Identification and Characterization of a Monocyte-derived Neutrophil-activating Factor in Corticosteroid-resistant Bronchial Asthma

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Abstract
Peripheral blood mononuclear cells (PBMC) were isolated from seven normal subjects, eight asthmatic subjects clinically sensitive to corticosteroids (CS), and eight asthmatic subjects clinically resistant to corticosteroids (CR). PBMC were cultured at 37°C for 24 h in the absence or presence of 10⁻¹⁴ to 10⁻⁴ M hydrocortisone. Calcium ionophore (A23187)-activated neutrophils (PMN) primed by supernatants of PBMC from asthmatic subjects cultured in the absence of hydrocortisone generated approximately threefold more leukotriene B₄ than PMN primed by supernatants of PBMC from normal subjects (P < 0.05). Incubation of PBMC derived from CS subjects with 10⁻⁸ M hydrocortisone completely inhibited the production of the enhancing activity (P < 0.01), whereas in CR subjects hydrocortisone at concentrations up to 10⁻⁴ M did not suppress the release of enhancing activity. The enhancing activity was produced by monocytes. Enhancing activity eluted with an Mᵋ of 3,000 D and a pI of 7.1. It eluted at 10% acetonitrile after reverse-phase HPLC. The activity was destroyed by heating to 60°C for 60 min and was sensitive to pronase treatment. The purified factor also enhanced superoxide generation by PMN which had been stimulated submaximally by phorbol myristate acetate.

Introduction
The airflow obstruction of the majority of patients with chronic and severe bronchial asthma will improve after treatment with corticosteroids. However, there are some patients in whom systemic or inhaled treatment with corticosteroids, even when given in very large doses, does not lead to any increases in forced expiratory volume in 1 s (FEV₁)¹. The asthma in such patients is usually severe, and they are seriously disabled for long periods of time. Carmichael et al. (3) defined corticosteroid responsiveness in asthma as an increase in FEV₁ of > 30% during a 7-d course of prednisolone treatment at 20 mg daily. Corticosteroid resistance in asthmatic subjects was defined as an improvement in the FEV₁ of < 15% after a similar course of prednisolone. Comparison of corticosteroid-resistant (CR) and -sensitive (CS) asthmatic subjects revealed that the CR asthmatic individuals tended to be older, with a longer history of asthma, and that their disease was frequently difficult to control. A family history of asthma was more frequent in CR asthmatic subjects, and their nonspecific bronchial responsiveness, as assessed by methacholine challenge, was increased relative to the CS asthmatic patients.

In CR asthmatic patients, complement receptor expression in monocytes was reduced after corticosteroid treatment as compared with cells from untreated patients (2). The reduction in complement receptor expression induced by corticosteroids was not observed in monocytes of CS asthmatic subjects. These observations suggest that there may be a defect in glucocorticoid responsiveness in the monocytes of CR asthmatic individuals. These studies have been extended recently by the demonstration that 10⁻⁸ to 10⁻⁹ M methylprednisolone, which substantially inhibited the growth of colonies from phytohemagglutinin-stimulated mononuclear cells of CS asthmatic subjects, had little effect on colony growth from the mixed mononuclear cells of CR asthmatic individuals (3).

Activated monocytes secrete cytokines which prime granulocytes for enhanced leukotriene generation after subsequent stimulation by calcium ionophore (4) or by IgG-coated Sepharose beads (5). Leukotrienes (LT) are derived from the metabolism of arachidonic acid by the 5-lipoxigenase pathway (6, 7) and are potent pro-inflammatory mediators. LTBa is a chemoattractant agent for granulocytes (8–10) and monocytes (11). LTC₃, LTD₄, and LTE₄ increase vascular permeability (12–15), contract nonvascular smooth muscle (12, 13), and augment nonspecific bronchial hyperresponsiveness in asthmatic subjects (16). Thus, a putative mechanism for corticosteroid action may be to inhibit the production of the mononuclear cell-derived cytokine(s) that prime granulocytes for enhanced leukotriene generation in CS but not in CR asthmatic patients. We have tested this hypothesis and have purified to homogeneity the major mononuclear cell-derived molecule in CR asthmatic patients, which enhances leukotriene generation in granulocytes.

Methods
Mononuclear cell donors. Peripheral blood mononuclear cells (PBMC) were obtained from three groups of subjects: normal healthy volunteers (n = 7), CS asthmatic subjects (n = 8), and CR asthmatic subjects (n = 8) (Table I). All asthmatic subjects were defined by a history of episodic wheezing and by a > 30% increase in FEV₁ after inhalation of 400 μg of albuterol. The clinical response to corticosteroid therapy was confirmed to be reproducible by testing the patients on at least three
occasions. None of the subjects were studied within 1 mo of using corticosteroids.

**Preparation of supernatants from cultured mononuclear cells.** PBMC were isolated from heparinized blood by sedimentation on dextran 110 (Dextraven 110, CP Pharmaceuticals Limited, Wrexham, England) and centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway) as previously described (17). The PBMC were washed three times in Hanks’ balanced salt solution without calcium or magnesium (HBSS−) (Flow Laboratories, Irvine, Scotland). The cells were then suspended at a concentration of 2 × 10⁷/ml in Eagle’s minimal essential medium (MEM) (Flow laboratories, Irvine, Scotland), and the plates were then incubated in a humidified atmosphere of 5% CO₂ at 37°C for 60 min. Nonadherent cells were then aspirated and the plates were washed twice with 2 ml of supplemented MEM without BSA. A further 2 ml of cell suspension was then added to the plates and incubated for 60 min, and the nonadherent cells were removed as described above. 10⁻⁴ to 10⁻⁶ M hydrocortisone sodium hemisuccinate (Upjohn Ltd., Crawley, England) in 2 ml of supplemented MEM containing 10% heat-inactivated fetal calf serum (FCS), or 2 ml of culture medium without hydrocortisone was added to separate plates and the plates were cultured in a humidified atmosphere of 5% CO₂ at 37°C for 24 h. At the end of the incubation period, cells were > 95% viable as assessed by trypan blue exclusion. The 24-h culture supernatants were removed by aspiration. The supernatants were then centrifuged at 200 g for 10 min at 4°C to remove any cells. The supernatants were decanted and stored at −20°C. Control samples consisted of MEM containing 10% FCS with or without hydrocortisone, in the absence of cells, which had been tested in an identical manner to the PBMC cultures.

