Human Monocyte-derived Mucus Secretagogue

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Abstract

Human peripheral monocytes were stimulated with opsonized zymosan or protein A-containing *Staphylococcus aureus* to examine whether factors might be released that were capable of stimulating mucous glycoprotein release from cultured human airways, as has recently been described with human pulmonary macrophages. While the supernatant from monocytes exposed to opsonized zymosan or protein A-containing *S. aureus* caused an impressive increase in mucous glycoprotein release, no secretagogue activity was found in the control samples that were cultured in parallel and exposed to nonactivated zymosan or *S. aureus* that was deficient in protein A. The responsible factor was termed monocyte-derived mucus secretagogue (MMS). The maximum MMS release was reached 4–8 h after stimulation, and the amount of MMS released was dependent on the dose of opsonized zymosan added. Chromatographic analyses of MMS indicate that its molecular weight was ~2,000 and that the isoelectric point (pI) was 5.2, with a smaller second peak of 7.4 on isoelectric focusing. MMS itself was not detected in monocyte lysates, nor was it formed by monocytes treated with the protein synthesis inhibitor, cycloheximide, before exposure to activating particles. MMS was not a prostaglandin, could not be extracted into organic solvents, and is probably not an eicosanoid. Based on these observations, we conclude that stimulated human peripheral monocytes synthesize a small, acidic molecule, termed MMS, that is capable of stimulating human airways to secrete mucus and in nearly every respect is identical to pulmonary macrophage-derived MMS.

Introduction

Many pulmonary inflammatory processes are accompanied by increased mucus secretion. Mechanisms currently recognized as being capable of stimulating mucus secretion from human airways include mast cell-derived mediators of allergy (1–5), neurohormones (1, 6–8), oxidative derivatives of arachidonic acid (2–5, 9), serum (10), and *Pseudomonas aeroginosa* filtrates (11). Very recent studies indicate that activated human pulmonary macrophages synthesize and release a factor termed macrophage-derived mucus secretagogue (MMS)[1] (12) that can stimulate mucus secretion in vitro. Since alveolar macrophages are derived from circulating blood monocytes (13, 14), it was of interest to examine whether human peripheral monocytes might also be capable of influencing mucus secretion.

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1. *Abbreviations used in this paper:* ETYA, eicosatetraenoic acid; MMS, monocyte-derived mucus secretagogue; PGE, prostaglandin E.

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Methods

CMRL-1066 medium, penicillin, streptomycin, and amphotericin B (Gibco Laboratories, Grand Island, NY); [^{3}H]glucosamine (20 Ci/mmol) (International Chemical and Nuclear Corp., Irvine, CA); ultrafluor (National Diagnostics, Somerville, NJ); trichloroacetic acid (J. T. Baker Chemical Co., Phillipsburg, NJ) and phosphotungstic acid (Mallinckrodt, St. Louis, MO) were obtained from the manufacturers. All other materials were obtained as cited (12).

Monocyte cultures

Monocytes were prepared by three different methods.

Ficoll-Hypaque density gradient (sp gr 1.074–1.078) separation (15). Heparinized peripheral blood from normal donors was first centrifuged at 500 *g* for 10 min at 22°C. The buffy coat was then layered onto Ficoll-Hypaque, the preparation was centrifuged at 500 *g* for 20 min at 22°C, and the mononuclear cells were removed from the interface. The cells were then washed three times in Hanks' balanced salt solution without Ca^{2+} or Mg^{2+} and resuspended in CMRL-1066 medium with penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (100 mM) without supplementary serum. The concentration of peripheral monocytes 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 medium to remove nonadherent cells. The adherent cells were incubated for 24 h in CMRL-1066 without serum at 37°C in a humidified atmosphere of 5% CO_2 before starting the experiments. The viability of the cells, which always exceeded 98%, was assessed by trypan blue dye exclusion after incubation in a 1% solution of the dye. Differential counts (Wright-Giemsa-stained cytocentrifuge preparations) revealed that the final cell preparation consisted of >90% monocytes and <10% lymphocytes. Adherent cells phagocytized both latex particles and activated zymosan (Sigma Chemical Co., St. Louis, MO) and stained positively for nonspecific esterases (16).

