CONCISE REPORT

Leukotriene B₄ is a Potent and Stereospecific Stimulator of Neutrophil Chemotaxis and Adherence

By Jan Palmblad, Curt L. Malmsten, Ann-Mari Udén, Olof Rådmark, Lars Engstedt, and Bengt Samuelsson

We studied the effects of leukotrienes on in vitro functions of neutrophil polymorphonuclear (PMN) granulocytes. Leukotriene B₄ (LTB₄) evoked a stimulated and directed migration of neutrophils under agarose with an optimum concentration of 10⁻⁴M, whereas two nonenzymatically formed isomers (compounds I and II) induced this response at 10⁻⁵M. Leukotriene C₄ (LTC₄) and 5-hydroxyeicosatetraenoic acid (5-HETE) did not affect this PMN migration. At the same optimum concentrations, LTB₄ and compounds I and II augmented PMN adherence to nylon fibers. The chemotactic and adherence responses were of the same magnitude as with formyl-Met-Leu-Phe (fMLP) at 10⁻⁷M. None of the leukotrienes influenced the spontaneous or phagocytosis-associated chemiluminescence or the ability to kill Staphylococcus aureus. The cyclooxygenase inhibitor, indomethacin, inhibited only partly the fMLP-induced migration at high concentrations and stimulated migration at 2.5 x 10⁻⁷M, suggesting that arachidonic acid was then mainly metabolized by the lipoxygenase pathways. The lipoxygenase and cyclooxygenase inhibitor, eicosatetraenoic acid, inhibited both spontaneous and stimulated migration at ≥2.5 x 10⁻⁷M, but not at lower concentrations. Thus, since LTB₄ and to a lesser degree compounds I and II, stimulated migration and adhesion, it is suggested that these mediators could be of importance for the emigration of neutrophils from blood vessels to areas of inflammation.

POLYMORPHONUCLEAR (PMN) neutrophil granulocytes exhibit increased adherence, chemotaxis, degranulation, oxidative metabolism, and bacterial killing after stimulation with substances formed within an inflammatory or infected site, e.g., the C₅a-fragment and bacterial products, such as fMLP. These substances are critical for host defense but are also responsible for tissue damage because of extrusion of PMN lysosomal enzymes and formation of toxic oxygen radicals.

A recently described group of mediators of inflammation are called leukotrienes (LT). These are formed within neutrophils after stimulation with fMLP or the calcium ionophore A 23187 by a lipoxygenase-catalyzed oxygenation of arachidonic acid. The hereby formed epoxide leukotriene Α₄ (LTA₄) is an unstable intermediate and is converted, by enzymatic hydrolysis, to leukotriene B₄ (LTB₄, 5, 12-dihydroxyeicosatetraenoic acid). Two other 5, 12-dihydroxy isomers (compounds I and II) can also be generated by a nonenzymatic hydrolysis of LTA₄. Further, leukotriene C₄ (LTC₄) is formed from LTA₄ by addition of glutathione, and by elimination of its γ-glutamyl residue, it is metabolized to LTD₄. LTC₄ and LTD₄ have been identified in SRS-A from various sources, being potent bronchoconstrictors and increasing permeability of the microvasculature.

We and others have recently shown that LTB₄ stimulates neutrophil migration (i.e., is a cytotaxin), aggregation, and to a lesser degree, degranulation. Also, the lipoxygenase product 5-hydroxyeicosatetraenoic acid (5-HETE) has been reported to exert a chemotactic effect and to cause release of lysosomal enzymes. This report concerns the further investigations of the effects on several neutrophil functions of LTB₄, compounds I and II, LTC₄, and 5-HETE as well as the effects on migration of two inhibitors of the lipoxygenase and cyclooxygenase pathways (indomethacin and eicosatetraenoic acid, ETYA).

MATERIALS AND METHODS

Healthy members of the laboratory staff served as donors of blood. None was on a drug regimen or had ingested acetylic salicylic acid during the preceding week.

Chemicals

5-HETE, LTB₄, compounds I and II, and LTC₄ were obtained after incubation of PMNs with arachidonic acid (Nu-Chek Prep. Inc., Elysian, Minn) and ionophore A 23187 (Eli Lilly, Indianapolis, Ind.). The incubate was extracted and purified by high-pressure chromatography.

Leukocyte Preparation

Twice washed, dextran-sedimented leukocytes were resuspended in HBSS containing 0.1% gelatin for the bactericidal and chemiluminescence assays; supplemented with 1% human albumin for adherence assays; and 3% Heps buffer (Sigma, St. Louis, Mo) for the chemotaxis assays. In some experiments PMNs were incubated with indomethacin (Sigma) or ETYA (a kind gift from Dr. J. Pike, Upjohn Co., Kalamazoo, Mich.) for 15 min at 37°C, both
dissolved in ethanol (at a final concentration of 2.5%). Cells incubated with solvent only served as controls.

