Comparison of leukotriene B_4 and D_4 effects on human eosinophil and neutrophil motility in vitro

C.S. Spada, A.L. Nieves, A.H-P. Krauss, and D.F. Woodward

Department of Biological Sciences, Pharmaceutical Research and Development, Allergan, Inc., Irvine, California

Abstract: The motility of isolated normal human peripheral blood eosinophils and neutrophils in response to exogenous leukotrienes B_4 and D_4 was examined by means of a modified under-agarose technique and a novel quantitative sampling strategy. Leukotriene D_4 was a potent chemoattractant for eosinophils, with a significant threshold chemotactic effect evident at 10^{-10} M. The abolition of eosinophil chemotaxis by the potent and selective peptide-leukotriene-antagonist SK&F 104353 indicated the pharmacological specificity of the leukotriene D_4-induced response. The chemokinetic response of eosinophils to leukotriene D_4 generally did not differ significantly from spontaneous migratory activity of unstimulated cells. Leukotriene D_4 did not, however, alter directed neutrophil motility until a very high concentration (10^{-5} M) was achieved, although significant neutrophil chemokinesis relative to unstimulated movement was observed over the tested concentration range. Directional emigration of both eosinophils and neutrophils was induced by leukotriene B_4 at concentrations as low as 10^{-8} M. Analysis of leukocyte orientations provided evidence that chemokinetic responses were not being interpreted as indications of chemotactic behavior. These studies suggest that leukotriene D_4 may behave as a potent and selective chemoattractant for human eosinophils at physiologically relevant concentrations. *J. Leukoc. Biol.* 55: 183-191; 1994.

Key Words: chemokinesis · chemotaxis · under-agarose · leukocyte orientation

INTRODUCTION

The peptide-containing leukotrienes (LTs), LTD_4 and LTE_4, have been shown to be more potent than LTB_4 in the elicitation of in vivo leukocyte ingress into the conjunctiva following local instillation [1]. Eosinophils are the predominant cell type in the resultant infiltrates induced by the sulfido-LTs, whereas both eosinophils and neutrophils are present following LTB_4 challenge, and cell movement is apparently directed toward the conjunctival epithelium. In addition, even though the separately administered LTs do not lead to any apparent leukocyte degranulation or fragmentation at any tested concentration, eosinophil-mediated tissue destruction is evident when LTB_4 and LTD_4 are administered in combination [1, 2]. The abolition of LTD_4-induced conjunctival eosinophil infiltration in guinea pigs by topical pretreatment with the selective peptide-LT antagonist SKF 104353 suggests that in vivo leukocyte emigration may be directly affected by the sulfido-LTs [3]. Furthermore, topical pretreatment with the LTD_4-receptor antagonist MK-571 completely inhibits conjunctival eosinophil ingress evoked by topical LTD_4 and significantly attenuates ovalbumin-induced conjunctival eosinophil infiltration at 17 h following challenge [4]. These in vivo findings suggest that the LTs could exert significant influences on the modulation of leukocyte behavior during the course of an allergic response in ocular tissues. However, the sulfido-LTs are not generally acknowledged as stimulators of leukocyte movement. Moreover, the results were obtained in experimental animal models utilizing guinea pigs, making their relevance to a potential therapeutic application in humans unclear, since significant biochemical and functional differences have been demonstrated between normal eosinophils from various animal species and humans [5].

The migratory behavior of purified leukocyte populations can be measured by a number of in vitro techniques that are based on different principles. The assessment of cell movement by the chemotaxis under-agarose method [6-8] appears to offer several advantages when compared to protocols which employ micropore filters (7, 9, 10). The under-agarose method is easy to perform, allows rapid evaluation of numerous samples, and provides an experimental milieu which more closely approximates some of the physical conditions that might occur in extravascular tissue regions. Performing the assay on glass coverslips also allows a permanent record for the detailed examination of individual cell dispositions relative to the net distribution of a larger population tested under a given condition. Measurement of more than one parameter, together with the availability of digital imaging technology, formed the basis for a novel quantitative sampling method to describe the general in vitro migratory activities of purified human leukocyte populations.

MATERIALS AND METHODS

Substrate preparation

24 × 50 mm glass coverslips were acid-cleaned, rinsed in three changes each of ultrapure water and absolute ethanol, and air-dried. Indelible ink was then used to inscribe a mark at one end of each coverslip to denote the side to receive the highest dose of LT. Each coverslip was also identified by code. The marked coverslips were then dipped in a sterile 0.5% gelatin solution and allowed to dry at room temperature. Although cells readily adhere to uncoated glass or plastic surfaces, such substrates retain a level of static charge that

---

Abbreviations: CD, chemokinetic differential; CI, chemotactic index; CPD, citrate-phosphate-dextrose; EDTA, ethylenediamine tetraacetic acid; EtBr, ethidium bromide; FCS, fetal calf serum; FDA, fluorescein diacetate; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; LT, leukotriene; MEM, Eagle's minimal essential medium; MeOH, absolute methanol; PBSS, phosphate-buffered saline solution; RBC, red blood cell.

Reprint requests: Clayton S. Spada, Department of Biological Sciences (RD-2C), Pharmaceutical Research and Development, Allergan, Inc., 2525B Dupont Drive, Irvine, CA 92715-1599.

Received July 15, 1993; accepted August 30, 1993.

will hamper or prevent cell movement [11]. The primary purpose of the gelatin coat is the elimination of this negative surface charge. However, leukocytes are also extremely sensitive to any surface imperfections, so great care was exercised to ensure that the gelatin coating was absolutely even over the entire coverslip.

Agarose preparation

Separate agarose and medium solutions were first prepared. 750 mg Indubiose A37 (Gallard-Schlesinger Industries, Inc., Carle Place, NY) per 50 ml ultrapure water were brought to boiling point in a water bath for 15–20 min and then allowed to cool to 48°C. The medium supplement consisted of 10 ml 10× Medium 199, with Hanks' balanced salts and L-glutamine (Gibco Laboratories, Grand Island, NY), 10 ml heat-inactivated fetal calf serum (FCS) (Gibco), 1 ml 200 mM L-glutamine (Gibco), 1 ml suspension of 100 μg/ml penicillin-100 μg/ml streptomycin (Gibco), 0.09 g $NaHCO_3$, and 28 ml ultrapure water. Equal volumes of agarose and medium solutions were mixed at 48°C immediately before plates were to be poured. The amounts of the components in each solution were adjusted according to the total working volume determined by the number of plates to be prepared.

Preparation of plates

Two coated coverslips were placed in the bottom of each 20 × 100 mm flat-bottomed polystyrene culture dish (Falcon #1005, Becton-Dickinson, Park Ridge, NJ) and overlain with 20 ml of the supplemented agarose mixture. The agarose was allowed to gel at room temperature and the covered plates were refrigerated overnight at 4°C to facilitate punching of wells. Three rows by four columns of wells were formed over each coverslip with the aid of a 3 mm gel punch (#19-3651-01, Pharmacia LKB, Piscataway, NJ) attached to a vacuum line. The rows were spaced so that companion wells in adjacent rows were separated by an edge-to-edge distance of 3 mm, and columns spaced at an edge-to-edge separation of 9 mm, to prevent diffusion from one column to contaminate a neighboring column during incubation (**Fig. 1**). Each well could easily accommodate a volume of 10 μl.

Preparation of leukotriene and LT-antagonist solutions

The 5S,6R stereoisomer of LTD_4 (Dr. J.G. Gleason, Smith Kline & French Medicinal Chemistry, Philadelphia, PA) was prepared in distilled water, from lyophilates that were stored under liquid nitrogen. 5S,12R-LTB_4 was prepared as the sodium salt which was then freeze-dried and stored under liquid nitrogen until use. LT solutions were prepared fresh and analyzed by UV spectroscopy immediately before use. The disodium salt of the peptide-LT-antagonist, SK&F 104353 [12] (Dr. J.G. Gleason), was dissolved in Eagle's minimal essential medium (MEM).

