Effects of Leukotriene B_4 in the Human Lung

Recruitment of Neutrophils into the Alveolar Spaces without a Change in Protein Permeability

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Abstract

Leukotriene B_4 (LTB_4) is a major product of human alveolar macrophages and has potent chemotactic activity for neutrophils (PMN) in vitro. To evaluate the effects of LTB_4 in the normal human lung, we instilled LTB_4 (5×10^{-7}M, 10 ml) into a subsegment of the right middle lobe and 0.9% NaCl (10 ml) into a subsegment of the lingula using a fiberoptic bronchoscope in 12 healthy human volunteers. 4 h later, we performed bronchoalveolar lavage of the same subsegments. Compared with the NaCl instillation, LTB_4 caused a large increase in lavage total cells (NaCl = $6.8 \pm 1.0 \times 10^6$ vs. LTB_4 = $26.4 \pm 5.0 \times 10^6$, $P < 0.01$), most of which were PMN (NaCl = 12.2±4.6% vs. LTB_4 = 55.7±6.0%, $P < 0.001$). In contrast, there was only a small increase in lavage total protein, and the lavage total protein correlated weakly with lavage total cells and PMN. The production of superoxide anion by the lavage PMN in response to phorbol myristate acetate was similar to that of peripheral blood PMN. The migration of lavage PMN was normal toward the chemotactic peptide FMLP, but reduced toward LTB_4 and zymosan-activated human serum. Morphometric analysis using transmission electron microscopy indicated a selective loss of small granules in the lung neutrophils as compared with peripheral blood neutrophils. The data indicate that in the normal human lung, LTB_4 can recruit active PMN into the airspaces without causing a significant change in the protein permeability of the epithelial barrier.

Introduction

Leukotriene B_4 (LTB_4) is the major product of arachidonic acid metabolism produced by the 5-lipoxygenase pathway in human alveolar macrophages and neutrophils (1–5). Unlike the other products of this pathway, leukotrienes C_4, D_4, and E_4, which are potent bronchoconstrictors in humans and animals, the major effects of LTB_4 are directed at phagocytes (reviewed in 6, 7). When studied in vitro, LTB_4 promotes chemotaxis of neutrophils (PMN), monocytes, and fibroblasts (2, 8–12). It also causes PMN degranulation (11, 13) and acts as a calcium ionophore, promoting calcium movement into cells (14). The injection of LTB_4 into human skin causes PMN accumulation and fibrin deposition within 6 h (15), indicating that LTB_4 can initiate an inflammatory reaction and cause cellular recruitment in the systemic circulation.

The effects of LTB_4 in the human lung have not been studied, and conflicting data exist about the effects of LTB_4 in the lungs of animals, which has made it difficult to predict the effects of LTB_4 in the human lung. LTB_4 can be produced in the airspaces of the human lung, because human alveolar macrophages produce large amounts of LTB_4 in vitro when stimulated with soluble and particulate stimuli (1–3). Indeed, LTB_4 accounts for most of the neutrophil chemotactic activity produced in vitro by normal human alveolar macrophages within the first 3 h of stimulation (3). However, when LTB_4 has been instilled into animal lungs to investigate its chemotactic effects in vivo, the results have varied. For example, in rat lungs LTB_4 caused only minor cellular recruitment into the airspaces as measured by lung lavage cell counts and pathology (2). It was found subsequently that rat neutrophils migrate poorly in response to LTB_4, which was attributed to deficient surface receptors for LTB_4 (16). These findings leave the function of LTB_4 in the rat lung uncertain, because rat alveolar macrophages can produce large amounts of LTB_4 in vitro (17, 18). When aerosolized into guinea pig lungs LTB_4 caused the accumulation of PMN in the walls of medium-sized airways, but not in the alveolar spaces, and no evidence of bronchospasm (19). In sheep, intratracheal instillation of LTB_4 caused a modest influx of neutrophils into the alveolar spaces with little increase in protein flux across the endothelial and epithelial barriers of the lung (20).

Because of the variability in the response to LTB_4 in the lungs of different animal species and the lack of any human data on the pulmonary response to LTB_4, our primary goal was to determine whether LTB_4 could recruit neutrophils and monocytes into the airspaces of human lungs. Our secondary goals were to determine the functional status of the newly recruited cells and to ascertain whether the migration of these cells into the airspaces was associated with an important change in the permeability of the pulmonary epithelial barrier to protein.

Methods

Subjects. We studied 12 healthy human volunteers, 9 males and 3 females, who ranged in age from 23 to 40 yr. All were free of clinical lung disease and none used cigarettes. All had medical or paramedical occupations and were familiar with flexible fiberoptic bronchoscopy before they volunteered for the study. None had used antiinflammatory agents of any kind for at least 1 wk before the study. The study was approved by the University of Washington Human Subjects Experimentation Committee, and all of the volunteers gave written informed consent.

Bronchoscopy procedures. Each subject underwent two fiberoptic bronchoscopies, either 4 h apart (11 subjects) or 1 h apart (1 subject). Each subject was premedicated with one double-strength tablet of tri-

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methaprim-sulfamethoxizole 12 h before and again 1 h before the first procedure to reduce the chance that any oropharyngeal flora aspirated during the bronchoscopy procedures would multiply in the lower airways. In addition, each subject took diazepam, 10 mg orally, 1 h before each bronchoscopy to minimize coughing and anxiety during and after the procedures. A peripheral venous blood sample was obtained from each subject on the morning of the study and the serum was separated and stored frozen at −70°C for protein determinations.

During the first bronchoscopy, the oropharynx was anesthetized topically with 2% lidocaine and a flexible fiberoptic bronchoscope (model FB 15A, Pentax Precision Instruments Corp., Orangeburg, NY) was passed orally into the trachea and wedged into a subsegment of the lingula. Then 10 ml of sterile pyrogen-free 0.89% NaCl (Abbott Laboratories, North Chicago, IL) containing 1:1,000 (vol/vol) absolute ethanol (the vehicle for the LTB_4) was instilled through the suction channel of the bronchoscope, followed by five separate 10-ml aliquots of air to disperse the liquid in the subsegment. The bronchoscope then was wedged in a subsegment of the right middle lobe and 10 ml of LTB_4 (Calbiochem-Behring Corp., La Jolla, CA), diluted to 5×10^{-7} M in sterile pyrogen-free 0.89% NaCl, was instilled followed by five separate 10-ml aliquots of air. To minimize cross-contamination, the saline was always instilled in the lingula before the LTB_4 was instilled into the right middle lobe. The instilled LTB_4 and NaCl solutions gave negative reactions for endotoxin by the Limulus amebocyte lysate test (Sigma Chemical Co., St. Louis, MO) and were sterile when cultured in trypticase soy broth. Two of the subjects were studied twice, at least 3 mo apart, once with 5×10^{-7} M LTB_4, and once with 5×10^{-6} M LTB_4 instilled in the right middle lobe in an identical fashion. In one subject who volunteered for an additional bronchoscopy, 10 ml of 10% $BaSO_4$ suspension was instilled in the same manner into a right middle lobe subsegment to permit radiographic evaluation of the distribution of the instilled fluid. The instillations were well-tolerated by all of the volunteers and did not provoke coughing.

