Recombinant Interferon-Gamma Primes Alveolar Macrophages Cultured In Vitro for the Release of Leukotriene B_4 in Response to IgG Stimulation

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Abstract

The capacity of interferon-gamma to regulate the generation and release of leukotriene B_4 (LTB_4) from human alveolar macrophages of normal nonsmoking individuals was evaluated. When alveolar macrophages were incubated for 60 min with heat aggregated IgG (HAIgG), they generated and released 5.7 ± 1.7 ng of LT B_4 per 10^6 cells compared to 1.9 ± 0.4 ng from cells incubated with buffer alone, $P = 0.02$. When alveolar macrophages were preincubated with interferon-gamma for 24 h before activation for 60 min with heat-aggregated IgG, the soluble IgG aggregates became a significantly more effective stimulus for LTB_4 release, 17.0 ± 3.9 ng/10^6 cells, $P = 0.001$, compared to cells incubated in the absence of interferon-gamma and challenged with HAIgG. Interferon-gamma did not alter the response to A23187. This effect of interferon-gamma was both time and dose dependent; it also was specific since neither interferon-alpha nor interferon-beta had a regulatory effect on the release of LTB_4 from cells in response to challenge with HAIgG. Preincubation of the alveolar macrophages with interferon-gamma augmented the density of IgG_1 receptors by $81.5\pm17.3\%$; neither interferon-alpha nor interferon-beta effected this parameter. Furthermore, monomeric IgG_1 blocked HAIgG induced LTB_4 release from alveolar macrophages primed with interferon-gamma. Therefore, at least one of the mechanisms by which interferon-gamma primes alveolar macrophages for the production and release of LTB_4 in response to stimulation by aggregates of IgG is that of increasing the number of receptors for this stimulus.

Introduction

Alveolar macrophages (AM)[1] reside on the epithelial surfaces of the lower respiratory tract and have been implicated as being central to the evolution of a variety of inflammatory events in the lung (1, 2). In orchestrating such reactions, human AM release numerous biologically active molecules, among which are included lipid metabolites of cellular membrane origin, which are synthesized de novo after cell activation (1–4). For these cells, only zymosan and the calcium ionophore A23187 are known to activate both phospholipase A_2 to cleave arachidonic acid from some pools of membrane phospholipids and also the calcium-dependent oxidative metabolism of arachidonic acid by the 5-lipoxygenase to leukotriene A_4. Leukotriene A_4 then is catalytically hydrolyzed to leukotriene B_4 (LTB_4) (5–7). As a proinflammatory mediator, LTB_4 is of particular interest because of its numerous effects on a variety of leukocytes. These effects include potent chemokinetic and chemotactic activity for human neutrophils (8–11), eosinophils (10–13), and monocytes (8, 14) in vitro, induction of human neutrophil aggregation (9), and stimulation of rabbit and human neutrophil lysosomal enzyme release (15). In addition, LTB_4 exerts potent immunoregulatory effects on human peripheral blood lymphocytes with the net effect being immunosuppression (16–18). In order to extend the relevance of these observations to the spectrum of human inflammatory lung diseases, we used a receptor-dependent soluble activator, heat aggregated IgG (HAIgG). In addition, we assessed the effect of an immune response regulator, human interferon-gamma (IF-γ), on human AM generation and release of LTB_4.

Human IF-γ is a 20,000 mol wt glycoprotein produced by lymphocytes (19, 20) and human AM (21) in response to a variety of stimuli (22). Many of the effects of IF-γ serve to intensify immune reactions. Macrophages and monocytes in particular, are targets for the actions of this molecule. Murine macrophages possess IF-γ receptors (23), and recently, receptors for IF-γ have been found on human peripheral blood monocytes (24). Some of the effects of IF-γ on macrophages or monocytes include augmenting expression of MHC class 2 antigens and antigen presentation to lymphocytes (25), enhancing cytotoxicity for tumor cells (26) and intracellular parasites (27), and augmenting the density of IgG Fc receptors (IgGFcR) (28). Based on these observations, we hypothesized that IF-γ might increase LTB_4 release from human AM when they were challenged with the immunologically relevant stimulus, HAIgG.

Methods

Preparation of AM cultures. Human AM from nonsmoking normal volunteers were retrieved by bronchoalveolar lavage (BAL) (29). Briefly, all subjects had normal medical histories, chest radiographs, spirometry, and complete blood counts, and none admitted to any illness including a respiratory tract infection for which any medication, including nonsteroidal antinflammatory agents, was required in the 4-wk period before participation. After topical 4% lidocaine anesthesia was applied to the oropharynx, BAL was performed through a fiberoptic bronchoscope, wedged in a segmental bronchus, by instilling sterile normal saline in 50-ml aliquots and gently aspirating after each

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1. *Abbreviations used in this paper:* AM, alveolar macrophages; BAL, bronchoalveolar lavage; HAIgG, heat aggregated IgG; IF-γ, interferon γ.

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instillate. A maximum of 300 ml was instilled into each lung of an individual volunteer. BAL aliquots were pooled, filtered through a single layer of gauze, and centrifuged at 500 *g* for 5–10 min at 24°C. The cells were washed twice in Ca^{2+} and Mg^{2+} free HBSS (Grand Island Biological Co., Grand Island, NY) and resuspended in McCoy's 5a medium (Grand Island) containing 5% heat-inactivated fetal bovine serum (HIFBS) (KC Biological Co., Lenexa, KS) supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin. Total cells were counted on a hemocytometer, viability was assessed by cellular exclusion of trypan blue, and differential cell counts were made on Wright-stained cytocentrifuge preparations. Lavage cells prepared in this way averaged (mean±SEM) 85.1±1.0% AM, 13.4±1.0% lymphocytes, 1.1±0.1% polymorphonuclear cells, 0.7±0.2% mast-basophiloid cells, and 0.5±0.1% eosinophils, n = 85. Macrophage viability averaged 88.7±0.6%. Platelet contamination, estimated from the small number of red blood cells, 9.8±1.7% of total cells recovered, present in the lavage fluid due to trauma of the airway epithelium by the tip of the bronchoscope, never exceeded one platelet per two nucleated cells. For the cell cultures used in the assessment of leukotriene release, lavage cells were adjusted to 2×10^6 cells per ml and placed into sterile 7-ml Teflon chambers (Scientific Specialties, Randallstown, MD).