Platelet-free human mononuclear cells. The usual Ficoll-Hypaque isolation procedures produce mononuclear cells contaminated with 10–1000 platelets/mononuclear cell (17). To reduce platelet contamination, a recently described technique was used (17). Briefly, EDTA-anticoagulated blood was first centrifuged through Hypaque (sp gr 1.060) at 400 *g* for 5 min at 22°C followed by the usual Ficoll-Hypaque (sp gr 1.077) gradient. This procedure reduced platelet contamination to <1 platelet/two mononuclear cells (17). After the initial isolation, the cells were processed and cultured as described above.

Enriched mononuclear cell preparation. Peripheral blood mononuclear cells were isolated on Ficoll-Hypaque gradients. The monocytes were further isolated with a J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA) equipped with a JE-6 elutriator rotor with two Sanderson separation chambers following a procedure modified from Lionetti et al. (18). The purity of the monocytes was >95% as determined by both morphology and nonspecific esterase staining. After the initial isolation, the cells were cultured as described above.

Preparation of human airways for culture

Human lung tissue was obtained at surgery primarily from tumor resection. Though all of the subjects from whom the lung tissue was

obtained were former or current smokers, no attempt was made to correlate their pulmonary function with mucus secretion. Normal appearing airways, 2–10 mm diam, were fragmented into 3 × 5-mm replicates and cultured as described (1, 2). The airway explants were maintained in CMRL-1066 medium with penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (0.5 μg/ml) in a controlled atmosphere chamber containing 45% O_2, 50% N_2, and 5% CO_2 and incubated at 37°C.

Radiolabeling of mucous glycoproteins

Mucous glycoproteins were radiolabeled by incorporating [^{3}H]glucosamine (1 μCi/ml) into the culture medium. Explants were initially incubated for 16 h in the absence of [^{3}H]glucosamine, washed twice with media, and then incubated with [^{3}H]glucosamine for a 16-h base-line period (period I). After the period I supernatants were harvested, fresh culture media without [^{3}H]glucosamine were added for an additional 4-h period (period II), and these supernatants were subsequently harvested. Airway cultures were subsequently cycled through 16-h base-line incubations (period I) and 4-h experimental incubations (period II) for up to 8 d. Histologic analysis of airways maintained in culture for 14 d revealed a normal appearing mucosa and submucosa. The culture media and washes from period I and II were stored at −70°C. 1-ml aliquots were added to 1 ml of a solution of 20% trichloroacetic acid and 2% phosphotungstic acid (19) in polypropylene disposable culture tubes (12 × 75 mm). After 16 h at 4°C, the samples were centrifuged at 3,000 rpm for 5 min, and the supernatant was decanted. The pellet was washed twice with a solution of 10% trichloroacetic acid and 1% phosphotungstic acid. The washed pellet was resuspended in 1 ml of 0.2 M NaOH. The suspension was vortexed and incubated at 22°C for 12 h. The suspension was vortexed a second time, and 0.50-ml aliquots were added to 10 ml of Ultrafluor and counted in a Beckman 8100 scintillation counter (Beckman Instruments, Inc.).

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 from period II/period I for each sample was determined and was termed the secretory index. Because of the relatively high secretory index observed in day 1 cultures, no experiments were done during the initial cycle. All experiments were instead 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. The control samples were derived from the same tissue, cultured in parallel, and handled identically to the experimental samples except that pharmacologic agents were not added. Each airway culture thus provided its own base-line period (period I) as well as stimulated period (period II), and the effects of each pharmacologic manipulation could be compared with matched controls.

Zymosan preparation

Zymosan particles 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 in a ratio of 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 0.15 M NaCl and finally resuspended in the incubation buffer prior to their use. Nonactivated zymosan was handled in an identical fashion except that these particles were not exposed to serum. Either activated zymosan or nonactivated zymosan was added to the cells in culture at a ratio of 150 particles per monocyte. Supernatants were removed at different intervals (as indicated), pooled, centrifuged (3,000 *g* at 22°C for 10 min), filtered through 0.22-μm Millex-GV sterilizing filter units (Millipore Corp., Bedford, MA), and applied to airways in culture, usually adding 2 ml/plate, at the beginning of period II.

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

Preparation of various particles for phagocytosis

Latex beads (polystyrene, average diam, 1.1 μm; Sigma Chemical Co.) were washed three times in saline before use. Preparations of formalin-fixed *Staphylococcus aureus* containing protein A (Bethesda Research Laboratories, Bethesda, MD) were washed five times in saline, resuspended at a 2% concentration in saline, and added to cultured monocytes at 10 bacteria per monocyte. *S. aureus* that were deficient in protein A were handled in an identical fashion.

