Effect of Arachidonic Acid Reacylation on Leukotriene Biosynthesis in Human Neutrophils Stimulated with Granulocyte-macrophage Colony-stimulating Factor and Formyl-methionyl-leucyl-phenylalanine

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Priming of human neutrophils with granulocyte-macrophage colony-stimulating factor (GM-CSF) followed by formyl-methionyl-leucyl-phenylalanine (fMLP) stimulates cells in a physiologically relevant manner with modest 5-lipoxygenase activation and formation of leukotrienes. However, pretreatment of neutrophils with thimerosal, an organomercury thiosalicyclic acid derivative, led to a dramatic increase (>50-fold) in the production of leukotriene B4 and 5-hydroxyeicosatetraenoic acid, significantly higher than that observed after stimulation with calcium ionophore A23187. Little or no effect was observed with thimerosal alone or in combination with either GM-CSF or fMLP. Elevation of [Ca\(^{2+}\)], induced by thimerosal in neutrophils stimulated with GM-CSF/fMLP was similar but more sustained compared with samples where thimerosal was absent. However, [Ca\(^{2+}\)], was significantly lower compared with calcium ionophore-treated cells, suggesting that a sustained calcium rise was necessary but not sufficient to explain the effects of this compound on the GM-CSF/fMLP-stimulated neutrophil. Thimerosal was found to directly inhibit neutrophil lypo-phospholipidacyl-CoA acyltransferase activity at the doses that stimulate leukotriene production, and analysis of lysates from neutrophil preparations stimulated in the presence of thimerosal showed a marked increase in free arachidonic acid, supporting the inhibition of the reincorporation of this fatty acid into the membrane phospholipids as a mechanism of action for this compound. The dramatic increase in production of leukotrienes by neutrophils when a physiological stimulus such as GM-CSF/fMLP is employed in the presence of thimerosal suggests a critical regulatory role of arachidonate reacylation that limits leukotriene biosynthesis in concert with 5-lipoxygenase and cytosolic phospholipase A\(_{2}\)α activation.

Neutrophils play an important role in natural immunity and function to eliminate microbes and dead tissues. They are also a major cell involved in the acute inflammatory response, since they respond rapidly to chemotactic stimuli and can be activated by cytokines produced primarily by macrophages to perform degradative and phagocytic functions. Upon activation at the site of inflammation, they release lysosomal enzymes and neutral proteinases, reactive oxygen species, including H\(_2\)O\(_2\) and O\(_2\)\(^{•-}\) (1, 2), and metabolites of arachidonic acid (AA)\(^{2}\), in particular leukotriene B\(_4\) (LTB\(_4\)) (3). Biosynthesis of leukotrienes involves the release of AA from membrane phospholipids by Ca\(^{2+}\)-dependent cytosolic phospholipase A\(_{2}\)α (cPLA\(_{2}\)α) (4) and its conversion into 5-hydroperoxyeicosatetraenoic acid and subsequently leukotriene A\(_{4}\) (LTA\(_{4}\)), catalyzed by 5-lipoxygenase (5-LO) (5). LTA\(_{4}\) is then enzymatically converted to LTB\(_{4}\) by LTA\(_{4}\)-hydrolase (6) or to leukotriene C\(_{4}\) (LTC\(_{4}\)) by the addition of a molecule of glutathione through the action of LTC\(_{4}\)-synthase (7). The site for most of these biochemical events is the perinuclear region (8).

Regulation of leukotriene production can occur at different levels; the increase in Ca\(^{2+}\) and release of free arachidonate by cPLA\(_{2}\)α are fundamental for 5-LO full activation. Specifically, Ca\(^{2+}\) binds to a calcium-dependent phospholipid binding domain (C2) on cPLA\(_{2}\)α that triggers its translocation from the cytosol to the membrane of the Golgi apparatus, nuclear envelope, and endoplasmic reticulum, where it can access its phospholipid substrate (9). 5-LO activity is enhanced by ATP-dependent phosphorylation, and micromolar concentrations of Ca\(^{2+}\) are required for its catalytic activity and translocation from the cytosol to the nuclear membrane, where it interacts with the 5-LO-activating protein, which facilitates the conversion of AA into leukotrienes by “preventing” the substrate to 5-LO (10).

Leukotriene synthesis is characterized by a high cellular specificity, with neutrophils synthesizing exclusively LTB\(_{4}\), since they contain only 5-LO and LTA\(_{4}\)-hydrolase. In these cells, LTB\(_{4}\) is also rapidly metabolized into biologically inactive α-oxidation products (20-OH-LTB\(_{4}\) and 20-COOH-LTB\(_{4}\)) by a specific cytochrome P-450 enzyme (11).

