CONCISE REPORT

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

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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 10⁻⁵M, whereas two nonenzymatically formed isomers (compounds I and II) induced this response at 10⁻⁴M. Leukotriene C₄ (LTC₄) and 5-hydroxyicosatetraenoic 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 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 Λ₄ (LTA₄) is an unstable intermediate and is converted, by enzymatic hydrolysis, to leukotriene B₄ (LTB₄, 5, 12-dihydroxyicosatetraenoic 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 eicosatetraynoic 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% Hepes 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 \times 10^4/liter) were mixed with suspensions of *Staphylococcus aureus* (70 \times 10^7 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.\(^3\)

The oxidative metabolism was quantitated with the luminol-augmented chemiluminescence.\(^12\) PMNs (2.5 \times 10^4/liter), serum (10%), luminol (2.6 \times 10^{-4}M) with or without living *S. aureus* (70 \times 10^4/liter), and simultaneously added leukotrienes were incubated at 37°C. The chemiluminescence was measured after 5, 10, and 15 min in a photomultiplier and the results are expressed in mV.

Adherence was assayed by a 40-mg 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 \times 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 lamellipodia 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 lamellipod 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 (\(\geq 2.5 \times 10^{-7}\)M) caused a dose-dependent inhibition of spontaneous and fMLP-stimulated migration (Fig. 1). However, with a lower concentration of indomethacin (2.5 \times 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 \(\geq 2.5 \times 10^{-7}\)M (Fig. 1). At final ETYA concentrations of 2.5 \times 10^{-6}M or below, spontaneous or stimulated migration was comparable to that of control cells.

When 5-HETE, LTC\(_4\), LTBA, and compounds I and II were added to the cytotaxin well, PMN migration was significantly stimulated by LTBA and compounds I and II (Fig. 2). When 5-HETE or LTC\(_4\) were utilized, the migration was not significantly different from that of spontaneously moving cells. In order to assess whether LTBA and compounds I and II stimulated directed migration 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.\(^12\) LTBA also stimulated directed migration at 30, 60, and 120 min when concentration was 10^{-5} M. After incubation for 3 hr, LTBA 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
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 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 mm 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 10⁻⁶ 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 5 min</th>
<th>Chemiluminescence as Percent of Controls After 10 min</th>
<th>Chemiluminescence as Percent of Controls After 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTB₄</td>
<td>10⁻⁶</td>
<td>25 ± 5‡</td>
<td>9.3 ± 1.9</td>
<td>99 ± 4</td>
<td>114 ± 13</td>
<td>130 ± 19‡</td>
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<tr>
<td></td>
<td>10⁻⁵</td>
<td>36 ± 5§</td>
<td>10.7 ± 2.3</td>
<td>98 ± 13</td>
<td>103 ± 9</td>
<td>102 ± 6</td>
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<tr>
<td></td>
<td>10⁻⁴</td>
<td>17 ± 5</td>
<td>12.4 ± 3.0</td>
<td>95 ± 8</td>
<td>104 ± 6</td>
<td>104 ± 6</td>
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<tr>
<td></td>
<td>10⁻⁻²</td>
<td>20 ± 7</td>
<td>—</td>
<td>83 ± 3</td>
<td>85 ± 4</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>LTC₄</td>
<td>10⁻⁷</td>
<td>8 ± 2</td>
<td>—</td>
<td>—</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>
<td>81 ± 4</td>
<td>84 ± 12</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷</td>
<td>14 ± 10</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>Compound I</td>
<td>10⁻⁵</td>
<td>27 ± 8‡</td>
<td>—</td>
<td>76 ± 5</td>
<td>81 ± 4</td>
<td>85 ± 10</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</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>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>10⁻⁶</td>
<td>16 ± 7</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>Controls</td>
<td>13 ± 2</td>
<td>10.9 ± 1.9</td>
<td>100</td>
<td>100</td>
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</tr>
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</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.
compounds elicited a chemiluminescence when added to neutrophils in absence of bacteria (data not shown).

**DISCUSSION**

This study has shown that leukotriene \( \text{B}_4 \) and, to a lesser degree its isomers, enhance neutrophil adherence and chemotaxis, but not bactericidal mechanisms or oxidative metabolism. Furthermore, \( \text{LTC}_4 \) or \( \text{5-HETE} \) did not stimulate any of these PMN functions. These results suggest a role for \( \text{LTB}_4 \) 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 once adherence has occurred. ETYA, on the other hand, had to be removed by neutrophils emigrating into the tissues, and is not a critical phenomenon for migration, it is noteworthy that \( \text{LTB}_4 \) affects both these functions in a similar way. However, in contrast to C5a and fMLP, \( \text{LTB}_4 \) 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**

8. Goetzl EJ, Pickett WC: The human PMN leukocyte chemo-

from PMNs (5-HETE, \( \text{LTC}_4 \), \( \text{LTB}_4 \), and compounds I and II) were studied. \( \text{LTB}_4 \) was found to induce both augmented adherence as well as directed and stimulated migration, i.e., chemotaxis. The response to \( \text{LTB}_4 \) 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 \( 10^{-9} \text{M} \). Moreover, both functions were hampered by \( \text{LTB}_4 \) at \( 10^{-5} \text{M} \). The nonenzymatic isomers of \( \text{LTB}_4 \) (compounds I and II) were considerably less active than the enzymatically formed \( \text{LTB}_4 \), and finally, 5-HETE and \( \text{LTC}_4 \) 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 \( \text{LTB}_4 \). It is hypothesized that \( \text{LTB}_4 \) 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 \( \text{LTB}_4 \) affects both these functions in a similar way. However, in contrast to C5a and fMLP, \( \text{LTB}_4 \) 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.
Leukotriene B4 is a potent and stereospecific stimulator of neutrophil chemotaxis and adherence

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