**Activation of neutrophils and assessment for mononuclear cell-derived enhancing activity for leukotriene generation.** Neutrophils (PMN) were purified to > 90% from heparinized blood of healthy volunteers by dextran sedimentation, centrifugation through Lymphoprep, and lysis of erythrocytes with isotonic ammonium chloride as previously described (17, 18). PMN were washed three times in HBSS− and resuspended in HBSS with calcium and magnesium (HBSS++/20 mM Hepes at 1 × 10⁶ PMN/ml. Separate portions of 5 × 10⁷ PMN in 50 μl HBSS++/20 mM Hepes were warmed to 37°C for 10 min and were then mixed with 25 μl of control buffer or a specified dilution (up to 1:192) of the PBMC culture supernatants. PMN were then incubated for 0–10 min before the addition of ionophore A23187 to final concentrations up to 10 μM in 50 μl HBSS++/20 mM Hepes, with di-methylsulfoxide at final concentrations of up to 0.1%. After incubation with ionophore for periods of 0–10 min the reaction was quenched by rapid cooling to 4°C followed by centrifugation at 10,000 g for 30 s. The supernatants were stored under argon at −20°C until used for measurement of immunoreactive LTB₄.

Preincubation of PMN with PBMC-derived supernatants did not affect viability of the cells as indicated by trypan blue exclusion.

**Quantitation of LTB₄.** The concentrations of immunoreactive LTB₄ in the PMN supernatants were assessed by separate specific radioimmunoassays (RIA) as previously described (19, 20).

Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis of the supernatants was performed on a C-18 ultrasil-O column (4.6 × 250 mm) (Altex-Beckman, Berkeley, CA) at a flow rate of 1 ml/min in a mobile phase solvent of 65% methanol/34.9% water/0.1% acetic acid, pH 5.6. 1-ml fractions were collected and the absorbance of the eluates was continuously monitored with an on-line spectrophotometer (Waters model 990 Photodiode Array Detector, Millipore Corp., Bedford MA) at 269 nm for the predominant PMN product, LTB₄. The column was calibrated for the retention times of synthetic leukotrienes, which were provided by Dr. B. Spur (Institut Henri Beaumont, Paris, France): (5S,12R)-6-trans-LTB₄, (15±0±1 min, n = 4), (5S,12S)-6-trans-LTB₄, (16±2±0±1 min, n = 4), and LTB₄, (20±3±0±4 min, n = 4). To carry out RIA on eluate fractions from RP-HPLC, the fractions were dried under reduced pressure and were resuspended in 200 μl Isogel-Tris buffer.

**Cellular origin of enhancing activity.** In three experiments, the monocytes or lymphocytes contaminating the adherent monocyte monolayers were lysed with specific antibodies and complement. After isolation and purification of mononuclear cells by adherence as already described, the culture medium was aspirated and replaced with 1 ml of supplemented MEM containing either a 1:10 final dilution of mouse anti-CD14 antibody directed against monocytes (Seralab Ltd., Crawley, Down, UK), or a 1:20 final dilution of mouse anti-CD5 antibody (Seralab Ltd.) plus a 1:100 final dilution of goat polyvalent anti-human immunoglobulin antiserum (Seralab Ltd.), directed against T and B lymphocytes, respectively. The cells were incubated for 60 min at 37°C before the addition of rabbit complement (Seralab Ltd.) to a final dilution of 1:10. Cells were cultured for a further 60 min at 37°C after which the supernatant was aspirated. The remaining adherent cells were washed twice with 2 ml of supplemented MEM and the supernatant was replaced with 2 ml of supplemented MEM containing 10% FCS. The remaining adherent mononuclear cells were cultured for 24 h at 37°C as described earlier. The mononuclear cells were stained with peroxidase stain (21) to quantitate monocytes. To identify lymphocytes, cells were reacted with fluorescein-conjugated anti-human Ig or fluorescein-conjugated anti-Leu-1 monoclonal antibody (Becton, Dickinson & Co., Sunnyvale, CA) and were examined under a fluorescence microscope.

In three experiments, the nonadherent mononuclear cells removed during monocyte purification were suspended at the same concentration as the adherent cells in supplemented MEM containing 10% FCS and cultured for 24 h at 37°C in an identical manner.

Supernatants from all experiments were tested in a 1:3 final dilution for LTB₄ release-enhancing activity in PMN.

**Purification and characterization of mononuclear cell-derived enhancing activity.** 300-μl samples of PBMC culture supernatant were applied to Sep Pak C-18 cartridges (Millipore Corp.) which had been prepared by priming with 2 ml of methanol followed by 2 ml of H₂O. The Sep Pak was then eluted with sequential 2-ml volume sof H₂O, followed by elution with 4 ml of methanol. Aqueous fractions were lyophilized and methanol extracts were dried under negative pressure. All samples were resuspended in 300 μl of HBSS− and tested for their activity in enhancing LTB₄ generation from A23187-activated PMN as described above. The percentage inhibition of activity by hydrocortisone was calculated using the following equation: \[1 - \left(\frac{LTB_4 \text{ generation by PMN primed by PBMC supernatants cultured}}{LTB_4 \text{ generation by PMN primed by PBMC supernatants cultured in the absence of PBMC supernatant}}\right)\] .