A common model for neutrophil activation in vitro has been the exposure to formyl-methionyl-leucyl-phenylalanine (fMLP), a bacterial chemotactic tripeptide that interacts with a specific membrane receptor (12), but the stimulation with this peptide alone does not lead to formation of detectable amounts of leukotrienes (13). The pretreatment of neutrophils with 5-lipoxygenase inhibitors such as indomethacin inhibits leukotriene synthesis in a dose-dependent manner 

\(^{2}\) The abbreviations used are: AA, arachidonic acid; LTA\(_{4}\), leukotriene A\(_{4}\); LTB\(_{4}\), leukotriene B\(_{4}\); LTC\(_{4}\), leukotriene C\(_{4}\); 5-LO, 5-lipoxygenase; cPLA\(_{2}\)α, cytosolic phospholipase A\(_{2}\)α; fMLP, formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte-macrophage colony-stimulating factor; LAT, lypoxygenatedphosphatidyllcholineacyl-CoA acyltransferase; AA-CoA, arachidonoyl-CoA; S-HETE, 5-hydroxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; Kdo, 3-deoxy-D-manno-octulosonic acid; MS, mass spectrometry; GC, gas chromatography; AM, acetoxymethyl ester; 16:0/OH-GPCho, 1-palmitoyl-2-hydroxy-sn-glycerol-3-phosphocholine; 19:0/OH-GPCho, 1-nondecanooyl-2-hydroxy-sn-glycerol-3-phosphocholine; 18:0/20:4-GPCho, 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine; 18:0/20:4-GPCho, 1-stearoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine; 19:0/20:4-GPCho, 1-nondecanooyl-2-arachidonoyl-sn-glycerol-3-phosphocholine.
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**Human Neutrophil Isolation and Stimulation**—Human neutrophils were isolated from whole blood by a Percoll gradient previously described (28). Briefly, blood was collected in 3.8% sodium citrate, and, after centrifugation for 20 min at 300 × g, the platelet-rich plasma was removed. The residual blood was combined with 6% dextran and 0.9% NaCl solution, mixed well, and left for about 30 min at room temperature for the red blood cells to settle. The upper layer was then removed and centrifuged at 280 × g for 10 min, layered over a Percoll gradient (42–51%), and centrifuged at 400 × g for 30 min. The neutrophil-enriched layer was collected and then washed in KRPD buffer (4.8 mM KCl, 0.97 mM CaCl2, 1.2 mM MgSO4, 3.1 mM NaH2PO4, 12.5 mM NaHPO4, 11 mM glucose). Cells were counted using a hemocytometer counting chamber. Neutrophil purity in these preparations was 95% with usually less than 2% eosinophils and cell viability greater than 98%.

Neutrophils were then centrifuged and resuspended in calcium/magnesium-free Hank’s balanced salt solution (Invitrogen) at a concentration of 10 × 10^6 cells/ml and primed with either GM-CSF (1 nM), tumor necrosis factor α (10 ng/ml), or Kdo2-lipid A (100 ng/ml) for 30 min at 37 °C. After the addition of CaCl2 (final concentration 2 mM) and MgCl2 (final concentration 0.5 mM), the cells were stimulated with fMLP ((10^-7 M) for 10 min at 37 °C. Thimerosal at different concentrations (5–100 μM) was added for 2 min at 37 °C before fMLP. Final incubation volume was 1 ml.

Alternatively, cells were stimulated with calcium ionophore A23187 (5 μM) or iomycin (5 μM) for 10 min at 37 °C after the addition of CaCl2 (2 mM) and MgCl2 (0.5 mM) and 2 min of thermal equilibration. Reactions were terminated by lysing cells in ice-cold MeOH (1 ml) containing 2 ng each of internal standards [d6]LTB4 and [d5]5-HETE. Samples were diluted with water to a final MeOH concentration lower than 15% and then extracted using a solid phase extraction cartridge (Strata C18-E, 100 mg/1 ml; Phenomenex, Torrance, CA). The eluate (1 ml of MeOH) was dried down and reconstituted in 40 μl of HPLC solvent A (8.3 mM acetic acid buffered to pH 5.7 with NH4OH) plus 20 μl of solvent B (AcCN/MeOH, 65/35, v/v).

**Metabolite Separation and Analysis by Reversed Phase HPLC and Electrospray Ionization-Mass Spectrometry**—An aliquot of each sample (35 μl) was injected into an HPLC system, and separation of the different metabolites was conducted using a C18 column (Columbus 150 × 1 mm, 5 μm; Phenomenex) eluted at a flow rate of 50 μl/min with a linear gradient from 25 to 100% of mobile phase B. Solvent B was increased from 25 to 85% in 24 min, increased to 100% in 2 min, and held at 100% for a further 12 min. The HPLC system was directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (Sciex API 2000, PE-Sciex, Thornhill, Canada) where mass spectrometric analyses were performed in the negative ion mode using multiple reaction monitoring of the specific transitions m/z 319 → 115 for 5-HETE, m/z 335 → 195 for LTB4 and Δ^2-trans-LTB5, m/z 351 → 195 for 20-OH-LTB4, m/z 365 → 195 for 20-COOH-LTB4, m/z 335 → 115 for 5,6-di-HETEs, m/z 303 → 205 for AA, m/z 327 → 116 for d5-5-HETE, and m/z 339 → 197 for [d6]LTB4. Quantitation was performed using a standard isotope dilution curve as previously described (29).

