airways, 2–10 mm diam, were fragmented into 3 × 5-mm replicates and cultured as described (4, 6). The airway explants were maintained in CMRL-1066 medium (Gibco Laboratories, Grand Island, NY) with penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (0.5 μg/ml) in a controlled atmosphere chamber gassed with 45% $O_2$, 50% $N_2$, and 5% $CO_2$, and were incubated at 37°C.

*Radiolabeling of Mucous Glycoproteins.* Mucous glycoproteins were radiolabeled by incorporating [$^3$H]glucosamine (1 μCi/ml) (New England Nuclear, Boston, MA) into the culture medium. Explants were initially incubated for 16 h in the absence of [$^3$H]-

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[1] *Abbreviations used in this paper*: CVF, cobra venom factor; ETYA, eicosa-5,8,11,14-tetraynoic acid; HBSS, Hanks' balanced salt solution; HETE, monohydroxyeicosatetraenoic acid; LT, leukotriene; MMS, macrophage-derived mucus secretagogue; PG, prostaglandin; PM, human pulmonary macrophages.

glucosamine, washed twice with media, and then incubated with [$^3$H]glucosamine for a 16-h baseline period (period I). After period I supernatants were harvested, fresh culture media without [$^3$H]glucosamine was added for an additional 4-h period (period II), and these supernatants were subsequently harvested. Glucosamine-labeled mucous glycoproteins were precipitated from the samples by adding an equal volume of 95% ethanol. The precipitates were filtered through 0.45-$\mu$m membrane filters (Gelman Sciences, Inc., Ann Arbor, MI) and the retained radioactivity was determined.

*Effect of Pharmacologic Manipulation upon the Release of Mucous Glycoproteins.* The effect of pharmacologic manipulations on the release of [$^3$H]glucosamine-labeled mucous glycoproteins was determined by adding agents to cultures at the beginning of period II. A ratio of the radiolabeled, precipitated counts per minute of period II to that of period I for each sample was determined and termed the secretory index. Due to the relatively high secretory index observed in day 1 cultures, no experiments were done during the initial cycle. Instead, all experiments were performed on the lung specimens between days 2 and 7 in culture.

The effects of pharmacologic agents were determined by comparing the secretory indices of manipulated samples with matched, unmanipulated control samples. These samples were derived from the same tissue, cultured in parallel, and handled identically to the experimental samples except that pharmacologic agents were not added. Thus, each airway culture provided its own baseline period (period I) as well as a stimulated period (period II), and the effects of each pharmacologic manipulation could be compared with matched controls.

*Macrophage Cultures.* PM were isolated from samples of human lung using a modification of a previously reported procedure (12). Human lung specimens were placed in 4°C sterile, heparinized saline immediately after surgical removal. The tissue was maintained at 22°C in Hanks' balanced salt solution (HBSS)[1] without $Ca^{2+}$ or $Mg^{2+}$. Fragments (0.5 by 0.5 cm) of human lung parenchyma were teased apart, filtered through two layers of sterile surgical gauze, and centrifuged at 500 *g* for 10 min at 22°C. Cell pellets were pooled, layered onto Hypaque-Ficoll (lymphocyte separation media; Pharmacia Fine Chemicals, Piscataway, NJ), and centrifuged at 1,000 *g* for 20 min at room temperature. The mononuclear cell preparations were removed from the interface, washed four times in HBSS, and resuspended in medium CMRL-1066 with penicillin (100 U/ml), streptomycin (100 $\mu$g/ml), and L-glutamine (100 mM) (Gibco Laboratories), without supplementary serum. The concentration of PM was adjusted to 3–3.5 × $10^6$ cells/2 ml. Cells were allowed to adhere to plastic dishes for 4 h and then washed vigorously three times with serum-free media to remove nonadherent cells. The adherent cells were incubated for 24 h in CMRL-1066 without serum at 37°C in room air with 5% $CO_2$ in a humidified atmosphere before starting experiments. The percent viability of the cells was assessed by trypan blue exclusion after incubation in a 1% solution of the dye, and the viability always exceeded 96%. Differential counts (Wright-Giemsa-stained cytocentrifuge preparation) revealed that the final cell preparations consisted of 95% macrophages and 5% lymphocytes. The cells were removed from the culture plates with a rubber policeman, washed, and stained by the nonspecific esterase procedure of Burston (13); 96% of the cells were positive. The combination of morphologic appearance, staining characteristics, and phagocytic capabilities (to be described below) indicate that at least 95% of the cells were PM. No attempt to separate alveolar and interstitial macrophages was made.

*Zymosan Preparation.* Zymosan particles (Sigma Chemical Co., St. Louis, MO) were suspended in sterile saline, boiled three times for 5 min, and washed three times with 30 vol of saline. The particles were then suspended 1:1 in normal human serum and incubated for 30 min at 37°C. The serum-treated zymosan particles were washed three times with 30 vol of 0.5 M NaCl and finally resuspended in the incubation buffer before their use. Nonactivated zymosan was handled in an identical fashion except that these particles were not exposed to serum. Activated or nonactivated zymosan was added to the cells in culture at a ratio of 150 particles per PM. Supernatants were removed at different intervals (as indicated), pooled, centrifuged (3,000 *g* at 22°C for 10 min), filtered through 0.22-$\mu$m

Millex-GV sterilizing filter units (Millipore Corporation, Bedford, MA), and applied to airways in culture (usually 2 ml/plate) at the beginning of period II.

An alternative experimental design was also used in which PM were incubated with activated zymosan (10 mg/ml) for 60 min, the cultures were washed extensively to remove free zymosan, and the incubation subsequently continued for 8 h. The supernatant was then processed in a fashion identical to that described above.

