Eosinophils Altered Phenotypically and Primed by Culture with Granulocyte/Macrophage Colony-stimulating Factor and 3T3 Fibroblasts Generate Leukotriene C_4 in Response to FMLP

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Abstract

Normodense eosinophils failed to generate leukotriene C_4 (LTC_4) in response to incremental concentrations of FMLP but did produce LTC_4 when stimulated with calcium ionophore A23187. Normodense eosinophils, maintained in culture with 10^{-11} M granulocyte/macrophage colony-stimulating factor (GM-CSF) in the presence of 3T3 fibroblasts, became responsive to transmembrane stimulation with FMLP by day 4 with a maximal effect by day 7. After 7 d of culture, hypodense eosinophils stimulated with 2×10^{-7} M FMLP generated 26 ng $LTC_4/10^6$ cells, and LTC_4 biosynthesis was blocked by *N*-tert-butoxy-carbonyl-L-methionyl-L-leucyl-L-phenylalanine (*N-t*-BOC-MLP). Neither calcium ionophore stimulation of LTC_4 from endogenous arachidonic acid nor substrate-initiated production of LTC_4 from incorporated LTA_4 changed when eosinophils were cocultured with GM-CSF and 3T3 fibroblasts. Furthermore, when incubated with 10^{-6} M FMLP, normodense eosinophils generated no net superoxide measured by the reduction of cytochrome *c*, whereas replicate eosinophils cultured for 7 d with 10^{-11} M GM-CSF and 3T3 fibroblasts reduced a net of 17 nmol of cytochrome $c/10^6$ cells. These studies suggest that primed and phenotypically altered eosinophils present at an extravascular site may exert pathobiologic effects by responding to soluble ligands in the tissues. (*J. Clin. Invest.* 1991. 87:1958–1963.) Key words: granulocyte • cytokine • connective tissue • superoxide • PAF

Introduction

After stimulation with the calcium ionophore A23187 or after incorporation of the terminal substrate leukotriene (LT)[1] A_4, peripheral blood eosinophils selectively generate LTC_4 and are the only human leukocytes to do so (1–4). Freshly isolated eosinophils from patients with hypereosinophilic states have a relatively lesser sedimentation density (hypodense) than eosinophils from healthy donors (normodense), and these phenotypically altered eosinophils are primed for enhanced antibody-dependent cytotoxicity against *Schistosoma mansoni* larvae, superoxide generation in response to FMLP, and increased LTC_4 generation in response to calcium ionophore A23187 or immunoglobulin-coated sepharose beads (5–10). Normodense eosinophils are converted to the hypodense phenotype in vitro by coculture over a period of 4–7 d, with picomolar concentrations of granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-3, or IL-5 in the presence of mouse 3T3 fibroblasts (11–13). This in vitro cytokine-dependent coculture system provides primed eosinophils that exhibit augmented antibody-dependent cytotoxicity, generate increased amounts of LTC_4 when stimulated by calcium ionophore, and express CD4 and MHC epitopes on their membrane (11–15). Because freshly isolated eosinophils generate only small quantities of LTC_4 in response to soluble ligands, even in the presence of cytochalasin *B* (3, 16), we studied the generation of LTC_4 in response to FMLP in eosinophils that were converted to the hypodense phenotype by culture with GM-CSF in the presence of 3T3 fibroblasts. In these eosinophils, the 5-lipoxygenase pathway exhibited a substantial response to FMLP after ~ 4 d, and this response increased to day 7. This is the first example of any cell system in which activation of the 5-lipoxygenase pathway with a soluble stimulus has been comparable to that obtained with calcium ionophore.

Methods

Isolation and culture of human eosinophils. Human eosinophils were isolated from the peripheral blood of 13 donors who were healthy or were diagnosed as having allergic rhinitis, conjunctivitis, and/or asthma. In brief, individual dextran (BDH Chemicals, Poole, UK) sedimented erythrocyte/leukocyte preparations were centrifuged through discontinuous cushions of metrizamide (Nyegaard and Co., Oslo, Norway) of 18–24% (wt/vol) (11). Eosinophils from the 22/23 and 23/24 metrizamide interfaces and the cell pellet (normodense eosinophils) were recovered. Initial cell viability in all experiments was > 98% as assessed by the exclusion of trypan blue dye (Gibco Laboratories, Grand Island, NY).