Determination if monocyte-derived mucus secretagogue is preformed or newly synthesized

Peripheral monocytes were isolated and divided into two portions, each having 30 × 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, and washed and cultured for 8 additional h. After the supernatants were collected, the cells were removed from the culture plates with a rubber policeman and frozen at −70°C. Thereafter, the cultured monocytes and the cells that had been frozen directly after isolation were handled in parallel. The monocytes were freeze-thawed three times, sonicated in ice (3 min at power setting 3) with the use of 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 isoelectric focusing (see below). The MMS was then mixed with monocyte lysates for 4 h at 37°C, filtered on 0.22-μm membranes, and added to airways in culture.

In a related experiment, mononuclear cells were cultured in the presence (or absence, in parallel) of cycloheximide at 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.

Relationship between eicosanoids and monocyte-derived mucus secretagogue

Supernatants rich in mucus-secretagogue activity were prepared by incubating mononuclear cells 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 (20). As used for this study, these assays were sensitive to 2.5 pg.

Partial purification of MMS

50 ml of MMS-rich supernatant was obtained from mononuclear cells 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) with 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 monocytes or that had been exposed to monocytes plus nonactivated zymosan was manipulated in parallel as controls.

Concentrated UM05 retentate material was filtered on a 1 × 60-cm Sephadex G-25 column (Pharmacia Fine Chemicals, Pistcataway, NJ) 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 of distilled water, and refiltered. 20 μl of this material was then added to the airways in culture. For isoelectric focusing, the active fractions were pooled (usually 2–4 ml), lyophilized, and resuspended in 1 ml of water. 1 ml of fractionated MMS material was mixed with 58 ml of Buffalyte solution (pH 3–10) (Pierce Chemical Co., Rockford, IL) and 111 g of Pevikon (Accurate Chemical and Scientific Corporation, Westbury, NY). The slurry was poured into a horizontal isoelectric focusing apparatus (LKB electrofocusing kit, LKB Instruments, Inc., Gaithersburg, MD), and, with the method of Harpel and Kuppers (21), a pH gradient was established in 4 h at a constant power of 400 W (LKB 2197 power supply). The anodal and cathodal solutions were 0.1 M phosphoric acid and sodium hydroxide, respectively. The slurry was divided into 15 fractions that were poured individually into 1 × 6 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 percent increase in mucous glycoprotein release as compared with control, as described in detail (2). Each experiment used quadruplicate airway cultures for the determination of each point, and all results represent data observed from several experiments. The results are generally provided as the mean±SEM. Statistical comparisons were derived from paired sample *t* tests.

Results

Effects of zymosan on the release of MMS. To examine whether supernatants from peripheral monocytes might influence mucus secretion, 3×10^6 monocytes/plate were exposed to either nonactivated zymosan particles (10 mg/ml) or activated zymosan particles. After 8-h 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 monocytes cultured with nonactivated zymosan had no significant effect on airway mucus release (−7±2.6%, $n = 5$). By contrast, the supernatants from monocytes incubated with opsonized zymosan increased mucus release by 45.8±5.0% ($n = 5$, $P < 0.001$). The enhancing effects on mucus secretion by the factor released by monocytes exposed to activated zymosan ranged from 22 to 59% above the control in these experiments. Neither activated nor nonactivated zymosan had any effect when added directly to airway cultures. The factor found in the supernatant from monocytes incubated with activated zymosan was present in every experiment and was not influenced by the procedure used to isolate the monocytes. Based on these observations, the factor causing increased mucus glycoprotein release was termed monocyte-derived mucus secretagogue (MMS).

Dose response of zymosan for MMS release. Monocytes were exposed to 0.01–100 mg/ml of activated zymosan for 8 h, and the supernatants were added to the airways (Fig. 1). In each of two experiments (which are pooled in Fig. 1), concentrations of zymosan >1 mg/ml produced near maximal release of MMS. For convenience, zymosan was used at 10 mg/ml (150 particles/monocyte) in all subsequent experiments.