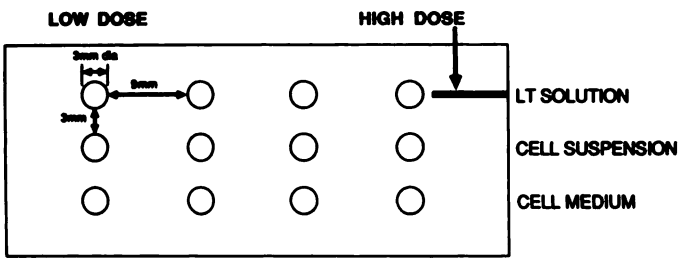

Fig. 1. Schematic of the pattern of wells on a 24 × 50 mm glass coverslip for the under-agarose leukocyte assay. Wells are 3 mm in diameter. Rows are separated by a distance of 3 mm and columns by 9 mm, measured from the closest edge of neighboring wells. The center row is seeded with the leukocyte suspension. The row denoted by the mark (arrow) receives the LT solutions, while the other row accommodates the cell medium as a control. The mark serves as a reference to identify the test solution row, as well as record the order of dosing (the highest dose nearest the mark).

Leukocyte isolation

Preparation of white cell concentrate

Collection of whole blood and preparation of white cell (buffy coat) concentrates were performed by laboratory personnel at a local chapter of the American Red Cross (Santa Ana, CA) according to standard protocols. Briefly, one unit (approximately 500 ml) whole blood was collected from each volunteer donor into sealed, sterile blood bags pretreated with citrate-phosphate-dextrose (CPD). The CPD/whole blood was then centrifuged at 4100*g* for 5.5 min, yielding 45–65 ml of buffy coat concentrate that was heavily contaminated with erythrocytes. The concentrate was recovered and sealed in a fresh blood bag, and stored at room temperature for no more than 18 h. Extensive preliminary evaluation of white cell concentrates prepared by this procedure showed >99% granulocyte viability, as determined by trypan blue exclusion and fluorescein diacetate (FDA) uptake. Light microscopic examination under intermediate and high magnifications revealed normal leukocyte morphology. Moreover, superoxide generation and elastase release stimulated by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) or LTB_4 in mixed granulocyte isolates prepared from Red Cross buffy concentrates were comparable to responses observed in human leukocytes from whole blood obtained on the same day (G.W. DeVries, L.D. Amdahl, unpublished observations).

Isolation of neutrophil-rich mixed-granulocyte fractions

45–65 ml white cell concentrates obtained from the American Red Cross were diluted 2:1 with phosphate-buffered saline solution (PBSS). In order to prevent leukocyte aggregation during the isolation procedure [11], ethylenediamine tetraacetic acid (EDTA) (Sigma Chemical Co., St. Louis, MO) was dissolved in the PBSS to result in a final concentration of 20 mM EDTA in the diluted buffy coat fraction. The diluted white cell concentrate was centrifuged at 700*g* for 30 min on a discontinuous Ficoll 400/sodium diatrizoate gradient (Histopaque, Sigma Chemical Co.), composed of 10 ml Histopaque 1077 layered over 20 ml Histopaque 1119. Granulocytes settled at the interface between the Histopaque layers, and were resuspended in 50 ml PBSS. Excess Histopaque was washed away by two centrifugation steps at 300*g* for 10 min each. Red blood cells (RBCs) were then lysed by resuspending the leukocytes in 4 ml Tris-NH_4Cl (5.0 ml 0.1 M Tris, pH 7.65; 45 ml 0.16 M NH_4Cl; mixture adjusted to pH 7.2) and incubating at 37°C for 8 to 10 min. Following lysis, a cushion of heat-inactivated FCS (1:1, v/v) was carefully pipetted under the leukocyte suspension and the tube centrifuged at 300*g* for 10 min. The pellet was then washed in PBSS through two additional centrifugation steps at 300*g* for 10 min each. All steps outlined above were carried out at room temperature unless indicated otherwise. Cells were quantitated with a hemacytometer, using a mixture of ethidium bromide (EtBr) and FDA dyes to visualize viable cells. A working solution of EtBr/FDA was prepared by mixing 1 ml EtBr (200 μg/ml in PBSS), 50 μl FDA (5 mg/ml in acetone), and 49 ml PBSS. This solution can be stored at

4°C for up to one month. One hundred μl of the leukocyte suspension were incubated with 10 μl EtBr/FDA working stain for 5 min at room temperature and subsequently examined under mercury illumination passed through a package comprised of 485/20 nm interference, 510 nm transmission, and 520 nm long-pass barrier filtration. Viable cells take up FDA and appear yellow-green, whereas damaged or otherwise compromised cells will appear orange due to EtBr staining. Cell viability in the neutrophil-rich, mixed-granulocyte isolates was typically greater than 95%. Giemsa and Luna [13] stains revealed that the leukocyte population consisted of approximately 96% neutrophils and 4% eosinophils, along with some erythrocytes. MEM was added to the remaining cell suspension to yield approximately 2.5×10^7 cells/ml.

Isolation of eosinophil-rich fractions

The sedimentation coefficients of normodense human neutrophils and eosinophils are very similar, precluding dependable separation of unstimulated cells by centrifugation with relatively high yield and purity [14, 15]. However, treatment of a mixed-granulocyte suspension with a very low (10 nM) concentration of fMLP has been shown to alter neutrophil density without causing any apparent presensitization or activation of eosinophils [16].

American Red Cross buffy coat concentrates were diluted and centrifuged through a discontinuous Histopaque gradient as outlined above for preparation of neutrophil-rich isolates. Following the first washing step with PBSS, the cell suspension was incubated with 10 nM fMLP (Sigma Chemical Co.) in PBSS/FCS (9:1, v/v) at 37°C for 10 min. The suspension was then centrifuged through 10 ml Histopaque 1083 layered over 20 ml Histopaque 1119 at 1000*g* for 15 min. The eosinophil-rich isolate settling at the interface between the two Histopaque layers was resuspended in 10 ml PBSS. A cushion of heat-inactivated FCS (1:1, v/v) was carefully pipetted underneath the suspension and the tube centrifuged at 300*g* for 10 min. The pellet was then resuspended in 10 ml PBSS and centrifuged at 300*g* for 10 min as a final wash. Loss of eosinophils could be minimized by eliminating the RBC lysis steps (which were found to be unnecessary in most cases due to the paucity of erythrocytes). The eosinophil-rich pellet was then suspended in a small volume of MEM which was determined by the number of wells to be seeded. Experience with this protocol revealed that exceeding a final volume of 0.6 ml of the eosinophil-rich suspension resulted in an inadequate number of cells to be plated for reliable quantitation of results. The isolation procedure consistently yielded about 1×10^6 cells/0.5 ml. Isolates comprised greater than 90% eosinophils and the remaining 10% of the cells were neutrophils; cell viability was >99%, as determined by trypan blue exclusion and uptake of EtBr/FDA dyes.

Assay conditions

LTB_4 and LTD_4 were each tested over a dose range of 10^{-9} M to 10^{-5} M for neutrophils. Eosinophil migration in response to LTB_4 was examined over a 10^{-10} M to 10^{-4} M dose range, while a 10^{-11} M to 10^{-4} M range was tested for LTD_4-induced eosinophil responses. The migratory activities of eosinophils incubated in the presence and absence of 10^{-6} M SK&F 104353 were also evaluated over a 10^{-10} M to 10^{-7} M LTD_4 dose range. It should be noted that the working LT concentrations delivered to the wells were 5×10^{-x}, but are reported as simple exponentials because actual concentrations forming the gradients in the agar were not determined. The distilled water LT vehicle, MEM, and the LT antagonist solution served as separate control conditions. Cells were also seeded without any treatment in companion wells to determine a baseline value for spontaneous unstimulated motility.

In order to ensure that chemokinetic responses could be readily distinguished from chemotactic responses in this assay, the LT gradient was allowed to extend to a distance equivalent to the distal edge of the central well, relative to the well into which the LT solution was loaded. A visual indication of the extent of diffusion was obtained by employing agarose medium supplement containing the colorimetric pH indicator phenolphthalein. Preliminary testing with LTB_4 and LTD_4 revealed that a gradient was formed over the desired distance in less than 30 min, and the solution levels in wells loaded with LT solution, medium, or cell suspension appeared to be depleted within the first 15 min following loading. The gradient did not appear to expand to any appreciable degree, as judged by colorimetric reaction of the pH indicator, during the remaining incubation period. Since the wells did not permit a milieu through which diffusion could occur, cells attached to the substrate far within the confines of the central well proper could not be stimulated by the gradient, whereas those that attached near the periphery of the well would be stimulated initially to obtain a chemokinetic response. Migration under-agarose would expose cells to an intact LT concentration gradient unless the cell happened to move toward the companion control well containing the control solution.