Adenosine released from neutrophils was analyzed by stable isotope dilution mass spectrometry (liquid chromatography/MS/MS). After GM-CSF/fMLP stimulation, cells (e.g. 5 × 10^6 neutrophils in 0.5 ml) were not lysed but centrifuged (1000 × g, 5 min), and [15N]adenosine (25 ng; Spectra Stable Isotopes, Columbia, MD) was added to the supernatant. An aliquot (20 μl) was directly injected onto a reversed phase HPLC column (150 × 1 mm, Synergi 4μ Hydro-RP C18 column; Phenomenex), which was eluted at 50 μl/min with a gradient from 95% solvent system C (20 mM ammonium acetate) and 5% solvent system D.
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(MeOH/20 mM ammonium acetate, 20/80, v/v) over an 8–min period to 70% solvent D. Adenosine and [3H]adenosine were detected by multiple-reaction monitoring (MRM) of m/z 268 → 136 and m/z 273 → 141, respectively. A standard curve between 6.25 and 100 ng of adenosine was linear (r² = 0.99) over this range.

Calcium Assay—Neutrophils (10 × 10⁶/ml in Hank’s balanced salt solution) were loaded with 5 μM Indo-1 AM and 0.02% Pluronic F-127 for 30 min at 37 °C. Cells were then washed and incubated with or without GM-CSF (1 nM) for an additional 30 min at 37 °C. After the addition of a 1 μM concentration of each of CaCl₂ and MgCl₂, primed cells were pretreated with thimerosal (50 μM) or vehicle 2 min prior to stimulation with FMLP (10⁻⁷ M). Cells that had not been treated with GM-CSF were stimulated with the calcium ionophore ionomycin (5 μM). Intracellular calcium changes were monitored by measuring the ratio of the fluorescence emissions at 405 and 485 nm (excitation wavelength 355 nm) in a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ).

Gas Chromatography (GC)/MS and Quantitation of Free Fatty Acids—One aliquot (corresponding to 1 × 10⁶ cells) of neutrophil lysate (1:2 H₂O/MeOH) was acidified with HCl (70 mM final concentration), and stable isotope-labeled fatty acid standards [²⁵C]palmitic, [d₆]stearic, [d₇]oleic, and [d₈]arachidonic acid were added. After mixing, samples were extracted with 1 ml of iso-octane. The extract was dried under a stream of N₂ and then derivatized by adding 25 μl each of 1% pentafluorobenzyl bromide and 1% diisopropylethylamine. After mixing, the vials were incubated at room temperature for 20 min, dried under a stream of N₂, and reconstituted in 100 μl of iso-octane. Analysis of the samples was performed by negative ion chemical ionization GC/MS (30) on a Finnigan DSQ GC/MS system (Thermo Finnigan, Thousand Oaks, CA), using a ZB-1 column (15 m × 0.25-mm inner diameter × 0.10-μm film thickness; Phenomenex). The GC was programmed to increase the temperature from 150 to 250 °C at 20 °C/min, 280 °C at 5 °C/min, and finally 310 °C at 30 °C/min and held at 310 °C for 1 min. The mass spectrometer was operated in the negative ion chemical ionization mode using methane as reagent gas. Data were acquired by selected ion monitoring of the following fatty acids: myristic (m/z 227), palmitic (m/z 255), stearic (m/z 283), linoleic (m/z 277), linoleic (m/z 279), oleic (m/z 281), eicosapentaenoic (m/z 301), and arachidonic (m/z 303). The ions at m/z 259, 286, 283, and 311 were monitored for [²⁵C]palmitic, [d₆]stearic, [d₇]oleic, and [d₈]arachidonic acids, respectively. Peak areas of each analyte were measured, and the ratio of the area from the fatty acid-derived ion to that from the internal standard was calculated for each. These ratios were then compared with calibration curves for each analyte prepared from commercially available standards to calculate fatty acid amounts.

Preparation of Microsomes from Human Neutrophils—The isolation of microsomes from human neutrophils was achieved following a previously described protocol for the extraction of LAT from pig spleen membranes (31) with modifications. Briefly, neutrophils, either resting or stimulated, were suspended in LAT homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20% w/v glycerol) containing a protease inhibitor mixture (Complete TT; 1 tablet dissolved in 20 ml of buffer) at a concentration of 100 × 10⁶ cells/ml. The cells were disrupted by sonication (4 × 15 s at 4 °C) using a high intensity ultrasonic processor (Vibracell VCX 600; Sonics and Materials Inc., Newtown, CT) equipped with a microtip (3-mm diameter) set at an amplitude of 23% and then centrifuged at 15,000 × g for 15 min at 4 °C to pellet the unbroken cells. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C, and the microsome pellet was resuspended in LAT assay buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) at a final protein concentration of 10 mg/ml. Protein content was determined by the bicinchoninic acid assay (Pierce), using bovine serum albumin dissolved in 0.9% NaCl as a standard.

LAT Activity Assay—Neutrophil microsomes (20 μg) were incubated in LAT assay buffer (200 μl final volume) containing bovine serum albumin (12.5 μM) with AA-CoA (30 μM) and with or without thimerosal (50 μM) for 2 min at 37 °C. A mixture of 16:0a/OH-GPCho and 19:0a/OH-GPCho (5 μM each) was added, and the reaction was allowed to proceed for 30 min at 37 °C. Samples were then extracted using chloroform/methanol according to the method of Bligh and Dyer (32), dried under N₂, and resuspended in 40 μl of HPLC solvent A (1 mM ammonium acetate in 60/20/20, MeOH/AcCN/H₂O), and an aliquot (30 μl) was injected into the liquid chromatography/MS/MS system. A gradient from 0% solvent A to 100% solvent B (1 mM ammonium acetate in MeOH) was utilized to elute a C18 column (Columbus 150 × 2 mm, 5 μm; Phenomenex) at a flow rate of 200 μl/min. Mobile phase B was increased from 0 to 100% in 42 min and kept at 100% for 23 min.