*Manipulation of Sera for Zymosan Activation.* Sera used for zymosan activation were obtained from healthy volunteers, permitted to clot 15–30 min at 4°C, centrifuged at 3,000 *g* at 4°C for 15 min, and stored as aliquots at −70°C. An aliquoted serum sample was heat inactivated at 56°C for 30 min. Rat sera were collected in a fashion similar to that described for human sera. Cobra venom factor (CVF)-treated sera were obtained from rats that had received CVF (250 U/kg; Cordis Laboratories, Miami, FL) in a single intravenous bolus. Blood was withdrawn 6 h later and had a $CH_{50}$ level of <1 U/ml (normal, 151 ± 3.5 U/ml) and a C3 titer of <5.0 hemolytic U/ml (normal, 48,959 ± 2844 hemolytic U/ml) (14). Sera from a C3-deficient patient was kindly provided by Dr. Carl Hammer, National Institutes of Health.

*Preparation of Various Particles for Phagocytosis.* Sepharose 4B (Pharmacia Fine Chemicals) was washed three times with saline, incubated with normal serum at a 1:1 dilution for 30 min at 37°C, washed three more times, and added to macrophages in culture. Latex beads (polystyrene, average diam 1.1 μm; Sigma Chemical Co.) were washed three times in saline before use. Preparations of formalin-fixed protein A containing *Staphylococcus aureus* (Bethesda Research Laboratories, Bethesda, MD) were washed five times in saline, resuspended at a 2% concentration in saline, and added to cultured macrophages at 10 bacteria per macrophage. *S. aureus* that were deficient in protein A were handled in an identical fashion.

*Determination if Macrophage-derived Mucus Secretagogue (MMS) Is Preformed or Newly Synthesized.* PM were isolated and divided into two portions, each of $30 \times 10^6$ cells. One portion of cells was frozen in culture media at −70°C immediately after isolation. The other portion was cultured, incubated with activated zymosan or *S. aureus* for 1 h, washed, and cultured for eight additional hours. After the supernatants were collected, the cells were removed from the culture plates with a rubber policeman and were frozen at −70°C. Thereafter, the cultured PM and the cells that had been frozen directly after isolation were handled in parallel. The PM were freeze-thawed three times, sonicated in ice (3 min at power setting 3, using a microtip, model W185-F; Heat Systems-Ultrasonics, Inc., Plainview, NY) and centrifuged at 3,000 *g* for 15 min at 4°C. The lysate was filtered in the cold through 0.22-μm membranes and added to airways in culture to assess mucus secretagogue activity.

Partially purified MMS was obtained by ultramembrane filtration, gel filtration, and isolectric focusing (see below). The MMS was then mixed with PM lysates for 4 h at 37°C, filtered on 0.22-μm membranes, and added to airways in culture.

In a related experiment, PM were cultured in the presence (or absence, in parallel) of cycloheximide 10 μg/ml (Sigma Chemical Co.) for 16 h. These sets of cells were then exposed to activated zymosan or *S. aureus* for 1 h, after which the cultures were washed and the cells cultured for an additional 8 h. The supernatant was assayed for MMS activity. The cells were removed from the plates, freeze-thawed, sonicated as described above, and assayed for mucus secretagogue activity.

To determine if the cycloheximide affected macrophage protein synthesis, its effects on leucine incorporation were studied. Two sets of four culture plates each of PM were maintained in parallel to those used for MMS release; one was incubated with cycloheximide, the other not. Both sets of cultures were exposed to $^3$[H]-L-leucine (59.2 Ci/mmol; New England Nuclear Co.) for 1 h simultaneously with the addition of activated zymosan to the cultures. These PM were manipulated in parallel to replicates used for assessment of MMS activity. After the incubations were completed, the cells used for leucine incorporation were scraped from the plates and lysed by sonication; the protein in the lysate was precipitated by the addition of an equal volume of 10% perchloric acid. The precipitate was collected by centrifugation (12,000 *g* for 2 min), washed twice, and

resuspended in water; the incorporated radioactivity was determined by liquid scintillation counting.

*Relationship Between Eicosinoids and MMS.* The PM were incubated with acetylsalicylic acid, indomethacin (Sigma Chemical Co.), or eicosa-5,8,11,14-tetraynoic acid (ETYA) (kindly provided by Dr. W. E. Scott, Hoffman LaRoche, Inc., Nutley, NJ) for 60 min before the addition of activated or nonactivated zymosan. The zymosan-PM incubation was continued for 60 min, the cells were washed three times, and the cultures were maintained for eight more hours after which the supernatant was examined for mucus secretagogue activity. The agents above were prepared as described (6).

Supernatants rich in mucus secretagogue activity were prepared by incubating PM with activated zymosan as described above. Such supernatants were adjusted to pH 3.0 with citric acid and extracted three times with 3 vol each of ethyl acetate or diethylether. The organic phase of each extraction was combined, evaporated to dryness under nitrogen, and resuspended in tissue culture medium before being assayed for mucus secretagogue activity.

Supernatants rich in MMS activity were partially purified by ultramembrane and gel filtration (see below). These partially purified preparations were assayed by radioimmunoassay for the presence of prostaglandin E (PGE), $PGF_{2\alpha}$, and thromboxane $B_2$ (15). As used for this study, these assays were sensitive to 2.5 pg.

Preparations of MMS were also fractionated on Amberlite XAD-7 (Mallinckrodt Inc., St. Louis, MO). A column of Amberlite (0.5 × 5 cm) was flushed alternatively with 20 vol of diethylether or water in preparation for the chromatography. Three different materials were fractionated and each was placed on the column in a volume of 20 μl. The materials were [$^3$H]leukotriene (LT) $B_4$ (kindly provided by Dr. Edward Goetzl, University of California, San Francisco, CA), 1 μg of $LTC_4$ (kindly provided by Dr. Michael Bach, Upjohn Co., Kalamazoo, MI), or MMS prepared by sequential fractionation using ultramembrane filtration, gel filtration, and isoelectric focusing (see below). After charging these materials onto the column, 5 vol of water followed by 5 vol of diethylether were added to the column. The MMS activity in the water fraction was assayed after lyophilization and resuspension in water; the ether eluate was evaporated to dryness under nitrogen and resuspended in water before assay.