Preparation of HAIgG. The gamma-globulin fraction from 30–50 ml of fresh human serum was obtained by precipitation with one-third saturated ammonium sulfate, extensively dialyzed against 0.05 M Tris-HCl, pH 8.0, and was partially purified by ion exchange column chromatography on diethylaminoethyl cellulose (DEAE 52, Whatman; H. Reeve Angel, Inc., Clifton, NJ). This was followed by dialysis against PBS 0.1 M, pH 7.2, and 0.1% sodium azide, with subsequent filtration on Sephadex G-200-gel bed volume 150 ml-(Pharmacia Fine Chemicals, Piscataway, NJ) that had been previously calibrated with the following standards: blue dextran, 11s-IgA (kindly provided by Dr. H. Y. Reynolds, Yale University School of Medicine), IgG, and albumin. The IgG containing eluates from each column were detected by absorbance at 280 nm and their known elution times for these columns. After the initial isolation of IgG from the G-200 column, the IgG was concentrated by positive pressure ultrafiltration through a UM010 membrane (Amicon Corp., Lexington, MA). HAIgG was prepared by incubating the IgG (10 mg/ml) at 63°C until slight opalesence appeared and then centrifuging at 3,000 rpm for 10 min (30). The supernatant then was refiltered over the Sephadex G-200 column and the void volume containing the IgG aggregates was retained, concentrated by positive pressure ultrafiltration, and dialyzed into Dulbecco's PBS, pH 7.2 (Gibco Laboratories, Grand Island, NY). The presence and purity of IgG aggregates was verified by immunoprecipitation diffusion-in-gel analysis using specific rabbit anti-human albumin, transferrin, whole serum, and immunoglobulin G and A antisera (Calbiochem-Behring Corp., San Diego, CA) and calculated to be > 99% pure. After sterilization by filtration through a 0.2-μm membrane, the amount of HAIgG was quantified by the method of Lowry (31) using bovine gamma globulin to generate the standard curve (Bio-Rad Laboratories, Richmond, CA). The presence of endotoxin in the HAIgG preparations and cell culture supernatants was quantitated using a Limulus assay sensitive to 1.0 ng/ml, as described (32).

Incubation of lavage cells with recombinant interferons-alpha (IF-α), beta (IF-β), or gamma (IF-γ). Bronchoalveolar lavage cells were incubated for 1 or 24 h at 37°C in a humidified atmosphere containing 5% CO_2 with 10, 100, or 1,000 U/ml of recombinant IF-γ or without interferon (Hoffmann-La Roche, Nutley, NJ). The units of the stock solution were determined by a WISH/VSV microtiter assay that utilized National Institutes of Health (NIH) standard Gg 23-901-530. The cells then, were centrifuged at 500 *g* for 5 min and resuspended in HBSS containing Ca^{2+} and Mg^{2+} immediately before stimulation. In those experiments that utilized IF-α and IF-β (Hoffmann-La Roche), the cell cultures were incubated under identical conditions at a single interferon concentration, 1,000 U/ml.

Stimulation of human AM. Bronchoalveolar lavage cells, after incubation with IF-γ as described above, were incubated for 30 min at 37°C, in an atmosphere of air and 5% CO_2 and with media containing 2 μM A23187 (Calbiochem-Behring Corp.), which was dissolved in dimethylsulfoxide and diluted to 0.1% (vol/vol) in the cell cultures. Then, the cells were centrifuged at 500 *g* for 5 min and the supernatants were harvested and stored at −70°C until assayed by RIA for LTB_4. Preliminary observations in our laboratory indicated that storage of macrophage supernatants under these conditions for as long as one year did not adversely affect the recovery of immunoreactive LTB_4. When reverse-phase high performance liquid chromatography (RP-HPLC) was to be performed for resolution of LTB_4, the supernatants were mixed with 4 vol of ethanol before being stored at −70°C. Just before chromatography, the samples were warmed to 4°C and centrifuged at 2,000 *g* for 10 min at 4°C to remove precipitated proteins. The supernatants were evaporated to dryness under reduced pressure in a Speed Vac Concentrator (Savant Products, Hicksville, NY) and resuspended in either RIA buffer for LTB_4 determination or mobile phase solvent for RP-HPLC.

Some of the selected cell cultures, which were to be stimulated by HAIgG, were first treated with IF-γ, then washed and resuspended in HBSS and challenged with 25–50 μg/ml of HAIgG for 1 h at 37°C, under 5% CO_2. The cell cultures were processed for LTB_4 by RIA with or without prior RP-HPLC resolution, as described for the cells activated with A23187. In some experiments lipopolysaccharide (LPS), type Salmonella Minnesota R595 (List Biological Laboratories, Campbell, CA) was added to IF-γ treated cell cultures in place of or in addition to HAIgG, to assess whether or not endotoxin was involved in the release of LTB_4. In addition, experiments were performed in which a monoclonal anti-IF-γ antibody (Meloy Laboratories, Springfield, VA) was added to AM cell cultures at the same time as the recombinant IF-γ. The AM then were cultured for 24 h before washing and stimulation with HAIgG. In other experiments monomeric IgG_1 was added at a final concentration of 50–100 μg/ml to AM cell cultures 10 min before the addition of HAIgG.

Quantitation of LTB_4 and LTC_4. LTB_4 was quantitated by either of two radioimmunoassays using immune rabbit plasmas provided by Dr. E. Hayes, Merck & Co., Rahway, NJ (33). The first was a double-antibody RIA. Briefly, 200 μl of immune rabbit serum, diluted to give 50% binding of [^{3}H]LTB_4, were added to a mixture of 200 μl of PBS/BSA/azide containing ~ 7,000 cpm of [^{3}H]LTB_4 (New England Nuclear, Boston, MA) and 100 μl of sample or a synthetic LTB_4 standard (J. Rokach, Merck Frosst, Québec, Canada). This mixture was incubated for 2 h at 4°C before the addition of 500 μl of a 1:5 dilution of goat anti-rabbit IgG (Bio-Rad Laboratories), and the incubation was continued for 18–20 h. The immune precipitates were sedimented at 12,000 *g* for 3 min and 800 μl of the solution phase added to 10 ml of scintillation cocktail, and assessed for radioactivity in a liquid scintillation counter. There was no difference in standard curves run in the presence or absence of 1,000 U/ml of IF-γ. The minimum quantities of LTB_4 detectable with this assay were 1.5 ng/ml. The second and more sensitive RIA (minimum detectable levels of 0.5 ng/ml) employed dextran-coated charcoal to separate antibody-bound LTB_4 from unbound LTB_4 (34). Results from the two assays were compared to each other and to results obtained with RP-HPLC on aliquots of cell supernatants. LTC_4 was quantitated using a charcoal RIA which was able to detect a minimum of 0.5 ng/ml and was purchased from New England Nuclear. Quantities of leukotriene in nanograms per milliliter were adjusted to nanograms/10^6 viable alveolar macrophages.