<table>
<thead>
<tr>
<th>Table I. Clinical Characteristics of PBMC Donors</th>
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<td>Normal</td>
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<tr>
<td>Number</td>
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<tr>
<td>Age (yr)</td>
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<td>FEV₁ (% predicted)</td>
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<tr>
<td>Albuterol response (%) increase in FEV₁</td>
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<td>Prednisolone response (%) increase in FEV₁</td>
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<td>Atopy</td>
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<td>Smokers</td>
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Albuterol response is the percent increase in FEV₁ after administration of 400 μg of albuterol via a metered dose inhaler. Prednisolone was taken for 2 wk at a dose of 40 mg a day.
in the presence of hydrocortisone + LTB₄ generation by PMN primed by PBMC supernatants cultured in the absence of hydrocortisone]) × 100. LTB₄ generation is the value (nanograms) obtained by subtracting the amount of LTB₄ generated by A23187-stimulated PMN which had been preincubated with the Sep Pak eluate derived from the culture medium, from the amount of LTB₄ generated by A23187-stimulated PMN, which had been preincubated with the corresponding Sep Pak eluate derived from the PBMC supernatant.

The active fractions after Sep Pak fractionation were subjected to gel filtration chromatography on a TSK 3000 HPLC column (Anachem Ltd., Luton, England), which had been equilibrated with 0.25% sodium phosphate buffer, pH 7.4, and eluted at a flow rate of 0.5 ml/min. l-ml fractions were collected. The column was calibrated with thyroglobulin (mol wt 443,000), albumin (mol wt 66,000), carbonic anhydrase (mol wt 29,000), and vitamin B₁₂ (mol wt 1,300). Eluate fractions were combined in groups of five consecutive fractions and were tested for enhancing activity.

Selected active fractions after TSK 3000 HPLC were combined, lyophilized, resuspended in 600 μl of H₂O, and stored at −20°C until used for desalting and further purification. 300-μl aliquots of the pooled and concentrated gel filtration fractions were desalted by passage through a Sephadex G10 column (0.9 × 20 cm) which was equilibrated with distilled H₂O. The desalted sample was then subjected to chromatofocusing (22-24) in a PBE 94 column (0.9 × 30 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated with 0.25% ethanolicamine, pH 8.6. The sample was eluted with Polybuffer 96 (Pharmacia LKB Biotechnology, Uppsala, Sweden; 1:10 dilution) pH 4.7 at 10 ml/h. 2-ml fractions were collected. The pH of eluent fractions was adjusted with 0.1 M HCl or 0.1 M NaOH as appropriate to pH 7.4. Each eluate fraction was tested for enhancing activity on PMN.

The active eluate fractions after chromatofocusing were combined and were applied with 0.25% sodium phosphate buffer, pH 7.4, at a flow rate of 0.5 ml/min. l-ml fractions were collected. The column was calibrated with ovalbumin (mol wt 40,000), chymotrypsinogen (mol wt 24,000), cytochrome (mol wt 12,000), insulin (mol wt 5,400), and vitamin B₁₂ (mol wt 1,300). Each eluate fraction was tested for enhancing activity as described.

Protein estimation was performed on eluant fractions following size exclusion chromatography using a bichinchonic acid (BCA) assay kit (Sigma Chemical Co., St. Louis, MO). Calibration was carried out using serial dilutions of BSA (Sigma Chemical Co.).

**RP-HPLC analysis of enhancing activity.** RP-HPLC analysis of biologic active peptides was performed on a C₁₈ spherisorb-ODS column (4.6 × 250 mm) (Anachem, London, UK) at a flow rate of 1 ml/minute. Mobile phase: solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile. The column was equilibrated with 10% solvent B in solvent A. After application of 100 μl of the sample, the column was washed with the equilibrating buffer for 10 min. Then a slope gradient was established from 10-40% solvent B over 30 min. 1-ml fractions were collected and the absorbance of the eluates at 215, 224, and 254 nm was continuously monitored with an on-line spectrophotometer (Waters 990 Photodiode Assay Detector). Each eluate function was lyophilized, resuspended in HBSS +, and tested for enhancing activity as described.

**Superoxide anion (O₂⁻) assay.** In six experiment with PMN from different donors the production of O₂⁻ by PMN preincubated with purified cytokine was measured by the capacity of O₂⁻ to reduce ferri-cytochrome C to ferro-cytochrome C (26). Control PMN preparations were preincubated with buffer only. Purified PMN were suspended at 4 × 10⁷/ml in HBSS +/20 mM Hepes. 50 μl of PMN suspension was warmed to 37°C for 10 min and then preincubated with either control buffer or a 1:3 dilution of cytokine for 2.5 min. 25 μl of HBSS +/Hepes with ferri-cytochrome C, type VI (6.25 mg/ml, Sigma Chemical Co.) either with or without superoxide dismutase (SOD, 500 μg/ml, Sigma Chemical Co.) and 25 μl of HBSS +/Hepes containing phorbol myristate acetate (PMA, 0.25-250 μg/ml, Sigma Chemical Co.) were then added to stimulate the respiratory burst. PMN reaction mixtures were then incubated at 37°C for 45 min, after which cells were sedimented at 10,000 g for 30 s and the supernatants were removed.

Supernatants were diluted 1:4 in HBSS + and assayed for reduction of ferri-cytochrome C by spectroscopic analysis. Nanomoles of cytochrome C reduced were calculated from the increase in absorbance measured using an absorption coefficient of 29.5 M⁻¹ cm⁻¹ at 550 nm. Superoxide-dependent cytochrome C reduced was calculated by subtraction of the value for cytochrome C reduced measured in the presence of hydrocortisone + LTB₄ generation by PMN primed by PBMC supernatants cultured in the absence of hydrocortisone].
reaction mixtures containing SOD from the value measured in the reaction mixtures without SOD. Results were expressed as nanomoles of cytochrome c reduced per $5 \times 10^6$ neutrophils.

Statistics. Statistics were performed by analysis of variance. A $P$ value of $<0.05$ was considered significant for each. Correlations were analysed by linear regression test.