After the first procedure, the subjects remained in the bronchoscopy suite and blood pressure, pulse, and temperature were monitored hourly. 4 h later, a second bronchoscopy was performed in 11 subjects using topical oral anesthesia with 2% lidocaine. The bronchoscope was passed orally into the lower airway and each subsegment into which saline or LTB_4 had been instilled was lavaged with five separate 30-ml aliquots of sterile pyrogen-free 0.89% NaCl and the lavage fluid was recovered by gentle suction. One additional subject was studied 1 h after the first bronchoscopy in order to investigate changes in lavage cells and proteins at an earlier time.

Immediately before the second bronchoscopy, each subject had postero-anterior and lateral chest radiographs, and 10 subjects had spirometry and lung volume measurements by the nitrogen washout technique using a Cybermedic pulmonary function system (Cybermedic, Louisville, CO). Five of these subjects had paired measurements performed before each bronchoscopy. After the second bronchoscopy, each volunteer was given a thermometer and asked to take his or her temperature at 4-h intervals. Each subject was interviewed by telephone by one of the investigators (T. R. Martin) during the evening after the study.

Analysis of bronchoalveolar lavage fluid. Differential cell counts were done on aliquots of the freshly recovered lavage fluids prepared by cytocentrifugation. The remainder of the lavage fluid was spun at 200 *g* and the supernatant was stored frozen at −70°C until assayed for protein content. The cell pellets were resuspended in HBSS and total cell counts were performed in a hemacytometer. Total protein in lavage fluid and serum was measured by a modification of the Lowry assay adapted for microtiter plates (21). Lavage aliquots were concentrated to ~ 10% of original volume by positive pressure filtration under nitrogen at 4°C using a 5,000-mol wt limit membrane (YM-5; Amicon Co., Danvers, MA). The concentrations of albumin, IgM, IgA, and IgG were measured in the concentrated lavage fluid and in the serum of each volunteer by radial immunodiffusion using commercially available reagents (Calbiochem). The concentration of C5a was measured in concentrated lavage fluid by radioimmunoassay as described (3) using antibodies that detect both C5a and $C5a_{desarg}$ (Upjohn Diagnostics, Kalamazoo MI). The sensitivity of this assay is ~ 0.5 ng C5a/ml in the unconcentrated lavage fluid. The concentrations of LTB_4 and TXB_2 were measured in the unconcentrated lavage fluids by radioimmunoassay using antibodies from New England Nuclear (Boston, MA). The sensitivity of the LTB_4 assay is 15 pg/ml of unconcentrated lavage fluid. The cross-reactivity of the antibody is 100% for LTB_4; < 0.4% for 20-OH-LTB_4 and 6-*trans*-LTB_4; and < 0.05% for LTC_4, LTD_4, 5-HETE, 12-HETE, 15-HETE, TXB_2, and arachidonic acid. The sensitivity of the thromboxane assay is ~ 10 pg/ml of unconcentrated lavage fluid. The cross-reactivity of the antibody is: TXB_2 100%; PGD_2 3.9%; PGE_2 0.23%; and < 0.1% for PGF_{2a}, PGE_1, 6k-PGF_{1a}, PGF_{1a}, PGA_2, and arachidonic acid.

Neutrophil ultrastructure and surface receptors. To compare the morphology of the cells in bronchoalveolar lavage fluid and in peripheral blood, 2.5×10^6 cells were fixed for 2 h in 2.5% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4, then washed three times with cacodylate buffer, post-fixed in 1.0% OsO_4, embedded in agar and processed routinely for electron microscopy. The cells were analyzed using a JEOL-100B transmission electron microscope at 60 kV as described (22). The size distributions of the PMN granules were determined morphometrically as described (23, 24). For each specimen, 15 PMN profiles were selected at random from at least 500 PMN profiles, taking care to select only PMN profiles that were cut similarly through the plane of the nucleus of the cell. All of the micrographs were enlarged to a final magnification of 20,000. In each PMN profile, the diameters of all granules were measured morphometrically (24) to produce a histogram of granule size for each profile.

In one of the three subjects in whom PMN granule size distributions were measured, we also measured the expression of a major surface adherence glycoprotein complex (CD11/CD18) on lavage and peripheral blood neutrophils by indirect immunofluorescence and flow cytometry using the murine monoclonal antibodies designated 60.1 and 60.3 (provided by Pat Beatty, Fred Hutchinson Cancer Institute, Seattle, WA). On neutrophils (25), this complex consists of an α_m subunit (CD11b, Mac-1a, Mo1a) noncovalently associated with a β subunit (CD18, Mac-1b, Mo1b). The 60.1 antibody detects the CD11b subunit (26) and the 60.3 antibody detects the CD18 subunit (27). The subject's peripheral blood was sampled before the instillation of LTB_4 and PMN were recovered by Ficoll-Hypaque density gradient centrifugation and dextran sedimentation (28). The freshly isolated lavage and peripheral blood PMN were incubated for 30 min at 4°C in microtiter plates with either 60.1, 60.3, or an isotype-matched antibody, 60.5, that binds to an HLA framework antigen present on all blood leukocytes and endothelial cells (26). Then they were washed, incubated for 30 min with fluoresceinated goat anti-mouse IgG, washed again, fixed in 1% paraformaldehyde, and stored in the dark until analyzed within 24 h. The fluorescence intensity of the labeled cells was measured on a fluorescence activated cell sorter (model 50 HH; Ortho Diagnostic Systems, Westwood, MA) using 400 mW light with an excitation wavelength of 488 nm and a detection wavelength of 520–540 nm.

Neutrophil function. In lavage fluids containing at least 80% neutrophils, we measured chemotaxis, superoxide anion production and protein kinase C content of the lavage cells. For comparison, the same assays were performed simultaneously using neutrophils recovered from the peripheral blood of a normal volunteer by Ficoll-Hypaque density gradient sedimentation followed by dextran sedimentation (28). Control experiments using human blood neutrophils and normal alveolar macrophages indicated that the assays for chemotaxis and superoxide anion production were unaffected by the presence of up to 20% alveolar macrophages.

Chemotaxis was measured in microchemotaxis chambers as previously described (2, 3, 29) using the formylated tripeptide FMLP, 10^{-9} to 10^{-6}M, or zymosan-activated human serum (ZAS,[1] 5% and 10%) as

1. *Abbreviations used in this paper:* ARDS, adult respiratory distress syndrome; PKC, protein kinase C; ZAS, zymosan-activated human serum.

the chemotactic stimuli. The upper and lower compartments of the microchemotaxis chambers were separated by nitrocellulose membranes with 3.0-μm pores (Neuroprobe Co., Bethesda MD). The cells were washed in HBSS and suspended at 2.5×10^6/ml in Gey's balanced salt solution (Gibco, Grand Island, NY) and 50 μl (1.25×10^5 cells) were added to each upper compartment and 25 μl of chemoattractant were added to each lower compartment of the microwell assembly. After 2 h incubation in 5% CO_2/air, the filters were removed, stained, and mounted on glass microscope slides. Chemotaxis was measured with a 1.0 mm^2 eyepiece grid as the total number of cells that migrated through each filter within the grid in 10 consecutive light microscopic fields (×540). In each experiment, the chemotactic response to each stimulus was measured in quadruplicate and the results were averaged.