*Partial Purification of MMS.* 50 ml of MMS-rich supernatant were obtained from PM incubated with activated zymosan. These supernatants were pooled and sequentially filtered on ultramembranes (YM10, molecular weight exclusion ~10,000, and UM05, molecular weight exclusion ~500; Amicon Corp., Danvers, MA) in a stirred chamber under nitrogen pressure (50 PSI) using water as the wash buffer. The UM05 retentate was concentrated to 1 ml, brought back to 50 ml with distilled water, and reconcentrated twice. The washed, concentrated UM05 retentate was lyophilized and resuspended in 1 ml of culture medium. In these experiments, 20 μl of the concentrate was added to the airways in culture to assess secretagogue activity. 50 ml of culture medium that had never been exposed to PM or which had been exposed to PM plus nonactivated zymosan were manipulated in parallel as controls.

Concentrated UM05 retentate material was filtered on a 1 × 60-cm Sephadex G-25 column (Pharmacia Fine Chemicals) at 4°C, and fractions of 1.2 ml were collected. Each fraction was separately filtered through 0.22-μm membranes, lyophilized, resuspended in 1 ml distilled water, and refiltered. 20 μl of this material was then added to airways in culture.

Concentrated UM05 retentate material was analyzed by gel filtration, the fractions were assayed for secretagogue activity, and the active fractions were pooled (usually 2–4 ml), lyophilized, and resuspended in 1 ml of water. One ml of fractionated MMS material was mixed with 58 ml Buffalyte solution (pH 3–10; Pierce Chemical Co., Rockford, IL) and 111 gm Pevikon (Accurate Chemical and Scientific Corp., Westbury, NY). The slurry was poured into a horizontal isoelectric focusing apparatus (electrofocusing kit; LKB Instruments, Inc., Gaithersburg, MD), and a pH gradient was established in 4 h using a constant power of 400 W (LKB 2197 power supply) using the method of Harpel and Kueppers (16). The anodal and cathodal solutions were 0.1 M phosphoric acid and sodium

hydroxide, respectively. The slurry was divided into 5 fractions that were poured individually into 1 × 6-cm columns and washed with 4 ml of water. The eluates were lyophilized, resuspended in 0.5 ml of water, and individually filtered over 1 × 60-cm Sephadex G-25 columns as described above.

*Statistics.* The results are expressed as the percent increase in mucous glycoprotein release as compared with control, as described in detail (6). Each experiment used quadruplicate airway cultures for determination of each point, and all results represent data observed from several experiments. The results are generally provided as the mean ± SEM. Statistical comparisons use paired sample *t* tests.

## Results

*Effect of Zymosan on the Release of MMS.* To examine if PM might influence mucus secretion, $3 \times 10^6$ PM/plate were exposed to either nonactivated or activated zymosan particles (10 mg/ml). After 8 h of incubation, the supernatants were collected and added to cultured human airways that were secreting [$^3$H]-glucosamine-labeled mucous glycoproteins. As shown in Table I, the supernatants from macrophages cultured with nonactivated zymosan had no significant effect on airway mucus release (−0.6 ± 3.0%, $n = 8$). By contrast, the supernatant from macrophages incubated with opsonized zymosan increased mucus release by 34.6 ± 7.8% ($n = 8$, $P < 0.001$). Overall, 22 individual macrophage preparations have been exposed to activated zymosan as described and in every instance there has been mucus secretagogue activity in the supernatant. The effects of mucus secretion range from 10 to 83% above control. Neither activated nor nonactivated zymosan had any effect when added to airway cultures directly. The factor in the supernatant from PM incubated with activated zymosan will henceforth be termed MMS.

*Dose Response of Zymosan for MMS Release.* Macrophages were exposed to 0.01–100 mg/ml activated zymosan for 8 h, and the supernatants were added to the airways (Fig. 1). In each of three experiments (pooled on Fig. 1), concentrations of zymosan >1 mg/ml produced near maximal release of MMS. For

TABLE I

*Effects of Supernatants from PM Cultures on Human Airway Mucous Glycoprotein Release*

| Source of supernatant | Mucus release (percent change from control) |
|---|---|
| PM + activated zymosan | 34.6 ± 7.8<br>$n = 8$<br>range, 12–83% |
| PM + nonactivated zymosan | −0.6 ± 3.0<br>$n = 8$<br>range, −1 to 13<br>$P < 0.001$ |

PM were incubated with nonactivated zymosan or activated zymosan for 8 h; the supernatants were pooled, centrifuged, filtered through 0.22-μm filters, and added to cultured airways. Results are the percent increase in mucous glycoprotein release as compared with control airways incubated in culture media alone. Zymosan at a concentration of 10 mg/ml was used in these experiments. Statistical comparisons are by paired sample *t* tests.

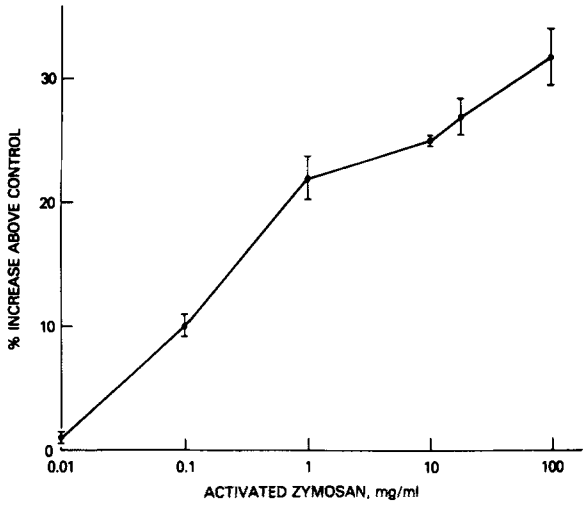


FIGURE 1. Dose response of activated zymosan on MMS release. PM were incubated with 0.01–100 mg/ml activated zymosan for 8 h. Supernatants were collected, pooled, centrifuged, filtered, and applied to human airways in culture. The results are the percent increase in mucus release as compared with control airways incubated in culture media alone. The results are the mean ± SEM of three separate experiments.