Results

Enhanced generation of $LTB_4$ by ionophore-stimulated PMN after preincubation in PBMC supernatants derived from asthmatic subjects. The effect of preincubating PMN for 1–10 min in a 1:3 dilution of PBMC culture supernatants on their subsequent maximal abilities to generate $LTB_4$ when stimulated by 2.5 $\mu$M A23187 is shown in Fig. 1. $LTB_4$ generation from A23187-activated PMN increased in a time-dependent manner to reach a maximum at 2.5 min of preincubation time and decreased rapidly thereafter. PMN preincubated with PBMC culture supernatants from each of six CS asthmatic, six CR asthmatic, and six normal subjects generated 405±45% ($P < 0.05$), 352±83% ($P < 0.05$), and 105±25% ($P < 0.05$) more $LTB_4$, respectively (mean±SEM), than PMN preincubated in control culture medium. PMN preincubated with control culture medium generated 10.0±3.5 ng of $LTB_4$ (mean±SEM, $n = 6$).

The dose-dependent enhancing effect of supernatants derived from cultures of PBMC isolated from two different CR asthmatic subjects on the subsequent $LTB_4$ generation by PMN stimulated by 2.5 $\mu$M A23187 for 5 min at 37°C was assessed in separate experiments (Fig. 2). Maximum enhancement of $LTB_4$ generation by A23187-activated PMN occurred at 1:3 dilution, with progressively less enhancement of $LTB_4$ biosynthesis with increasing dilutions of the PBMC culture supernatants. The enhancement was lost at dilutions > 1:24.

Time course and dose dependency of ionophore stimulation after incubation of PMN with PBMC supernatants. The dose and time dependence of A23187 stimulation was evaluated for PMN from normal donors preincubated for 2.5 min in a 1:3 dilution of PBMC culture supernatants derived from eight CS asthmatic subjects, eight CR asthmatic subjects, and seven normal volunteers. In seven experiments, $LTB_4$ generation in PMN which had been preincubated with PBMC culture supernatants from normal donors peaked at 2.5 min (Fig. 3). In 16 further experiments on PMN preincubated with PBMC culture supernatants from eight CS and eight CR asthmatic subjects, maximum quantities of $LTB_4$ were detected at 5 min and these were on average 2.5-fold greater than those generated under optimal conditions by PMN preincubated with PBMC culture supernatants from normal subjects ($P < 0.05$).

In three experiments, PMN were stimulated with increasing concentrations of A23187 for 2.5 and 5 min in normal and asthmatic subjects, respectively, after 2.5 min of preincubation with PBMC culture supernatants derived from each of three CS asthmatic, three CR asthmatic, and three normal subjects or culture medium alone (Fig. 4). $LTB_4$ generation increased

![Figure 1](image1.png)

**Figure 1.** Time-dependent enhancing effects of PBMC culture supernatants on the subsequent generation of $LTB_4$ by ionophore-activated neutrophils (PMN). PMN were pretreated with a 1:3 dilution of PBMC culture supernatants from six CS asthmatic subjects (●), six CR asthmatic subjects (○), and six normal donors (△), and then stimulated with 2.5 $\mu$M ionophore for 2.5 and 5 min in PMN preincubated with normal PBMC supernatants and asthmatic supernatants, respectively. Values are mean±SEM. Six normal subjects served as donors for PMN.

![Figure 2](image2.png)

**Figure 2.** Concentration-dependent enhancing effects of PBMC culture supernatants on the subsequent generation of $LTB_4$ by ionophore-activated neutrophils (PMN). PMN were incubated with culture supernatant for 2.5 min and then stimulated with 2.5 $\mu$M ionophore. Symbols represent different PBMC donors.
in a dose-dependent manner, with maximal generation occurring at 2.5 μM A23187 in all experiments. At all concentrations of ionophore studied, PMN preincubated with PBMC culture supernatants from CS and CR asthmatic subjects generated more LTB₄ than those preincubated with PBMC supernatants from normal subjects (P < 0.05, < 0.05, respectively). In the absence of A23187 stimulation, there was no enhancement of LTB₄ generation by culture supernatants derived from either asthmatic or normal subjects.

**Figure 3.** Effect of pretreatment of neutrophils (PMN) for 2.5 min with a 1:3 dilution of PBMC culture supernatants from eight CS (●) asthmatic subjects, eight CR (○) asthmatic subjects, and seven normal (△) subjects on the time course of subsequent LTB₄ generation after the addition of 2.5 μM A23187. Values are mean±SEM. Eight normal subjects served as donors for PMN.

**Analysis by RP-HPLC of immunoreactive LTB₄.** To establish that the immunoreactive LTB₄ represented a single product, the supernatants from 10⁶ PMN activated by 2.5 μM ionophore for 5 min at 37°C after pretreatment with 1:3 dilution of PBMC culture supernatants derived from each of three CS asthmatic, three CR asthmatic, and three normal donors, or control medium for 2.5 min were resolved by RP-HPLC, and the fractional eluates were assessed by RIA for immunoreactive LTB₄. In all experiments, there was only one peak of immunoreactivity which eluted at the same retention time as that of a synthetic LTB₄ reference standard. The supernatants derived from PMN activated in the presence of PBMC culture supernatants derived from the three CS asthmatic subjects contained 75, 52, and 46 ng of LTB₄ after RP-HPLC, whereas the supernatants from PMN activated after pretreatment with control medium contained 21, 17, and 9 ng of LTB₄, respectively, after RP-HPLC.

The supernatants derived from PMN pretreated with PMBC culture supernatants from the three CR asthmatic subjects contained 64, 39, and 37 ng of LTB₄ after RP-HPLC, whereas the supernatants from PMN activated after pretreatment with control medium contained 22, 15, and 12 ng of LTB₄, respectively, after RP-HPLC.