The production of superoxide anion (O_2^-) by resting and stimulated neutrophils was measured as the superoxide dismutase inhibitable reduction of ferricytochrome *c* in a microtiter assay using phorbol myristate acetate as the stimulus (PMA; Cancer Research Chemicals Consolidated, Brooklyn, NY) (30). Neutrophils (2.5×10^5/well) were incubated for 1 h with ferricytochrome *c* (2.7 mg/ml; Sigma Chemical Co.) with or without PMA (100 ng/ml) in the presence or absence of superoxide dismutase (SOD, 1.0 mg/ml; Sigma Chemical Co.). The reduction of ferricytochrome *c* was measured as the change in absorbance at 550 nm in each microtiter well using an eight channel photometer that measures absorbance vertically through each individual well of the microtiter plate (Dynatech Co., Chantilly, VA). To measure sequential O_2^- production in each well, each microtiter plate was read repeatedly over 60 min, at which time sodium dithionite (Sigma) was added to completely reduce the ferricytochrome *c* in each well. This yielded an absorbance value for each well that corresponded to 100% ferricytochrome *c* reduction. The difference in ferricytochrome *c* reduction with and without SOD was taken as a measure of O_2^- production. The production of O_2^- in at each time was determined by the following formula: O_2^- = 10.9 nmol × (% ferricytochrome *c* reduced without SOD − % ferricytochrome *c* reduced with SOD).

The protein kinase C (PKC) content of lavage and peripheral blood neutrophils was measured as the specific binding of a radiolabeled phorbol ester, [^{3}H]phorbol dibutyrate ([^{3}H]PDBu, New England Nuclear), to whole neutrophils as described (31, 32). Peripheral blood neutrophils or lavage cells were incubated in a final volume of 0.5 ml containing 1×10^6 cells in assay buffer (140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 0.5 mg/ml bovine serum albumin, 15 mM Hepes buffer, pH 7.4) with [^{3}H]PDBu (10 nM final concentration). Nonspecific binding of [^{3}H]PDBu was measured as the binding that occurred in the presence of excess cold PDBu (10 mM). After incubation at 4°C for 2 h, the reaction was stopped by the addition of 3 ml ice-cold buffer and the reaction mixtures were aspirated onto glass fiber filters (GF/C, Whatman Ltd., Maidstone, England) that were washed and counted by liquid scintillation.

Statistics. The differences between LTB_4 and NaCl treatments were analyzed using Student's two-tailed paired *t* test. The results from each individual volunteer were treated as one "*n*" with paired observations on the saline and the LTB_4 sides. The granule distribution data were analyzed by one way analysis of variance, using Scheffe's test for secondary differences. A *P* value of < 0.05 was considered to be significant.

Results

All of the volunteers tolerated the bronchoscopy procedures well. None of the subjects had wheezing, chest tightness, or changes in heart rate or blood pressure from baseline during the 4-h observation period. In all of the subjects, the pulmonary function measurements (airflow rates and lung volumes) were ≥ 94% of predicted immediately before the second bronchoscopy. In the five subjects in whom pulmonary function was measured immediately before and 4 h after the instillation of LTB_4, no significant changes occurred in either flow rates or lung volumes. The forced expiratory volume (FEV_1) increased 1.2±5.1%; the forced vital capacity (FVC) fell −1.3±4.3%; the total lung capacity (TLC) fell −3.5±7.0%; the vital capacity (VC) fell −2.5±1.4%; and the functional residual capacity (FRC) fell −5.1±6.1% (mean±SD). In addition, none of the volunteers had infiltrates on chest radiographs taken ~ 3.5 to 4 h after the instillation of the LTB_4. Fig. 1 shows the anatomic location of an aqueous solution containing 10% barium sulfate as a marker that was instilled in a fashion identical to the LTB_4 in one volunteer. The volunteer did not cough during or after this instillation. Within 5 min after instillation, the marker is visible in the distal bronchoalveolar units.

Lavage cells. The lavage fluid findings are shown in Table I. The total recovery of lavage fluid was similar on both sides (NaCl = 61.1±2.7% vs. LTB_4 = 65.7±3.1%, $P > 0.05$), so that expressing cell and protein recoveries as absolute values or as concentrations gives similar results.

The fluid recovered from the LTB_4 side contained significantly more total cells. Most of this increase was accounted for by a large increase in both the percentage and the total number of PMN. On the LTB_4 side, 3.9 times more cells were recovered than on the NaCl side ($P = 0.002$). On the LTB_4 side 55.7±6.0% of the recovered cells were PMN ($P = 0.001$). By contrast, on the NaCl-treated side only 12.2±4.6% of the cells were PMN. Compared with the NaCl side, 17.1 times more total PMN were recovered from the LTB_4-treated side ($P = 0.005$). The percentage of PMN recovered on the NaCl treated side (12.2±4.6%) exceeds that normally found in lavage fluid of normal volunteers (mean±SD of 21 normal volunteers = 1.8±0.5% PMN), indicating that NaCl instillation alone caused the accumulation of some PMN in the airspaces (3, 33).

The percentage of alveolar macrophages in the lavage fluid was significantly lower on the LTB_4-treated side, but the total yield was 1.8 times greater, reflecting the increased total cell recovery ($P = 0.008$). The percentage of lymphocytes did not differ significantly between the two sides, but the total recovery of lymphocytes was 1.9-fold higher on the LTB_4 side, because of the greater number of total cells ($P = 0.026$).

Lavage proteins. Unlike the effect on PMN recovery, the LTB_4 did not have a significant effect on the recovery of total protein (Table I). The mean total protein concentration was increased slightly on the LTB_4 side, but this did not reach statistical significance. As with total protein, the albumin recovery was somewhat higher on the LTB_4 side, but this difference also did not reach statistical significance. The ratio of albumin to total protein in the lavage fluid was similar on both sides. In addition, we found no difference between the two sides in the lavage-to-serum ratios for total protein and albumin.

When total protein recovery was compared with total cell recovery, there was significantly less protein per cell on the LTB_4 side ($P = 0.019$). Overall, the protein recovery per cell on the LTB_4 side was only 47.8% of that on the NaCl side ($P = 0.019$). On the NaCl side, there was no correlation between the amount of total protein and the total number of PMN recovered. On the LTB_4 side, the correlation between total protein and total PMN was weak ($r = 0.5$, $P < 0.05$). Similarly, only weak correlations were found for total protein vs. total cells ($r = 0.6$) and for total protein vs. the percentage PMN in the lavage fluid ($r = 0.5$).