Freshly isolated eosinophils ($1–2 \times 10^6$ cells) were suspended in 2 ml of enriched medium (RPMI 1640 supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 10 μg/ml of gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% fetal bovine serum [Sigma Chemical Co., St. Louis, MO]) supplemented with 10^{-10}–10^{-12} M GM-CSF (Genetics Institute, Cambridge, MA), and the cells were maintained in 35-mm plastic culture dishes in the presence or absence of a confluent monolayer of mouse 3T3 fibroblasts (line CCL 92, American Tissue Culture Collection, Rockville, MD) at 37°C in a 5% CO_2 atmosphere (11). The culture medium was changed every 48 h, and fresh GM-CSF was added to the cultures. Neutrophils, which were the primary cellular contaminant, did not survive in culture for longer than 48 h. At the desired time, the eosinophils were washed from the dishes, and their survival was determined by compar-

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1. *Abbreviations used in this paper:* ECF-A, eosinophil chemotactic factor of anaphylaxis; GM-CSF, granulocyte/macrophage colony-stimulating factor; LT, leukotriene; *N-t*-BOC-MLP, *N*-tert-butoxy-carbonyl-L-methionyl-L-leucyl-L-phenylalanine; PAF, platelet activating factor.

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ing the number of viable cells recovered with the number seeded. The cells were counted in a Neubauer counting chamber, and their viability was determined by the exclusion of trypan blue dye (11). For any time point, all functional data were based upon the number of viable eosinophils, and the data were expressed per 10^6 viable cells.

Stimulation of LTC_4 production by eosinophils and assay. Freshly isolated eosinophils and their cultured replicates were washed into 3 ml of modified Tyrode's buffer, pH 7.5, containing 0.3 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% (wt/vol) gelatin, and 20 mM L-serine. L-Serine was added to minimize the hypochlorous acid–dependent oxidative metabolism of any newly formed and released sulfidopeptide leukotrienes (3). After two successive washes with modified Tyrode's buffer at 100 *g* for 10 min at 4°C, the eosinophils were resuspended in the same buffer at a density of 0.4–2.0 × 10^6 cells/ml. Samples (500 μl) of the cell suspension were prewarmed for 10 min at 37°C in a humidified atmosphere of 5% CO_2. Duplicate or triplicate tubes of cells were stimulated with FMLP by the addition of 500 μl of prewarmed Tyrode's buffer containing twice the desired final concentration of FMLP. FMLP (Sigma Chemical Co.) was stored as a stock in DMSO, and the final concentration of DMSO in experiments with FMLP was < 0.1%. For some experiments with FMLP, the eosinophils were preincubated for 15 min at 37°C with *N*-tert-butoxy-carbonyl-L-methionyl-L-leucyl-L-phenylalanine (*N-t*-BOC-MLP). As a positive control, cells were stimulated with calcium ionophore A23187 by the addition of 500 μl of Tyrode's buffer containing 5 μM A23187, stored as a stock in DMSO (3). For some experiments, eosinophils were stimulated with an equal volume of Tyrode's buffer containing incremental concentrations of alanyl-glycyl-seryl-glutamate (Sigma Chemical Co.) (17) or platelet-activating factor (PAF) (Calbiochem, La Jolla, CA) (18). At the desired time, the activation of the eosinophils by the agonist was terminated by the addition of 2 ml of chilled methanol. The methanolic suspension of the entire reaction mixture was stored overnight under argon at 4°C to separate the supernatant from the cell pellet. For some experiments, the incubation with FMLP was terminated by immersing the tube containing the cell suspension in an ice slurry for 15 min followed by centrifugation at 1,000 *g* for 10 min at 4°C. The individual cell pellets, containing intracellular LTC_4, and the supernatants, containing released LTC_4, were individually extracted overnight with methanol and processed (3).

Immunoreactive LTC_4 was quantitated by RIA of the methanolic extracts (3, 11). In brief, the methanolic extracts were evaporated to dryness under negative pressure and resuspended in Tris-Isogel buffer. Duplicate samples were incubated for 60 min at 37°C with ^3H-labeled LTC_4 (40 Ci/mmol, New England Nuclear, Boston, MA) and immune rabbit plasma (ID_{50} values of 0.2 ng for LTC_4, 0.4 ng for LTD_4, and 0.58 ng for LTE_4). Unbound LTC_4 was removed by incubating the suspensions for 15 min at 4°C with charcoal/dextran T-40 (Sigma Chemical Co.). Synthetic LTC_4 was detectable on the linear portion of the radioligand inhibition-binding curve at concentrations ranging from 0.4 to 4.0 ng/ml. The identity of immunoreactive LTC_4 was confirmed by reverse-phase HPLC of the methanolic extract of FMLP-stimulated cells. PGB_2 (100 ng) (Cayman Chemical Co., Ann Arbor, MI) was added as an internal standard. Each sample was applied to a 5-μm, 4.6 × 250–mm C_{18} Ultrasphere reverse phase column (Beckman Instruments, Inc., Wakefield, MA) and eluted at a flow rate of 1 ml/min with a solvent of methanol/water/acetic acid (65:34.9:0.1, vol/vol), pH 5.6, containing 0.02% disodium EDTA (4). On-line ultraviolet absorbance was monitored at 280 nm for LTC_4 (retention time, 10.0±0.1 min; mean±SEM, n = 14).