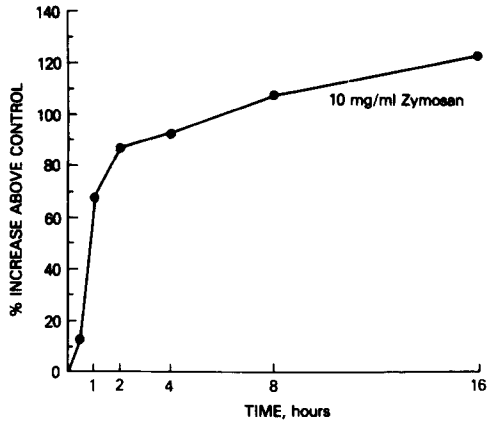


FIGURE 2. Time course of the appearance of MMS. PM were incubated with activated zymosan (10 mg/ml) for 30 min to 16 h before supernatants were removed and added to airway cultures.

convenience, zymosan was used at 10 mg/ml (150 particles per PM) in all subsequent experiments.

*Time Course of MMS Release from PM.* To assess the time course of MMS release, PM were exposed to activated zymosan for from 30 min to 16 h before removal of the supernatant (Fig. 2). MMS was evident in cultures within 30 min of incubation and the release of MMS appeared to peak after 2–4 h. The time course of MMS release was repeated on several other occasions and cultures exposed to zymosan for 24 h appeared to have less activity than those at 4–16 h. Therefore, 4–to 8-h incubations with activated zymosan were considered

optimal for macrophages to release MMS. The time course of MMS release in response to exposure to 10 or 20 mg/ml of activated zymosan were compared, and equivalent kinetics were observed. Therefore, within this narrow dose range, concentration did not seem to affect kinetics.

*Dose Response of MMS.* MMS collected from PM incubated with activated zymosan for 8 h was added to airway cultures in dose response experiments (Table II). In two separate experiments, MMS, 0.5–2 ml, added to airways caused a dose-related increase in mucus release. In both experiments, adding 1 ml of MMS-containing culture medium to 1 ml of ordinary culture medium resulted in significant increases in the release of mucous glycoproteins.

*Effect of Washing Macrophages on MMS Release.* Microscopic analysis of PM exposed to activated zymosan indicated that the zymosan was rapidly taken up by the cells. We therefore examined whether a short initial exposure to activated zymosan followed by a longer incubation (to optimize MMS release) might be an acceptable design. In these experiments, PM were exposed to activated zymosan for either 8 h (the usual experimental design) or for 1 h followed by washing and an additional 8-h incubation. The supernatants from three experiments using the two procedures were compared, and each generated equivalent degrees of increased mucus release (+31.1 ± 1.3% for continuous zymosan exposure and +34 ± 2.3% for 1 h of zymosan exposure). Therefore, it appeared that PM generate equivalent MMS either in the continuous presence of activated zymosan or after a 1-h exposure.

*Opsonization and Phagocytosis.* To analyze the requirements for zymosan activation, zymosan was exposed to various sera before being incubated with PM (Table III). PM exposed to saline-treated zymosan generated no MMS. Zymosan that had been incubated in sera obtained from C3-deficient humans, CVF-treated rats, or after heat inactivation failed to stimulate macrophages to generate MMS. Only zymosan that was activated in normal rat or human sera caused the macrophages to release MMS. Thus, complement products dependent on C3 activation are required for zymosan to induce MMS release from PM.

TABLE II

*Dose Response of MMS-containing Culture Media on Mucus Release*

| Experiment | Supernatant added | Mucus release (percent increase above control) | *P* |
|---|---|---|---|
| | *ml* | | |
| A | 0.5 | +1 ± 1.3 ($n$ = 5) | NS |
| | 1 | +18 ± 2.0 | <0.025 |
| | 1.5 | +22 ± 4.0 | <0.05 |
| | 2 | +23 ± 0.5 | <0.0005 |
| B | 1 | +29 ± 0.2 ($n$ = 5) | <0.005 |
| | 2 | +45 ± 0.1 | <0.0005 |

PM were incubated with activated zymosan for 8 h and the supernatants were collected. The supernatants were diluted with culture media to a total final volume of 2 ml and added to cultured airways. Results are the percent increase over airways incubated in culture media alone. Statistical comparisons are by paired sample $t$ tests. NS, not significant.

TABLE III
*Role of Complement in the Generation of MMS*

| PM plus: | Mucus release (percent change from control) |
|---|---|
| Zymosan incubated in normal human serum | +23 ± 0.2 |
| Zymosan incubated in heat-inactivated human serum | +5 ± 1.3 |
| Zymosan incubated in rat serum | +32 ± 1.0 |
| Zymosan incubated in CVF-treated rat serum | +3 ± 2.0 |
| Zymosan incubated in C3-deficient human serum | −5 ± 1.5 |
| Nonactivated zymosan | 0 ± 0.4 |

All zymosan and serum incubations were done at 37°C for 30 min as described. PM were incubated with these zymosan particles (10 mg/ml) for 1 h. The supernatants were then removed and the cells were washed three times. Fresh medium was added and the cells were incubated for 8 h, after which the supernatants were pooled, centrifuged, filtered, and added to human airways in culture. The results are the percent increase in mucus release as compared with control airways incubated in culture media alone.