The supernatants derived from PMN activated after pretreatment with PBMC culture supernatants derived from the three normal subjects contained 27, 25, and 22 ng of LTB₄ after RP-HPLC, whereas those from PMN activated after pretreatment with control medium contained 18, 13, and 9 ng, respectively, after RP-HPLC. In all experiments, there was >84% recovery of immunoreactive LTB₄ applied for resolution by RP-HPLC. The quantities of LTB₄ as assessed by integrated UV absorbance at 269 nm were virtually identical to those measured by RIA in all experiments (data not shown).

**Generation of lipoxygenase products by [³H]arachidonic acid-labeled PMN activated with A23187.** The enhancement in the quantities of LTB₄ detected in supernatants from PMN preincubated with cytokine derived from CR subjects could have been due to increased biosynthesis, decreased omega oxidation, or both. Therefore, the radioactive counts originating from membrane-derived [³H]arachidonic acid and eluting with the ω-oxidation products of LTB₄ during HPLC were compared with those eluting with LTB₄ (Table II). In PMN preincubated with cytokine, there was enhancement of the total quantities of LTB₄, as indicated by the sum of LTB₄ and ω-oxidation products of LTB₄, as compared with PMN preincubated with control buffer (P < 0.05). There was no significant difference in the percentage of total LTB₄ measured as ω-oxidation products between PMN preincubated with cytokine and those preincubated with control buffer. Thus, the increased appearance of LTB₄ was attributed to increased biosynthesis.

**Cellular origin of enhancing activity.** To determine the cell of origin of the enhancing activity, mononuclear cells were fractionated into adherent and nonadherent cells and these were cultured separately for 24 h (Table III). In three experiments with mononuclear cells isolated from three different CR subjects, the enhancing activity was found in culture supernatants from adherent mononuclear cells but not in supernatants from nonadherent cells. When 90% of the lymphocytes that contaminated the adherent monocytes were lysed with anti-CD5 and anti–human Ig antibodies plus complement, the enhancing activity of supernatants for ionophore-stimulated
Table II. Analysis of LTB₄ and ω-Oxidation Metabolites of LTB₄ Produced by [³H]Arachidonic Acid-labeled PMN Stimulated with 2.5 μM A23187

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>&quot;Total LTB₄&quot;*</th>
<th>ω-Metabolites</th>
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<tr>
<td></td>
<td>cpm</td>
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<tr>
<td>Control buffer</td>
<td>252±26</td>
<td>58±4</td>
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<tr>
<td>Cytokine</td>
<td>602±90</td>
<td>52±24</td>
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In three experiments with PMN from different donors, 2 × 10⁶ PMN labeled with [³H]arachidonic acid, were incubated with either control buffer or a 1:3 dilution of cytokine (sequentially purified by TSK 3000 HPLC, chromatofocusing, Sephadex G50, and RP-HPLC) for 2.5 min, then stimulated with 2.5 μM A23187 for 5 min. Supernatants were resolved by RP-HPLC and quantitated as counts per minute (cpm)/10⁶ PMN. Values are mean ± SEM.

* Total LTB₄ represents the sum of the values for LTB₄ and ω metabolites of LTB₄ in each experiment. NS, not significant.

LTB₄ generation was not diminished (Table III). When > 85% of the adherent monocytes were lysed with anti–CD 14 antibody plus complement, the enhancing activity was abolished.

Suppression of enhancing activity from cultured PBMC by hydrocortisone. The effects of incubating PBMC from eight CR and eight CS asthmatic subjects for 24 h in the absence or presence of 10⁻¹⁰ to 10⁻⁶ M hydrocortisone on the production of the enhancing activity are shown in Fig. 5. In the absence of hydrocortisone, PMN preincubated with a 1:3 dilution of PBMC culture supernatants derived from eight CR asthmatic, eight CS asthmatic, or seven normal subjects or control culture medium alone, produced 61.9±26.1, 49.2±11.2, 21.6±8.6, and 10.8±3.5 ng LTB₄/10⁶ PMN (mean±SEM), respectively, after stimulation with A23187. PMN which had been preincubated with PBMC culture supernatants from CS and CR asthmatic subjects generated significantly more LTB₄ than those preincubated with PBMC culture supernatants from normal subjects (P < 0.05). PMN pretreated with PBMC supernatants from normal subjects produced more LTB₄ than PMN pretreated with control medium (P < 0.05). There was no significant difference between the CS and CR asthmatic individuals. In CS asthmatic subjects, hydrocortisone suppressed the production of the enhancing activity in a dose-dependent manner. There was significant inhibition at 10⁻⁸, 10⁻⁶, and 10⁻⁴ M hydrocortisone (P < 0.01, < 0.05, < 0.01, respectively) and complete inhibition of enhancing activity occurred at 10⁻⁸ M hydrocortisone. In CR asthmatic subjects there was no inhibition of PBMC-derived enhancing activity with increasing concentrations of hydrocortisone up to 10⁻⁴ M.

Increasing concentrations of hydrocortisone up to 10⁻⁴ M did not alter significantly the quantities of LTB₄ generated by ionophore-activated PMN preincubated with control culture medium (data not shown).

Correlation between suppression of PBMC-derived enhancing activity by hydrocortisone and improvement in FEV₁ after treatment with prednisolone. There was a correlation between the percent suppression of PBMC-derived enhancing activity by 10⁻⁸ M hydrocortisone and the percentage increase in FEV₁ in asthmatic subjects after a 2-wk course of prednisolone at a dose of 40 mg per day. (r = 0.68, P = 0.005). There was a similar correlation between suppression of activity at 10⁻⁴ M hydrocortisone and the increase in FEV₁ (r = 0.67, P = 0.006).