Assay of cellular LTC_4 synthase activity. LTA_4 methyl ester was synthesized and provided by Drs. E. J. Corey and B. Spur (Harvard University) (19), and was hydrolyzed as described (20). Freshly isolated, cultured eosinophils were washed twice with PBS, pH 7.4, containing 2 mg/ml BSA, and resuspended in the same buffer at 2–5 × 10^6 cells/ml. Then, 1-ml portions of this suspension were prewarmed to 37°C for 5 min, mixed with 2.5 × 10^{-5} M LTA_4 (dissolved in 1 μl of methanol), and incubated for 15 min at 37°C (4). The incubation was terminated by immersing the tube containing the cell suspension in an ice slurry for 15 min, followed by centrifugation at 1,000 *g* for 10 min at 4°C. The cell pellet and supernatant were extracted overnight with methanol, and LTC_4 was quantitated by integrative optical density.

Stimulation and assay of superoxide production by eosinophils. Freshly isolated eosinophils and their cultured replicates were washed into HBSS with calcium and magnesium supplemented with 5% FBS, 1 mM Hepes, and 0.1% BSA, and were resuspended at a density of 6.25–12.5 × 10^5 cells/ml. Samples (800 μl) of the cell suspension were combined with 100 μl of freshly prepared cytochrome *c* (Sigma Chemical Co.; type V, 10 mg/ml) in HBSS without calcium and magnesium ($HBSS^-$) with or without the addition of 20 μl of superoxide dismutase (Sigma Chemical Co.; 1 mg/ml), and the tubes were placed in a shaking water bath at 37°C for 10 min. Various concentrations of FMLP were added to produce a final volume of 1 ml, and incubations were continued for an additional 15 min (21). The reactions were terminated by rapidly cooling the samples and centrifuging them at 1,000 *g* for 15 min at 4°C. The supernatants were immediately transferred to individual wells of a 96-well microtiter plate for measurement of reduced cytochrome *c* at an absorbance of 550 nm using an extinction coefficient of 18.5 mM^{-1}.

Statistical analysis. The statistical significance of differences between sample means for each set of eosinophils was based upon comparisons as determined by the two-tailed Student's *t* test.

Results

LTC_4 production by FMLP-stimulated eosinophils. Freshly isolated eosinophils, incubated for 10 or 60 min at 37°C with incremental concentrations of FMLP up to 2 × 10^{-5} M generated < 1 ng of $LTC_4/10^6$ cells (n = 3). Replicate suspensions of eosinophils stimulated for 10 min with 2.5 × 10^{-6} M calcium ionophore A23187 generated 60±18 ng of $LTC_4/10^6$ cells (mean±SEM, n = 3). Replicate eosinophils cultured for 7 d with 10^{-10}, 10^{-11}, and 10^{-12} M GM-CSF in the presence of 3T3 fibroblasts and washed before the addition of 2 × 10^{-6} M FMLP generated 13±2, 24±5, and 23±5 ng of $LTC_4/10^6$ cells (mean±SEM, n = 3), respectively. Eosinophil survival to 7 d in culture did not occur using GM-CSF concentrations < 10^{-12} M (data not shown). Because 10^{-11} M GM-CSF was the concentration which resulted in optimal eosinophil survival ex vivo to 7–14 d (12), and no significant improvement in FMLP-stimulated LTC_4 generation occurred using higher concentrations of this cytokine, all subsequent experiments were performed using eosinophils maintained in culture with 10^{-11} M GM-CSF.