When a higher concentration of LTB_4 (5×10^{-6} M) was

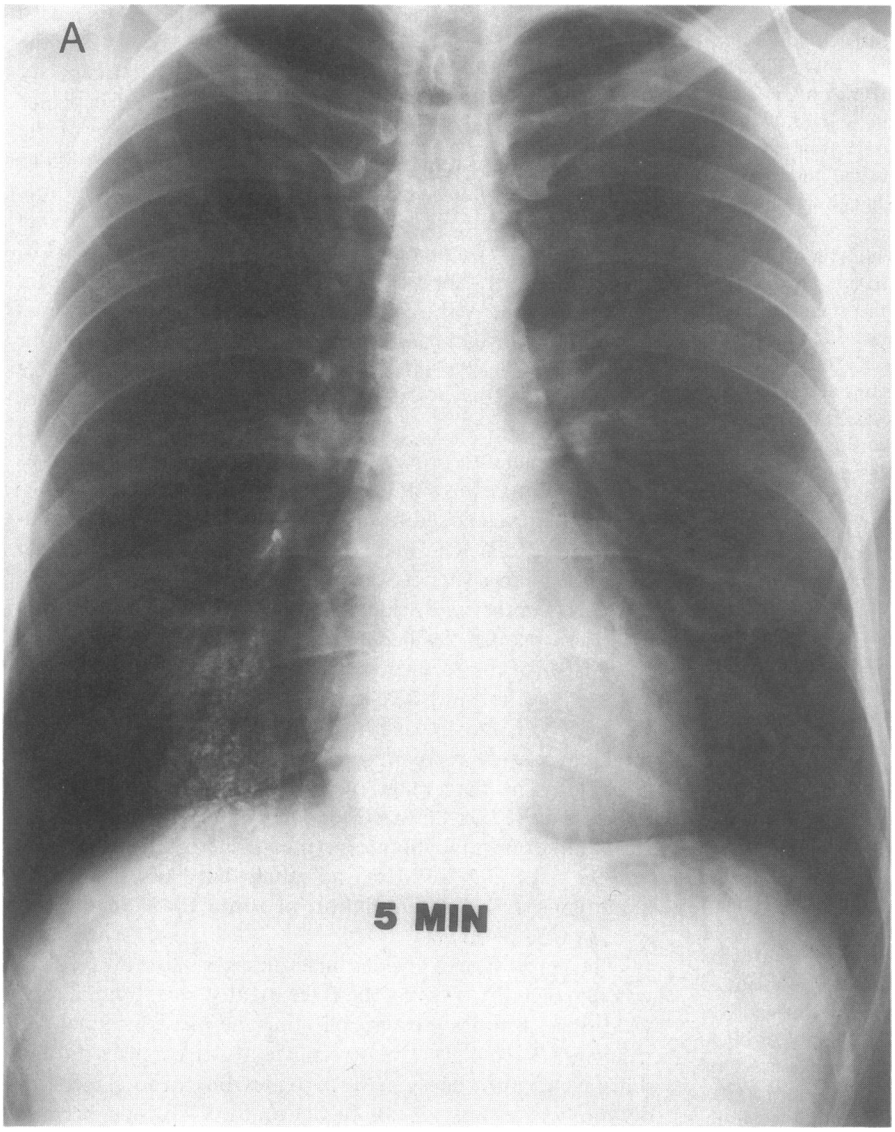

Figure 1. Posteroanterior (*A*) and lateral (*B*) chest radiographs taken 5 min (*A, B*) after the instillation of 10 ml of 10% $BaSO_4$ in a subsegment of the right middle lobe of a normal volunteer, using the identical technique used to instill LTB_4. The $BaSO_4$ is visible in an alveolar pattern in an anterior subsegment of the right middle lobe. Repeat films taken 4 h later showed no appreciable change in the radiographic distribution of the $BaSO_4$.

instilled at a different time in two of the subjects, the recovery of total cells (mean = 11.6×10^6 total cells) and total PMN (mean = 5.5×10^6 PMN, or 47.4% of total cells) were increased as compared with the NaCl-treated side, but the increase was not as great as with 5×10^{-7} M LTB_4. Similarly, the recovery of total protein did not increase more than with 5×10^{-7} M LTB_4 (mean = 13.5 mg total protein recovered on the LTB_4 side vs. 9.7 mg on the NaCl side). In one additional subject, we performed the second lavage 1 h after the instillation of LTB_4 to determine whether the protein values might be higher at an earlier time. Although there was a 2.2-fold increase in total cells (5.1×10^6 vs. 11.0×10^6 for NaCl vs. LTB_4) and a 10-fold increase in total PMN (5.8×10^5 vs. 55.8×10^5 for NaCl vs. LTB_4) as compared with the NaCl-treated side, the total protein was not increased compared with the NaCl instilled side (9.1 mg/dl vs. 10.4 mg/dl for NaCl vs. LTB_4).

The high molecular weight protein IgM (900,000 mol wt) was not detected by radialimmunodiffusion in any of the concentrated lavage fluids from either the NaCl or the LTB_4-treated sides. IgA (440,000 mol wt) was detected in 8 of 11 subjects on the NaCl side (mean = 3.44±0.73 mg/dl) and in 8 of 11 subjects on the LTB_4 side (mean = 5.48±1.01 mg/dl), and this difference was not significant ($P = 0.15$).

The complement fragment C5a was detectable in the concentrated lavage fluid from both sides in all of the subjects at a concentration that corresponded to ~ 0.1 nM (1.0 ng/ml) in the unconcentrated lavage fluids (Table I). The difference in concentration between the NaCl- and the LTB_4-treated sides was not significant.

LTB_4 was not detectable in any of the lavage fluids by radioimmunoassay. By contrast, TXB_2, a cyclooxygenase metabolite of arachidonic acid that causes pulmonary vasoconstriction, was detectable in 7 of 11 subjects on the LTB_4 side (range 36.0–330 ng/ml), but in only 2 of 11 subjects on the NaCl side (26.0 and 32.0 pg/ml, respectively).

PMN morphology. Fig. 2 compares the morphology of lung PMN recovered from the LTB_4 side of one of the subjects with peripheral blood PMN from the same subject obtained before the instillation of LTB_4. The peripheral blood PMN are rounded and have prominent granules. In contrast, many of the lavage PMN are elongated, or polarized, and the granules