Table III. Identification of the Cell Source of LTB₄ Generation-enhancing Activity in Cultures of PBMC

<table>
<thead>
<tr>
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<th>Anti-CD5 +anti-Ig</th>
<th>Anti-CD14</th>
<th>Generation of LTB₄</th>
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<td>Expt. 1</td>
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<tr>
<td>Adherent PBMC</td>
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<td>10.4</td>
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<td>+</td>
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<td>1.8</td>
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<td>Nonadherent PBMC</td>
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<td>2.4</td>
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<td>Control medium</td>
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<td>0.9</td>
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</table>

Adherent PBMC were treated with either anti–CD5 plus anti-Ig for 1 h, or anti–CD14 for 1 h, at 37°C followed by incubation with complement for 1 h. Remaining cells were washed twice and then cultured for 24 h. Nonadherent PBMC were suspended at the same concentration as adherent cells and cultured for 24 h. Supernatants were tested by preincubation with PMN for 2.5 min at a 1:3 dilution for enhancement of LTB₄ generation. PMN were obtained from three separate, normal donors and stimulated with 2.5 μM ionophore at 37°C for 5 min. Each value is the mean of duplicate determinations by radioimmunoassay of duplicate experiments.
Fractionation of PBMC-derived culture supernatants by Sep Pak C-18 cartridges. As maximal suppression of the PBMC-derived enhancing activity in CS asthmatic subjects occurred at $10^{-8}$ M hydrocortisone, initial fractionation of the supernatants on Sep Pak C-18 cartridges was performed on each of the supernatants of PBMC isolated from three CS and three CR asthmatic subjects which had been cultured in the presence or absence of $10^{-8}$ M hydrocortisone. All eluant fractions after Sep Pak fractionation contained enhancing activity (Table IV). The activities in the aqueous fractions 2 and 3 were suppressed by an average of 91% and 95%, respectively, by $10^{-8}$ M hydrocortisone in CS but not in CR asthmatic subjects. The activities of fraction 1 derived from both CS and CR patients were sensitive to corticosteroid preincubation whereas the activities of fraction 4 were not attenuated by hydrocortisone.

Size and pI of PBMC-derived enhancing activity. The size of the molecule(s) carrying enhancing activity in PBMC culture supernatants, and the effects of hydrocortisone on the production of this activity by PBMC, were assessed by chromatography of the combined Sep Pak eluant fractions 2 and 3 derived from each of three CS and three CR asthmatic subjects on a TSK 3,000 HPLC column. Representative experiments from one subject in each group are shown in Fig. 6. In all CS asthmatic subjects there was one major peak of enhancing activity which eluted with particles of between $M$, 1,300 and 10,000 D. In each of these subjects the peak of activity was reduced by $>90\%$ in the samples derived from PBMC which were cultured in the presence of $10^{-8}$ M hydrocortisone as compared to PBMC samples cultured in the absence of corticosteroids.

In CR asthmatic subjects, enhancing activity was present in all the groups of eluant fractions tested and, similarly to the CS asthmatic individuals, the major peak of activity eluted with particles of between $M$, 1,300 and 10,000 D. However, unlike the samples from CS asthmatic subjects, none of the peaks of activity eluting from the column were suppressed by culturing the PBMC in the presence of hydrocortisone.

The eluant fractions 24–28 after TSK 3000 HPLC of samples derived from PBMC, which were isolated from three CR asthmatic subjects and then cultured in the absence of hydrocortisone, were combined and then chromatofocused on a PBE column with a pH gradient of 9–6. Enhancing activity focused between pH 6.5 and 7.3, with a major peak of activity eluting at pH 7.1 and a smaller activity peak eluting at pH 6.7 (Fig. 7).

To obtain a more accurate estimate of molecular size, the fractions eluting at pH 7.1 and containing the maximal activity after chromatofocusing were combined and applied to a Sephadex G-50 column (Fig. 8). After Sephadex G-50 chromatography there were four peaks of enhancing activity which eluted in fractions corresponding with molecular sizes of 10,000, 8,000, 3,000, and 2,000 D. The activity peak which consistently occurred at ~3,000 D coeluted with the major protein peak.

Heat stability and enzyme digestion. The stability of the PBMC-derived enhancing activity, which had been partially purified by TSK 3000 HPLC, to heat degradation and enzyme digestion was assessed in five experiments (Table V). Pronase produced a mean 95% inhibition and heat denaturation produced a mean 76% inhibition of enhancing activity. Neuraminidase produced no significant inhibition of activity.

RP-HPLC analysis of enhancing activity. The fractions corresponding with the major 3,000-D peak of activity found after Sephadex G-50 gel filtration were further purified by RP-HPLC on a C-18 Spherisorb-ODS column which was eluted with a gradient of 10–40% acetonitrile in water. A representative experiment is shown in Fig. 9. There was a single peak of activity after RP-HPLC, which eluted at 10% acetonitrile.

SDS PAGE. SDS PAGE of the purified 3,000-D peptide revealed a single band corresponding with an $M$, of between 2,500 and 3,000 D on staining with Coomassie Blue (Fig. 10). Autoradiography of the $^{125}$I-labeled peptide after SDS-PAGE demonstrated a single band corresponding with that found on Coomassie staining (Fig. 10).

Influence of 3,000-D peptide on superoxide production by neutrophils stimulated by PMA. To ascertain whether the priming activity of the 3,000-D peptide was specific to leukotriene generation or represented a more widespread cellular activation involving other pro-inflammatory products, the influence of the purified peptide on superoxide ($O_2^-$) production by resting and stimulated PMN was evaluated in six experiments (Fig. 11). Preincubation of PMN with the peptide for 2.5 min did not result in increased production of $O_2^-$ in PMN subsequently exposed to buffer alone. However, PMN preincubated with the peptide and then stimulated by exposure to PMA demonstrated enhanced $O_2^-$ production ($P < 0.05$) com-

| Table IV. Activity of Eluant Fractions after Sep Pak C-18 Filtration |
|-----------------|-----------------|-----------------|
| Subject group   | Fraction number | Generation of LTB4 |
|                 |                 | No hydrocortisone | Hydrocortisone $10^{-8}$ M |
|                 |                 | ng/10^5 PMN       |                               |
| Corticosteroid sensitive | 1             | 79.6±17.2         | 32.6±7.0                     |
|                   | 2             | 79.4±9.2          | 15.4±2.8                     |
|                   | 3             | 150.0±40.8        | 16.0±9.2                     |
|                   | 4             | 40.2±21.6         | 65.6±22.4                    |
|                   | Original      | 89.6±32.8         | 27.6±5.6                     |
| Corticosteroid resistant | 1            | 70.4±21.6         | 24.8±8.2                     |
|                   | 2             | 76.5±9.8          | 87.4±5.6                     |
|                   | 3             | 74.0±6.8          | 84.2±27.8                    |
|                   | 4             | 60.0±41.0         | 67.6±13.2                    |
|                   | Original      | 70.4±11.2         | 105.2±20.6                   |