All wells received a total volume of 10 μl. Covered agarose plates were incubated at 37°C for 2 h in a humidified 5% CO_2 atmosphere. All experiments were conducted under sterile conditions, which were maintained through the completion of incubation. After the incubation period, each plate was flooded with approximately 5 ml of 10% neutral-buffered formalin and fixed at 37°C for 30 min in a 5% CO_2 atmosphere. The formalin was then aspirated and the agarose layer hardened with approximately 5 ml absolute methanol (MeOH) for 5 min at room temperature. The agarose layer could then be peeled away, and the coverslips briefly washed in MeOH, air-dried, and stained by the Luna technique for eosinophil granules [13]. The stained coverslips were mounted with Permount (Fisher Scientific, Fair Lawn, NJ) diluted with xylene (1:1, v/v) face-down on standard microscope slides and observed under brightfield illumination.

Quantitative analysis

Quantitation of results was accomplished with a PSICOM 327 digital imaging workstation (Perceptive Systems, Inc., League City, TX), using a novel sampling strategy that is not influenced heavily by the more rapidly moving leukocytes that may comprise only a small portion of the total cell population in a given sample (**Fig. 2**). Briefly, the outermost cells that migrated from the central well along the vertical axis, defined by the column of companion wells, were located and centered in the visual field with the aid of a calibrated ocular linear scale. This marked the starting point from which successive counting fields were defined back toward the edge of the central well. Location of each subsequent field was accomplished by moving the microscope stage a predetermined distance in the *y*-direction, such that adjacent fields overlapped by a known distance at each end. The resulting linear distance of this "active measurement window" (f, the actual field analyzed = 106 μm × 106 μm) was calibrated into the host processor of the imaging system. Image processing techniques were employed to isolate features of interest, and object counts were automatically obtained

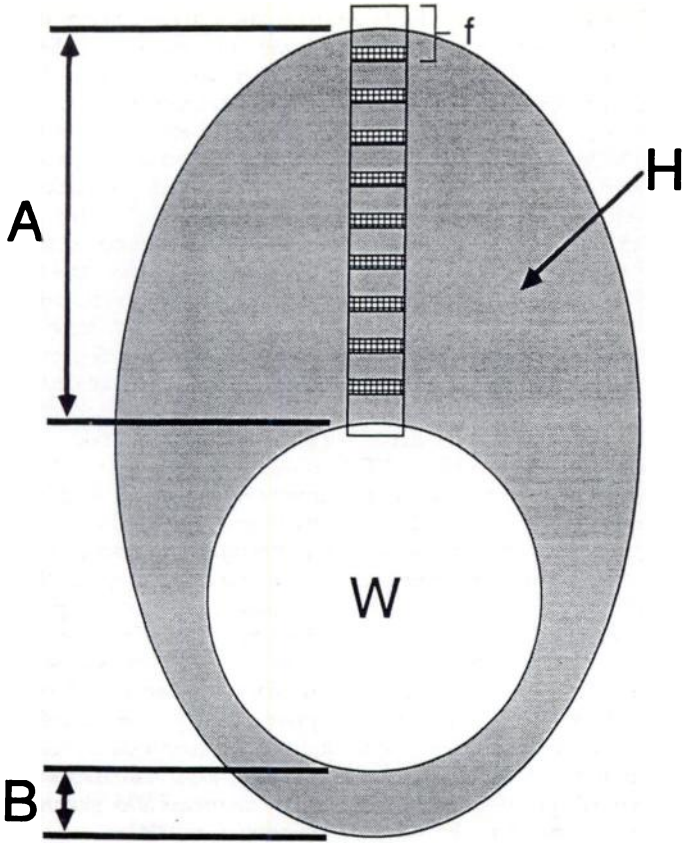

Fig. 2. The sampling strategy employed for the quantitation of under-agarose results is depicted in this schematic diagram. The outer boundary of the well (W) is clearly defined by a halo (H) of leukocytes which have attached to the substrate. The outermost cell that has moved in the direction of the test solution well (direction A) is located and centered along a linear scale placed in the back focal plane of the microscope ocular. This marks the starting point from which counting fields will be determined back toward the edge of W. Subsequent fields are located by moving along the axis extending from the outermost cell to the center of W. The microscope stage is moved a predetermined distance in the *y*-direction, such that adjacent fields overlap by a known distance on each side (cross-hatched areas). The resulting linear distance of the active measurement window (f) is calibrated into the host processor of the imaging system. Object counts are automatically obtained for each field. This process is repeated for the control side of W (direction B). The field in each direction with object counts closest to half the maximal count (i.e., the maximal count observed in the field adjacent to the edge of W) is identified and used to calculate migration distance.

for each field. This process was repeated on the opposite side of the central well. Successive object counts obtained on each side of the central well were then plotted into two curves, one representing counts along the axis toward the test substance (indicating chemotaxis) and the other representing counts in the direction of the control substance (indicating chemokinesis). The field in each direction closest to half the area under the curve was identified, and the corresponding migration distance was then calculated based on the calibrated linear dimension of the active window.

Each condition was replicated three to five times for each donor sample. Results for test and control conditions are expressed directly as the mean migration distance in μm. Statistical analysis was performed with Student's *t* test, comparing migration induced by the LT solutions (chemotaxis, or direction A in Fig. 2) relative to that observed on the opposite side of the well in the direction of the control well (chemokinesis, or direction B in Fig. 2).

RESULTS

Comparison of neutrophil migratory responses to LTB_4, examined over a concentration range of 10^{-9} M to 10^{-3} M, revealed the threshold for significant chemotaxis to be at 10^{-8} M, relative to the chemokinetic response (**Fig. 3a**). LTB_4-induced neutrophil chemotaxis and chemokinesis both achieved maximal levels at 10^{-5} M, the chemotactic response nearly double that for chemokinesis, using baseline unstimulated random activity as unity. LTD_4 evoked a sudden, marked neutrophil chemotaxis at the 10^{-5} M dose, followed by a declining response over the remaining concentration range (Fig. 3b). There was essentially no difference between chemotaxis and chemokinesis to LTD_4 below the 10^{-5} M threshold dose. Although LTB_4 and LTD_4 elicited somewhat different chemotaxis profiles, these LTs gave rise to similar chemokinetic responses over the same dose range.

The responses of eosinophils to LTB_4 and LTD_4 differed dramatically from those of neutrophils (**Fig. 4**). LTB_4-