Eosinophils cultured for 7 d with 10^{-11} M GM-CSF in the presence of 3T3 fibroblasts and washed before the addition of agonist generated LTC_4 in a dose-dependent fashion in response to FMLP (Fig. 1). The observed ED_{50} for LTC_4 generation was ~ 2 × 10^{-8} M FMLP, and maximum LTC_4 generation occurred at concentrations of 2 × 10^{-7} M FMLP or greater. This dose-response to FMLP was not changed by increasing the duration of incubation with the agonist to 60 min (data not shown). In order to be on the plateau of the dose-response curve, a concentration of 2 × 10^{-6} M FMLP was selected as the routine dose. When stimulated for 10 min at 37°C with 2 × 10^{-6} M FMLP, the replicate cultured eosinophils generated 42±6 ng of $LTC_4/10^6$ cells (n = 3), which was ~ 70% the amount generated by replicate eosinophils stimulated with calcium ionophore A23187.

To confirm the identity of the immunoreactive LTC_4, 6 × 10^6 eosinophils that had been cultured for 7 d with GM-CSF in the presence of 3T3 fibroblasts were stimulated for 10 min with 2 × 10^{-6} M FMLP, and a portion of the methanolic ex-

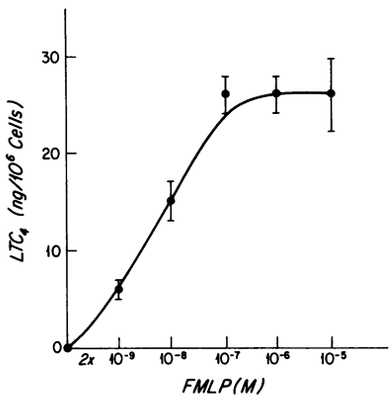

Figure 1. Dose-dependent FMLP-initiated LTC_4 production by eosinophils cultured for 7 d in 10^{-11} M GM-CSF in the presence of 3T3 fibroblasts. The eosinophils were stimulated for 10 min, the total reaction mixture was extracted with methanol, and LTC_4 was measured by RIA. Data are expressed as the mean±SEM for three experiments.

tract of the reaction mixture was analyzed for the quantity of sulfidopeptide leukotrienes by RP-HPLC. An ultraviolet absorbing peak was detected at the retention time of synthetic LTC_4; no other peaks were detected (data not shown). 1-ml fractions of the eluant were collected, evaporated to dryness under negative pressure, resuspended in aqueous buffer, and subjected to RIA. A small quantity of immunoreactive material eluted at the retention time of LTD_4 and no immunoreactivity was detected at the retention time of LTE_4. By integrated optical density and by RIA, the amounts of LTC_4 present were 58 and 42 ng, respectively.

LTC_4 was generated by eosinophils cultured for 7 d with GM-CSF and 3T3 fibroblasts in a time-dependent manner; near maximum biosynthesis was observed 5 min after the addition of FMLP (Fig. 2). In an experiment, at 1, 5, 10, and 15 min after the addition of FMLP to the cell suspension 100, 61, 39, and 30%, respectively, of the total amount of LTC_4 generated remained intracellular, and 50, 43, 43, and 28%, respectively, was intracellular in another experiment.

Kinetics of the GM-CSF-mediated acquisition of the eosinophil response to FMLP. Freshly isolated eosinophils were cultured for 1–336 h with 10^{-11} M GM-CSF and 3T3 fibroblasts, then washed and stimulated for 10 min with 2×10^{-6} M FMLP or 2.5×10^{-6} M calcium ionophore A23187. LTC_4 generation by the eosinophils in response to FMLP was acquired over several days, accelerated after day 4, and did not approach a plateau until ~ 14 d of culture (Fig. 3*A*). For calcium ionophore-stimulated cells, an ~ 2.5-fold augmentation in LTC_4 generation was noted after a 60-min exposure to GM-CSF, and remained significantly increased over the subsequent 7 d of culture (Fig. 3*B*) ($P < 0.05$ for LTC_4 generation by eosinophils exposed to GM-CSF as compared with that by freshly isolated cells). For some experiments, replicate freshly isolated eosinophils were cultured for 7 d in 10^{-11} M GM-CSF in the presence or absence of a monolayer of 3T3 fibroblasts. The cells were washed, and equal numbers of viable cells were stimulated for 10 min with 2×10^{-6} FMLP. Eosinophils cultured in the presence of a fibroblast monolayer generated 51±8 ng of $LTC_4/10^6$ cells, whereas replicate eosinophils cultured in the absence of a monolayer generated only 12±4 ng of $LTC_4/10^6$ cells (mean±SEM, $n = 3$) ($P < 0.05$).