Culture supernatants derived from PBMC cultured in the absence or presence of $10^{-8}$ M hydrocortisone were filtered on C-18 cartridges which had been equilibrated with H$_2$O and methanol and eluted with 2 × 2 ml of H$_2$O followed by 4 ml methanol. Aqueous eluant fractions were dried under negative pressure and similarly resuspended. Fractions were tested in a 1:3 dilution for LTB4 generation-enhancing activity in PMN obtained from four different, normal donors. PMN were preincubated for 2.5 min with the fractions and then stimulated with 2.5 $\mu$M A23187 for 5 min. Original fractions are unprocessed supernatant. The values in parentheses represent LTB4 generation by PMN incubated with a 1:3 dilution of control culture medium and then stimulated with 2.5 $\mu$M A23187. Values are mean±SEM ($n = 4$). Fraction 1, flow-through effluent; fractions 2 + 3, sequential 2-ml aqueous eluant fractions; fraction 4, 4-ml methanol eluate.
CORTICOSTEROID SENSITIVE

![Absorbance at 240 nm](image)

Fraction number

- 29,000 Carbonic Anhydrase
- 66,000 Albumin
- 1300 Vitamin B12

CORTICOSTEROID RESISTANT

![Absorbance at 240 nm](image)

Fraction number

- 29,000 Carbonic Anhydrase
- 66,000 Albumin
- 1300 Vitamin B12

Figure 6. TSK 3000 gel filtration of PBMC culture supernatants from one CS asthmatic subject and one CR asthmatic subject. Column fractions were tested for enhancement of LTB4 synthesis by neutrophils (PMN) obtained from two normal donors. Open bars represent activity of supernatant derived from PBMC cultured in the absence of hydrocortisone. Shaded bars represent supernatant derived from PBMC cultured in the presence of 10^(-8) hydrocortisone. For each patient, the experiments with and without hydrocortisone were performed with PMN from the same donor.

Figure 7. Chromatofocusing of PBMC culture supernatant after TSK 3000 gel filtration. Active eluant fractions from three CR subjects were combined before chromatofocusing. Eluate from chromatofocusing was adjusted to pH 7.4 and then assessed for enhancement of LTB4 generation by neutrophils obtained from one normal donor.

Figure 8. Sephadex G-50 gel filtration of the major peak of activity which eluted at pH 7.1 after chromatofocusing. Each point is the mean of duplicate leukotriene determinations on ionophore-activated neutrophils (PMN) obtained from one normal donor. (c) Activity peaks; and (a) denote protein peaks.
Cytokine partially purified by Sep-Pak C-18 fractionation and TSK 3000 HPLC from five asthmatic subjects was concentrated 10-fold and then treated with pronase for 15 min at 37°C, with neuraminidase for 1 h at 37°C, or heated to 60°C for 1 h. Samples were then diluted 1:30 and tested for enhancing activity in PMN obtained from two normal donors. PMN were preincubated with the treated cytokine for 2.5 min and then stimulated with 2.5 μM A23187 for 5 min. The values are mean±SEM of five experiments.

**Discussion**

We have demonstrated that the supernatants of cultures of PBMC derived from asthmatic patients prepared PMN for enhanced LTD₄ generation in response to activation by the calcium ionophore A23187. The short duration for the incubation period that produced maximal enhancement suggests that protein synthesis was not required for the effect. Prolonging the incubation period from 2.5 to 10 min reduced the enhancement of LTD₄ generation and release by PMN as previously described for stimulation of the PMN respiratory burst by formylmethionylleucylphenylalanine (FMLP) (27–29) and

**Figure 9.** RP-HPLC resolution of the 3,000-D peak of enhancing activity from Sephadex G-50 gel filtration chromatography. Fractions were lyophilized, resuspended in HBSS⁺, and assessed for enhancement of LTD₄ synthesis by neutrophils (PMN) obtained from one normal donor.

**Figure 10.** SDS-PAGE of the 3,000-D factor after RP-HPLC resolution. 125I-labeled samples were subjected to electrophoresis on a 20% acrylamide/SDS flat bed gel. Gels were fixed, dried, autoradiographed, and then stained with Coomassie blue. Molecular mass standards given in kilodaltons: track a, low molecular mass standards (Sigma Chemical Co.); track b, 3,000-D factor (60 ng); track c, 3,000-D factor (600 ng); track d, autoradiograph of 125I-labeled factor (600 ng).

**Figure 11.** Production of superoxide anion (expressed as nanomoles of cytochromes reduced) by neutrophils preincubated for 2.5 min with either control buffer (c) or a 1:3 dilution of cytokine purified by TSK 3000 HPLC, chromatofocusing, Sephadex G50, and RP-HPLC (e) and then stimulated with incremental concentrations of PMA for 45 min. Values are mean±SEM for six separate experiments. Six normal subjects served as donors for PMN.
for the enhancement of PMN LTB₄ generation by a group of monocyte-derived molecules (4).

The enhancing effect of the asthmatic PBMC supernatants on PMN LTB₄ generation was significantly greater than that of PBMC supernatants from normal individuals, suggesting that asthmatic PBMC may already have been activated in vivo. Analysis of ω-oxidation metabolites of LTB₄ suggests that the apparent difference in the enhancement of LTB₄ generation is at the level of biosynthesis rather than due to reduced ω-metabolism of generated LTB₄. Authenticity of the immunoreactive LTB₄ was confirmed by subjecting the immunoreactive material to RP-HPLC and assessment of the fractional eluates by a specific LTB₄ radioimmunoassay. There was only one peak of immunoreactive material which eluted at the retention time of the synthetic standard.