Table I. Effects of Supernatants from Human Monocytes on Mucous Glycoprotein Release

Source of supernatant	Mucus release
	% change from control
Monocytes plus activated zymosan ($n = 5$)	+45.8±5.0*
Monocytes plus nonactivated zymosan ($n = 5$)	−7.0±2.6‡

* Range, +22–59%; ‡ Range, −10–+5%; $P < 0.001$.
Human peripheral monocytes were incubated with activated zymosan or nonactivated zymosan for 8 h. The supernatants were centrifuged, filtered through 0.22-μm filters, and added to cultured airways. Results are from monocytes isolated by the elutriation technique (see text). In separate experiments, with monocytes isolated by Ficoll-Hypaque sedimentation, the increase in mucus release averaged +39.0±2.1% ($n = 3$) and +43.6±2.0% ($n = 3$) for monocytes isolated by the platelet-depletion technique.

Time course of MMS release from monocytes. To assess the time course of MMS release, monocytes were exposed to activated zymosan for 30 min to 16 h before removal of the supernatant (Fig. 2). Within 4 h of incubation, the cultures showed evidence of MMS release, which plateaued between 6 and 16 h. The time course study for MMS release was repeated on several other occasions, and the cultures exposed to zymosan for 24 h appeared to have less activity than those at 4–16 h. Therefore, 4–8-h incubations of mononuclear cells with activated zymosan were considered optimal for MMS release.

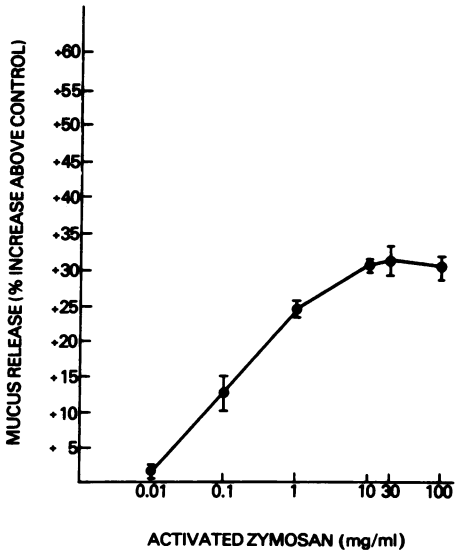

Figure 1. Dose response of activated zymosan on MMS release. Monocytes were incubated with 0.01–100 mg/ml of activated zymosan for 8 h. Supernatants were collected, pooled, centrifuged, filtered, and then applied to human airways in culture. The results are the percent increase in mucus release as compared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

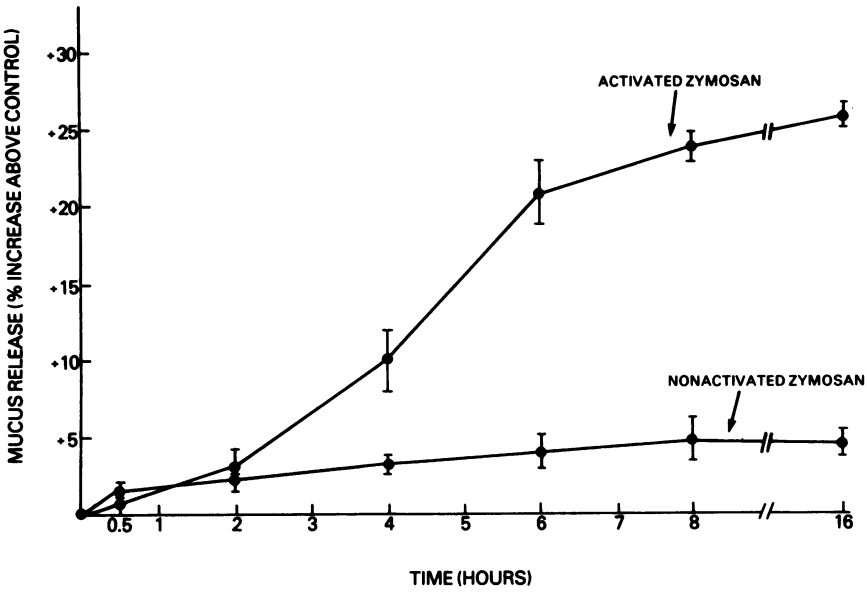

Figure 2. Time course of the appearance of MMS. Monocytes were incubated with activated zymosan or nonactivated zymosan (10 mg/ml) for 30 min to 16 h before supernatants were removed and added to airways in culture.