**Bactericidal Capacity**

Neutrophils (2.5 x 10^6/liter) were mixed with suspensions of *Staphylococcus aureus* (70 x 10^9 colony-forming units/liter) and serum (10%). After 90-min incubation at 37°C, samples were removed for quantification of viable bacteria. The results are given as the percentages of living bacteria of the initial counts. This bacterial concentration has been shown to disclose enhancements of the bactericidal capacity better than a traditional assay. The details and reproducibility of the method have been presented elsewhere.13 The oxidative metabolism was quantitated with the luminol-augmented chemiluminescence.12 PMNs (2.5 x 10^6/liter), serum (10%), luminol (2.6 x 10^-4 M) with or without living *S. aureus* (70 x 10^9/liter), and simultaneously added leukotrienes were incubated at 37°C. The chemiluminescence was measured after 5, 10, and 15 min in a photomultiplicator and the results are expressed in mV.

Adherence was assayed by a 40-μg nylon fiber column to which washed leukocytes (preincubated with LTs for 2 min at room temperature) were added. The results are given as the percentage of neutrophils adhering to the fibers. The reproducibility and details of the method have been given.12 The spontaneous and stimulated locomotion were assayed with an agarose method.12 Wells were punched in a gelled agarose solution, with an albumin concentration of 0.08%. The distance between the centers of two adjacent wells was 5 mm. Ten microliters of the leukocyte suspension (10 x 10^6 PMNs/liter) was filled into the central well (diameter 3 mm). The chemotactic factors (either 10^-7 M fMLP, 5-HETE, or the leukotrienes, dissolved in 10% ethanol in HBSS) were filled into the outer wells and controls into the inner wells. After 0.5–3 hr, incubation cells were fixed with methanol and stained with hematoxylin. The distance migrated by the leading front neutrophils was measured by microscopy and is given in millimeters.

In order to assess whether locomotion was directed, i.e., whether chemotaxis predominated, the orientation of lamellopodia and nuclei of 600 neutrophils migrating towards the cytotaxin wells was estimated by microscopy.12 The cells were considered oriented in the gradient when the nuclei were in the rear of the cell and the anterior lamellopod located within a 90° sector, open towards the cytotaxin well. The viability of incubated PMNs was >95% as evidenced by trypan blue exclusion.

Statistical analyses were performed with Wilcoxon’s signed rank test.

**RESULTS**

Preincubation of PMNs with indomethacin (≥2.5 x 10^-7 M) caused a dose-dependent inhibition of spontaneous and fMLP-stimulated migration (Fig. 1). However, with a lower concentration of indomethacin (2.5 x 10^-7 M), a significant enhancement of stimulated migration was observed. Lower concentrations of indomethacin exerted no significant effects on stimulated or spontaneous migration compared with controls.

PMNs pretreated with ETYA showed markedly impaired migration when final inhibitor concentration was ≥2.5 x 10^-7 M (Fig. 1). At final ETYA concentrations of 2.5 x 10^-6 M or below, spontaneous or stimulated migration was comparable to that of control cells.

When 5-HETE, LTC4, LTB4, and compounds I and II were added to the cytotaxin well, PMN migration was significantly stimulated by LTB4 and compounds I and II (Fig. 2). When 5-HETE or LTC4 were utilized, the migration was not significantly different from that of spontaneously moving cells. In order to assess whether LTB4 and compounds I and II stimulated directed or random migration, i.e., chemotaxis or chemokinesis, the degree of orientation at 30, 60, and 120 min after incubation began was analyzed and compared with fMLP as cytotaxin (Fig. 2). PMNs stimulated by fMLP showed the highest degree of orientation at 30 and 60 min, but decreased after 120 min to control values.12 LTB4 also stimulated directed migration at 30, 60, and 120 min when concentration was 10^-5 M. After incubation for 3 hr, LTB4 at 10^-5 M was associated with a regression of the leading front cells and a stimulation of the previously spontaneously moving cells facing the control well, a pattern...
PMN LOCOMOTION & ORIENTATION DURING MIGRATION

![Graph showing PMN locomotion and orientation during migration.](image)

Fig. 2. The migration of neutrophils towards LTB₄, LTC₄, 5-HETE, and compounds I and II, measured as the distance to the leading front cells in millimeters after incubation for 3 hr. Mean and SD values for triplicates performed on PMNs from 4–6 subjects. The mean distance migrated after stimulation with fMLP at 10⁻⁷ M is 0.80 mm (SD ± 0.27 mm) and is depicted as ....... The mean value for spontaneously migrating PMNs is 0.13 mm (± 0.02 mm) and is given as -----. The inserted figure shows the orientation of PMNs migrating towards the leukotriene or fMLP-containing agarose wells. The shaded area represents the normal value (mean ± 2 SD) for spontaneously moving cells, 13.5%–30.9%. (1) With LTB₄ at 10⁻⁶ M, the cells facing the LT well were densely packed round the PMN well, and cells migrating toward the well containing tissue culture medium had advanced to a mean value of 0.29 ± 0.16 mm, a pattern recognized as desensitization. With LTB₄ and compounds I and II, directed migration was observed at 10⁻⁷ M and to a lesser degree at 10⁻⁶ M (Fig. 2).