TABLE IV
*Capacity of Various Particles to Cause MMS Release*

| Stimuli | Concentration | Mucus release (percent increase above control) |
|---|---|---|
| Activated zymosan | 10 mg/ml | +28 ± 1.7 |
| Nonactivated zymosan | 10 mg/ml | +5 ± 1.0 |
| Latex | 0.05% | 0 ± 1.3 |
| Latex | 1% | 0 ± 1.8 |
| Sepharose 4B and serum | 0.05% | +26 ± 3.1 |
| Sepharose 4B without serum | 0.05% | +8 ± 4.8 |
| *S. aureus*-protein A | 10 bacteria/macrophage | +29 ± 2.5 |
| *S. aureus*-nonprotein A | 10 bacteria/macrophage | +7 ± 3.0 |

PM cultures were exposed to the various particles for 60 min, after which the cells were washed and the incubation continued with fresh media (that did not contain phagocytic particles) for 8 h. The supernatants were prepared as described for assessment of MMS activity. Each result is the mean ± SEM for three separate experiments.

Phase microscopy of PM showed that phagocytosis occurred within 1 h when zymosan particles opsonized in normal human or rat serum were used. Within a 1-h period, there was little or no phagocytosis of zymosan particles that had been incubated with saline, C3-deficient human serum, heat-inactivated human serum, or serum from rats treated with CVF. Thus, phagocytosis of appropriately opsonized zymosan particles appeared to be associated with the release of MMS.

To address whether phagocytosis itself was related to MMS release, the following experiments were performed (Table IV). PM were incubated with Sepharose 4B that had been exposed to normal human serum or buffer alone.

The serum-treated Sepharose 4B induced MMS release from PM whereas the non–serum-treated Sepharose did not. As Sepharose is known to activate complement (17) but is too large for ingestion, this observation suggests that attempted phagocytosis is sufficient for secretagogue release. However, incubation of PM with latex particles (either 0.05 or 1%) led to apparent active phagocytosis of the particles, as judged by light microscopy, without MMS release. Thus, phagocytosis by PM without concomitant opsonization is not an adequate stimulus for MMS release. To examine if complement-derived factors were the only opsonins adequate for MMS release, the effect of adding *S. aureus* containing protein A on their surface was examined. A ratio of 10 bacteria per macrophage was used, and the staphylococci were observed to be actively ingested by the PM. In contrast to latex ingestion, the phagocytosis of bacteria with protein A led to MMS release. However, incubation of PM with staphylococci that were deficient in protein A failed to generate MMS release.

To determine if the actual act of phagocytosis of activated zymosan was a prerequisite for PM generation of MMS, the effects of cytochalasin B were examined. Cytochalasin B disrupts microfilaments and inhibits phagocytosis (18, 19). The PM were exposed to cytochalasin B for 1 h (either 5 or 15 μg/ml), incubated with activated zymosan for 1 h, and washed; the incubation was then continued for 8 h. The cultures were examined at 1 and 8 h by light microscopy; rosettes of zymosan were observed on the surface of the PM but no obvious ingestion was apparent. While the cytochalasin B impaired the phagocytosis of zymosan by PM, there was no reduction in MMS generation by either 5 or 15 μg. Thus, the supernatants from zymosan-exposed PM increased mucus release by 22 ± 2.3%, and cytochalasin B-treated and zymosan-exposed PM increased mucus release by 22 ± 2.6%. Cytochalasin B in the absence of zymosan had no effect on PM viability or MMS generation. It appeared that cell-surface activation, but not actual phagocytosis, was sufficient to generate MMS release.

*Is MMS Preformed or Newly Synthesized?.* PM were exposed to activated zymosan or *S. aureus* for 1 h and the cultures were washed and then incubated for 8 h to produce a supernatant with MMS activity (Table V). After the collection of the supernatant, the PM were removed from the culture plate and lysed, and the lysate was also assayed for MMS activity. The supernatants and lysates from PM that were exposed to nonactivated zymosan were also assessed for MMS activity as were lysates of purified PM that had never been cultured in vitro. Only the supernatants from PM exposed to opsonized zymosan or *S. aureus* contained MMS activity; none was observed in control cultures or in cultures exposed to nonactivated zymosan. PM lysates from all of these preparations failed to affect mucous glycoprotein release.

Since it was possible that enzymes contained in the PM lysates might be able to degrade MMS and thereby falsely obscure MMS activity in the lysates, PM lysates were also incubated with partially purified MMS. This partially purified MMS was obtained from supernatants of cultured PM after exposure to opsonized zymosan; it was then sequentially filtered on ultramembranes and Sephadex G-25 and focused on a horizontal isoelectric focusing apparatus (see below). Such partly purified material increased mucous glycoprotein secretion from cultured airways by 17 ± 6%. The partly purified MMS was incubated for 4 h with lysates

TABLE V
*Effects of PM Supernatants and Lysates on Mucous Glycoprotein Release*

| PM exposed to: | Preparation | Mucus release (percent change from control) |
|---|---|---|
| Activated zymosan | Culture supernatant | +24 ± 2 |
| | Lysate | −3 ± 8 |
| Culture media alone | Culture supernatant | 0 ± 1 |
| | Lysate | −3 ± 1 |
| Nonactivated zymosan | Culture supernatant | +4 ± 2 |
| | Lysate | +3 ± 1 |
| *S. aureus* | Culture supernatant | +32 ± 4 |
| | Lysate | +1 ± 3 |
| Lysate of PM not cultured in vitro | | −9 ± 13 |

Results from three separate experiments, each in quadruplicate, are pooled and presented as the mean ± SEM.