Effect of washing monocytes on MMS release. Microscopic analysis of monocytes 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 (in order to optimize MMS release) might be an acceptable design. In these experiments, monocytes 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. Using this procedure, we compared the supernatants from three experiments, and each generated equivalent degrees of increased mucus release (+30.2±1.0% for continuous zymosan exposure and +29.7±1.4% for 1 h of zymosan exposure). Therefore, it appeared that peripheral monocytes generate equivalent amounts of 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 human sera that had been heat inactivated (56°C/30 min) (Table II). Monocytes exposed to saline-treated zymosan generated no MMS. Zymosan that had been incubated in heat-inactivated sera failed to stimulate monocytes to generate MMS. Only zymosan that was activated in normal human sera caused the monocytes to release MMS. Thus, complement products are required for zymosan to induce MMS release from peripheral monocytes.

To examine whether complement-derived factors were the only opsonins adequate for inducing MMS release, we examined the effect of using *S. aureus* that contains protein A. A ratio of 10 bacteria per monocyte was used (Table II), and this incubation also led to MMS release. However, incubation of monocytes with staphylococci that were deficient in protein A failed to cause MMS release.

Dose response of MMS. MMS collected from monocytes incubated with activated zymosan for 8 h was added to airway cultures in dose-response experiments (Table III). In two separate experiments, MMS in amounts ranging from 0.5 to 2 ml (diluted to a final volume of 2 ml) added to airways caused a dose-related increase in mucus release.

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

As it was possible that enzymes contained in the monocyte lysates might be capable of degrading MMS and thereby falsely obscure MMS activity in the lysates, monocyte lysates were

Table II. Role of Complement and Cell Surface Activation in the Generation of MMS

Peripheral monocytes plus	Mucus release
	% change from control
Nonactivated zymosan	+1±0.4
Zymosan incubated in normal human serum	+33±0.2
Zymosan incubated in heat-inactivated human serum	+3±2.0
S. aureus-protein A	+73±5.1
S. aureus-deficient in protein A	+5±2.0

Peripheral monocytes in cultures were exposed to the various particles for 60 min after which the cells were washed and the incubation was continued with fresh media (without phagocytic particles) for 8 h. Thereafter, the supernatants were prepared for assessment of MMS activity as described. Each result is the mean±SEM for three separate experiments.

Table III. Dose Response of MMS

	MMS concentration (dilution)	Mucus release
		% increase above control
Experiment A	1:3	16±1.7
	1:1	21±1.6
	Undiluted	40±2.1
Experiment B	1:3	21±1.0
	1:1	30±0.9
	Undiluted	45±1.2

Monocytes were incubated with activated zymosan for 8 h, and the supernatants were collected. The supernatants were diluted with culture media to a final volume of 2 ml and added to cultured airways.

also incubated with partially purified MMS. This partially purified MMS was obtained from supernatants of cultured monocytes 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 44±3.0%. The partly purified MMS was incubated for 4 h with lysates from monocytes, which had no effect on mucous glycoprotein release on their own (+3±2.0%). Despite the exposure to monocytes lysates, the partially purified MMS was still capable of increasing mucus release by 47±4.0%. MMS is thus not degraded by mononuclear lysates, and the inability to detect MMS in mononuclear lysates probably means MMS is neither preformed nor stored intracellularly.

These data suggested that MMS activity, as was found with macrophage-derived secretagogues (12), might be newly synthesized after exposure to the phagocytic stimuli. To examine this possibility further, mononuclear cells were incubated for

Table IV. Effects of Monocyte Supernatants and Lysates on Mucous Glycoprotein Release

Monocytes exposed to	Preparation	Mucus release
		% change from control
Activated zymosan	Culture supernatant	+33±2.0
	Lysate	+1±1.2
Nonactivated zymosan	Culture supernatant	+8±3.0
	Lysate	+3±1.5
S. aureus	Culture supernatant	+58±6.0
	Lysate	+9±5.2
Culture media alone	Culture supernatant	0±1.0
	Lysate	−1±1.5
Lysate of mononuclear cells not cultured in vitro		+7±4.0

Results are from three separate experiments presented as the mean±SEM.