RP-HPLC. RP-HPLC was performed for some experiments using a Waters system with a NOVA PAK C18 column (3.9 × 150 mm) and for other experiments on an Altex-Beckman system (35) with a 10-μm C-18 Ultrasil ODS column (4.6 × 250 mm) (Altex Rainin, Berkeley, CA) and eluted isocratically with a mobile phase consisting of methanol/water/acetic acid (67;33;0.08, vol/vol; pH adjusted to 5.85 with ammonium hydroxide) at a flow rate of 1 ml/min with continuous online monitoring at 280 nm (36). The performance of the two chromatography systems was essentially equivalent. Synthetic LTC_4, LTD_4, and LTE_4 standards were provided by J. Rokach.

IgG rosette assay. Rosette assays were performed as described by Spiegelberg and co-workers (37). Briefly, ox erythrocytes (Colorado

Serum, Denver, CO) were washed thoroughly in PBS and were sensitized with that subagglutinating concentration of rabbit IgG anti-ox red blood cell antibody (Cappel Laboratories, Cochranville, PA) which resulted in rosette formation of ~ 40% of fresh human alveolar macrophages. The rosette assays were performed by mixing 50 μl of 1% ox erythrocytes, suspended in McCoy's 5a medium supplemented with 5% HIFCS, with 50 μl of lavage cells (5×10^6/ml) suspended in the same medium. The mixture was centrifuged at 200 *g* for 1 min and incubated on ice for 1 h. The cells then were agitated to gently resuspend them, and the number of cells bearing three or more erythrocytes counted as IgG receptor positive. Assays performed using ox erythrocytes not coated with anti-ox cell antibody served as controls.

Purification and iodination of IgG_1 monomer. IgG_1 monomer was prepared from the serum of a patient with monoclonal IgG_1 myeloma as summarized previously (38). Briefly, serum gamma globulin was twice precipitated, first with the addition of dry weight ammonium sulfate to 30% concentration, and then the supernatant was adjusted to 50% concentration. The precipitates were dialyzed extensively against 0.01 M PBS/azide, pH 7.4, to remove ammonium sulfate and then against 0.02 M Tris-HCl in preparation for anion exchange column chromatography on DEAE-52. The IgG was eluted with a logarithmic gradient of 0.5 M NaCl in 0.02 M Tris-HCl. The IgG-rich fractions were gel filtered on Sephadex G-200. The fraction that eluted after an 11S-IgA standard was collected, concentrated, and iodinated with ^{125}I (New England Nuclear) by the lactoperoxidase-glucose oxidase method (Enzymobeads; Bio-Rad). Briefly, 15 μl of 11.0 mg/ml monomeric IgG_1 were added to 50 μl of preswollen enzymobeads and 50 μl of 0.2 M phosphate buffer, pH 7.2. After adding 1.0 mCi of ^{125}I, the reaction was initiated by adding 50 μl of 1% B-D-glucose, and continued for 30 min at room temperature; the reaction was terminated by the addition of 50 μl of 1% sodium azide. Iodinated IgG_1 was separated from unbound ^{125}I by filtration over a Bio-Gel P-6DG (Bio-Rad) column. In all instances over 95% of the radioactivity was precipitable with 20% TCA. Ultracentrifugation of a trace quantity of the iodinated IgG_1 on a 10–40% sucrose density gradient at 35,000 *g* for 18 h verified that the IgG_1 was monomeric and that the iodination procedure did not result in aggregation of the ligand. IgG_1 concentrations in the final preparations were determined by the method of Lowry (31) using an IgG standard (Bio-Rad). The mean±SEM specific activity was 1.43±0.37 μCi/μg IgG_1. ^{125}I-IgG_1 was stored at 4°C and was used within 2 wk of preparation.

Binding studies of monomeric ^{125}I-IgG_1 to AM. The binding of radioligand to alveolar macrophages was carried out in PBS (Dulbecco's; Gibco) pH 7.2, supplemented with 0.1% sodium azide, 0.1% EDTA, and 0.1% human serum albumin (39). For Scatchard analysis, duplicate suspensions of alveolar macrophages (200,000–400,000 cells per sample) were incubated with radioligand concentrations ranging from 1 to 250 nM in polypropylene microfuge tubes at 37°C for 1 h, since preliminary kinetic studies demonstrated that equilibrium was established at these conditions. At the end of the incubation period the tubes were mixed gently and 50-μl aliquots centrifuged through 200 μl of a 4:7 (vol/vol) mixture of dioctyl phthalate/dibutyl phthalate oil (Aldrich Chemical Co., Milwaukee, WI) for 1 min at 10,000 *g* in a microfuge (Fisher Scientific Corp., Springfield, NJ).

The tubes then were frozen in methanol, which was cooled with dry ice, and the tubes were cut above the cell pellet. Both cell pellet and supernatant were counted in a gamma counter. Nonspecific binding to cells always was determined at the same time by incubating duplicate cell suspensions with radioligand and a 100-fold excess of unlabeled IgG_1. Binding of $^{125}IgG_1$ was calculated in molecules per macrophage present in the cell suspensions. The binding of radioligand to lymphocytes was ignored because lymphocyte contamination of the macrophage cultures used for binding studies was limited to 15.5±3.4%, $n = 9$, but more notably because lymphocytes possess minimal numbers of IgG receptors (39). Binding data were calculated as described by Scatchard (40). Linear regressions (TI-55; Texas Instruments, Lubbock, TX) were performed by plotting the ratio of bound/free ^{125}I counts per minute against the nanomolar amount of bound ^{125}I-IgG_1. The number of binding sites per cell was calculated from the *x* axis intercept. The equilibrium association constant (K_a), expressed in liters/mol (M^{-1}), was determined from the negative slope.

Determination of AM cell volume. Macrophage cell volume was determined by incubating a 1-ml aliquot of lavage cells (1×10^6 cells) with 2.5 μCi [^{3}H]H_2O (New England Nuclear) and 0.5 μCi of [^{14}C]-inulin (Amersham Corp., Arlington Heights, IL) for 1 min at room temperature. 50, 100, and 200 μl aliquots were placed in 400-μl eppendorf microfuge tubes, which contained 120 μl of a mixture of paraffin and silicone oil (16:84 vol/vol) layered over 35 μl of 15% TCA in water. The cells were centrifuged into the TCA in a Beckman model B microfuge at 10,000 *g* for 1 min. The difference between the total pellet volume of the water (^{3}H-related dpm) and the extracellular volume (^{14}C-related dpm) was taken as the intracellular macrophage volume (41).

Statistical analyses. All data are expressed as the mean±SEM. For normally distributed data statistical calculations were performed using a paired *t* test (two tailed). For nonparametric data, the Wilcoxon's signed rank test or the sign test were used for paired data. Comparisons between groups of subjects were made using the Wilcoxon two-group rank sum test. All analyses were performed using the NIH sponsored CLINFO project. *P* values ≤ 0.05 were considered significant.