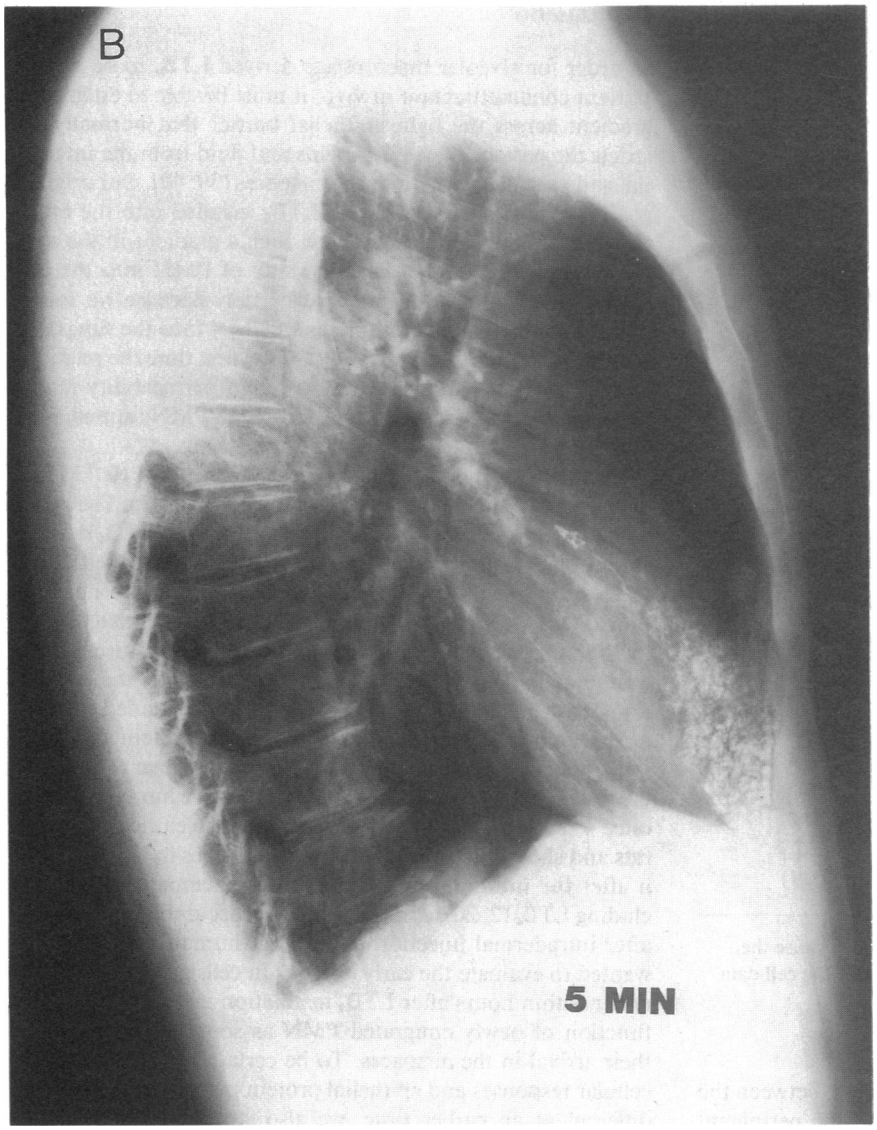

Figure 1. (Continued)

have moved to the ends of the cells, as occurs in vitro when PMN are exposed to chemotactic stimuli (34). Fig. 3 compares the size distribution of granules from peripheral blood PMN with those of PMN from the LTB_4-treated segment of five subjects, and the NaCl-treated segment of two additional subjects. Although the distribution of larger granules (> 0.3 μm diam) was similar in the lung PMN from both sides and the blood PMN, the lung PMN from both the NaCl and the LTB_4-treated sides had significantly fewer small granules than the blood PMN. The granule distributions were similar in PMN recovered from the NaCl- and the LTB_4-treated sides. These findings suggest that the lung PMN from both sides had selectively lost the smaller secondary granules during the process of migration into the lung (35).

Fig. 4 shows the expression of the major adherence glycoprotein complex (CD11/CD18) on simultaneously studied lung and peripheral blood PMN from one of the three subjects in whom the granule measurements were made. The lung lavage and peripheral blood PMN were labeled with the monoclonal antibodies 60.1 (CD11b subunit) and 60.3 (CD18 subunit), and 60.5, which binds an HLA framework antigen. As compared with the blood PMN, the lung PMN had a threefold increase in peak fluorescence intensity with 60.3, suggesting a threefold increase in the peak expression of the CD18 subunit. Using 60.1, the peak fluorescence intensity of lung and blood PMN was similar, but the tail of the fluorescence curve was shifted to the right, indicating that some of the PMN expressed increased amounts of the CD11b subunit. The expression of the HLA framework antigen labeled with 60.5 was not increased on the lung PMN as compared with the blood PMN. These data are consistent with the morphometric data indicating that some degranulation had occurred in the lung PMN, because the CD11 and CD18 epitopes increase on the surface of PMN after degranulation (36). The CD11 subunit has been found in specific granules of PMN (37, 38) although it may occur in other locations as well (39). The CD18 subunit may also be present in specific granules (38).

PMN function. The production of O_2^- by neutrophils obtained from the LTB_4 side of three of the subjects is shown in Fig. 5. There was no difference in either the rate or the total

Table I. Lavage Fluid Cells, Proteins, and Thromboxane

	NaCl	LTB_4	*P* value
Lavage return (%)	61.1±2.7	65.7±3.1	NS
Lavage cells			
Total cells ($\times 10^6$)	6.8±1.0	26.4±5.0	0.002
Total cells/ml ($\times 10^4$)	7.5±1.0	27.5±5.3	0.002
Neutrophils			
%	12.2±4.6	55.7±6.0	0.001
Total ($\times 10^5$)	9.4±4.7	160.3±39.7	0.005
Macrophages			
%	82.7±5.9	40.5±6.1	0.001
Total ($\times 10^6$)	5.4±0.9	9.5±1.9	0.008
Lymphocytes			
%	5.8±3.0	3.7±2.0	NS
Total ($\times 10^5$)	4.2±2.7	7.8±3.6	0.025
Lavage proteins and thromboxane			
Total protein (*mg*)	15.4±4.8	23.4±3.5	NS
Protein concentration (*mg/dl*)	15.9±4.2	24.5±3.7	NS
Total albumin (*mg*)	8.7±2.7	13.9±1.8	NS
Albumin concentration (*mg/dl*)	9.1±2.4	14.4±1.9	NS
Albumin:total protein ratio	0.58±0.04	0.62±0.05	NS
Lavage:serum protein concentration ratio (*mg/g*)	2.0±0.5	3.4±0.5	NS
Lavage:serum albumin concentration ratio (*mg/g*)	2.0±0.6	3.2±0.4	NS
Total protein/cell (*ng/cell*)	2.3±0.6	1.1±0.2	0.019
C_{5a} (*ng/ml*)	0.8±0.1	1.03±0.01	NS
TXB_2 (*pg/ml*)	29.0±2.1 (n = 2/11)	93.1±37.7 (n = 7/11)	—

The data are the mean±SE of data from 11 subjects. Because the total volume of fluid recovered was similar, expressing the cell data as concentration (cells/ml) does not change the results.

production of O_2^- in response to PMA, 100 ng/ml, between the simultaneously studied lung lavage PMN and the peripheral blood PMN during the 60-min incubation. In the absence of PMA, the unstimulated lung and blood PMN made negligible amounts of O_2^- during the assay.

Fig. 6 compares the chemotactic responses of the lung lavage PMN of four of the subjects with normal peripheral blood PMN. For each subject, the lung and blood PMN were studied simultaneously, so that the comparisons between the cell populations and the stimuli would be valid. The lavage and the blood PMN migrated equally well toward a range of concentrations of the chemotactic peptide FMLP. In contrast, the response of the lavage PMN to human ZAS was significantly reduced at both 5% and 10% concentrations of ZAS. In one additional subject, the response of lung PMN to LTB_4 was also significantly blunted at 10^{-7} M and 10^{-8} LTB_4 as compared with simultaneously tested blood PMN from this subject. In the absence of chemotactic stimuli, the random migration of the blood and lung PMN was negligible.