Characterization of the ligand specificity for LTC_4 generation. Competition studies were performed with FMLP and its structural antagonist, *N-t*-BOC-MLP (3, 22). After culture for 7 d, eosinophils were washed, resuspended in buffer containing *N-t*-BOC-MLP, and incubated for 15 min at 37°C. FMLP was added, and the incubation was continued for 10 min more. Replicate eosinophils were preincubated with buffer alone and stimulated in parallel with FMLP. Eosinophils preincubated in

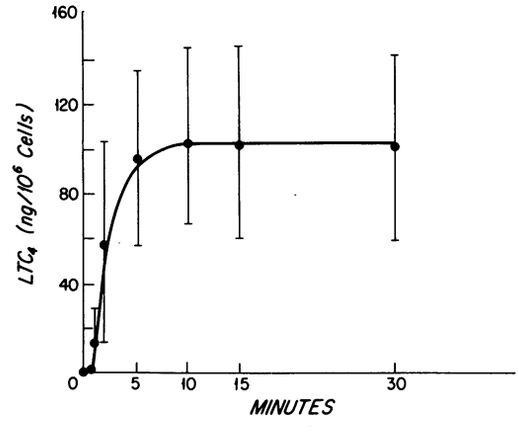

Figure 2. Time course of LTC_4 generation by eosinophils cultured for 7 d in GM-CSF in the presence of 3T3 fibroblasts. The eosinophils were stimulated with 2×10^{-6} M FMLP, and the total reaction mixture was extracted with methanol. Data are expressed as the mean±SEM for five experiments, except for the data points at 1 and 2 min, which are the mean of four experiments.

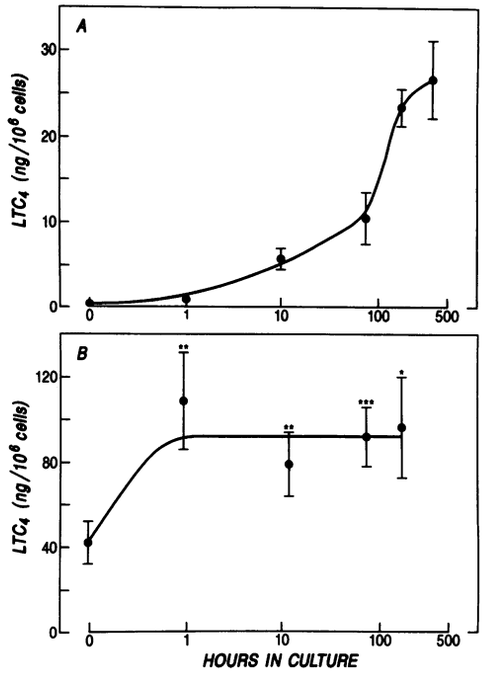

Figure 3. Time course of the change in FMLP-stimulated LTC_4 production by eosinophils maintained in coculture with GM-CSF and 3T3 fibroblasts. After the designated culture intervals, replicate eosinophils were stimulated for 10 min with 2×10^{-6} M FMLP (*A*) or 2.5×10^{-6} M calcium ionophore A23187 (*B*), and the total reaction mixture was extracted with methanol and assayed for LTC_4 by RIA. Data are expressed as the mean±SEM for four experiments in *A* and five experiments in *B*. Statistically significant increases in A23187-stimulated LTC_4 generation as compared to that of freshly isolated replicate eosinophils (*$P < 0.05$; **$P < 0.02$; ***$P < 0.01$).

buffer and stimulated with 2×10^{-6} and 0.2×10^{-6} M FMLP generated 55±7 and 45±11 ng of $LTC_4/10^6$ cells (n = 3, mean±SEM), respectively, whereas eosinophils preincubated with 2×10^{-5} M *N-t*-BOC-MLP and stimulated with the same concentrations of FMLP generated the reduced amounts of 33±7 and 16±11 ng of $LTC_4/10^6$ cells, respectively. When the *N-t*-BOC-MLP concentration was decreased 10-fold to 2×10^{-6} M, FMLP-stimulated LTC_4 generation increased to 45±6 and 36±9 ng/10^6 cells, respectively. Thus, equimolar concentrations of FMLP and *N-t*-BOC-MLP (2×10^{-6} M) permitted LTC_4 to be generated in an amount equal to that obtained with a one log lesser dose of FMLP alone.