Results

LTB_4 release from human AM stimulated with calcium ionophore or HAIgG. Human AM cultures stimulated for 30 min at 37°C with 5 μM A23187 released a mean±SEM of 86.9±14.9 ng of immunoreactive $LTB_4/10^6$ viable AM, range 24–178 ng, (Fig. 1). Preliminary A23187 dose-response experiments determined that maximal LTB_4 release occurred with between 2 and 10 μM A23187. Previous experiments in our laboratory using rat AM (42) demonstrated that maximal LTB_4 release occurs between 10 and 30 min after challenge with A23187; hence the 30-min time point was used in our experiments. As assessed by the double-antibody RIA, incubation with 25–50 μg/ml of HAIgG for 60 min resulted in the release of 1.6±0.5 ng/10^6 viable AM, which was not significantly different than that released from cells incubated in medium alone, 1.2±0.8 ng/10^6 viable AM ($n = 12$). However, when LTB_4 in cell supernatants of AM from a second group of volunteers was quantitated using the more sensitive dextran-coated charcoal RIA, HAIgG was found to elicit the release of LTB_4, 8.9±2.8 ng/10^6 cells, that was significantly greater ($P < 0.01$) than that seen in supernatants from alveolar macro-

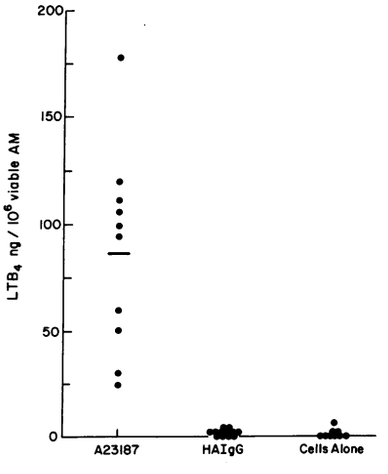

Figure 1. Human alveolar macrophage release of immunoreactive LTB_4, as assessed by the double-antibody RIA, in response to the addition of A23187, HAIgG or medium alone. Each point represents the mean of duplicate determinations for a single experiment.

phages incubated with medium alone, 2.5±0.4 ng/10^6 cells (n = 15).

To determine if these differences were due to variances in measurements by the two RIAs, we compared LTB_4 concentrations in aliquots of 10 additional supernatants from cells stimulated with A23187, HAIgG, or buffer alone. We found no significant difference ($P < 0.6$) in the amounts of LTB_4 determined with either RIA. The correlation coefficient between values obtained with the two assays was excellent, $r = 0.967$, $P < 0.001$. More importantly, LTB_4 concentrations quantitated using the more sensitive dextran-charcoal RIA were within a mean of 33.0±6.3% of the determinations made with the double-antibody RIA. The differences in LTB_4 determined with each RIA were similar at both high and low LTB_4 ranges. When the results of all supernatants from both groups of subjects were combined without regard to the RIA used, AM challenged with HAIgG generated and released 5.7±1.7 ng/10^6 viable AM compared with cells incubated with buffer alone, which released 1.9±0.4 ng/10^6 viable AM, $P = 0.02$, (n = 27). Thus, HAIgG is an effective stimulus for the release of LTB_4 by AM from some normal nonsmoking subjects.

Release of LTB_4 from human AM preincubated with IF-γ. The time curve for release of immunoreactive LTB_4 from AM after incubation with 1,000 U/ml of IF-γ for 24 h at 37°C and subsequent to challenge with HAIgG was assessed using the double-antibody RIA (Fig. 2). When macrophage cell cultures were challenged at 37°C with 25–50 μg/ml of HAIgG for times varying between 2.5 min and 3 h, net release of immunoreactive LTB_4 occurred in some instances at the earliest time point measured, 2.5 min. Maximal release of LTB_4 varied between experiments, occurring between 2.5 min and 1 h after incubation with HAIgG. Therefore, a 1-h incubation period was utilized for all subsequent experiments involving HAIgG challenge. Detection of immunoreactive LTB_4 decreased progressively in all experiments by 2 h. The decline in the quantities of LTB_4 detected over time in the macrophage supernatants was not due to absorption of leukotriene onto the Teflon chamber because, when 5,000 cpm of [^{3}H]LTB_4 were added to 1 ml of macrophage culture medium containing either A23187 or HAIgG and the mixtures were incubated at 37°C for 90 min,

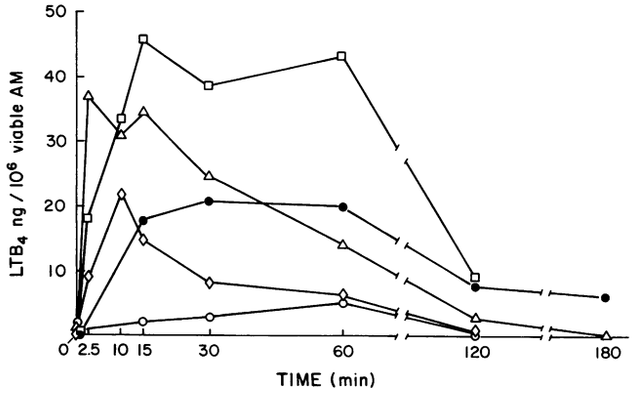

Figure 2. Time-response curve for release of immunoreactive LTB_4, as assessed by the double-antibody RIA, from AM preincubated with 1,000 U/ml of IF-γ for 24 h and then challenged with HAIgG. Each symbol represents determinations made from a separate subject. Each point represents the mean of duplicate determinations.

> 90% of the counts were recovered in the supernatants of mixtures in all instances. In addition, when 40,000 cpm of [^{3}H]LTB_4 were incubated for either 15 min or 2 h with IF-γ primed AM, no conversion to either 20-hydroxy-LTB_4 or 20-carboxy-LTB_4 was observed. However, after 2 h, > 50% of the counts were converted to two products more polar than the omega oxidation metabolites of LTB_4. Further investigations into the identities of these metabolites currently are being performed.

HAIgG dose-response curves were performed using concentrations ranging from 2.5 ng to 250 μg/ml on AM preincubated with 1,000 U/ml of IF-γ (Table I). The HAIgG dose-response of LTB_4 release was progressive up to a concentration of 25 μg/ml and remained constant at the highest concentration, 250 μg/ml.