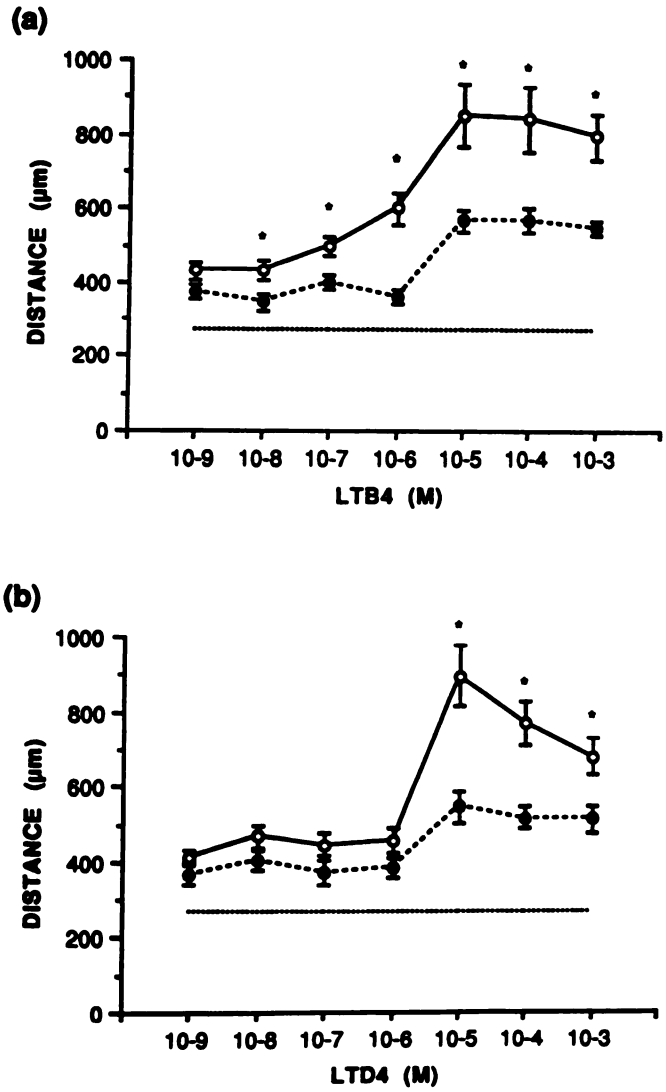

Fig. 3. The effects of LTB_4 (a) and LTD_4 (b) on the chemotactic (—○—) and the chemokinetic (---●---) responses of isolated human neutrophils, measured by the under-agarose leukocyte assay. The horizontal dotted line indicates the baseline spontaneous activity of unstimulated cells. Results are expressed as the mean migration distance in linear μm from the nearest edge of the center well, ± S.E.M. * = $P < 0.05$, comparing chemotaxis relative to chemokinesis. n = 8–10 donors.

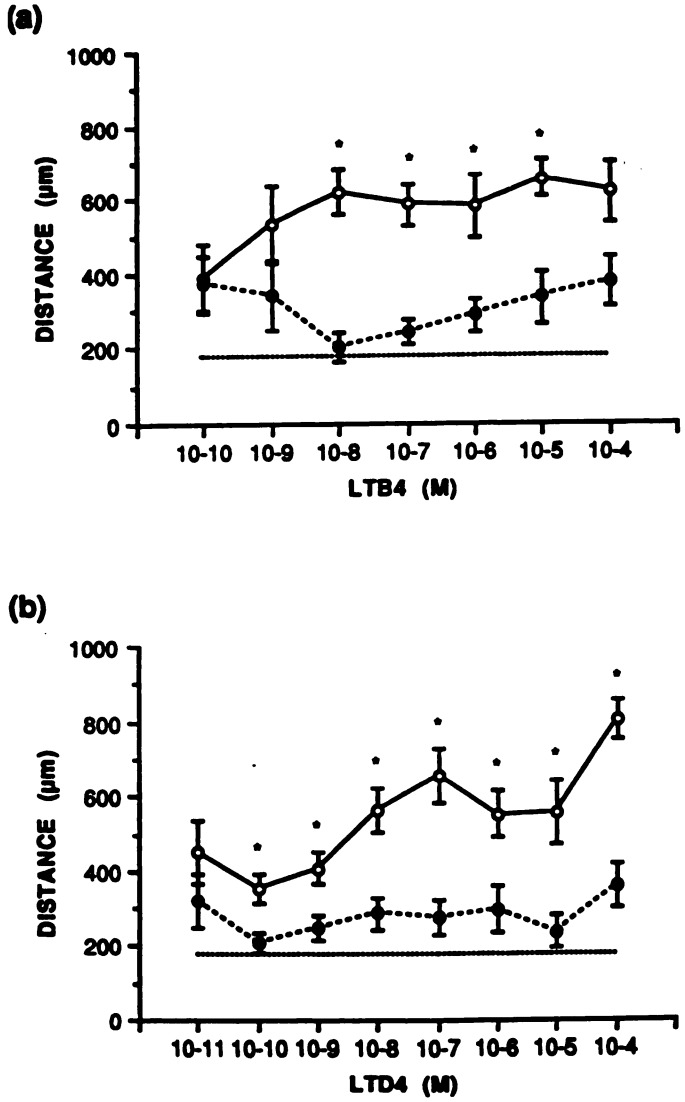

Fig. 4. The effects of LTB_4 (a) and LTD_4 (b) on the chemotactic (—O—) and the chemokinetic (---●---) responses of isolated human eosinophils, measured by the under-agarose leukocyte assay. The horizontal dotted line indicates the baseline spontaneous activity of unstimulated cells. Results are expressed as the mean migration distance in linear µm from the nearest edge of the center well, ± S.E.M. * = $P < 0.05$, comparing chemotaxis relative to chemokinesis. n = 8–10 donors.

induced eosinophil chemotaxis was evident over a concentration range of 10^{-10} M to 10^{-4} M, with a steep rise to a maximum effect occurring at 10^{-8} M (Fig. 4a). Significance relative to chemokinesis was obtained only between 10^{-8} M and 10^{-5} M. Eosinophil chemokinesis exhibited a sudden decrease at the 10^{-8} M dose of LTB_4, followed by a steady dose-related increase, but these fluctuations were not significant relative to one another. LTD_4 induced significant eosinophil chemotaxis at doses extending from 10^{-10} M to 10^{-4} M with a gradual dose-related increase in response that leveled off at 10^{-7} M (Fig. 4b). The eosinophil chemokinetic profile elicited by LTD_4 did not differ significantly from spontaneous activity of unstimulated cells.

Unstimulated neutrophils appeared to display a generally higher level of spontaneous movement than unstimulated eosinophils, with a difference of 100 µm in mean migration distance. Migratory behavior of neutrophils and eosinophils was not significantly influenced by LT vehicle or medium only. Examination under brightfield and phase-contrast illumination at high magnification showed unstimulated cells to be oriented in all directions in relation to the axis formed by each column of companion wells. Similar patterns of orientation were observed with cells that were exposed to LT vehicle or MEM only. Neutrophils and eosinophils migrating toward wells containing LT solutions exhibited polarization which generally coincided with an alignment along the columnar axis. Such a pattern of orientation was not apparent for cells that had moved in the direction of the opposite control substance well. Photographs obtained directly from one of the imaging system's video monitors depicting typically observed leukocyte orientations are shown in **Figures 5 to 7.** Digital image analysis of cell orientation is described by Krauss et al. [17].

SK&F 104353 abolished eosinophil chemotaxis and chemokinesis evoked by LTD_4 doses of 10^{-10} M to 10^{-7} M (**Fig. 8**), and responses were suppressed to a level that was comparable to the baseline activity of unstimulated cells (see Fig. 4 for comparison with LTD_4 effects). Spontaneous random movement of drug-treated and naive eosinophils that were not stimulated with LTD_4 was neither inhibited nor enhanced by SK&F 104353 itself. Fluorescence microscopy of drug-treated cells that had been double-stained with EtBr/FDA revealed nearly 100% viability, and no adverse morphological effects were apparent when eosinophils stained by the Luna technique were examined at high magnification under brightfield illumination. Furthermore, no significant degree of eosinophil or neutrophil degranulation was noted under any test or control condition.

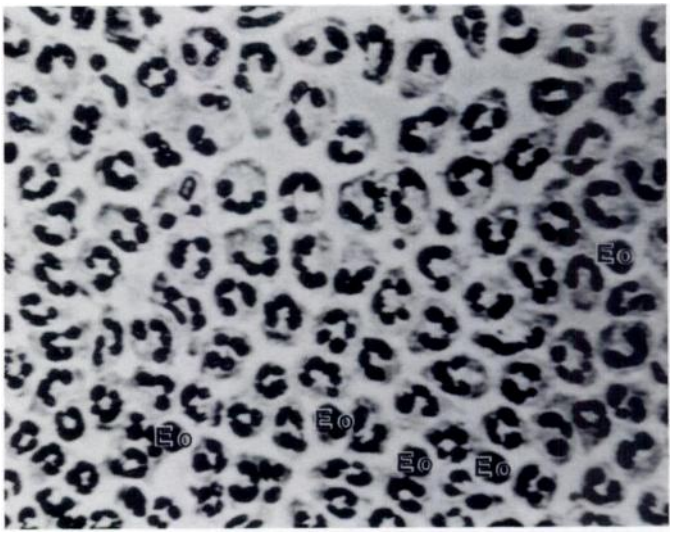

Fig. 5. A representative example of attached, non-motile cells within the perimeter of the central well proper situated in a LTD_4 concentration gradient formed under-agarose. Cell shape is essentially spherical, with no particular nuclear-to-cytoplasmic disposition discernible. Leukocytes in fields observed within the central wells of columns exposed to gradients formed by LTB_4, the LT-antagonist SK&F 104353, MEM medium, or in native agarose (i.e., no loading of neighboring wells), exhibited similar characteristics. The majority of cells shown in this particular example are neutrophils, although a few eosinophils (Eo) are present. The field was photographed directly from the imaging system's video monitor display. Samples were viewed under brightfield illumination through a nominal optical magnification of 50×, plus a 1.5× increase in image scale effected by the video pickup device. The image was digitized and processed to enhance subject-to-ground contrast in order to make cytoplasmic boundaries clearly visible. The vertical linear distance visible on the display screen is approximately 120 µm.