Identification of the different species formed was carried out using a Sciex API 2000 triple quadrupole mass spectrometer. Mass spectrometric analysis was performed in the positive ion mode using multiple reaction monitoring of the specific transitions m/z 496 → 184 for 16:0a/OH-GPCho, m/z 538 → 184 19:0a/OH-GPCho, m/z 782 → 184 for 16:0a/20:4-GPCho, m/z 810 → 184 for 18:0a/20:4-GPCho, and m/z 824 → 184 for 19:0a/20:4-GPCho. The rate of conversion of the lysophosphatidylcholines into the corresponding phosphatidylcholines was expressed as a ratio between the peak areas of the product diacylphospholipid to the lysophospholipid substrate.

RESULTS

Leukotriene Production and Thimerosal—Leukotriene synthesis in the human neutrophil can be initiated through different stimuli. In our study, neutrophils (10 × 10⁶/ml) were pretreated with 1 nM GM-CSF for 30 min at 37 °C and then stimulated with 10⁻⁷ M FMLP for 10 min at 37 °C. Metabolite production was assessed by liquid chromatography followed by mass spectrometry through measurement of the ion abundances for the specific collision-induced transformations for 20-OH-LTB₄, LTB₅, and Δ⁸-trans-LTB₅, LTB₆, 5,6-di-HETEs, 5-HETE, and AA and the internal standards [d₆]LTB₄ and [d₅]5-HETE.

The stimulation with GM-CSF and FMLP resulted in a modest production of total LTB₅ (0.24 ± 0.09 ng/10⁶ cells) and 5-HETE (0.22 ± 0.08 ng/10⁶ cells) (Fig. 1A, inset), with total LTB₄, corresponding to the sum of LTB₄ and the ω-oxidized metabolite 20-OH-LTB₄. The small amount of LTB₅ detected was probably due to the presence of eosinophils in the cell preparation, since neutrophils do not express LTC₄-synthase, the enzyme responsible for the conversion of LTA₄ into LTC₄. The combination of GM-CSF and FMLP was necessary to achieve a detectable amount of 5-LO metabolites, since the analysis of lysates obtained from cells stimulated with either GM-CSF or FMLP alone did not show any measurable quantity of LTB₄ or 5-HETE (Fig. 2). Pretreatment of GM-CSF–primed cells with the organomercury compound thimerosal (50 μM) resulted in a dramatic increase (50-fold) in the synthesis of both total LTB₄ and 5-HETE (12.22 ± 0.35 and 10.12 ± 2.52 ng/10⁶ cells, respectively) after stimulation with FMLP (Fig. 1B and 2). Thimerosal alone or with GM-CSF did not induce the synthesis of detectable amounts of 5-LO metabolites, whereas the combination of thimerosal and FMLP yielded an increase in synthesis compared with samples from cells stimulated with GM-CSF and FMLP (0.81 ± 0.13 and 2.4 ± 0.3 ng/10⁶ cells for LTB₄ and 5-HETE, respectively) (Fig. 2), in agreement with previously published data (26). The dramatic effects on leukotriene synthesis observed when neutrophils were primed with GM-CSF and stimulated with FMLP in the presence of thimerosal sug-
gested that full cell stimulation and activation of cPLA₂ and 5-LO were required for this organomercury compound to exert its activity. The translocation of cytosolic 5-LO to nuclear membranes was also assessed in GM-CSF/fMLP-challenged neutrophils in the presence and absence of thimerosal (50 μM) by SDS-PAGE and immunoblotting. Thimerosal treatment led to a substantial increase in membrane associated 5-LO, comparable with A23187-treated cells (see supplemental Fig. 1).

The production of 5-LO metabolites in the presence of GM-CSF, fMLP, and thimerosal was induced very rapidly, with a maximal production of eicosanoids observed already at 3 min following the addition of fMLP (data not shown), and this level of total LTB₄ did not change after 30 min. In subsequent experiments, cells were challenged for 10 min to be able to make comparisons with other stimuli, such as ionomycin, that require longer incubation times. Surprisingly, the absolute quantity of leukotriene production in thimerosal-treated neutrophils was higher than that observed when these cells were challenged with the calcium ionophore A23187 (8.08 ± 0.74 ng/10⁶ cells).

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The same increase in leukotriene production by thimerosal was observed when neutrophils were primed with tumor necrosis factor α or Kdo₂-lipid A (bacterial endotoxin) and challenged with fMLP (Table 1). This effect of increasing LTB₄ production in activated neutrophils was also observed in the presence of other sulfhydryl-modifying reagents such as p-chloromercuribenzoic acid and Alexa Fluor phenylmercury (data not shown).