In paired experiments, the effects of incubating alveolar macrophages without IF-γ or with 10 U/ml, 100 U/ml, and 1,000 U/ml of IF-γ for either 1 h or 24 h before stimulation with either A23187 or HAIgG were assessed using the double-antibody RIA. None of these concentrations of IF-γ alone could elicit release of immunoreactive LTB_4 after 1 or 24 h in two separate experiments. The results (not shown) reveal that preincubation of macrophages for 1 h in the absence or presence of IF-γ (1,000 U/ml) did not affect the release of immunoreactive LTB_4 induced with either HAIgG (n = 7) 3.0±2.7 vs. 3.6±2.3 ng/10^6 AM $P = 0.5$ or with A23187 (n = 8) 69.4±19.4 vs. 105.2±26.0 ng/10^6 viable AM without or with IF-γ, respectively. In contrast, HAIgG augmented LTB_4 release when macrophages were preincubated for 24 h with IF-γ, Fig. 3 *A*. This effect of IF-γ on macrophage release of immunoreactive LTB_4 was dose dependent. A statistically significant increase in immunoreactive LTB_4 release was observed from cells incubated with as little as 10 U/ml of IF-γ ($P = 0.04$). The effect of IF-γ was maximal at the highest concentration utilized in this study, 1,000 U/ml, (n = 12), 11.3±3.1 ng vs. 1.6±0.5 ng/10^6 viable AM for cells not primed with IF-γ, Fig. 3 *A*. In 9 of the 12 experiments the increase in LTB_4 release from primed AM exceeded 100%. In an additional 15 experiments where AM were cultured either without IF-γ or with 1,000 U/ml of IF-γ, and LTB_4 was assayed with the dextran-coated charcoal RIA, HAIgG induced the release of 8.9±2.8 and 21.6±6.5 ng of LTB_4/10^6 viable AM, respectively (data not shown, $P = 0.01$). In these later experiments concentrations of LTB_4 rose by > 100% in 8 of the 15 experiments. The ability of IF-γ to augment HAIgG induced LTB_4 release was not due to a stimulus-independent increase in the overall capacity of the cell to generate LTB_4, because A23187-induced release of LTB_4 from unprimed AM, 86.9±15.0 ng/10^6 viable AM, did not change statistically after macrophages were incubated with IF-γ under similar conditions, 60.9±11.9 ng/10^6 viable AM (n = 12) (Fig. 3 *B*).

Because stimulation of polymorphonuclear leukocytes with unopsonized zymosan results in the cellular retention of ~ 80% of the generated LTB_4 (35), we assessed the cellular and extracellular distribution of LTB_4 generated from macrophages incubated with 1,000 U/ml of IF-γ for 24 h and subsequently challenged with HAIgG or A23187. In two experiments A23187 stimulated macrophages released into the supernatants 110.0 and 102.7 ng of immunoreactive LTB_4/10^6 viable AM, whereas 4.7 ng (4%) and 5.3 ng (5%) remained cell associated (assessed by the double-antibody RIA). Similarly, cells incubated with IF-γ and stimulated with HAIgG released

*Table I. Dose-dependent Effect of HAIgG on the Release of Immunoreactive LTB_4 by AM Primed for 24 h with 1,000 U/ml of IF-γ**

Macrophage donor	HAIgG (μg/ml)					
	0.0025	0.025	0.25	2.5	25	250
1	0	0	0.9	0	0.92	1.9
2	0	0	1.1	3.3	40.0	10.8
3	0	0	0	0	8.5	3.3
4	ND‡	0	0	0	10.7	11.5
5	ND	ND	1.2	6.9	37.2	22.0
Mean±SEM	0	0	0.6±0.3	2.0±1.4	19.5±7.9	9.9±3.6

* LTB_4 expressed in ng/10^6 viable AM and assessed by either the double-antibody or dextran-charcoal RIA. ‡ ND not determined.

13.5 and 10.3 ng of immunoreactive $LTB_4/10^6$ viable AM into the supernatants with 0.4 ng (3%) and 0.7 ng (7%) remaining cell associated.

Cell supernatants of macrophages cultured for 24 h in the absence or presence of IF-γ (1,000 U/ml) and subsequently challenged with A23187 or HAIgG were examined for the presence of LTC_4 by a charcoal RIA and RP-HPLC. LTC_4 was not detected by RP-HPLC and was detected in only minimal quantities by RIA in supernatants from A23187 stimulated cells preincubated in the absence of IF-γ, 0.9±0.6 ng, or presence of IF-γ, 0.7±0.6 ng $LTC_4/10^6$ viable AM (n = 9) and in only minimal quantities in supernatants from macrophages stimulated with HAIgG after preincubation in the absence of IF-γ, 0.3±0.2 ng or presence of IF-γ, 0.2±0.1 ng/10^6 viable AM (n = 9), respectively.

Effect of cytochalasin B and endotoxin on HAIgG-induced LTB_4 release from AM cultured with IF-γ. Experiments were performed to determine whether phagocytosis of HAIgG was required for LTB_4 release. In three paired experiments, alveolar macrophages were incubated with or without cytochalasin B, 5 μg/ml, for 10–15 min at room temperature after culture with IF-γ for 24 h and before challenge with HAIgG. In preliminary experiments we had observed that this concentration of cytochalasin B eliminated the capacity of human AM to ingest three or more IgG coated ox red blood cells. Cytochalasin B at this concentration and for this incubation period did not, however, decrease the quantity of immunoreactive LTB_4 released. Rather, cells cultured with HAIgG in the presence of cytochalasin B released 88.6±47 ng of immunoreactive $LTB_4/10^6$ viable AM, whereas cells not treated with cytochalasin B released 12.1±2.8 ng/10^6 viable AM as assessed by the double-antibody RIA. A similar effect of cytochalasin B on leukotriene release from human peripheral blood monocytes (43) and leukocytes (44) has been observed.

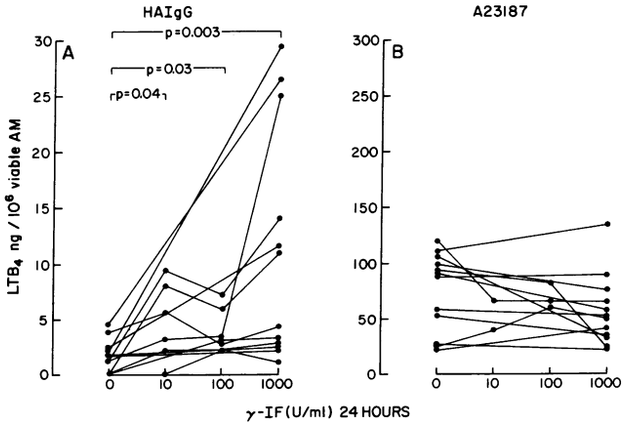

Figure 3. Dose-dependent effect of IF-γ on release of immunoreactive LTB_4, as assessed by the double-antibody RIA, from AM in response to HAIgG or A23187. AM were cultured in the presence of 0-1,000 U/ml of IF-γ for 24 h before challenge with HAIgG (*A*) or with A23187 (*B*). Each point represents the mean of duplicate determinations.