Eosinophils were cultured for 7 d with GM-CSF in the presence of 3T3 fibroblasts, and replicate samples were stimulated for 10 min with 2×10^{-6} M FMLP or incremental concentrations of other eosinophil chemotactic agonists, eosinophil chemotactic factor of anaphylaxis (ECF-A) and PAF. In two experiments, no LTC_4 was detected from the eosinophils stimulated with concentrations of ECF-A as great as 2×10^{-5} M, whereas the FMLP-stimulated cells produced 24 and 48 ng of $LTC_4/10^6$ cells, respectively. Even in the presence of concentrations of PAF as great as 10^{-5} M, no LTC_4 was detected, whereas the FMLP-stimulated replicate eosinophils produced 14 and 42 ng of $LTC_4/10^6$ cells, respectively.

LTC_4 synthase activity of freshly isolated and cultured eosinophils. To determine LTC_4 synthase activity, freshly isolated eosinophils and replicate cells maintained in culture with GM-CSF in the presence of 3T3 fibroblasts were incubated with LTA_4 (Fig. 4). Freshly isolated eosinophils generated 206±21 ng of $LTC_4/10^6$ cells, whereas cells maintained in culture for 7 and 14 d produced 180±22 and 175±14 ng of $LTC_4/10^6$ cells, respectively. LTC_4 production from endogenously generated substrate by replicate eosinophils stimulated with 2×10^{-6} M FMLP was 0, 19, and 20 ng/10^6 cells on days 0, 7, and 14 of culture, respectively (Fig. 4) for one experiment, and 0, 21, and 20 ng/10^6 cells, respectively, in another experiment. Thus, the latency noted for the membrane-transduced LTC_4 response to FMLP was not apparent for the circumstance in which exogenous substrate interacted directly with LTC_4 synthase.

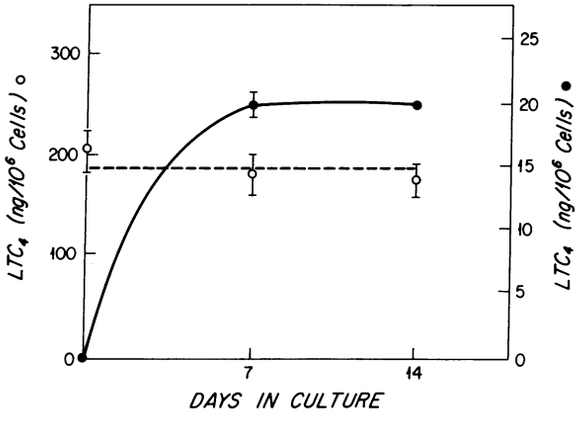

Figure 4. Influence of the duration of coculture with GM-CSF and 3T3 fibroblasts on LTC_4 synthase activity (○, left scale) and FMLP-stimulated LTC_4 production (●, right scale) by replicate eosinophils. The eosinophils were cultured for the desired intervals. To measure LTC_4 synthase activity, 2.5×10^{-5} M LTA_4 was added to the cells for 15 min, the total reaction mixture was extracted with methanol, and LTC_4 generation was quantitated by integrative optical density. FMLP-stimulated LTC_4 production was assayed by RIA of the total reaction mixture after the cells were incubated for 10 min with 2×10^{-6} M FMLP. The data are expressed as the mean±SEM for LTC_4 synthase (n = 4, except for day 14 when n = 3), and as the mean±half-range for FMLP-stimulated LTC_4 production (n = 2).

Superoxide generation by freshly isolated and cultured eosinophils. In five experiments in which freshly isolated eosinophils were stimulated with incremental concentrations of FMLP, only minimal net reduction of cytochrome c was observed at FMLP doses of $\leq 10^{-6}$ M. When stimulated with 10^{-6} M FMLP, replicate eosinophils which had been cultured for 7 d with 10^{-11} M GM-CSF in the presence of 3T3 fibroblasts reduced 21±7 nmol of cytochrome $c/10^6$ cells, and the extrapolated ED_{50} was $\sim 10^{-8}$ M FMLP (Fig. 5). In the absence of FMLP stimulation, freshly isolated eosinophils reduced 3±1 nmol of cytochrome $c/10^6$ cells, and their cultured replicates reduced 4±2 nmol of cytochrome $c/10^6$ cells. Superoxide dismutase completely abolished the spontaneous and FMLP-stimulated reduction of cytochrome c (data not shown).