When we analyzed the PKC content of whole PMN recovered from the LTB_4-treated side of four subjects, we found that the PKC content of these cells was similar to that of simultaneously tested normal peripheral blood PMN (lung PMN = 400.2±28.8 fmol/10^6 PMN; blood PMN = 375.8±47.2 fmol/10^6 PMN, $P > 0.05$).

Discussion

In order for alveolar macrophage derived LTB_4 to be an important chemoattractant in vivo, it must be able to establish a gradient across the tight epithelial barrier that normally restricts the passage of cells, proteins and fluid from the interstitial and vascular spaces into the airspaces (39, 40). Our original goal was to determine whether LTB_4 instilled into the bronchoalveolar spaces could establish such a gradient in the normal human lung and cause an influx of PMN into the airspaces, across the tight epithelial barrier. Because we found that LTB_4 recruits large numbers of PMN into the lungs, we also had an opportunity to study for the first time the relationship between cell migration and protein permeability in the human lung, and the functional activity of PMN immediately after emigration into the airspaces of the lung.

We instilled the LTB_4 at a concentration of 5×10^{-7} M in a volume of 10 ml and studied the subjects 4 h later. The rationale for selecting this concentration of instilled LTB_4 derived from in vitro studies in which the peak chemotactic effect for PMN occurs at $\sim 1 \times 10^{-7}$ M (2, 8, 10). Also, this concentration allows for up to a fivefold dilution of the instillate locally in the airspace without a substantial reduction in expected chemotactic activity. In two additional subjects, the instillation of 5×10^{-6} M LTB_4 in an identical fashion failed to cause a further increase in total cells, PMN, or protein recovery, suggesting that the instilled concentration was near the peak of the dose-response relationship. We studied the human subjects once 4 h after the initial instillation because earlier studies in rats and sheep had shown substantial changes in lavage cells 4 h after the intratracheal instillation of chemoattractants, including LTB_4 (2, 20), and because PMN accumulate within 6 h after intradermal injection of LTB_4 in human skin (15). We wanted to evaluate the early changes in cells and proteins that occur within hours after LTB_4 instillation and to evaluate the function of newly emigrated PMN as soon as possible after their arrival in the airspaces. To be certain that the profile of cellular responses and epithelial protein permeability were not different at an earlier time, we also studied one additional subject 1 h after instillation of LTB_4.

The data indicate that LTB_4 instilled into the normal human lung results in the recruitment of large numbers of PMN into the airspaces without substantially altering the permeability of the lung epithelial barrier to proteins within the first 4 h after instillation of LTB_4. This demonstrates that LTB_4 can function selectively as a chemotactic stimulus for PMN in the normal lung without causing the increase in permeability that is characteristic of inflammatory lung injury. The data also suggest that PMN migration in the normal human lung is not necessarily associated with a major change in lung epithelial permeability. The lung PMN selectively lose the small specific granules, but they function normally in two respects, superoxide anion production in response to a phorbol ester, and chemotaxis toward a potential bacterial signal, FMLP. The PMN also can migrate toward activated human serum, containing the complement component C5a, but the magnitude of this response is significantly reduced as compared with simultaneously tested blood PMN. The lung PMN from one additional subject also had a blunted chemotactic response to LTB_4, which could be consistent with migration into the lung in response to LTB_4 as the stimulus.

Two lines of evidence support the conclusion that LTB_4

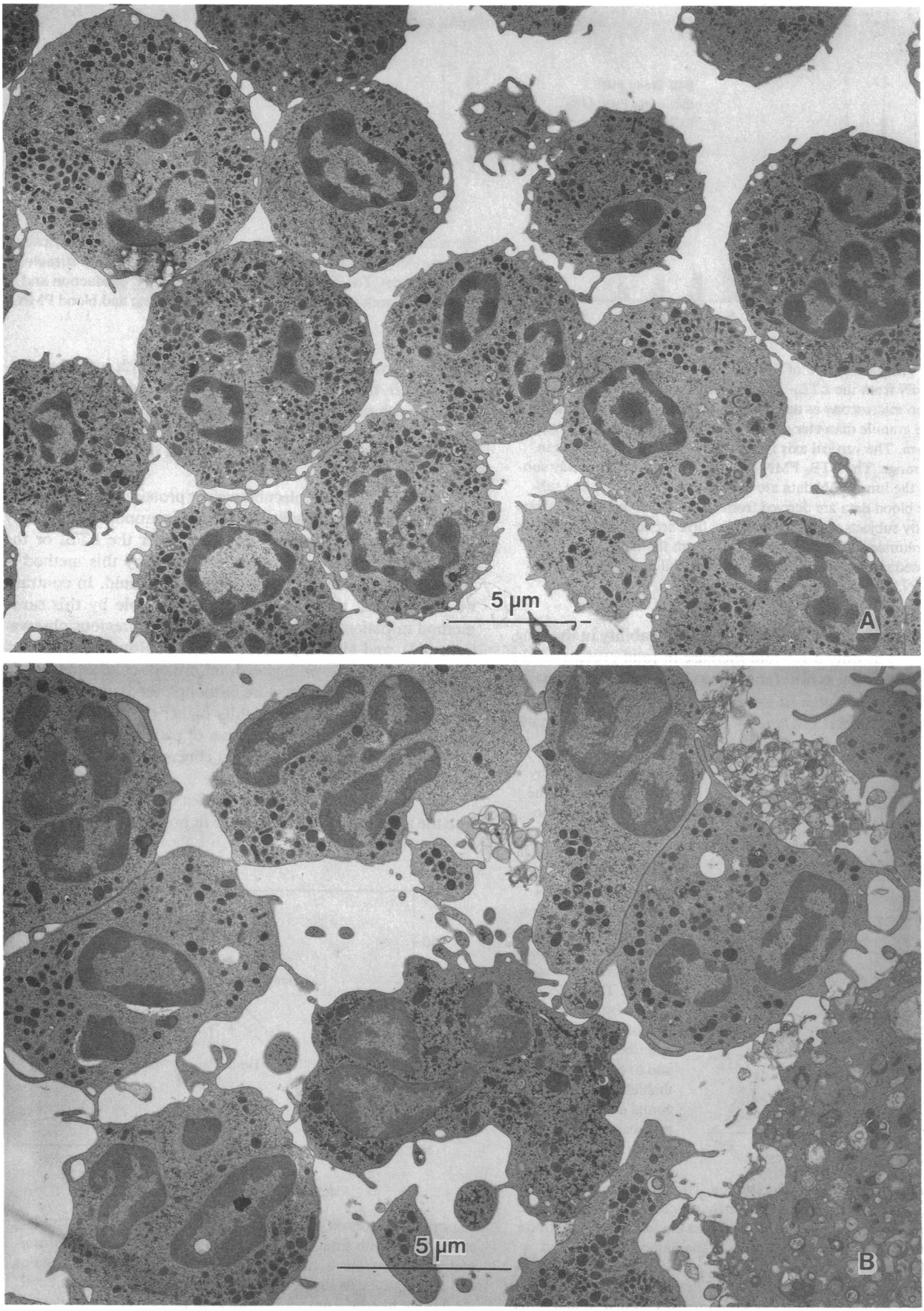

Figure 2. Electron micrographs of PMN from the peripheral blood (*A*) and the lung lavage fluid (*B*) of one of the volunteers in whom granule size distributions were measured. The blood PMN are rounded and contain numerous granules distributed throughout the cytoplasm (×4,500). The lavage PMN are elongated and the granules are more eccentrically located in the cytoplasm (×6,000).