TABLE VI
*Effects of Cycloheximide on MMS Release from PM*

| PM exposed to: | Preparation | Mucus release (percent change from control) |
|---|---|---|
| *S. aureus* | Culture supernatant | +43 ± 3 |
| | Lysate | −9 ± 3 |
| Cycloheximide + *S. aureus* | Culture supernatant | −11 ± 2 |
| | Lysate | +10 ± 2 |
| Activated zymosan | Culture supernatant | +49 ± 5 |
| | Lysate | −2 ± 1 |
| Cycloheximide + activated zymosan | Culture supernatant | +9 ± 2 |
| | Lysate | +12 ± 6 |

Results from three separate experiments, each done in quadruplicate, are pooled and presented as mean ± SEM.

from PM, which had no effect on mucous glycoprotein release on their own (−2 ± 3%). Despite the exposure to PM lysate, the partially purified MMS was still able to increase mucus release by 16 ± 4%. Thus, MMS is not degraded by PM lysates, and the inability to detect MMS in PM lysates probably means MMS is neither preformed nor stored intracellularly.

These data suggest that MMS activity might be newly synthesized after exposure to the phagocytic stimuli. To examine this possibility further, PM were incubated for 16 h in the presence or absence of cycloheximide (20) and then exposed to either activated zymosan or *S. aureus*, and the capacity of these cells to generate MMS activity was compared with non–cycloheximide-exposed controls (Table VI). PM that were exposed to phagocytic stimuli released MMS activity while cycloheximide-exposed PM released much less. While these data suggested that cycloheximide interfered with MMS synthesis, cell lysates were also examined to examine if intracellular MMS was generated and then not released. No intracellular MMS was detected. Protein synthesis by these cells was

measured simultaneously to confirm one of the actions of cycloheximide (20). Control cells incorporated [$^3$H]leucine into precipitable protein (1,208 ± 134 cpm/$10^6$ cells), while cycloheximide-treated cells incorporated only 106 ± 30 cpm. Thus, cycloheximide reduced leucine incorporation into precipitable protein by 91%. These observations strongly suggest that MMS is newly synthesized through a cycloheximide-sensitive pathway.

*Do Eicosinoids Contribute to MMS Activity?* PM were exposed to the cyclooxygenase inhibitors, acetylsalicylic acid (100 μg/ml) or indomethacin (10 μg/ml), for 1 h before the addition of activated zymosan and subsequent culture for MMS generation. In both instances, the cyclooxygenase inhibitors failed to affect MMS release. The tissue culture medium was assayed for the presence of immunoreactive $PGF_{2\alpha}$, PGE, and thromboxane $B_2$ with assays sensitive to 2.5 pg/ml for each. The tissue culture medium itself had no detectable PG but stimulation of the PM with activated zymosan resulted in the generation of 99 ± 10 pg/ml $PGF_{2\alpha}$, 170 ± 18 pg/ml PGE, and 157 ± 3 pg/ml $TxB_2$. Preincubation of the PM with acetylsalicylic acid or indomethacin reduced immunoreactive PG generation below 10 pg/ml. Thus, two cyclooxygenase inhibitors failed to influence MMS generation by PM but did prevent prostaglandin formation by these cells. These experiments were extended to examine the actions of ETYA (35 μg/ml), which inhibits both cyclooxygenase and lipoxygenase pathways. ETYA preincubation also failed to influence MMS release by zymosan-activated PM.

To be certain that eicosinoids were not contributing to the secretagogue activity, a 50-ml preparation of MMS-rich culture supernatant was generated from the culture of 75 × $10^6$ PM and extracted, after acidification, into the organic solvents, ethyl acetate or diethylether. Neither of the solvents, despite their capacity to extract PG, monohydroxyeicosatetraenoic acids (HETE), and leukotrienes, was able to remove MMS activity from the aqueous phase. Thus, MMS generation is not affected by inhibition of arachidonate metabolism nor extracted into the organic solvents commonly used in eicosinoid purification.

The capacity of Amberlite XAD-7 to fractionate eicosinoids was also explored. Preparations of [$^3$H]$LTB_4$, $LTC_4$, or MMS were added to Amberlite and eluted with water or ether (Table VII). Both $LTB_4$ and $LTC_4$ fractionated into the organic phase, as reflected by scintillation counting or increased mucus release, respectively. MMS activity eluted with the aqueous extract, reflecting its nonpolarity and strengthening the conclusions that MMS does not derive from arachidonic acid.

*Chromatographic Analyses of MMS.* To obtain a rough estimate of the size of MMS, supernatants obtained from PM incubated with activated zymosan were filtered on ultramembranes. MMS was found to filter through a YM10 membrane (indicating a size of <10,000 daltons) and to be retained by a UM05 membrane, suggesting that the size of MMS is between 500 and 10,000 D. Because of the ease of this procedure, ultramembrane filtration was subsequently used as the initial step in all purifications. Tissue culture media obtained from cultures of nonactivated PM failed to generate any MMS activity before or after fractionation on ultramembranes. Such tissue culture media controls (devoid of MMS activity) were carried through all chromatographic analyses as negative controls.

Over the course of these experiments, 10 separate preparations of MMS (after

TABLE VII

*Fractionation of MMS on Amberlite XAD-7**

| A. Fractionation of [$^3$H]$LTB_4$ | cpm/ml |
|---|---|
| $LTB_4$ added to column | 13,980 |
| $LTB_4$ in aqueous extract | 1,800 |
| $LTB_4$ in organic extract | 11,184 |
| **B. Fractionation of $LTC_4$** | **Mucus release (percent increase above control)** |
| Starting activity | +56 ± 3 |
| Aqueous extract | +14 ± 4 |
| Organic extract | +42 ± 3 |
| C. Fractionation of MMS | |
| Starting activity | +35 ± 1 |
| Aqueous extract | +28 ± 1 |
| Organic extract | +10 ± 1 |

* $LTB_4$, $LTC_4$, or MMS were charged onto 0.5 × 5-cm Amberlite XAD-7 that was washed with 5 vol of water (aqueous extract) and 5 vol diethylether (organic extract). Results from single, representative experiments are presented. Results in parts B and C are mean ± SEM of quadruplicates.