Table V. Effects of Cycloheximide on MMS Release from Mononuclear Cells

Monocytes exposed to	Preparation	Mucus release
		% change from control
Activated zymosan	Culture supernatant	+26±1.0
	Lysate	+2±1.0
Cycloheximide + activated zymosan	Culture supernatant	+3±1.2
	Lysate	+5±3.1
S. aureus-protein A	Culture supernatant	+31±2.0
	Lysate	−2±1.0
Cycloheximide + *S. aureus*-protein A	Culture supernatant	+5±3.0
	Lysate	+3±2.0

Results from three separate experiments are pooled and presented as mean±SEM.

16 h in the presence or absence of cycloheximide and then exposed to either activated zymosan or *S. aureus*. The capacity of these cells to generate MMS activity was then compared to noncycloheximide-exposed controls (Table V). Mononuclear cells that were exposed to phagocytic stimuli released MMS activity, while cycloheximide-exposed mononuclear cells released much less. While these data suggested that cycloheximide interfered with MMS synthesis, cell lysates were also examined to determine whether intracellular MMS was generated and then not released. No intracellular MMS was detected. These observations strongly suggest that MMS is newly synthesized through a cycloheximide-sensitive pathway.

Is MMS a lipid or eicosanoid? Mononuclear cells were exposed to the cyclooxygenase inhibitors, acetylsalicylic acid (100 μg/ml) or indomethacin (10 μg/ml) (Table VI), for 1 h

Table VI. Effects of Inhibitors of Arachidonic Acid Metabolism on MMS Release by Mononuclear Cells

Mononuclear cells exposed to	Mucus release
	% change from control
ASA (100 μg/ml)	+4±0.3
Activated zymosan	+31±0.8
Activated zymosan + ASA	+29±0.3
Nonactivated zymosan	+6±0.1
Indomethacin (10 μg/ml)	+7±0.6
Activated zymosan	+22±0.1
Activated zymosan + indomethacin	+26±0.3
Non-activated zymosan	−2±0.2
ETYA (100 μM)	+2±0.5
Activated zymosan	+28±0.2
Activated zymosan + ETYA	+31±0.3
Nonactivated zymosan	+1±0.7

ASA, acetylsalicylic acid.
The results from single, representative experiments in which mononuclear cells were exposed to ASA, indomethacin, or ETYA for 1 h before the addition of activated zymosan and subsequent culture for MMS generation. Results are each the mean±SEM of five plates.

before the addition of activated zymosan and subsequent culture for MMS generation. In both instances, the cyclooxygenase inhibitors failed to affect MMS release. These experiments were extended to examine the effect of eicosatetraynoic acid (ETYA) (100 μM), which inhibits both cyclooxygenase and lipoxygenase pathways. ETYA also failed to influence MMS release by zymosan-activated mononuclear cells (Table VI).

To be certain that other eicosanoids were not contributing to the secretagogue activity, a 50-ml preparation of MMS-rich supernatant was generated from the culture of 75×10^6 monocytes and after acidification, extracted into the organic solvents ethylacetate or diethylether. Neither of the solvents, despite their capacity to extract prostaglandins, monohydroxyeicosatetraenoic acids (HETEs), and leukotrienes, was able to remove MMS activity from the aqueous phase. In two experiments where diethylether was used, the original crude MMS activity on mucus release was +32±6.0% before extraction. After extraction with diethylether, the aqueous phase maintained most of the MMS activity (+28±4.0% above control), whereas the organic phase contained very little activity (+5±3.0% above control). In the two experiments where ethylacetate was used as the organic solvent, the original MMS activity, when assayed on human airways, showed a +35±1.0% increase in mucus release above control. After extraction with ethylacetate, the aqueous phase contained all of the MMS activity (34±3.0% above control) and the organic phase had none (+2±2.0% above control). MMS generation is thus not affected by inhibition of arachidonate metabolism nor extracted into the organic solvents commonly used in eicosanoid purification. Partially purified MMS (see below) was assayed for the presence of immunoreactive $PGF_{2\alpha}$, PGE, and thromboxane B_2 using radioimmunoassays sensitive to 2.5 pg/ml for each. No prostaglandins could be detected in any of the three partially purified MMS preparations that were assayed. It thus appears that MMS is neither a lipid nor an eicosanoid and that eicosanoids do not contribute to MMS activity.