Undetectable quantities (< 1.0 ng/ml) of endotoxin were present in 25–50 μg/ml dilutions of two separate HAIgG preparations. The highest concentration of LPS detected in macrophage supernatants tested after challenge with HAIgG was 100 ng, n = 12. Therefore, we assessed whether or not LPS contaminating our cultures was responsible in part, or totally for HAIgG-induced LTB_4 release. In three sets of paired experiments AM preincubated with IF-γ and challenged with HAIgG released 14.0±7.0 ng of immunoreactive $LTB_4/10^6$ viable AM, while macrophages preincubated with IF-γ and challenged with HAIgG plus 100 ng LPS released 8.7±10.5 ng/10^6 viable AM; macrophages incubated with IF-γ and challenged with 100 ng of LPS alone for 60 min released minimal quantities of LTB_4, 0.7±0.5 ng/10^6 viable AM. To assess whether or not LPS present in the cell cultures served as a cofactor with IF-γ in priming AM for enhanced LTB_4 release, five paired experiments were performed in which AM were preincubated for 24 h with IF-γ (1,000 U/ml) in the presence or absence of a monoclonal anti-IF-γ antibody. That portion of the LTB_4 response augmented by preincubation with IF-γ was completely eliminated in three of the five experiments and was decreased by 44% and 70% in the remaining two, suggesting, but not proving, that IF-γ alone was responsible for the priming effect observed.

Reverse-phase HPLC. RP-HPLC was utilized to verify that the immunoreactive material in macrophage supernatants was LTB_4. For these experiments aliquots of supernatants from noninterferon and IF-γ (1,000 U/ml)-treated cell cultures which had been challenged with HAIgG, n = 3, were pooled, extracted with methanol, and subjected to RP-HPLC (Fig. 4). Chromatographic peaks which coeluted with synthetic LTB_4 standard were observed in both aliquots. As determined by double-antibody RIA, 17.0 ng of LTB_4 from supernatants

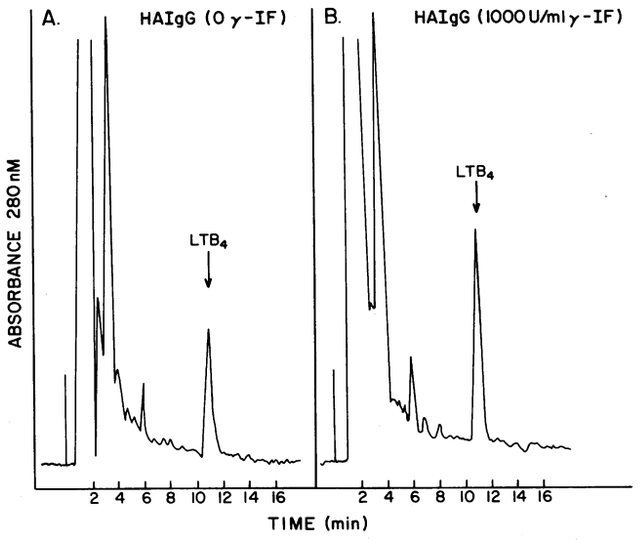

Figure 4. RP-HPLC resolution of methanol-extracted aliquots of supernatants from cell cultures incubated for 24 h in the absence of IF-γ before challenge with HAIgG (*A*), or in the presence of 1,000 U/ml of IF-γ (*B*) before challenge with HAIgG. The retention times of pure synthetic LTB_4 standard are indicated by the arrow.

from macrophages that were not treated with IF-γ and 32.4 ng of LTB_4 from IF-γ-treated macrophages were injected. 74.8% and 63.7% of these amounts, respectively, were recovered, as determined by optical density at 280 nM. No LTC_4 or LTD_4 was observed by ultraviolet absorbance after elution from RP-HPLC.

Specificity of effect for IF-γ. To assess the specificity of the effect of IF-γ on macrophage release of LTB_4 in response to HAIgG, paired experiments were performed in which macrophages were incubated alone or with 1,000 U/ml of either IF-α, IF-β, and with IF-γ for 24 h and then challenged with

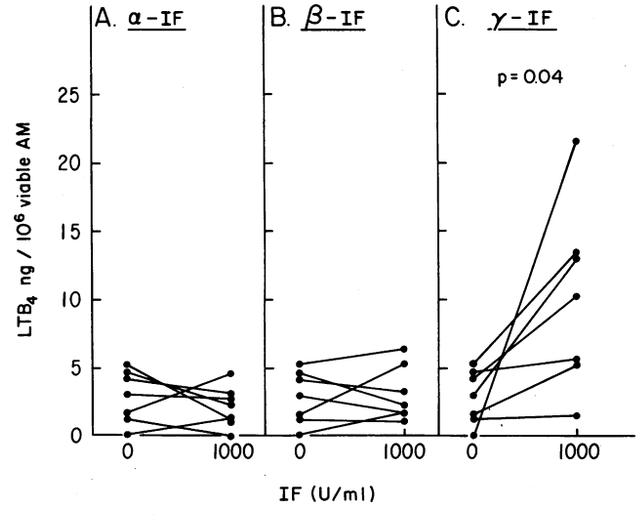

Figure 5. Specificity of the effect of IF-γ. Comparison of the effect of IF-α, (*A*), IF-β, (*B*) and IF-γ, (*C*) on AM release of immunoreactive LTB_4, as assessed by the double-antibody RIA, in response to stimulation with HAIgG. Each point represents the mean of duplicate determinations.

HAIgG (Fig. 5). As assessed by the double-antibody RIA, macrophages challenged with HAIgG after culture with IF-α released 2.2±0.6 ng of immunoreactive $LTB_4/10^6$ viable AM and after culture with IF-β, 3.2±0.8 ng/10^6 viable AM, which was not significantly different from that released from cells challenged with HAIgG after culture in the absence of either interferon, 2.8±0.8 ng/10^6 viable AM, $n = 7$. In contrast, in these same experiments macrophages incubated with IF-γ and challenged with HAIgG released 10.2±2.6 ng/10^6 viable AM, which was significantly different from that released without interferon, 2.9±0.8 ng/10^6 viable AM, ($P = 0.04$), $n = 7$.

Effect of interferons on alveolar macrophage IgG receptors. In three experiments we assessed the effect of IF-γ on the percentage of alveolar macrophages forming rosettes with IgG-coated ox red blood cells. After incubation in the absence of IF-γ for 24 h at 37°C, 39.3±10.6% of macrophages were IgGFcR+, whereas incubation of macrophages with 1,000 U/ml of IF-γ for 24 h resulted in a statistically significant increase in the percent of IgGFcR+ macrophages to 61.3±8.3%, $P < 0.04$. These data suggested that either the number or the density of IgGFcR receptors on the cell surface increased or that the binding affinity of receptor for ligand increased in response to incubation with IF-γ.