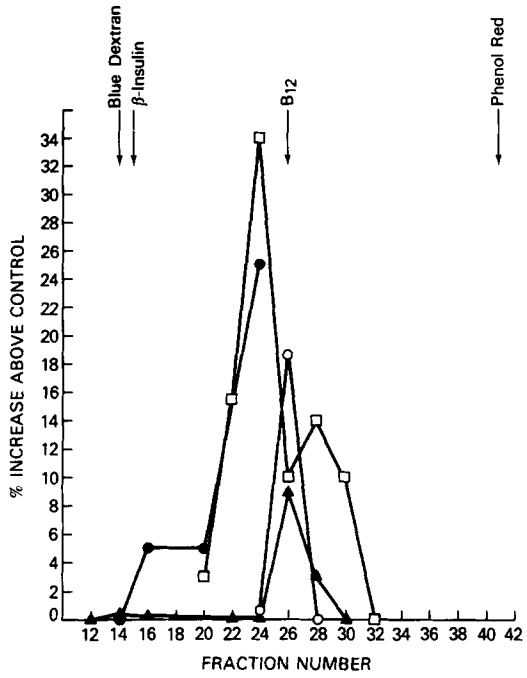


FIGURE 3. Sephadex G-25 chromatography. 50–100 ml of supernatant obtained from PM incubated with activated zymosan were pooled and concentrated by ultramembrane filtration. The sample was then applied to a 1 × 60-mm Sephadex G-25 column that was eluted with distilled water. The figure represents a summary of four chromatograms. Each fraction from the column was filtered, lyophilized, and resuspended in 0.5 ml of distilled water for assay of MMS activity.

ultramembrane fractionation) have been filtered through Sephadex G-25. Fig. 3 presents the chromatographic pattern from the first four preparations, which suggests that the major portion of MMS activity fractionated with an approximate molecular size of 2,000 D. The MMS activity of Sephadex G-25 fractions 24–26 have been analyzed in six subsequent fractionations, and the mean (±SEM) increase in mucus release by these fractions is 39.6 ± 3.1%. Material prepared by sequential ultramembrane and gel filtration was examined to determine if MMS activity was dose dependent (Table VIII*A*).

Preparations of MMS were sequentially prepared by ultramembrane and gel filtration before horizontal isoelectric focusing on a Pevikon bed. Two separate preparations of MMS after isoelectric focusing are presented in Fig. 4. In both instances, the major peak of activity focused at pH 5.15. A third preparation of MMS also focused at pH 5.15 (data not shown) indicating that this is a highly reproducible finding. This third preparation of partially purified MMS was examined to determine if the MMS activity was dose dependent (Table VIII*B*), which it was.

## Discussion

Human PM release a factor, MMS, which is able to cause human airways to release increased amount of biosynthetically radiolabeled mucous glycoproteins. This factor was released over a 4–8-h period after stimulation of the PM and was released by 22 of 22 human PM preparations examined. Zymosan was used as a convenient stimulus, but protein A-containing *S. aureus*, C3b-coated Sepharose beads, and concanavalin A (data not presented) are also effective stimuli. It initially appeared that phagocytosis was a prerequisite for MMS release, but several lines of evidence suggest that cell surface activation of macrophages is a

TABLE VIII

*Effects of Various Doses of MMS on Mucus Release**

| A. MMS activity after ultramembrane and gel filtration | |
|---|---|
| MMS added | Mucus release (percent increase above control) |
| *μl* | |
| 1 | 15 ± 1 |
| 10 | 28 ± 2 |
| 20 | 32 ± 2 |
| **B. MMS activity after sequential ultramembrane filtration, gel filtration, isoelectric focusing, and gel filtration** | |
| MMS added | Mucus release (percent increase above control) |
| *μl* | |
| 20 | 8 ± 7 |
| 50 | 24 ± 7 |
| 75 | 62 ± 15 |

* Results present the data from single representative dose response experiments ($\bar{X}$ ± SEM of quadruplicates).

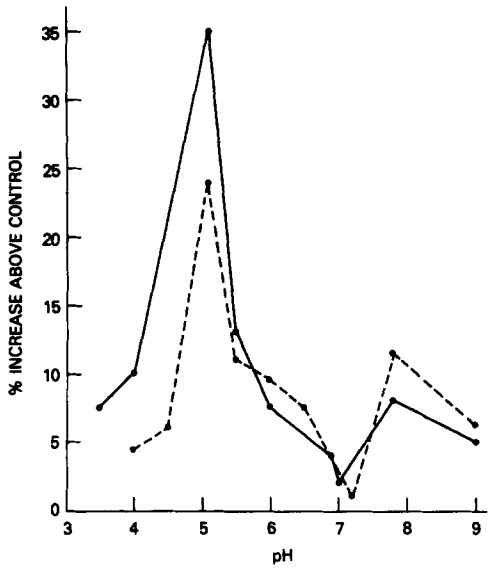


FIGURE 4. Isoelectric focusing of MMS on Pevikon. Two preparations of MMS (— and ---) were filtered on ultramembranes and Sephadex G-25 and then applied to a horizontal bed of Pevikon into which were incorporated ampholytes. The material was focused for 4 h, resulting in a linear pH gradient. The bed was divided into 15 fractions that were individually eluted with water. The eluates were lyophilized, each was refiltered on Sephadex G-25, and the MMS activity in fractions 24–26 was determined.

sufficient stimulus: (*a*) nonphagocytosable particles, such as C3b-coated Sepharose beads, are effective; (*b*) latex beads, while actively phagocytosed, do not cause MMS release; and (*c*) cytochalasin B-treated PM release MMS after zymosan exposure but do not internalize the zymosan. Thus, surface activation appears to be a sufficient stimulus for MMS release.