When different concentrations of thimerosal (5–100 μM) were tested on neutrophil preparations stimulated with a standard GM-CSF/fMLP protocol, a significant increase in total LTB₄ production was observed even at the lowest dose of thimerosal tested (5 μM) (1.81 ± 0.11 ng/10⁶ cells), with a maximal effect at ~75 μM thimerosal (13.6 ± 0.63 ng/10⁶ cells) (Table 1). When 100 μM thimerosal was added to the neutrophil samples, LTB₄ synthesis dropped to values lower (1.65 ± 0.3 ng/10⁶ cells) than those observed with 5 μM thimerosal, suggesting that at higher concentrations in the dose-response curve alternative effects of thimerosal could be observed that decreased 5-LO biosynthesis of LTA₄ from arachidonate. At these higher concentrations, thimerosal (~75 μM) has been reported to be quite cytotoxic to cells (33). The initiation of events at thimerosal concentrations below 75 μM appeared to be very fast and irreversible, since the same effects on leukotriene synthesis could be observed when thimerosal 50 μM was added to the cells 30 min before (together with GM-CSF) or at the same time as fMLP and also in experiments where the compound was removed by washing thimerosal-pretreated neutrophils with buffer to remove nonbound drug before
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TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LTB₄ (ng/10⁶ cells)</th>
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<tbody>
<tr>
<td>Resting</td>
<td>ND</td>
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<tr>
<td>GM-CSF/fMLP</td>
<td>0.24 ± 0.09</td>
</tr>
<tr>
<td>GM-CSF/fMLP/thimerosal (5 µM)</td>
<td>1.81 ± 0.11</td>
</tr>
<tr>
<td>GM-CSF/fMLP/thimerosal (25 µM)</td>
<td>9.06 ± 0.82</td>
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<td>GM-CSF/fMLP/thimerosal (50 µM)</td>
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<tr>
<td>GM-CSF/fMLP/thimerosal (75 µM)</td>
<td>13.6 ± 0.63</td>
</tr>
<tr>
<td>GM-CSF/fMLP/thimerosal (100 µM)</td>
<td>1.65 ± 0.3</td>
</tr>
<tr>
<td>Tumor necrosis factor α/fMLP</td>
<td>0.02 ± 0.0002</td>
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<tr>
<td>Tumor necrosis factor α/fMLP</td>
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<tr>
<td>kdo₂-lipid A/fMLP</td>
<td>0.06 ± 0.009</td>
</tr>
<tr>
<td>kdo₂-lipid A/fMLP/thimerosal (50 µM)</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>A23187</td>
<td>8.08 ± 0.74</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>0.52 ± 0.06</td>
</tr>
</tbody>
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stimulation with fMLP (data not shown). When neutrophils were stimulated with GM-CSF/fMLP in the absence of calcium ion (1 mM EGTA), there was no leukotriene production either in the presence or the absence of thimerosal (data not shown).

Thimerosal and Intracellular Ca²⁺—The effects of thimerosal on [Ca²⁺], in neutrophils was studied in cells loaded with Indo-1 AM. In all experiments, primed neutrophils responded to fMLP (10⁻⁷ M) with a rapid rise in [Ca²⁺], (Fig. 3). The calcium ionophore A23187 was not used in these experiments due to its intrinsic autofluorescence, but rather ionophore stimulation was carried out using ionomycin. When fMLP was added in the presence of 50 µM thimerosal, an increase in [Ca²⁺], similar to that obtained with fMLP alone was observed, but the Ca²⁺ level was consistently more sustained. Neutrophils stimulated with ionomycin (5 µM) showed a higher increase in [Ca²⁺], compared with GM-CSF/fMLP-stimulated cells in the presence of thimerosal (Fig. 3). Since leukotriene production following ionomycin stimulation of human neutrophils was very modest and below the levels observed with even the lowest concentration of thimerosal tested in GM-CSF/fMLP-treated cells, 0.52 ± 0.06 ng/10⁶ cells compared with 1.81 ± 0.11 ng/10⁶ cells for LTB₄ (Table 1), it can be concluded that the sustained calcium response in the presence of thimerosal would be necessary, but it is not sufficient to explain the effects observed on eicosanoid synthesis.

Adenosine Release and Thimerosal—Adenosine is a known regulator of leukotriene biosynthesis (34), and the effect of thimerosal on adenosine present in the extracellular media of neutrophil incubations was separately investigated. A slight increase in released adenosine was observed from unstimulated neutrophils (23.3 ng/ml) relative to GM-CSF/fMLP-treated cells (31.6 ng/ml). A similar elevation of released adenosine was observed when thimerosal was present in the control incubation (29.8 ng/ml) as well as in stimulated neutrophils in the presence of thimerosal (34.9 ng/ml).