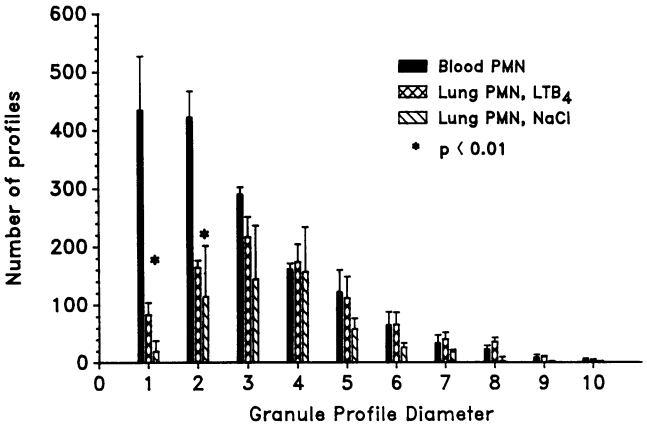

Figure 3. The distribution of PMN granule sizes in blood and lung lavage PMN from the LTB_4- and the NaCl-treated sides, measured by electron microscopy as described in Methods. The horizontal axis represents granule diameter measured in millimeters, where 1 mm = 0.065 μm. The vertical axis is the number of granule profiles in each size range. The LTB_4 PMN data are from five of the study subjects, and the lung PMN data are from two of these same five subjects. The blood data are derived from the PMN of five subjects, one of the study subjects sampled before the first bronchoscopy and four normal volunteers. The lung PMN from both the LTB_4- and the NaCl-treated sides had significantly fewer small granules than the blood PMN.

did not substantially increase protein permeability in the lung. First, there was only a 1.4-fold increase in total protein on the LTB_4 side (which was not statistically significant), as compared with a 3.9-fold increase in total cells ($P = 0.002$) and a 17.1-fold increase in total PMN ($P = 0.005$). This contrasts markedly with lavage protein values in patients with increased permeability in the lung. For example, in four separate studies of patients with the adult respiratory distress syndrome (ARDS), the lavage total protein concentration averaged 97.4 mg/dl (40–43), compared with 24.5±3.7 mg/dl in response to LTB_4 in this study.

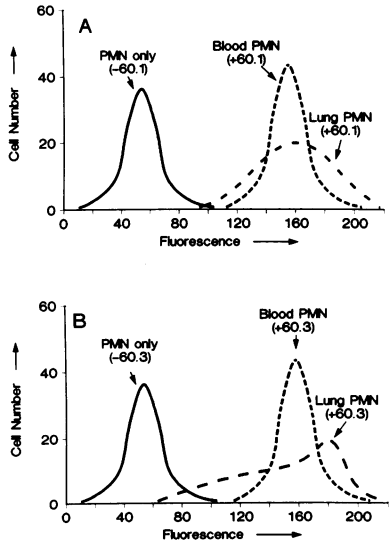

Figure 4. Analysis by flow cytometry of the binding of the monoclonal antibodies 60.1, and 60.3 by the lung lavage PMN and the peripheral blood PMN from one of the volunteers in whom PMN granule size distributions were measured (Fig. 3). The PMN were labeled by indirect immunofluorescence as described in Methods and fixed in paraformaldehyde. The horizontal axis in a logarithmic scale of fluorescence intensity on which an increase of 18 U represents a doubling of fluorescence. The vertical axis represents the number of PMN detected at each fluorescence intensity. In each panel, the fluorescence profile of unlabeled blood PMN is shown for comparison. The fluorescence curves of the unlabeled blood and lung PMN were virtually identical. (*A*) PMN labeled with the monoclonal antibody 60.1. There was a broad fluorescence curve for the lung PMN with the mean fluorescence intensity similar to that of the blood PMN. (*B*) PMN labeled with the monoclonal antibody 60.3. There was a threefold increase in the fluorescence peak of the lung PMN as compared with the simultaneously tested blood PMN.

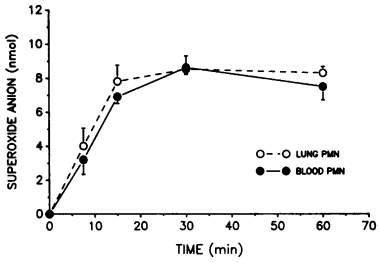

Figure 5. Superoxide anion production by the lung lavage PMN from three of the subjects, as compared with simultaneously tested blood PMN from three normal volunteers. Superoxide anion production was measured as the reduction of ferricytochrome *c*, detected spectrophotometrically at a wavelength of 550 nm, using PMA (100 ng/ml) as the stimulus (see Methods). The initial rate of superoxide anion production and the total amount produced were similar using lung and blood PMN.

Second, the high molecular weight protein IgM (> 900,000 mol wt) was not detectable by radial immunodiffusion in any of the concentrated lavage fluids on either the LTB_4 or the saline sides. The lower limit of detection by this method is ~ 0.1 mg/dl in the unconcentrated lavage fluid. In contrast, we have found that IgM is easily detectable by this same method in patients with ARDS, supporting previous observations (43), and also in patients with high altitude pulmonary edema, who have even higher total protein concentrations in lavage fluid (44). Furthermore, although the concentration of IgA (440,000 mol wt) was slightly higher on the LTB_4 side, it was detectable in the same number of patients on each side ($n = 8$) and the apparent increase in concentration on the LTB_4 side was not statistically significant.

Our observation that LTB_4 causes a large influx of PMN into the airspaces with little change in protein recovery extends

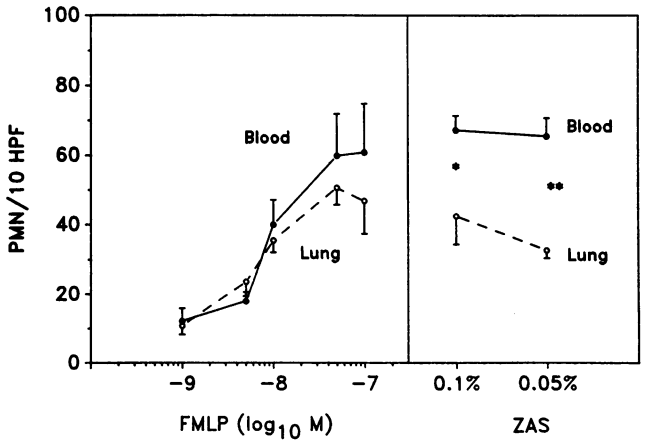

Figure 6. Chemotaxis of lung lavage PMN from four of the subjects, as compared with simultaneously tested blood PMN from four normal volunteers. The horizontal axis shows the chemotactic stimulus and the vertical axis shows the number of PMN migrating through the 3.0-μm filters during a 2-h incubation period. The migration of lung and blood PMN was similar toward FMLP, but the migration of the lung PMN to ZAS was significantly reduced at the two concentrations tested (*$P < 0.05$, **$P < 0.01$).