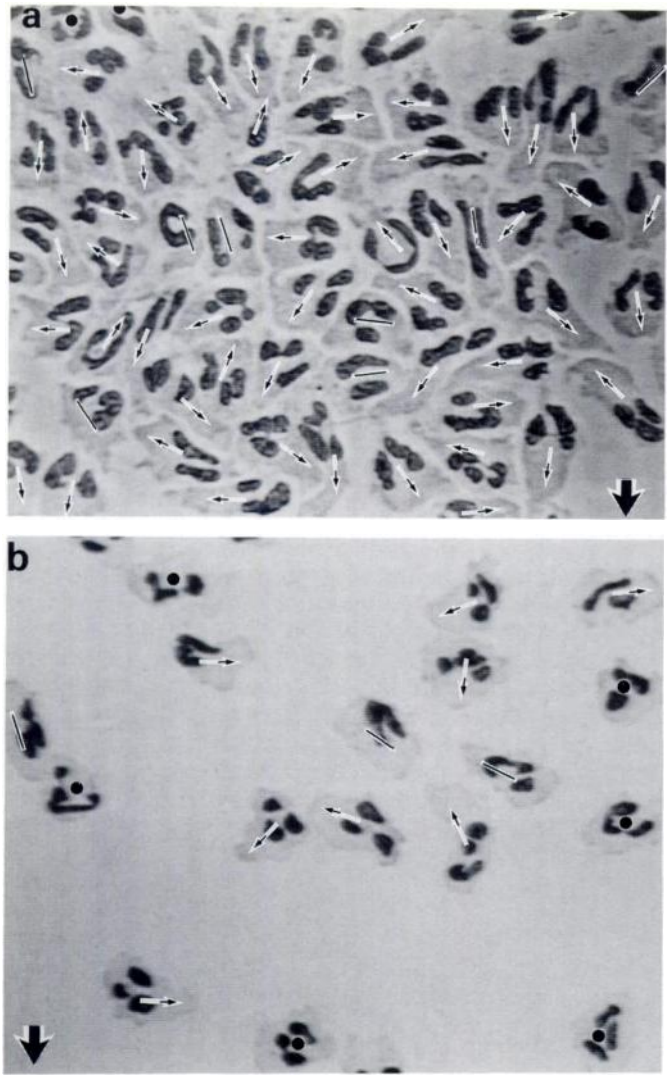

Fig. 6. Representative examples of LTD_4-stimulated random migration in leukocytes found under-agarose near the periphery of the central well (a) and near the "leading edge" of migration (b) show evidence of cell movement, but no clear overall tendency of leukocyte axial orientation toward the control well (large solid arrow). The small arrows indicate nuclear-to-pseudopod orientations for which a directional component could be derived, whereas cells with axial alignment of uncertain directional tendencies are denoted by a line lacking an arrowhead. Solid dots mark cells in which no clear axial or directional migration could be shown by image analysis of nuclear-to-cytoplasmic disposition. Fields were photographed directly from the imaging system's video monitor display. Samples were viewed under brightfield illumination through a nominal optical magnification of 50×, plus a 1.5× increase in image scale effected by the video pickup device. Images were digitized and processed to enhance subject-to-ground contrast in order to make cytoplasmic boundaries clearly visible. The vertical linear distance visible on the display screen is approximately 120 μm.

DISCUSSION

The present under-agarose investigations demonstrate that LTD_4 can potently attract isolated normodense human eosinophils. While concentration-related eosinophil emigration is evoked by LTD_4 doses as low as 10^{-10} M, this peptido-LT has little effect on the directional movement of neutrophils at physiological concentrations. In contrast, LTB_4 induces substantial chemotactic responses in eosinophils and neutrophils over comparable concentration ranges. These studies are congruent with previous in vivo experiments involving local administration of LTs to guinea pig conjunctival tissues, in which LTD_4 and LTE_4 elicited a virtually exclusive eosinophilic infiltration, whereas neutrophils and eosinophils were present following treatment with LTB_4 [1, 2].

Our observations of LTB_4-induced leukocyte emigration are in agreement with previously published results obtained by different experimental techniques for the assessment of in vitro leukocyte chemotaxis. Micropore filter assays have yielded results indicating that LTB_4 induces polymorphonuclear leukocyte chemotaxis at concentrations as low as 10^{-10} M to 10^{-8} M, with significant responses relative to

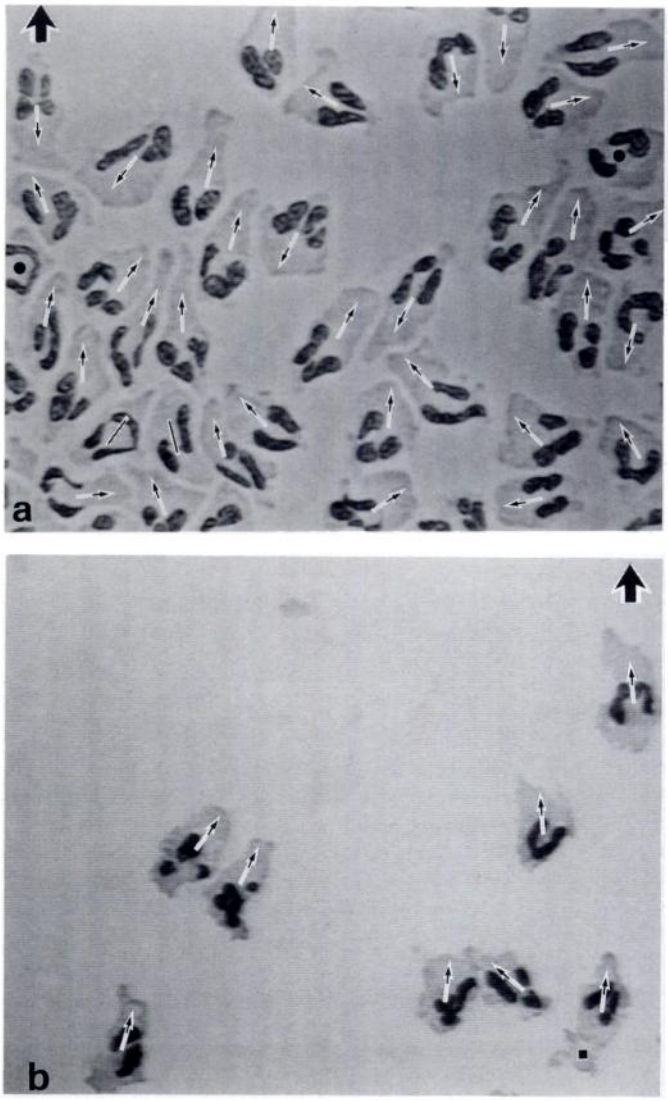

Fig. 7. Representative examples of LTD_4-stimulated directional migration in leukocytes found under-agarose within an intact gradient of LTD_4 near the periphery of the central well (a), showing alignment of a majority of cells toward the source of the LTD_4 gradient (large solid arrow), and near the "leading edge" of migration (b), where all cells are oriented toward the gradient source. Similar results were observed with LTB_4. The small arrows indicate nuclear-to-pseudopod orientations for which a directional component could be derived; cells with axial alignment of uncertain directional tendencies are denoted by a line without arrowhead. Solid dots mark cells in which no clear axial or directional migration could be shown by image analysis of nuclear-to-cytoplasmic disposition. Fields were photographed directly from the imaging system's video monitor display. Samples were viewed under brightfield illumination through a nominal optical magnification of 50×, plus a 1.5× increase in image scale effected by the video pickup device. Images were digitized and processed to enhance subject-to-ground contrast in order to make cytoplasmic boundaries clearly visible. The vertical linear distance visible on the display screen is approximately 120 μm.