Discussion

The hematopoietic generation of eosinophils from bone marrow progenitors depends upon the action of a group of glycoprotein cytokines that are segregated to the short arm of chromosome 5: GM-CSF, IL-3, and IL-5 (23, 24). These same three cytokines also alter the viability and function of mature eosinophils in vitro (11–13). This regulatory role for IL-5 has been noted in vivo in the pathologic state of the idiopathic hypereosinophilic syndrome (IHES) (25). In IHES, the cytokine-dependent priming of the eosinophils is so marked that, in comparison to the eosinophils from healthy donors, the normodense eosinophils exhibit greatly augmented antibody-dependent cytotoxicity, and the hypodense eosinophils mediate cytotoxicity in the absence of antibody directed against the target *S. mansoni* (25). In the case of eosinophils of the hypodense phenotype that are generated in vitro by culture with IL-3, GM-CSF, or IL-5, the augmentation of antibody-dependent cytotoxicity exhibited at 7 d is fully dependent upon the presence of 3T3 fibroblasts during culture, whereas the enhancement of calcium ionophore-stimulated LTC_4 generation is mediated by the cytokine alone (11, 13). Because the effect of

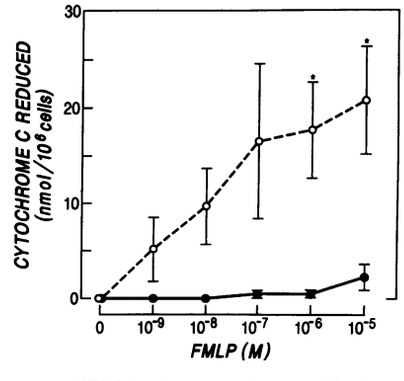

Figure 5. Effect of incremental concentrations of FMLP on net superoxide generation by freshly isolated eosinophils (●) and replicate eosinophils cultured for 7 d with 10^{-11} M GM-CSF and 3T3 fibroblasts (○). Cytochrome c reduction was monitored at 550 nm and values shown represent the mean±SEM for five experiments. *Statistically significant increase in FMLP-stimulated superoxide generation as compared with that of freshly isolated replicate eosinophils ($P < 0.05$).

cytokine priming on antibody-dependent cytotoxicity involves the transduction of a transmembrane stimulus, we examined the influence of cytokine-driven alterations in eosinophil phenotype on their capacity to generate LTC_4 in response to the soluble transmembrane agonist, FMLP.

Freshly isolated normodense eosinophils did not respond to micromolar concentrations of FMLP with the production of detectable quantities of LTC_4. However, as the eosinophils were maintained in culture with 10^{-10}–10^{-12} M GM-CSF in the presence of 3T3 fibroblasts, they became increasingly more responsive to FMLP as an agonist for LTC_4 generation. When maintained in coculture with 10^{-11} M GM-CSF and 3T3 fibroblasts, a gradual increase in FMLP-stimulated LTC_4 biosynthesis occurred over the first 4 d of culture, followed by an accelerated increase to day 7 (Fig. 3 *A*). This effect of coculture is temporally associated with a progressive conversion of the eosinophils to the hypodense phenotype (11–13, 26). LTC_4 biosynthesis in response to FMLP occurred in a dose-dependent manner, with an observed ED_{50} of $\sim 2 \times 10^{-8}$ M FMLP and was maximum at FMLP concentrations of 2×10^{-7} M or greater (Fig. 1). The quantity of LTC_4 elaborated by 7 d cultured eosinophils which were stimulated for 10 min with 2×10^{-6} M FMLP was 69±19 ng/10^6 cells ($n = 6$); replicate cultured eosinophils stimulated with calcium ionophore A23187 elaborated 111±17 ng/10^6 cells ($P < 0.10$). FMLP-stimulated biosynthesis of LTC_4 was rapid with initial intracellular accumulation (3, 4), and total LTC_4 generation was completed within 5 min (Fig. 2). Analogous to the situation for another transmembrane-mediated response, antibody-dependent cytotoxicity (11, 13), optimal expression of the FMLP-stimulated LTC_4 generation required that the eosinophils be cultured in the presence of a fibroblast monolayer.

For all experiments, there was a fivefold range for LTC_4 production by eosinophils which were cocultured with GM-CSF and 3T3 fibroblasts and stimulated with FMLP. This is comparable with previous data which revealed similar variability for LTC_4 generation by A23187-stimulated freshly-isolated normodense eosinophils (3, 11). The biochemical basis of this variability in LTC_4 generation for eosinophils stimulated with either FMLP or A23187 suggests that donor differences may exist in enzymatic activity or substrate availability for the 5-lipoxygenase pathway. The role of differential responsiveness to GM-CSF and 3T3 fibroblasts as priming factors for FMLP includes receptor number and affinity, and signal transduction may further contribute to eosinophil donor variability in LTC_4 synthesis in response to FMLP.