These possibilities were explored further using Scatchard analyses of radioligand ^{125}I-IgG_1 binding. Insufficient numbers of cells were retrieved by BAL from the lungs of nonsmoking normal volunteers to permit the performance of radioligand binding studies on cells cultured in the absence of interferon and with each class of interferon in a single experiment. Therefore, paired experiments were performed on cells cultured in the absence or presence of IF-γ. Additional paired experiments then were performed on AM cultured without interferon and with cells incubated with IF-α or IF-β. Scatchard analysis of ^{125}I-IgG_1 binding to AM revealed a single binding site on IF-γ treated cells and on control cells (Fig. 6). The mean K_a for cells incubated in the absence of IF-γ was $4.5\pm1.1 \times 10^8$ M^{-1}, which was not significantly different from that of cells incubated with 1,000 U/ml of IF-γ for 24 h, $3.3\pm0.6 \times 10^8$ M^{-1} (Table II). Similarly, no change in the K_a was observed for cells cultured with either IF-α or IF-β (Table II). However, the number of IgG_1FcR on macrophages which were not pretreated with interferon, 168,700±16,995 increased approxi-

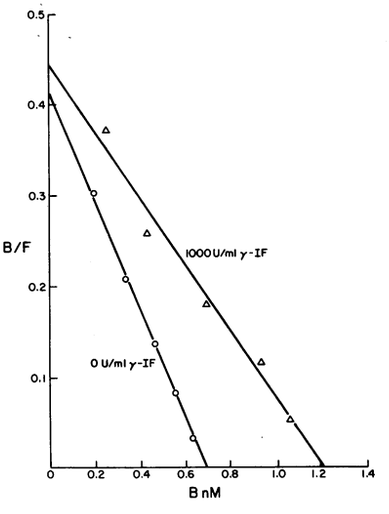

Figure 6. Representative Scatchard plot of data from a single experiment for ^{125}I-IgG_1 binding to AM. Human AM were cultured in the presence (△) or absence (○) of 1,000 U/ml of IF-γ for 24 h and the binding characteristics were determined.

*Table II. Normal Human Alveolar Macrophage IgG_1 Receptor Data**

IF (U/ML)	K_a (M^{-1})	IgGFcR/cell	Cell volume
			($\mu l \times 10^{-7}$/cell)
Gamma- (n = 7)§			
0	4.5±1.1 × 10^8	168,700±16,995	0.71±0.2
1,000	3.3±0.6 × 10^8	391,340±51,982‡	0.90±0.1
Alpha- (n = 5)			
0	2.9±0.4 × 10^8	107,810±14,948	—
1,000	2.6±0.3 × 10^8	128,450±19,177	—
Beta- (n = 5)			
0	2.9±0.4 × 10^8	107,810±14,948	—
1,000	3.1±0.3 × 10^8	110,680±21,891	—

* Expressed as mean±SEM.
‡ $P < 0.02$.
§ (n) number of experiments.

mately twofold to 319,340±51,982 after incubation for 24 h with IF-γ ($P < 0.02$). Culture of macrophages with IF-α or IF-β at 1,000 U/ml did not induce a statistically significant increase in IgG_1FcR on the AM (Table II). This increase in receptor number represented an increase in surface IgG_1 receptor density because the relative macrophage cell volumes did not change after incubation with IF-γ (Table II).

To assess whether or not HAIgG induced LTB_4 release from IF-γ primed AM occurred via binding to the IgG_1FcR on AM, experiments were performed in which IF-γ prepared AM were stimulated with HAIgG (25 μg/ml) in the presence or absence of 50–100 μg/ml of IgG_1 monomer (Table III). In 6/6 experiments IgG_1 monomer inhibited HAIgG-induced LTB_4 release, supporting the contention that HAIgG effected LTB_4 release as assessed by the dextran-coated charcoal RIA, in large part through coupling with AM IgG_1FcR.

Discussion

The results of this study demonstrate that soluble aggregates of human IgG are an effective stimulus for LTB_4 release from normal human AM, and that release due to HAIgG is enhanced significantly when these cells are preincubated with IF-γ. Under the conditions employed in our experiments, HAIgG was a potent cell activator. HAIgG induced the generation and release of 5.7±1.7 ng (n = 27) of LTB_4 from 10^6 viable and not purposefully activated AM. After incubation with IF-γ for 24 h, 1 × 10^6 AM released a mean of 17.0±3.9 ng of LTB_4 (n = 27), P = 0.001, with a range of 1 to 88 ng and corresponding to a concentration range of ~ 6 × 10^{-9} – 5.6 × 10^{-7} M. These concentrations are functionally significant, considering that LTB_4-mediated chemokinesis of neutrophils has an EC_{50} in the range of 1 to 10 × 10^{-10} M (45), and only slightly higher concentrations, 3 × 10^{-9} M, are chemotactic for neutrophils (46). LTB_4 also demonstrates chemokinetic and chemotactic activity for eosinophils (10–13) and monocytes (8, 14) at concentrations found in the supernatants of 10^6 viable AM. Furthermore, concentrations of LTB_4 in the picomolar to nanomolar range exert profound immunosuppressive effects on immunoglobulin synthesis, which is due to induced differentiation of T-suppressor cells (16, 18). Therefore, it is reasonable to conclude that IgG immune complexes can, under appropriate conditions, stimulate human AM release of LTB_4 in sufficient quantities to profoundly influence lung inflammatory events.

*Table III. IgG_1 Monomer Inhibition of HAIgG-induced LTB_4 Release**

Macrophage donor	IF-γ 1,000 U/ml		
	HAIgG	HAIgG + IgG_1	HAIgG
1	68.6	39	1.9
2	38.7	22.9	3
3	14.8	3.7	5.6
4	18.9	15.3	9.2
5	8.0	2.2	3.5
6	18.5	6.7	15.1

* LTB_4 expressed in ng/10^6 viable AM, as assessed by the dextran-charcoal RIA.

The suspensions of BAL cells used in our experiments contained predominately AM and much smaller relative numbers of lymphocytes, neutrophils, mast-basophiloid cells and eosinophils. It is unlikely that these cells contributed significantly to the LTB_4 present in the cell supernatants because lymphocytes synthesize comparatively little LTB_4 (47) and because the other cells were not present in sufficient numbers to contribute significant quantities of LTB_4.

Other investigators have reported that human peripheral blood monocytes, precursors to alveolar macrophages, also will release LTB_4 by IgG dependent mechanisms (43, 48). In these experiments, on nanogram/10^6 cells basis, the quantities of product released in response to an IgG agonist were in the range of 2 ng, which is approximately one-half of that which we observed from 10^6 AM that were not primed with IF-γ or one-eighth of that released from IF-γ primed cells. Human AM produce ~ 10 times the amount of LTB_4 as peripheral blood monocytes when stimulated with A23187 (49). Therefore, it is possible that the amounts of LTB_4 generated by AM stimulated with HAIgG is large relative to peripheral blood monocytes because of a greater expression of the enzymes of the 5-lipoxygenase pathway or a more efficient processing of substrates.