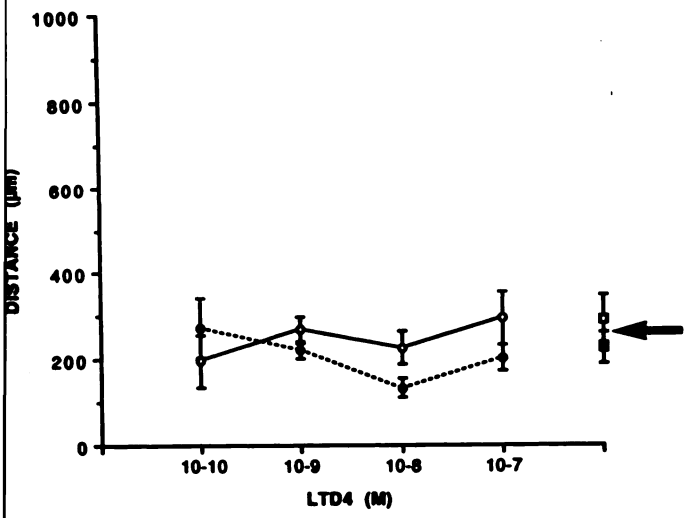

Fig. 8. The effect of 10^{-6} M SK&F 104353 on the chemotactic (—○—) and the chemokinetic (---●---) responses evoked in isolated human eosinophils by graded doses of LTD_4. Results obtained by the under-agarose leukocyte assay are expressed as the mean migration distance in linear μm from the nearest edge of the center well, ± S.E.M. The arrow indicates the migratory activity of unstimulated eosinophils toward companion wells containing SK&F 104353 only (□) or medium only (■). * = $P < 0.05$, comparing chemotaxis relative to chemokinesis. n = 5–10 donors.

buffer control values still evident at the highest dose tested, usually 10^{-6} M to 10^{-5} M [18–26]. Studies involving agarose methods or cell polarization techniques have demonstrated chemotaxis at low doses ranging from 10^{-9} M to 10^{-8} M through the highest tested doses of 10^{-6} M to 10^{-5} M [27–29]. Human eosinophil data has proven to be more equivocal, since significant quantities of relatively pure cells are difficult to obtain. Until the description of several reliable methods for obtaining highly purified normodense eosinophil fractions, investigators had to rely on mixed-leukocyte populations, or on cells that were obtained from patients with blood eosinophilia that was consequential to various disease states. One or both cell types in a mixed-granulocyte preparation may release factors upon stimulation that can influence the responses of either or both types of leukocytes, and it is known that eosinophils from subjects with hypereosinophilia differ in cell density and functional activity compared with those isolated from normal donors [15, 30–37]. Thus, LTB_4-stimulated eosinophil chemotaxis has been reported at low doses that range from 10^{-9} M to 10^{-7} M, and through high doses of 10^{-6} M to 10^{-5} M [18–27]. In any event, differential chemotactic responses and release of β-glucoronidase in purified human neutrophils and eosinophils in response to exogenous LTB_4 has been reported recently [26].

Although recently published in vivo studies suggest that the peptide-containing LTs are involved in the mediation of leukocyte infiltration into conjunctival tissues [1–5], LTD_4 is not yet generally acknowledged as a cell chemotaxin. The present in vitro studies demonstrate that human eosinophils possess chemotactic sensitivities to LTD_4, and the complete inhibition of this response by the selective peptide-LT-antagonist, SK&F 104353 [12], provides further evidence that human leukocyte migratory behavior can be directly influenced by LTD_4. Moreover, the comparative LTD_4 dose-response profiles of neutrophils and eosinophils are consistent with in vivo findings in the guinea pig conjunctiva that show a relative lack of neutrophil infiltration in response to topically administered LTD_4 [2]. Thus, eosinophils respond to LTD_4 at a dose that is five orders of magnitude less than that required to elicit the first indication of directed neutrophil motility.

The lack of previously published accounts describing in vitro leukocyte movement caused by the peptide-LTs may be regarded as surprising, considering the unambiguous chemotactic responses to LTD_4 which were observed in the present studies. A recent report has suggested that LTD_4 does not produce eosinophil chemotaxis [38]. One possible explanation for these conflicting results is that the previous study employed eosinophils obtained from patients with various conditions resulting in high peripheral eosinophil levels, whereas normodense eosinophils from healthy donors were used in the experiments reported herein. A method for the rapid purification of human eosinophils involving the incubation of whole blood with 1 μM fMLP was first described by Roberts and Gallin [39]. This concentration of fMLP has been shown to activate eosinophils [40, 41], and neutrophils and eosinophils may also be primed to suboptimal concentrations of another stimulus by lower fMLP concentrations ($\approx 10^{-8}$M) that do not directly stimulate cellular oxygen consumption [41, 42]. It has been subsequently demonstrated that a very low concentration (10 nM) of fMLP can be used to isolate eosinophils from peripheral blood of normal individuals without cell activation or priming to a number of stimuli [16]. The possibility that the use of fMLP for the purification of eosinophils in these present studies may have served to prime cells to LTD_4 cannot be entirely ruled out, since LTD_4 was not included in the panel of factors tested in the investigation by Koenderman et al., mentioned above [16].

Recent critiques of the in vitro methodologies used to assess leukocyte chemotaxis have brought to light several pitfalls in the use of filter assays that were not fully appreciated in earlier studies [7, 10, 11, 22, 43–50], but there are no recent reports in which the peptide-LTs have been re-examined in light of this new information. Limitations are also recognized for agarose assays. A number of investigators have modified the under-agarose method in order to accommodate special needs, but experimental results are usually quantitated according to a previously published description [6]. Migration distances toward the chemoattractant and toward the control medium are first determined by locating the "leading edge," or most outlying cells, in each direction. Dividing the former distance by the latter yields a number, the so-called chemotactic index (CI), which gives an indication of the chemotactic activity of the test substance. A chemokinetic differential (CD) is obtained by subtracting the response to the control medium from that observed for the putative chemoattractant, thereby conferring a quantifiable expression for stimulated non-directional cellular movement. However, reliance on measurements obtained from the "leading edge" assumes a uniform response under a set of homogeneous conditions, while in vivo and in vitro evidence indicates that normal human blood neutrophil and eosinophil populations show heterogeneity of granulopoiesis kinetics, as well as a range of functional responses such as locomotor behavior and phagocytic and cytotoxic activities [51–57]. Moreover, even in the presence of a chemoattractive concentration gradient, individual cells frequently change their direction and rate of movement, sometimes even assuming a spherical shape and ceasing locomotion for several minutes before resuming active movement [44, 46, 49, 58–62]. The quantitative analysis of randomly selected samples at four different LT concentrations, derived from a concept introduced in a recently published method for the determination of neutrophil orientation under agarose [61], also suggests that such cell behavior occurred in the present studies, since there was some variability in terms of both ax-

ial and directional tendencies within an intact LT gradient at a position close to the starting point of migration relative to those observed at the "leading edge" (see ref. 17). It is thus apparent that sampling at the "leading edge" will tend to favor those cells that respond more readily under a given set of conditions.

In the under-agarose assay, there is the often-overlooked danger that measurement of outlying cells can result in underestimation of a response if few cells are initially seeded, as would be the case with eosinophils. Although the validity of "leading edge" sampling in the agarose method has been questioned in the literature [45, 47, 57], it is still widely employed, primarily because this is the most expedient way of obtaining numerous measurements. We believed that the concerns associated with this sampling strategy could be avoided by employing digital image analysis to define the response of the "average" cell. This novel method of quantitation yielded highly reproducible results in comparison to those obtained by measurements based on the "leading edge," indicating that the behavior of outlying cells is not necessarily representative of the response in the remaining population. The latter contention conflicts with the conclusion of a recent study in which micropore filters were employed [23], so it is possible that the sampling strategy is dependent to some degree on the experimental technique. Taking the foregoing technical points into account, it becomes understandable why leukocyte chemotaxis has not been previously demonstrated with LTD_4, whereas the present studies show clear responses induced by concentrations that apparently were not explored in prior investigations. Moreover, there are to our knowledge no reports of attempts to examine the effects of LTD_4 on the in vitro migratory activity of normal human eosinophils in eosinophil-rich isolates.