LTC_4 synthase, which adducts glutathione to LTA_4, is the terminal enzyme in the biosynthesis of LTC_4 (3, 20), and, analogous to the xenobiotic-metabolizing glutathionyl S-transferases, it may be inducible (27). The ability of intact eosinophils to take up LTA_4, and to further metabolize this substrate to LTC_4 in the absence of an added agonist (4), allowed a functional estimate of the cellular LTC_4 synthase activity. Over 14 d of culture in GM-CSF in the presence of 3T3 fibroblasts, no change occurred in LTC_4 synthase activity (Fig. 4). Furthermore, after 1 h of coculture with GM-CSF and 3T3 fibroblasts, the calcium ionophore-stimulated generation of LTC_4 was augmented for 7 d of observation (Fig. 3 *B*). During the same interval in which FMLP-initiated LTC_4 production was increasing progressively, the finding that coculture did not alter either the response to the substrate for LTC_4 synthase, or the postmembrane response to ionophore, suggests that this cytokine-dependent upregulation of FMLP-stimulated LTC_4 generation must occur at steps proximal to the 5-lipoxygenase.

The demonstration that the FMLP-stimulated generation of LTC_4 by eosinophils is time-dependent and cytokine-dependent suggests the occurrence of new protein synthesis relevant to signal transduction. Another study has demonstrated the biosynthesis of new proteins by hypodense eosinophils (28). In the neutrophil, a 30-min preincubation with GM-CSF augments the rise in cytosolic calcium in response to FMLP (29). Furthermore, several studies have suggested that the short-term exposure of neutrophils to GM-CSF may directly stimulate or augment phospholipase activity in response to FMLP (30–32). Even if these effects of GM-CSF are operative in the hypodense eosinophil, activation by FMLP would still require the presence of FMLP receptors, as suggested by the blocking action of *N-t*-BOC-MLP. *N-t*-BOC-MLP, a stereospecific antagonist of FMLP-induced chemotaxis, as defined in a neutrophil system (22), attenuated the ability of FMLP to stimulate LTC_4 generation. The addition of an equimolar concentration of *N-t*-BOC-MLP to the assay resulted in a decrease in LTC_4 generation to the level achieved with stimulation by a one log lesser concentration of FMLP alone. As compared to FMLP, the failure to demonstrate significant LTC_4 generation in response to stimulation with PAF and ECF-A implies some selectivity in the up-regulation of the capacity to respond to soluble ligands in the presence of a change in phenotype (16).

In contrast to freshly isolated normodense eosinophils which exhibited little capacity to elaborate superoxide in response to FMLP, replicate hypodense eosinophils generated in vitro by coculture with GM-CSF and 3T3 fibroblasts demonstrated a strikingly augmented capacity to generate superoxide when stimulated with FMLP (Fig. 5). The extrapolated ED_{50} for FMLP-stimulated superoxide generation was similar to that observed for FMLP-stimulated LTC_4 biosynthesis ($\sim 10^{-8}$ and $\sim 2 \times 10^{-8}$ M, respectively). Therefore, the eosinophils are primed to elaborate other proinflammatory mediators, in response to this soluble ligand. The greatly augmented capacity of eosinophils isolated from hypereosinophilic donors to catabolize endogenously generated sulfidopeptide leukotrienes to their subclass-specific diastereoisomeric sulfoxides and to 6-*trans*-LTB_4 diastereoisomers (1, 3) may relate to the predominant hypodense phenotype of the eosinophils from such patients and their cytokine-driven priming for superoxide generation.

The ability of the 3T3 fibroblasts to significantly augment FMLP-stimulated LTC_4 generation by hypodense eosinophils provides further evidence that the fibroblast microenvironment actively participates in the postmitotic determination of eosinophil phenotype. However, it is the GM-CSF component that fulfills the critical role of maintaining cell viability within the microenvironment, which is mandatory for the expression of functional priming by these phenotypically altered cells. Transmembrane activation of the 5-lipoxygenase pathway of human neutrophils and monocytes by particulate activators which are ingested (33, 34), human eosinophils by immunoglobulin-coated beads (35), and murine IL-3-dependent bone marrow–derived mast cells by cross-linking of the IgE receptor (36, 37) results in the generation of 7–40% of the leukotriene which is generated by calcium ionophore activation of these cells. Thus, the response of the cytokine-cultured eosinophils appears to provide a model system for the evaluation of soluble ligand–initiated transmembrane stimulation of the 5-lipoxy-

genase pathway in the absence of phagocytosis (38) or cross-linking of cell surface receptors (35).

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