Chromatographic analyses of MMS. To estimate the size of MMS, supernatants obtained from mononuclear cells incubated with activated zymosan were filtered on ultramembranes. MMS was found to filter through a YM10 membrane (indicating a size of <10,000 D) 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 mononuclear cells 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, eight separate preparations of MMS (after ultramembrane fractionation) have been filtered through Sephadex G-25. Fig. 3 presents the chromatographic pattern from the first three preparations, which suggested 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 five subsequent fractionations and the mean (±SEM) increase in mucus release by these fractions is 43±2.0%. Material from a single isolation prepared by sequential ultramembrane and gel filtration was examined and found to stimulate mucus secretion in a dose-dependent fashion.

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 5.20. A third preparation of MMS also focused at pH 5.20 (data not shown), indicating that this is a highly reproducible finding. A second smaller peak of activity at pH 7.4 was also consistently noted. Two preparations of partially purified MMS, after ultramembrane and gel filtration and isoelectric focusing, were examined and also found to stimulate mucus secretion in a dose-related fashion.

Discussion

It has recently been demonstrated that human pulmonary macrophages synthesize a factor (MMS) that is capable of

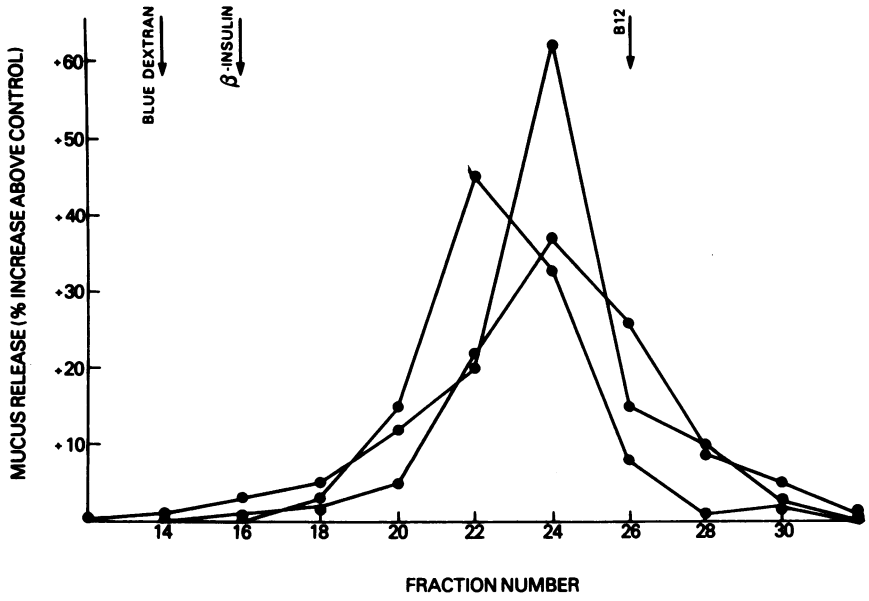

Figure 3. Sephadex G-25 chromatography. 50–100 ml of supernatant that was obtained from monocytes incubated with activated zymosan were pooled and concentrated by ultramembrane filtration. The sample was then applied to a 1 × 60-cm Sephadex G-25 column that was eluted with distilled water. The figure presents a summary of three separate chromatograms.

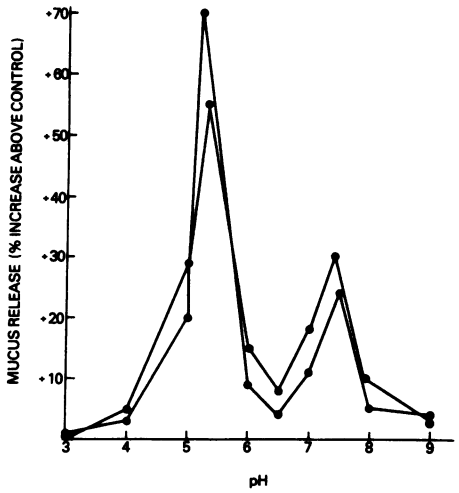

Figure 4. Isoelectric focusing of MMS on Pevikon. Two preparations of MMS were filtered on ultramembranes and Sephadex G-25 and then applied to a horizontal bed of Pevikon into which ampholytes were incorporated. The material was focused for 4 h, resulting in a linear pH gradient. The bed was divided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

causing human airways to release increased amounts of biosynthetically radiolabeled mucous glycoproteins (12). As alveolar macrophages are derived from circulating blood monocytes (13, 14), it was logical to examine circulating blood monocytes to determine whether they were also capable of releasing the same (or a similar) factor. Monocytes were isolated in three different techniques, with some of the preparations having a purity of >95%, as determined by both morphology and nonspecific esterase staining. Peripheral blood monocytes require 7 d in culture to mature into macrophages (22, 23). Therefore, all the experiments described were performed within 3–5 d of the monocyte isolation. However, monocytes cultured for 1–2 d were equally as active as 3- to 5-d cultures.