The opsonic factor operant in serum-treated zymosan activation of PM appears to be dependent on C3 activation. Again, several observations support this suggestion: (*a*) heat-inactivated sera failed to opsonize the zymosan; (*b*) C3-deficient sera were ineffective; (*c*) CVF-treated rat sera were ineffective; and (*d*) serum-treated Sepharose, which binds C3b to its surface (17), was as active as serum-treated zymosan.

Thus, cell surface activation of PM by complement-derived opsonins or through Fc receptors (as suggested by the protein A experiments) initiates a process that results in the release of a mucus secretagogue over a 4–8-h period. The time course of release suggested the possibility that synthesis or activation of this molecule is necessary before its release.

The capacity of cycloheximide, which inhibits protein synthesis by reducing peptide bond formation (20), to affect MMS release was examined. Preincubating PM with cycloheximide prevented PM that were exposed to either *S. aureus* or opsonized zymosan from releasing MMS. These cycloheximide-treated cells remained viable, as indicated by trypan blue dye exclusion, and phagocytically active, as reflected in apparent uptake of particles observed by phase microscopy. To determine if cycloheximide might have impaired macrophage export of

MMS, cell lysates were also examined and found largely devoid of MMS activity. The capacity of cycloheximide to inhibit new protein synthesis was monitored in parallel by following [$^3$H]leucine incorporation into precipitable proteins; cycloheximide inhibited >90% of new protein synthesis. Thus, MMS is a newly synthesized molecule whose formation involves a cycloheximide-sensitive step.

PM contain large amounts of esterified arachidonic acid in membrane phospholipids (21) and are prolific sources of many eicosinoids, including PG, thromboxanes, HETE, and LT (22–25). Human PM primarily produce $LTB_4$ after stimulation with the calcium ionophore A-23187 (25).

Earlier work using human airways demonstrated that arachidonic acid and many of its oxidative derivatives possess MMS activity. These secretagogues include $PGE_1$, $PGF_{2\alpha}$, $PGD_2$, $PGI_2$, and other PG (6); 5-, 8-, 9-, 11-, 12-, and 15-HETE, 5- and 9-hydroperoxyeicosatetraenoic acid (HPETE) (9); and $LTC_4$ and $LTD_4$ (7). It was therefore critically important to examine the relationship between MMS and arachidonic acid. The first approach was to modulate cyclooxygenase and lipoxygenase enzymes by pharmacologic inhibitors. Two nonsteroidal antiinflammatory drugs, aspirin and indomethacin, did not influence MMS release and nor did ETYA. Thus, impairment of either or both enzymatic pathways involved in the oxidation of arachidonic acid failed to influence PM MMS release.

While these data mitigate strongly against MMS being a derivative of arachidonic acid, two additional experimental approaches were tried. MMS was extracted on three separate occasions with ethyl acetate or diethylether, and MMS failed to enter the organic phase of either solvent. Moreover, MMS failed to adhere to Amberlite XAD-7 resin in experiments in which $LTB_4$ and $LTC_4$ did adhere. Thus, MMS is not synthesized by cyclooxygenase or lipoxygenase enzymes, is not extractable into organic solvents, and does not adhere to lipophilic resin. Therefore, it is very unlikely that MMS is an eicosinoid.

Thereafter, MMS was characterized as to size and charge by ultramembrane filtration, gel filtration, and horizontal isoelectric focusing. MMS filtered through YM10 membranes was retained by UM05 membranes, and filtered on Sephadex G-25 with an apparent molecular weight of 2,000. 10 separate preparations were sized on Sephadex G-25 with consistent results. Isoelectric focusing of MMS revealed an acidic molecule (pI, 5.15). Preparations of MMS after these purification steps displayed dose-related secretagogue activity, indicating that MMS is relatively stable. Thus, MMS is a small, acidic molecule, probably an oligopeptide. To our knowledge, the generation of similar molecules by macrophages have never previously been described.

## Summary

Human pulmonary macrophages (PM) obtained from surgically removed human lung tissue released a factor after exposure to activated zymosan that caused cultured human airways to release increased amounts of radiolabeled mucous glycoproteins. The factor was released maximally after 4–8 h of zymosan exposure and caused a dose-related increase in glycoprotein release; it was termed macrophage-derived mucus secretagogue (MMS). MMS release was produced in a dose-dependent fashion by activated but not by nonactivated zymosan. The

activation of zymosan was C3 dependent, and C3b-coated Sepharose was also an effective stimulant. The data suggested that cell surface activation of the PM was a sufficient stimulus to cause MMS release and that both C3-dependent activation as well as Fc receptor activation were effective. The synthesis of MMS was sensitive to cycloheximide, and no active MMS was detectable intracellularly. To determine if MMS might be one of the oxidative derivatives of arachidonic acid, PM were incubated with cyclooxygenase and lipoxygenase inhibitors before activation. These manuevers did not influence MMS generation. MMS-rich supernatants were then extracted into organic solvents or exposed to lipophilic resin; in both cases, MMS remained in the aqueous phase. Thus, MMS is not a derivative of arachidonic acid. Sequential fractionation of MMS on ultramembrane and gel filtration followed by isoelectric focusing and gel filtration indicated that MMS is a small (~2000 daltons), acidic (pI, 5.15) molecule. Therefore, surface activation of human PM results in the synthesis and release of a small acidic molecule that causes airway mucous glands to secrete increased quantities of mucous glycoproteins.

We gratefully acknowledge the kind cooperation of the surgeons and pathologists in the Washington metropolitan area for providing human lung tissue for these experiments; Carl Hammer, Ph.D. and Michael Frank, M.D., National Institutes of Health, for their advice and provision of sera; Shirley Starnes for typing the manuscript; and Karen Leighty for editorial assistance.

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