The difference in random activities of unstimulated neutrophils and eosinophils is also of interest in this regard. Effects arising from the preparative techniques used for cell isolation cannot be entirely ruled out, but respective baseline values remained remarkably consistent in the presence of either the distilled water LT vehicle or cell medium, as well as in the absence of any treatment whatsoever, indicating that the observed leukocyte responses to the LTs were not unduly influenced by purification procedures. The clear separation of chemotactic, chemokinetic, and baseline responses also tends to support this argument. It is also relevant to note that results from the quantitative sampling of cellular orientation provide evidence that chemotaxis could be readily distinguished from chemokinesis by the methodologies reported herein, making it unlikely that stimulated random migration was mistaken for true directional motility. Finally, treatment with SK&F 104353 reduced both eosinophil chemotaxis and chemokinesis to a level comparable with baseline activity, and the random unstimulated motility of naive eosinophils was unaffected by the LT antagonist.

In summary, differential sensitivities of isolated human neutrophils and eosinophils to LTB_4 and LTD_4 have been demonstrated with a modified under-agarose technique. Neutrophils appear to have a higher level of intrinsic nonspecific motile activity than eosinophils, but eosinophils possess a significantly lower threshold at which directional migration is triggered by LTs. In addition, evidence has been presented which shows that chemotaxis of human eosinophils can be directly influenced by LTD_4, and that this stimulation can be inhibited with a selective antagonist for the peptide-LTs. Leukocyte emigration into extravascular tissue regions is one of a host of salient pathobiologic responses that the LTs are believed to modulate during the process of inflammation. Ablation of cell ingress is viewed as a desirable clinical effect, since the presence of a substantial cellular infiltrate can be detrimental to surrounding tissues. The observations reported here indicate that earlier in vivo studies in the guinea pig bear some relevance to human ocular inflammation in particular, and underscore the possible therapeutic utility of a peptide-LT-antagonist in the treatment of immediate-type allergic reactions arising from a variety of disease states.

REFERENCES

1. Spada, C.S., Woodward, D.F., Hawley, S.B., Nieves, A.L. (1986) Leukotrienes cause eosinophil emigration into conjunctival tissue. *Prostaglandins* **31,** 795–809.
2. Spada, C.S., Woodward, D.F., Hawley, S.B., Nieves, A.L., Williams, L.S., Feldman, B.J. (1988) Synergistic effects of LTB_4 and LTD_4 on leukocyte emigration into the guinea pig conjunctiva. *Am. J. Pathol.* **130,** 354–368.
3. Woodward, D.F., Krauss, A.H-P., Nieves, A.L., Spada, C.S. (1991) Studies on leukotriene D_4 as an eosinophil chemoattractant. *Drugs Exp. Clin. Res.* **XVII,** 543–548.
4. Chan, C-C., McKee, K., Tagari, P., Chee, P., Ford-Hutchinson, A. (1990) Eosinophil-eicosanoid interactions: inhibition of eosinophil chemotaxis in vivo by a LTD_4-receptor antagonist. *Eur. J. Pharmacol.* **191,** 273–280.
5. Sun, F.F., Crittenden, N.J., Czuk, C.I., Taylor, B.M., Stout, B.K., Johnson, H.G. (1991) Biochemical and functional differences between eosinophils from animal species and man. *J. Leukoc. Biol.* **50,** 140–150.
6. Nelson, R.D., Quie, P.G., Simmons, R.L. (1975) Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.* **115,** 1650–1656.
7. Territo, M.C. (1981) Chemotaxis. In *Leukocyte Function* (M.J. Cline, ed), Churchill-Livingstone, New York, New York 39–52.
8. Cutler, J.E. (1974) A simple in vitro method for studies of chemotaxis. *Proc. Soc. Exp. Biol. Med.* **147,** 471–474.
9. Boyden, S. (1962) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J. Exp. Med.* **115,** 453–466.
10. Wilkinson, P.C. (1988) Micropore filter methods for leukocyte chemotaxis. *Methods Enzymol.* **162,** 38–50.
11. Chenoweth, D.E., Rowe, J.G., Hugli, T.E. (1979) A modified method for chemotaxis under agarose. *J. Immunol. Methods* **25,** 337–353.
12. Hay, D.W.P., Muccitelli, R.M., Tucker, S.S., Vickery-Clark, L.M., Wilson, K.A., Gleason, J.G., Hall, R.F., Wasserman, M.A., Torphy, T.J. (1987) Pharmacologic profile of SK&F 104353: a novel potent and selective peptidoleukotriene receptor antagonist in guinea pig and human airways. *J. Pharmacol. Exp. Ther.* **243,** 474–481.
13. Luna, L.G. (1968) *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology,* Third Edition McGraw-Hill Book Co., New York, New York 111–112.
14. Gartner, I. (1980) Separation of human eosinophils in density gradients of polyvinylpyrrolidone-coated silica gel (Percoll). *Immunology* **40,** 133–136.
15. Kloprogge, E., de Leeuw, A.J., de Monchy, J.G.R., Kauffman, H.F. (1989) Hypodense eosinophilic granulocytes in normal individuals and patients with asthma: generation of hypodense cell populations in vitro. *J. Allergy. Clin. Immunol.* **83,** 393–400.
16. Koenderman, L., Kok, P.T.M., Hamelink, M.L., Verhoeven, A.J., Bruijnzeel, P.L.B. (1988) An improved method for the isolation eosinophilic granulocytes from peripheral blood of normal individuals. *J. Leukoc. Biol.* **44,** 79–86.
17. Krauss, A.H.-P., Nieves, A.L., Spada, C.S., Woodward, D.F. (1994) Determination of leukotriene effects on human neutrophil chemotaxis in vitro by differential assessment of cell motility and polarity. *J. Leukoc. Biol.* **55,** 201–208.
18. Goldman, D.W., Goetzl, E.J. (1983) Mediation and modulation of immediate hypersensitivity and inflammation by products of the oxygenation of arachidonic acid. In *Handbook of Inflammation, Vol. 4. Immunology of Inflammation* (P.A. Ward, ed) Elsevier/North Holland, New York, New York 163–187.
19. Payan, D.G., Goldman, D.W., Goetzl, E.J. (1984) Biochemical and cellular characteristics of the regulation of human leukocyte function by lipoxygenase products of arachidonic acid. In *The Leukotrienes: Chemistry and Biology* (L.W. Chakrin and D.M. Bailey, eds) Academic Press, New York, New York 231–245.
20. Palmblad, J., Udén, A-M., Friberg, I., Ringerz, B., Rådmark, O., Lindgren, J-A., Hansson, G., Malmsten, C.L. (1983) Effects of novel lipoxygenase products on neutrophil and eosinophil functions in vitro. In *Advances in Prostaglandin, Thromboxane, and Leukotriene Research. Vol. 12* (B. Samuelsson, R. Paoletti, and P. Ramwell, eds) Raven Press, New York, New York 25–31.