Human peripheral blood mononuclear cells were found to release a factor, termed MMS, that is a potent mucus secretagogue. Simply culturing monocytes, in the absence of surface activation, resulted in neither MMS release into the supernatant nor its presence in cell lysates. However, culturing monocytes with either opsonized zymosan or *S. aureus* containing protein A results in MMS release into the media. MMS is released over a 4–8-h period after stimulation and was found in every stimulated monocyte preparation examined. Similar to earlier observations with human pulmonary macrophages (12), cell surface activation of monocytes by complement-derived opsonins (activated zymosan) or through Fc receptors (as suggested by the *S. aureus* containing protein A) initiated the process of MMS release. The time course of release suggested the possibility that synthesis or activation of this molecule is necessary before its release. Lysis of MMS-producing cells failed to reveal any detectable intracellular MMS, suggesting that the MMS is newly synthesized before release rather than being stored. However, another possibility is that intracellular MMS may be stored in an inactive form.

Cycloheximide inhibits protein synthesis by reducing peptide bond formation (24) and is capable of inhibiting >90% of new protein synthesis by human pulmonary macrophages as measured by [^{3}H]leucine incorporation into precipitable proteins (12). The capacity of cycloheximide to affect MMS release was examined. Preincubating monocytes with cycloheximide before exposure to either *S. aureus* or activated zymosan significantly reduced MMS release. To determine whether cycloheximide might simply have impaired monocyte export of MMS, cell lysates were also examined and found to contain no MMS activity. MMS is thus probably a newly synthesized molecule whose formation is inhibited by cycloheximide.

When stimulated in vitro with zymosan or endotoxin, human peripheral blod mononuclear cells release prostaglandins, predominantly PGE (25, 26). Earlier work with human airways demonstrated that arachidonic acid and many of its oxidative derivatives express mucus secretagogue activity. Among others, these secretagogues include PGE, $PGF_{2\alpha}$, PGD_2, PGI_2, and other prostaglandins (2). It was therefore important to examine the relationship between MMS and arachidonic acid derivative. Several lines of evidence suggest that MMS is not one of the oxidative derivatives of arachidonic acid: (*a*) Monocytes were incubated with cyclooxygenase (aspirin and indomethacin) and lipoxygenase (ETYA) inhibitors before activation; these maneuvers did not influence MMS generation. Thus, impairment of either or both enzymatic pathways involved in the oxidation of arachidonic acid failed to influence MMS formation. (*b*) MMS was extracted on three separate occasions with ethylacetate or diethylether and failed to enter the organic phase of either solvent. Thus, MMS is not extractable into organic solvents. (*c*) Partially purified MMS was assayed for the presence of immunoreactive $PGF_{2\alpha}$, PGE, and thromboxane B_2 with the use of radioimmunoassays sensitive to 2.5 pg/ml for each (20). No prostaglandins could be detected. Therefore, it is very unlikely that MMS is an eicosanoid.

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. Eight separate preparations were sized on Sephadex G-25 with consistent results. Isoelectric focusing of MMS revealed an acidic molecule (isoelectric point [pI] = 5.20). Preparations of MMS after these three sequential purification steps displayed dose-dependent secretogogue activity, indicating that MMS is relatively stable. The second, smaller peak of activity noted on isoelectric focusing had a pI of 7.40 and did not exhibit any dose-related secretagogue activity, indicating that it is most probably not related to MMS. Therefore, we conclude that monocyte-derived MMS (like macrophage-derived MMS) is a small, acidic molecule that is synthesized by monocytes after surface activation and has the capability of influencing mucus secretion. In nearly every respect, monocyte-derived MMS resembles pulmonary macrophage-derived MMS. Indeed, one must conclude that these MMS molecules are most likely identical products released by cells in the same lineage under similar conditions. Taken together with earlier studies of pulmonary macrophges, these data suggest that attention needs to be directed at the role monocytes/macrophages play in human diseases in which excess mucus secretion is important.

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