The enhancing activity was produced by adherent blood mononuclear cells that resisted complement-dependent lysis by anti–CD5 and anti–human Ig and were sensitive to treatment with anti–CD 14 and complement. The cells were therefore presumably monocytes. In the presence of increasing concentrations of hydrocortisone, there was a significant suppression of the production of enhancing activity from the PBMC derived from CS asthmatic subjects but not from the cells derived from the CR asthmatic individuals. The release of enhancing activity by PBMC was suppressed by 10⁻¹⁰ to 10⁻⁴ M hydrocortisone. The Kᵣ of a specific glucocorticoid receptor in monocytes and macrophages ranges from 2 to 10 nM prednisolone and from 10 to 80 nM hydrocortisone (30–32). Glucocorticoids at comparable concentrations have previously been shown to suppress a number of mononuclear cell functions including generation of thromboxane B₂, LTB₄, and LTC₄ (33, 34); Iα expression and IL-1 generation by macrophages (35); and IL-1 generation (36) and colony formation (3) by monocytes. The dose which produced 50% inhibition (ID₅₀) for such suppressive effects range from ~ 5 x 10⁻⁸ M hydrocortisone for leukotriene generation to 10⁻⁶ M for arachidonic acid release (37). Paradoxical stimulatory effects of low concentrations of glucocorticoids on mediator release have been reported (34), but suppressive effects at concentrations lower than 10⁻⁸ M have not previously been described. The low effective concentrations of hydrocortisone observed in this study suggest that either the mechanism involved in the inhibitory effect does not require a high degree of binding to the glucocorticoid receptor, or that the response is mediated via a high-affinity receptor.

The PBMC from CR subjects were resistant to the suppressive effects of hydrocortisone at concentrations which were similar to those achieved clinically in the treatment of acute asthma (38). There was no difference between the CS asthmatic subjects and the CR asthmatic subjects in the time interval between isolation of the PBMC for the in vitro experiments and the last ingestion of corticosteroid therapy. Thus, the response of each individual group of patients was not influenced by the administration of exogenous corticosteroid. The significant positive correlation between the suppression of priming activity by hydrocortisone in vitro and the clinical improvement in FEV₁ after treatment with prednisolone suggests that the cell types involved in generating the enhancing activity may be similar to those contributing to the pathogenesis of asthma in these patients.

In view of the potential importance of a molecule which could enhance the pro-inflammatory functions of granulocytes which was not suppressed by corticosteroids, we elected to focus on the characterization of the activity produced by PBMC of CR asthmatic subjects. Thus, the major peak of activity eluting from the TSK 3,000 HPLC column was sequentially purified by chromatofocusing, gel filtration, and RP-HPLC. After chromatofocusing, there was one major peak of activity which eluted with an estimated pl of 7.1. When this peak of activity was subjected to gel filtration chromatography on columns of Sephadex G-50 it eluted in four peaks of activity with estimated molecular sizes of between 12,400 and 2,000 D. This is equally compatible with several distinct and unrelated molecules carrying the activity, as with a family of molecules related as aggregates or cleavage products. When the major peak of activity which coeluted with the major protein peak from Sephadex G-50 in fractions corresponding with an Mₛ of 3,000 D, was subjected to further purification by RP-HPLC (39), there was only one peak of activity and this eluted prior to the start of the linear acetonitrile gradient. SDS-PAGE of this material demonstrated a single protein band both on autoradiography and on staining with Coomassie Blue. The finding that the material was pronase and heat-sensitive, but resistant to neuraminidase, suggested that it had a peptide structure. The size and charge of the molecule suggests that it was not IL-1 (40) or interferon-α (41), both of which have been reported to modulate prostaglandin biosynthesis in fibroblasts and macrophages (42), or granulocyte/macrophage colony-stimulating factor and tumor necrosis factor which increase leukotriene biosynthesis in neutrophils and eosinophils (43, 44).

To evaluate whether the enhancing activity of the 3,000-D molecule was selective for leukotriene generation, or represented a more widespread cellular activation, the capacity of the molecule to enhance generation of superoxide anion by PMN subsequently stimulated with PMA was assessed. The purified molecule also enhanced superoxide generation by PMA stimulated PMN, but did not stimulate superoxide generation in the absence of PMA (Fig. 11).

A substantial body of evidence implicates cells of the monocyte lineage in the mechanisms of asthma (2, 3, 45–49). There are several ways in which improvement in the clinical condition of asthmatic patients treated with corticosteroids may depend upon the effects of these drugs on such cells. There is well-documented inhibition of production of cytokines by low concentrations of corticosteroids (3, 35, 36), and inhibition of arachidonic acid release in leukocytes (33, 34, 37). Our results, therefore, would support the view that cells of monocyte lineage within the lungs of CR asthmatic patients enhance the pro-inflammatory potential of infiltrating PMN and that they exist in a hyperreactive state with increased cytokine production despite the presence of inhibitory concentrations of corticosteroids.

We have studied corticosteroid resistance in chronic asthma by selecting patients of two polar extremes on the basis of their clinical response to corticosteroid treatment. It is likely, however, that corticosteroid sensitivity and resistance forms a continuous spectrum (3). It is, nevertheless, critical to recognize patients whose asthma is highly resistant to systemic corticosteroids, since these individuals may otherwise be needlessly exposed to high doses of corticosteroid treatment with little or no benefit. This study indicates one way in which such patients may be identified. The mechanism of the cellular basis of corticosteroid resistance remains unknown. A success-
ful definition of the mechanism(s) will allow further understanding of not only corticosteroid resistance, but also the manner in which corticosteroids function in asthma.

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References