Free Fatty Acid Levels and Thimerosal—Gas chromatography coupled to mass spectrometry was utilized to determine the exact quantity
of free fatty acids in human neutrophils stimulated with GM-CSF and FMLP in the presence or the absence of thimerosal as well as A23187. Whereas there were no significant differences in the levels of saturated fatty acids (myristic, palmitic, and stearic acid), striking differences in the levels of polyunsaturated fatty acids were observed (Table 2). In neutrophils stimulated with GM-CSF and FMLP in the presence of thimerosal (50 μM), the level of free AA was 30 times higher than in control samples (30.2 ± 5.1 ng versus 1.1 ± 0.03 ng/10^6 cells) and 3 times the amount found in cells stimulated with 5 μM A23187 (10.1 ± 0.03 ng/10^6 cells). Eicosapentaenoic acid (20:5) was 20 times higher in thimerosal-treated cells compared with control and more than double compared with A23187-treated cells (10.2 ± 3.3 versus 0.5 ± 0.1 ng/10^6 cells). Modest increases of oleic, linoleic, and linolenic acids were observed, but far below the increases observed for the polyunsaturated fatty acids arachidonate and eicosapentaenoate.

In separate experiments, exogenous arachidonate (0.3–100 μM) was added to GM-CSF–primed neutrophil suspension followed by treatment with FMLP. A dose-dependent increase in leukotriene product was observed, and at the highest arachidonate concentrations employed (100 μM), leukotriene biosynthesis was found to be 13.4 ± 1.8 ng/10^6 cells. Monodistearoylphosphatidylcholine decreased exponentially with concentration on diacylphosphatidylcholine synthesis from lysophosphatidylcholines to their corresponding tandem mass spectrometric ion transformation at corresponding retention times (Fig. 4). LAT activity was also determined in microsomes isolated from intact cells. Noncovalently attached thimerosal was washed away as a consequence of the microsome preparation. LAT activity appeared to be calcium-independent, since no significant changes in activity were observed when free Ca^2+ was removed from the incubation buffer before the addition of the lysophosphatidylcholines (data not shown). Neutrophil microsomes appeared to be more efficient in converting 16:0a/OH-GPCho rather than 19:0a/OH-GPCho into the corresponding diacylphospholipid, but in both cases, pretreatment with thimerosal (50 μM) profoundly inhibited the reincorporation of the AA-CoA into the lysophosphatidylcholines, almost 90% (Table 3). Very similar inhibitory effects on LAT in vitro activity were observed with 50 μM p-chloromercuribenzoic acid or 50 μM Alexa Fluor phenylmercury (data not shown).

Using the LAT activity assay, the dependence of thimerosal concentration on diacylphosphatidylcholine synthesis from lysophosphatidylcholine and AA-CoA was measured (Fig. 5). The production of diacylphosphatidylcholines decreased exponentially with ~50% inhibition at 20 μM thimerosal. This concentration of thimerosal was very close to the estimated concentration of thimerosal which stimulated LTB4 production to the half-maximal level (Table 1).

Since thimerosal was found to elevate intracellular Ca^2+ levels in a prolonged fashion (Fig. 3), the activity of LAT was measured at different Ca^2+ concentrations. Since EDTA was present in the assay buffer, CaCl_2 was added in amounts calculated to yield the concentrations of free calcium needed (35). LAT activity appeared to be calcium-independent, since no significant changes in activity were observed when free Ca^2+ concentration was changed in a stepwise manner from 0 to 5 μM (data not shown).

The activity of LAT was also determined in microsomes isolated from neutrophils treated with and without thimerosal (50 μM) under the same conditions used to stimulate leukotriene production (see above) in intact cells. Noncovalently attached thimerosal was washed away as a consequence of the microsome preparation. LAT was significantly inhibited in microsomes from thimerosal-exposed neutrophils compared with microsomes from untreated neutrophils (Fig. 6), supporting the assumption that thimerosal covalently binds to LAT to irreversibly inhibit its activity.

**DISCUSSION**

The biosynthesis of leukotrienes in the neutrophil is known to be the result of a complex series of reactions. Considerable focus has centered on the release of AA from membrane phospholipids by cPLA2α following translocation as well as translocation of 5-LO to the perinuclear environment. It is there that 5-LO-activating protein assembles with the other two enzymes in a manner that permits AA to enter the active site of 5-LO and be converted into LTA4 (36). The initiation of these events on the release of AA from membrane phospholipids by cPLA2α was illustrated in Fig. 4C. LAT enzyme activity in the neutrophil microsomes was expressed as a ratio between the ion abundance areas of the diacylphosphatidylcholines to their corresponding lysophosphatidylcholines precursors. The addition of thimerosal (50 μM) 2 min prior to the addition of the lysophosphatidylcholine resulted in almost a complete inhibition of the formation of phospholipids (Fig. 4D). The inhibitory effect of thimerosal on the membrane LAT enzyme was irreversible, as observed in experiments where thimerosal was removed from the incubation buffer before the addition of the lysophosphatidylcholines (data not shown). Neutrophil microsomes appeared to be more efficient in converting 16:0a/OH-GPCho rather than 19:0a/OH-GPCho into the corresponding diacylphospholipid, but in both cases, pretreatment with thimerosal (50 μM) profoundly inhibited the reincorporation of the AA-CoA into the lysophosphatidylcholines, almost 90% (Table 3). Very similar inhibitory effects on LAT in vitro activity were observed with 50 μM p-chloromercuribenzoic acid or 50 μM Alexa Fluor phenylmercury (data not shown).