observations made in sheep (20) to humans. In sheep, LTB_4 caused a linear increase in the percentage of PMN in lavage fluid over the first 16 h after instillation (20). The total number of PMN was not reported, however. As in our study, the apparent increase in PMN occurred with little increase in lymph or lavage protein, which suggested that neither endothelial nor epithelial permeability had been altered. Other studies of the alveolar clearance of serum proteins instilled into the lungs of sheep provide further support for the conclusion that large numbers of PMN can migrate across the epithelial barrier with little effect on protein flux into the airspaces (45). This is consistent with observations made in vitro, showing that PMN can migrate through the normal pulmonary vasculature without altering the morphology or the permeability of the endothelium (46, 47). Although an earlier study showed that LTB_4 altered venular permeability in the hamster cheek pouch model (48), a recent study using this model showed that in response to topical LTB_4, circulating PMN can migrate between venular endothelial cells with no apparent change in permeability to albumin (67,000 mol wt) or dextran (150,000 mol wt) (47). These observations apply to normal circulating PMN; we have not tested the possibility that when PMN are activated in the circulation before migration, their migration into the lungs in response to LTB_4 might be associated with microvascular injury and an increase in permeability (49).

Although other studies have measured the function of human PMN in periodontal tissue (50, 51), joint fluids (52, 53) and skin blisters (54), this is the first study that has measured the function of newly emigrated PMN in human lungs, and the first study to investigate both PMN activity and the effects of PMN migration on the permeability of the epithelial barrier in the human lung. The data indicate that PMN can migrate across the tight epithelial barrier of the lungs without a major loss in function. The PMN lose some of their smaller secondary granules during transit, which has been observed in PMN from skin blisters and gingival tissue (51, 54, 55). This effect is not necessarily specific for LTB_4, inasmuch as the PMN from the NaCl- and the LTB_4-treated sides had similar granule size distributions. We did not confirm the morphometric measurements by measuring specific granular enzyme content because of the limited numbers of PMN recovered, but we did find that the loss of smaller specific granules was associated with an increase in surface expression of CD18 and CD11b on the lung PMN, consistent with translocation of these subunits to the surface of the PMN during degranulation (36–39). Despite the loss of some of the small granules, we found that the lung PMN are capable of recognizing and responding to gradients of FMLP, an important bacterial chemotactic factor (56). This indicates that PMN that enter the lungs in response to a major alveolar macrophage-derived chemoattractant still have the capacity to migrate toward bacterial signals.

In contrast to the FMLP response, we found that the migratory response of lung PMN was reduced toward both LTB_4 and zymosan-activated serum, in which C5a is the predominant chemoattractant. The downregulation of the lung PMN toward LTB_4 is consistent with the PMN having migrated toward LTB_4 in the lungs. Because of the small numbers of PMN present on the NaCl-treated side, it was impossible to study the response of these PMN to test the specificity of this finding for the LTB_4-treated side. The finding that the PMN also have an impaired response to ZAS raises the possibility that the PMN also may have been exposed to C5a in the lungs during transit, with resulting downregulation of the chemotactic response to this agent as occurs with prior exposure to C5a in vitro (57). In support of this interpretation, we found that low levels of C5a were detectable in lavage fluids from both sides of the lungs, suggesting that the PMN might have been exposed to C5a during migration into the lungs. We cannot be certain exactly how much C5a actually was present in the alveolar spaces, however, because of the uncertain amount of dilution of the alveolar fluid during the lavage process. The low numbers of PMN on the NaCl side and the equivalent concentrations of C5a on both sides suggest that the alveolar concentration of C5a was not sufficient to result in major migration of PMN into the airspaces. This makes it unlikely that C5a could have been a second signal for PMN accumulation in the lung during the course of this study. Because the lavage concentrations of C5a were equal on the saline and the LTB_4 sides, it is likely that the presence of C5a was not a specific result of the LTB_4 instillation. For example, C5a could have been formed as a response to minor trauma in the airway during the bronchoscopy procedures.

Our findings provide new relevance for studies of PMN obtained from experimental skin blisters, because the lung PMN share some features with PMN in this experimental model (54, 55). Like the lung PMN, the PMN recovered from skin blisters have lost secondary granules, as indicated by reduced vitamin B_{12} binding protein, a constituent of specific granules. They also have increased C3bi and FMLP receptors consistent with translocation of these receptors to the cell surface as specific granules are lost during migration (54). The skin blister PMN had an enhanced chemotactic response to FMLP that was consistent with the enhanced receptor expression, and a reduced response toward endotoxin-activated serum. We found that the lung PMN had normal, rather than enhanced chemotactic responsiveness to FMLP, but the reduced migration toward activated serum was found in both studies. This same differential responsiveness to FMLP and activated serum has been found in studies of peritoneal exudate PMN in guinea pigs (54) and rabbits (58) and in mononuclear cells from joint fluids of patients with arthritis (53). In contrast, crevicular PMN from periodontal tissue showed reduced migration to both stimuli (51).

When we studied superoxide anion production, we found that the lung PMN retained their capacity to produce superoxide anion in response to a maximal concentration of PMA (100 ng/ml), consistent with studies of gingival PMN, showing normal production of superoxide anion in response to PMA (10 ng/ml) (51). When PMA is used as the trigger, superoxide anion production is mediated by protein kinase C translocated from the cytosol to the cell membrane (59). Consistent with this, we found that lung PMN have normal amounts of total protein kinase C. The assay that we used does not separate cytosolic from translocated protein kinase C in the cell membrane, however, so that the data do not provide evidence about the location of protein kinase C in the PMN, or its total activity. The finding that superoxide anion generation is normal, however, indicates that sufficient quantities of activatable protein kinase C are present to provide normal superoxide formation at this concentration of PMA. Because of limitations on the number of cells recovered from the subjects, we did not study a range of concentrations of PMA and we cannot be certain that the dose response to PMN has not shifted in the lung PMN. It is also possible that superoxide production in

response to a membrane stimulus such as FMLP could be altered in the lung PMN, because such stimuli may activate the membrane oxidase by a different mechanism than PMA (59). The number or the affinity of FMLP receptors could have changed during degranulation (54); however, we did not find evidence of increased chemotactic responsiveness of the lung PMN at low concentrations of FMLP, which occurred together with upregulation of FMLP receptors and increased superoxide anion production in skin blister PMN (54).

In summary, the results of this study indicate that in the first 4 h after the instillation into the lungs of normal human volunteers, LTB_4 causes the recruitment of large numbers of PMN without altering the protein permeability of the tight epithelial barrier of the lung. This suggests that alveolar macrophage-derived LTB_4 can function as a chemoattractant for PMN without by itself initiating an inflammatory cascade within the lungs. The newly emigrated PMN are functionally active and are capable of migrating toward a bacterial signal and producing normal amounts of superoxide anion in response to PMA. These findings provide a definite role for LTB_4 in the recruitment of neutrophils from the bloodstream into the lungs of normal humans.

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