21. Czarnetzki, B.M., Rosenbach, T. (1986) Chemotaxis of human neutrophils and eosinophils towards leukotriene B_4 and its 2-ω-oxidation products in vitro. *Prostaglandins* **31**, 851-858.
22. Clancy, R.M., Dahinden, C.A., Hugli, T.E. (1987) Effect of structural modification at carbon atom 1 of leukotriene B_4 on the chemotactic and metabolic response of human neutrophils. *Anal. Biochem.* **161**, 550-558.
23. Hakansson, L., Westerlund, D., Venge, P. (1987) New method for the measurement of eosinophil migration. *J. Leukoc. Biol.* **142**, 689-696.
24. Lee, T.H., Sethl, T., Crea, A.E.G., Peters, W., Arm, J.P., Horton, C.E., Walport, M.J., Spur, B.W. (1988) Characterization of leukotriene B_3: comparison of its biological activities with leukotriene B_4 and leukotriene B_5 in complement receptor enhancement, lysozyme release and chemotaxis of human neutrophils. *Clin. Sci.* **74**, 467-475.
25. Czarnetzki, B.M., Csato, M. (1989) Comparative studies of human eosinophil migration towards platelet-activating factor and leukotriene B_4. *Int. Arch. Allergy Appl. Immunol.* **88**, 191-193.
26. Morita, E., Schröder, J-M., Christophers, E. (1989) Differential sensitivities of purified human eosinophils and neutrophils to defined chemotaxins. *Scand. J. Immunol.* **29**, 709-716.
27. Palmblad, J., Malmsten, C.L., Udén, A-M., Rådmark, O., Engstet, L., Samuelsson, B. (1981) Leukotriene B_4 is a potent and stereospecific stimulator of neutrophil chemotaxis and adherence. *Blood* **58**, 658-661.
28. Palmblad, J., Udén, A-M., Venizelos, N. (1981) The quantification of neutrophil orientation and migration under-agarose—a new method for detecting directed and random movements. *J. Immunol. Methods* **44**, 37-53.
29. Scheja, A., Fosgren, A. (1986) Effect of LTB_4 and its isomers on human leucocyte migration into skin chambers. *Clin. Exp. Rheumatol.* **4**, 323-329.
30. Tai, P-C., Spry, C.J.F. (1975) Studies on blood eosinophils. I. Patients with a transient eosinophilia. *Clin. Exp. Immunol.* **24**, 415-422.
31. Bass, D.A., Grover, W.H., Lewis, J.C., Szejda, P., DeChatelet, L.R. (1980) Comparison of human eosinophils from normals and patients with eosinophilia. *J. Clin. Invest.* **65**, 1265-1271.
32. Winqvist, I., Olofsson, T., Olsson, I., Person, A-M., Hallberg, T. (1982) Altered density, metabolism and surface receptors of eosinophils in eosinophilia. *Immunology* **47**, 531-539.
33. Kajita, T., Yui, Y., Mita, H., Taniguchi, N., Saito, H., Mishima, T., Shida, T. (1985) Release of leukotriene C_4 from human eosinophils and its relation to the cell density. *Int. Arch. Allergy Appl. Immunol.* **78**, 406-410.
34. Peters, M.S., Gleich, G.J., Dunnette, S.L., Fukuda, T. (1986) The morphologic basis of hypodense eosinophils. *Clin. Res.* **34**, 774A.
35. Kauffman, H.F., van der Belt, B., de Monchy, J.G.R., Boelens, H., Koeter, G.H., de Vries, K. (1987) Leukotriene C_4 production by normal-density and low-density eosinophils of atopic individuals and other patients with eosinophilia. *J. Allergy Clin. Immunol.* **79**, 611-619.
36. Shult, P.A., Lega, M., Jadidi, S., Vrtis, R., Warner, T., Graziano, F.M., Busse, W.W. (1988) The presence of hypodense eosinophils and diminished chemiluminescence response in asthma. *J. Allergy Clin. Immunol.* **81**, 429-437.
37. Gruart, V., Balloul, J-M., Prin, L., Tomassini, M., Loiseau, S., Capron, A., Capron, M. (1989) Variations in protein expression related to human eosinophil heterogeneity. *J. Immunol.* **142**, 4416-4421.
38. Nagy, L., Lee, T.H., Goetzl, E.J., Pickett, W.C., Kay, A.B. (1982) Complement receptor enhancement and chemotaxis of human neutrophils and eosinophils by leukotrienes and other lipoxygenase products. *Clin. Exp. Immunol.* **47**, 541-547.
39. Roberts, R.L., Gallin, J.I. (1985) Rapid method for isolation of normal human peripheral blood eosinophils on discontinuous Percoll gradients and comparison with neutrophils. *Blood* **65**, 433-440.
40. Yazdanbakhsh, M., Eckmann, C.M., Koenderman, L., Verhoeven, A.J., Roos, D. (1987) Eosinophils do respond to fMLP. *Blood* **70**, 379-383.
41. Pretlow, T.P., Wilk, A.I., Davis, L.A., Ortmann, R.A. (1988) Comparison of different methods for the purification of eosinophils from human peripheral blood. *Anal. Biochem.* **175**, 334-341.
42. Shaw, R.J., Walsh, G.M., Cromwell, O., Moqbel, R., Spry, C.J.F., Kay, A.B. (1985) Activated human eosinophils generate SRS-A leukotrienes following IgG-dependent stimulation. *Nature* **316**, 150-152.
43. Schmalstieg, F.C., Rudolff, H.E., Hillman, G.R., Anderson, D.C. (1986) Two-dimensional and three-dimensional movement of human polymorphonuclear leukocytes: two fundamentally different mechanisms of location. *J. Leukoc. Biol.* **40**, 677-691.
44. Zigmond, S.H., Lauffenburger, D.A. (1986) Assays of leukocyte chemotaxis. *Ann. Rev. Med.* **37**, 149-155.
45. Lackie, J.M., Chaabane, N., Crocket, K.V. (1987) A critique of the methods used to assess leukocyte behavior. *Biomed. Pharmacother.* **41**, 265-278.
46. Pederson, M.M. (1987) Comparison of two methods for measurement of chemotaxis of neutrophil polymorphonuclear leukocytes in vitro. *Acta Pathol. Microbiol. Immunol. Scand.* Sect C **95**, 189-193.
47. Bignold, L.P. (1988) Measurement of chemotaxis of polymorphonuclear leukocytes in vitro. The problems of the control of gradients of chemotactic factors, of the control of the cells and of the separation of chemotaxis from chemokinesis. *J. Immunol. Methods* **108**, 1-18.
48. Wilkinson, P.C. (1988) Chemotaxis and chemokinesis: confusion about definitions (letter). *J. Immunol. Methods* **110**, 143-144.
49. Bignold, L.P. (1988) Chemotaxis and chemokinesis of neutrophils: a reply. *J. Immunol. Methods* **110**, 145-148.
50. Lauffenburger, D.A., Tranquillo, R.T., Zigmund, S.A. (1988) Concentration gradients of chemotactic factors in chemotaxis assays. *Methods Enzymol.* **162**, 85-101.
51. Pawaresch, M.R., Walle, A.J., Arndt, D. (1976) The peripheral kinetics of human radiolabelled eosinophils. *Virchows Arch. B Cell. Pathol.* **21**, 57-66.
52. Klempner, M.N., Gallin, J.I. (1978) Separation and functional characterization of human neutrophil subpopulations. *Blood* **51**, 659-699.
53. Prin, L., Capron, M., Tonnel, A-B., Bletry, O., Capron, A. (1983) Heterogeneity of human peripheral blood eosinophils: variability in cell density and cytotoxic ability in relation to the level and the origin of hypereosinophilia. *Int. Arch. Allergy Appl. Immunol.* **72**, 336-346.
54. Sorice, F., DeSimone, C. (1986) Human eosinophil heterogeneity. *Ric. Clin. Lab.* **16**, 429-434.
55. Molinari, A., Rosati, F., Donelli, G., DiFabio, S., Sorice, F., DeSimone, C. (1988) Expression of receptors for C3b and IgG-Fc in human eosinophil subpopulations. *EOS-J. Immunol. Immunopharmacol.* **8**, 42-46.
56. Spielberg, H., June, C., Cereb, N., Nystrom-Rosander, C., Deeg, H.J. (1989) Differential sensitivity of human leukocyte subpopulations to ultraviolet light. *Transplant Proc.* **21**, 180-181.
57. Terpstra, G.K., Houben, L.A.M.J. (1989) Directional cellular movement of cell populations: its description by chemotactic assays. *Agents Actions* **26**, 224-226.
58. Wilkinson, P.C. (1982) *Chemotaxis and Inflammation. Second Edition* Churchill-Livingstone, Edinburgh 18-21.
59. Lackie, J.M. (1986) *Cell Movement and Cell Behavior* Alan & Unwin, London 219-251.
60. Noble, P.B., Levine, M.D. (1986) *Computer-Assisted Analyses of Cell Locomotion and Chemotaxis* CRC Press, Inc., Boca Raton, Fla. 17-48.
61. Pederson, J.O., Hassing, L., Grunnet, N., Jersild, C. (1988) Real-time scanning and image analysis. A fast method for the determination of neutrophil orientation under agarose. *J. Immunol. Methods* **109**, 131-137.
62. Tranquillo, R.T., Lauffenberger, D.A., Zigmond, S.H. (1988) A stochastic model for leukocyte random motility and chemotaxis based on receptor binding fluctuations. *J. Cell Biol.* **106**, 303-309.