Using the LAT activity assay, the dependence of thimerosal concentration on diacylphosphatidylcholine synthesis from lysophosphatidylcholine and AA-CoA was measured (Fig. 5). The production of diacylphosphatidylcholines decreased exponentially with ~50% inhibition at 20 μM thimerosal. This concentration of thimerosal was very close to the estimated concentration of thimerosal which stimulated LTB4 production to the half-maximal level (Table 1).

Since thimerosal was found to elevate intracellular Ca^2+ levels in a prolonged fashion (Fig. 3), the activity of LAT was measured at different Ca^2+ concentrations. Since EDTA was present in the assay buffer, CaCl_2 was added in amounts calculated to yield the concentrations of free calcium needed (35). LAT activity appeared to be calcium-independent, since no significant changes in activity were observed when free Ca^2+ concentration was changed in a stepwise manner from 0 to 5 μM (data not shown).

The activity of LAT was also determined in microsomes isolated from neutrophils treated with and without thimerosal (50 μM) under the same conditions used to stimulate leukotriene production (see above) in intact cells. Noncovalently attached thimerosal was washed away as a consequence of the microsome preparation. LAT was significantly inhibited in microsomes from thimerosal-exposed neutrophils compared with microsomes from untreated neutrophils (Fig. 6), supporting the assumption that thimerosal covalently binds to LAT to irreversibly inhibit its activity.

**TABLE 2**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Resting</th>
<th>GM-CSF</th>
<th>FMLP</th>
<th>GM-CSF/FMLP/thimerosal</th>
<th>A23187</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/10^6 cells</td>
<td>ng/10^6 cells</td>
<td>ng/10^6 cells</td>
<td>ng/10^6 cells</td>
<td>ng/10^6 cells</td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>3.1 ± 0.6</td>
<td>4.6 ± 2.3</td>
<td>4.4 ± 2.1</td>
<td>5.1 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>19.1 ± 0.5</td>
<td>24.4 ± 8.2</td>
<td>25.4 ± 5.9</td>
<td>26.2 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>Steric acid (18:0)</td>
<td>20.8 ± 1.5</td>
<td>29.1 ± 9.5</td>
<td>32.2 ± 5.2</td>
<td>28.6 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.5</td>
<td>6.1 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>1.1 ± 0.3</td>
<td>1.8 ± 1.0</td>
<td>7.3 ± 2.2</td>
<td>3.6 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Linolenic acid (18:3)</td>
<td>0.7 ± 0.6</td>
<td>1.3 ± 0.9</td>
<td>4.7 ± 2.4</td>
<td>4.7 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid (20:4)</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.03</td>
<td>30.2 ± 5.1</td>
<td>10.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Eicosapentaenoic acid (20:5)</td>
<td>0.3 ± 0.01</td>
<td>0.5 ± 0.1</td>
<td>10.2 ± 3.3</td>
<td>3.7 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>
generation of detectable quantities of LTB₄ in the isolated human neutrophil. The elevation of intracellular calcium is critical for the translocation events as well as for the activation of 5-LO (4, 10). The central importance of cPLA₂/H₉₂₅₁ was clearly established through studies by the groups of Shimizu (37) and Bonventre (38), who independently observed that cells derived from animals that did not express cPLA₂/H₉₂₅₁ failed to generate leukotrienes as well as other metabolites of AA and platelet-activating factor.

The results of the experiments described here suggest that other control mechanisms are in place that limit the extent to which AA is converted into LTA₄ within the neutrophil. Previous papers have investigated the effects of thimerosal on eicosanoid production in different cell types and by different stimuli with alternative hypotheses proposed to explain the mechanism of action of this organomercury compound. However, the dramatic effects of this compound on leukotriene production and polyunsaturated fatty acid release in neutrophils stimulated by the combination of GM-CSF and fMLP have not been reported. Previous reports suggest that thimerosal elevated the production of prostaglandins and leukotrienes by increasing intracellular calcium (39) or by

**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Area of phosphatidylcholine/area of lysophosphatidylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>Thimerosal (50 μM)</td>
<td>0.03 ± 0.007</td>
</tr>
</tbody>
</table>

**FIGURE 4.** Mass spectrometric analysis of LAT activity in human neutrophil microsomes. Mass spectrometric analysis of Bligh-Dyer extracts from samples of neutrophil microsomes (20 μg) (A), 16:0a/OH-GPCho and 19:0a/OH-GPCho (50 μM each) plus AA-CoA (30 μM) (B), membranes plus 16:0a/OH-GPCho and 19:0a/OH-GPCho plus AA-CoA (C), and membranes plus 16:0a/OH-GPCho and 19:0a/OH-GPCho plus AA-CoA plus thimerosal (50 μM) (D), all incubated for 30 min at 37 °C. 16:0a/OH-GPCho, 19:0a/OH-GPCho, 16:0a/20:4-GPCho, and 19:0a/20:4-GPCho were detected by electrospray ionization-mass spectrometry in the positive ion mode by monitoring the ion transitions m/z 496 → 184, m/z 538 → 184, m/z 782 → 184, and m/z 824 → 184, respectively.
Inhibiting reacylation of AA into cellular phospholipids (33). Becker et al. (39) speculated that thimerosal only slightly increased liberated AA and that its mechanism of action in increasing leukotriene biosynthesis in the neutrophil was the result of further activation of cPLA₂. Kaever et al. (33) suggested that the increased production of PGE₂ in P388D1 cells was a consequence of interfering with the fatty acid deacylation-reacylation cycle that would result in elevated concentrations of AA. Such an effect was thought to be a result of direct inhibition of LAT, as evidenced by the decrease in [14C]AA incorporated into the different phospholipid classes, most predominantly glycerophosphocholine (33).