Neutrophil adherence was increased after 2 min of incubation with LTB₄ and compounds I and II, but not with 5-HETE and LTC₄ (Table I). As with chemotaxis, 10⁻⁶ M of LTB₄ was as potent a stimulator as fMLP at 10⁻⁷ M, whereas compounds I and II were most effective at 10⁻⁵ M.

Neither LTs nor 5-HETE did stimulate phagocytosis-associated chemiluminescence nor the bactericidal capacity (Table I). Furthermore, none of the

Table 1. PMN Adherence, Bactericidal Capacity, and Phagocytosis-Associated Chemiluminescence

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar Concentration</th>
<th>Adherence (%)</th>
<th>Bactericidal Capacity (% CFU at 90 min)</th>
<th>Chemiluminescence as Percent of Controls After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>LTB₄</td>
<td>10⁻⁶</td>
<td>25 ± 5‡</td>
<td>9.3 ± 1.9</td>
<td>99 ± 4</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷</td>
<td>36 ± 5§</td>
<td>10.7 ± 2.3</td>
<td>98 ± 13</td>
</tr>
<tr>
<td></td>
<td>10⁻⁸</td>
<td>17 ± 5</td>
<td>12.4 ± 3.0</td>
<td>95 ± 8</td>
</tr>
<tr>
<td>LTC₄</td>
<td>10⁻⁶</td>
<td>20 ± 7</td>
<td>—</td>
<td>83 ± 3</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷</td>
<td>8 ± 2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5-HETE</td>
<td>10⁻⁶</td>
<td>9 ± 3</td>
<td>—</td>
<td>78 ± 5</td>
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<tr>
<td></td>
<td>10⁻⁻</td>
<td>14 ± 10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Compound I</td>
<td>10⁻⁵</td>
<td>27 ± 8‡</td>
<td>—</td>
<td>78 ± 5</td>
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<tr>
<td></td>
<td>10⁻⁶</td>
<td>18 ± 2‡</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Compound II</td>
<td>10⁻⁵</td>
<td>29 ± 9‡</td>
<td>—</td>
<td>—</td>
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<td></td>
<td>10⁻⁶</td>
<td>16 ± 7</td>
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<tr>
<td>Controls</td>
<td>13 ± 2</td>
<td>10.9 ± 1.9</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Mean and SE values of 4–6 determinations in duplicates on PMNs from 3–4 donors.
†fMLP (10⁻⁷ M) augmented adherence with 25% ± 7% compared with controls.
‡p < 0.05.
§p < 0.01.
LEUKOTRIENE B₄

compounds elicited a chemiluminescence when added to neutrophils in absence of bacteria (data not shown).

DISCUSSION

This study has shown that leukotriene B₄ and, to a lesser degree its isomers, enhance neutrophil adherence and chemotaxis, but not bactericidal mechanisms or oxidative metabolism. Furthermore, LTCA₄ or 5-HETE did not stimulate any of these PMN functions. These results suggest a role for LTBA in the inflammatory response.

The observation that ETYA caused a dose-dependent decrease of PMN migration induced by fMLP indicates that oxygenation of arachidonic acid is essential for the chemotactic response of human PMNs to the peptide, since ETYA inhibits both the lipoxygenase and cyclooxygenase pathways. High concentrations of indomethacin (previously shown to inhibit mainly cyclooxygenase and, to a lesser extent, lipoxygenase) also hampered part of the chemotactic effect of fMLP, whereas lower concentrations (inhibiting only the cyclooxygenase) stimulated migration. These findings are compatible with a role of lipoxygenase products for neutrophil locomotion, e.g., because more arachidonic acid derivates are available to leukotriene synthesis. When some recently discovered lipoxygenase metabolites of arachidonic acid from PMNs (5-HETE, LTC₄, LTBA, and compounds I and II) were studied, LTBA was found to induce both augmented adherence as well as directed and stimulated migration, i.e., chemotaxis. The response to LTBA was of the same magnitude as to fMLP but required a tenfold higher molar concentration. Interestingly, the adherence and migration responses showed similar dose–response curves over the range of concentrations studied here, with an optimum at 10⁻⁶M. Moreover, both functions were hampered by LTBA at 10⁻⁵M. The nonenzymatic isomers of LTBA (compounds I and II) were considerably less active than the enzymatically formed LTBA, and finally, 5-HETE and LTC₄ did not show any significant effects on any of the neutrophil functions studied here. The stereospecificity in the migration and adherence responses provides support for a physiologic role for LTBA. It is hypothesized that LTBA is formed in order to attract more neutrophils to an inflammatory area. Since adherence to endothelial cells is the first step taken by neutrophils emigrating into the tissues, and is also a critical phenomenon for migration, it is noteworthy that LTBA affects both these functions in a similar way. However, in contrast to C5a and fMLP, LTBA did not augment the production of cytotoxic oxygen radicals and caused only a marginal extrusion of lysosomal enzymes, thereby possibly avoiding attacks on the tissues of the host.

REFERENCES

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