"Spontaneous" release of LTB_4 into each milliliter of culture media by 1 × 10^6 unprimed and not purposely activated AM averaged 1.2±0.8 ng, range 0–7 ng (Fig. 1). This is much higher than the amounts reported to be released from unstimulated peripheral blood monocytes (43), and suggests that in the "resting state," AM are in a higher state of activation with respect to LTB_4 production than are peripheral blood monocytes, a conclusion supported by others (49). Assuming that the differences are not due to differences in the methods of cell harvesting, this would not be surprising in view of the fact that alveolar macrophages are constantly in contact with gasses, fumes, and particulate matter present in the atmosphere and inhaled each day.

Release of LTB_4 by HAIgG from IF-γ primed AM was generally rapid but the total immunoreactive LTB_4 had decreased by 2 h in the supernatants of AM from all of five subjects (Fig. 2). This was not due to a decrease in cell viability,

nor was it because of loss of immunoreactive LTB_4 in the cell supernatants due to adherence of product to the culture chamber surfaces. Analysis of AM supernatants with added [^{3}H]LTB_4 by RP-HPLC did not reveal omega-hydroxy-LTB_4 or omega carboxy-LTB_4 but did reveal two more polar unidentified radiolabeled peaks. Thus, whereas human peripheral blood monocytes do not catabolize LTB_4 over similar time periods to a significant degree (43, 48), human AM do. The identities of the unknown metabolites presently are being sought.

Endotoxin was present in low quantities in our cell cultures. Concentrations as low as 20 ng/ml have been reported to elicit the release of small quantities of LTC_4 from human polymorphonuclear leukocytes (50) and bacterial lipopolysaccharide (LPS) can stimulate LTC_4 release by mouse peritoneal macrophages (51). However, the concentrations of endotoxin present in our cell suspensions were not directly responsible for the LTB_4 synthesis and release from AM challenged with soluble IgG complexes. Our results obtained from AM preincubated with IF-γ and a specific antibody to IF-γ before stimulation with HAIgG suggest, but do not prove, that endotoxin played no significant role as a cofactor with IF-γ to prepare our cells for release of LTB_4. Species differences or other undefined conditions may account for the observation that preincubation of mouse peritoneal macrophages with LPS has been shown to prime these cells for the release of prostaglandins and LTC_4 by IgG-coated beads (52).

The production of LTB_4 in response to HAIgG was not blocked by cytochalasin B, suggesting that phagocytosis of cell bound IgG aggregates was not necessary for LTB_4 synthesis and release. The effect of cytochalasin B on LTB_4 release in response to IgG aggregates was rather one of enhancement, which is similar to its observed effect on release of eicosanoids from human peripheral blood monocytes by immunoglobulin aggregates (43) and to that on polymorphonuclear cell generation of LTB_4 in response to FMLP (44). Neither this effect nor that of IF-γ priming of the AM for LTB_4 release in response to IgG aggregates is completely and satisfactorily explained by a single mechanism. By analogy with the proposed mechanism for cytochalasin B enhancement of the superoxide generating response of neutrophils to FMLP, it is possible that cytochalasin B maintains IgGFcR in a high activity state for signal transduction (53).

Along the same lines, several of our observations suggest that the IF-γ effect was mediated, in part, by the increase in density of cell surface IgG receptors. The ability of IF-γ to prime AM for enhanced LTB_4 release in response to IgG aggregates was not apparent after one hour but was readily detectable by 24 h. This time-related difference is consistent with an effect that requires protein synthesis. Because IF-γ-induced augmentation of peripheral blood monocyte IgGFcR is inhibitable by cycloheximide (Guyre, P., personal communication), it is likely that the expression of immunoglobulin receptors on AM surfaces is at least partially dependent on the synthesis of new receptors. IF-γ primed alveolar macrophages for enhanced LTB_4 release and simultaneously induced are approximately twofold increase in the density of AM IgG_1 receptors. In addition, IgG_1 monomer inhibited HAIgG-induced AM release of LTB_4. Finally, neither the priming effects nor the receptor-inducing effects were observed in cells preincubated with IF-α or with IF-β. Thus, it is probable that the IF-γ prepared the AM by providing more sites for ligand binding. It is also likely that additional post-IgGFcR mechanisms could be induced by IF-γ, since the several other macrophage functions, which are altered by IFs are not obviously regulated by a single mechanism (54).

Peripheral blood monocytes, precursors to alveolar macrophages, release both LTC_4 and LTB_4 when challenged with A23187 (43, 48). Several investigators now have observed that human AM release large quantities of LTB_4 (5–7) but only minimal quantities of LTD_4 (55). While IF-γ clearly primed AM for enhanced release of LTB_4 in response to HAIgG, it did not augment release of LTC_4 from AM challenged with either A23187 or HAIgG.

Our observations on human alveolar macrophages differ from the results of other studies that examined the effects of IF-γ on arachidonic acid metabolism in murine macrophages. Resident mouse peritoneal macrophages after preincubation with murine IF-γ and subsequent challenge with either A23187 or Zymosan, released less LTC_4 than peritoneal macrophages not primed with murine IF-γ (56). Furthermore, elicited mouse peritoneal macrophages primed with murine IF-γ released no more LTC_4 after challenge with IgG immune complexes than unprimed cells (57). These differences may reflect any of a number of variables between the studies, including those of species, culture conditions, and the nature of the agonists employed.

The findings of our study have potential relevance to host lung defense mechanisms. One classification divides these mechanisms into two broad groups: surveillance mechanisms and augmenting mechanisms (1). Our data suggest that in conditions where local surveillance mechanisms prove insufficient, augmenting mechanisms are initiated, with the activation of T-lymphocytes to release IF-γ. It is reasonable to hypothesize that in such circumstances AM may become "primed." Since the fluid that lines the epithelial surfaces of the lower respiratory tract is rich in IgG (1), AM under specified conditions then would be stimulated by IgG immune complexes to release LTB_4. Thus, the overall defense mechanisms might be further augmented through the recruitment of neutrophils, eosinophils, and monocytes.

Furthermore, the observations that IF-γ augments the number of IgEFcR on the macrophage-like U-937 cell line (58), that normal rat AM release substantial quantities of LTB_4 when activated by IgE immune complexes (59), and that IF-γ primes human AM for the release of LTB_4 in response to an IgG stimulus, raise the possibility that IF-γ may also increase the density of IgE receptors on human AM and similarily prime these cells for LTB_4 release by IgE-dependent mechanisms.

Acknowledgments

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