In our studies, an induction of LTB₄ production was achieved by priming neutrophils with GM-CSF and stimulating them with fMLP. This protocol has been used by many investigators to generate leukotrienes in a physiologically relevant manner, as opposed to incubation with calcium ionophores. One of the surprising findings was that when thimerosal was present in GM-CSF- and fMLP-stimulated neutrophils, there was a greater than 50-fold increase in LTB₄ production as well as in AA release and that these increases were significantly higher than those achieved through stimulation with the calcium ionophore A23187. These studies demonstrated that the level of leukotriene production was parallel to the level of AA present in these cells. Thus, the concentration of free arachidonic acid within a cell (Table 2) can drive 5-lipoxygenase formation of LTA₄. This, as well as the observed increase in membrane associated 5-LO measured by immunoblotting in thimerosal-treated cells, was consistent with the recent observation that free arachidonate can increase 5-LO translocation to the nuclear envelope (40). However, the level of intracellular calcium ion did not follow this pattern. Whereas thimerosal increased the duration of the [Ca²⁺], spike initiated by fMLP, it was not to the extent observed for ionomycin. However, the quantity of leukotrienes produced in the presence of ionomycin or A23187 was substantially lower than that with GM-CSF/fMLP treated neutrophils in the presence of thimerosal. Together with the EGTA experiments, these results suggested that whereas the elevation of [Ca²⁺] is critically involved in the generation of leukotrienes as previously reported (5), it is not sufficient to account for the extent to which LTB₄ biosynthesis was elevated when thimerosal was incubated with neutrophils.

Since the most likely explanation for these effects was the failure of thimerosal-treated cells to reacylate AA, a direct assay to follow AA reacylation into lysophospholipids was investigated in our experimental paradigm. It was clear that the quantity of free AA present within the neutrophil was directly related to the quantity of the LTB₄ produced, no matter what stimulus was employed to release AA, elevate [Ca²⁺], and activate 5-LO. This would be the expected result if substrate availability for 5-LO were a major component driving the absolute level of leukotriene production. Since the reacylation pathway catalyzed by LAT reduces the absolute level of free AA by converting lysophosphatidylcholine into arachidonate-containing phospholipids, the reacylation pathway could be a fundamental control mechanism regulating the total quantity of leukotrienes produced within the neutrophil stimulated by receptor occupation or phagocytosis. Thimerosal, in the concentration range used for intact neutrophil studies, caused a dose-dependent inhibition of LAT activity in neutrophil microsome preparations. Moreover, the increased leukotriene synthesis observed in thimerosal-treated neutrophils correlated with the irreversible LAT inhibition.

The fact that thimerosal and other organomercury compounds studied had such a profound effect would also suggest that the oxidation of sulfhydryl groups within the active site of critical enzymes, such as LAT, may significantly alter the overall quantity of leukotrienes that result from neutrophil activation. This level of regulation of leukotriene biosynthesis has been suggested but has not been widely recognized. Rather, the activation of cPLA₂ and translocation of 5-LO are generally thought to be the critical events in the regulation of this biosynthetic pathway. Reactive oxygen species generated as a result of neutrophil activation of the NADPH oxidase respiratory burst could have an effect on the sulfhydryl-dependent reacylation pathway. For some time, it has been recognized that the reacylation pathway is sensitive to the presence of reduced sulfhydryl groups for full activity (41). In fact, when H₂O₂ was added to alveolar macrophages, a dose-dependent decrease in arachidonate reacylation could be measured (42). This effect was not due to inhibition of LAT but rather to a decrease in ATP, a cofactor required for AA-CoA generation. GM-CSF is known to induce a respiratory burst with generation of reactive oxygen species (43), and this may decrease the availability of AA-CoA, which is a substrate for LAT.

There are additional mechanisms that could reduce AA-CoA generation such as mutations of AA-CoA ligase, FACL4, which leads to mental retardation (44). However, there have not been reports of aberrant eicosanoid production in such individuals with this genetic defect.
In summary, thimerosal irreversibly interacts with LAT to inhibit reacylation of AA into phospholipids. When neutrophils were primed with GM-CSF followed by stimulation with fMLP, there was a reproducible release of AA as well as production of LTB₄. However, when thimerosal was present, there was a significant increase in the measured amounts of free AA and LTB₄. Whereas there was a prolonged increase in [Ca²⁺]ᵢ, this effect alone could not explain the enhanced synthesis of leukotrienes mediated by thimerosal. These experiments support the hypothesis of a regulatory role of the reacylation pathway on leukotriene biosynthesis and indicate that alterations in this pathway, which could involve AA-CoA ligase as well as LAT, play a central role in leukotriene production in the human neutrophil. Another feature that may explain differences in the extent to which leukotrienes are made in individuals could be genetic diversity in the reacylation pathway either through differences in the extent to which leukotrienes are made in individuals.

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REFERENCES