LEUKOTRIENE PRODUCTION IN HUMAN NEUTROPHILS PRIMED BY RECOMBINANT HUMAN GRANULOCYTE/ MACROPHAGE COLONY-STIMULATING FACTOR AND STIMULATED WITH THE COMPLEMENT COMPONENT C5A AND FMLP AS SECOND SIGNALS

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Leukotrienes are potent cell-derived lipid mediators that play an important role in allergic and inflammatory reactions (1, 2). Leukotriene B_4 (LTB_4)[1] and its Ω hydroxylated metabolite 20-OH LTB_4 are highly active chemotactic factors and interact with specific high-affinity receptors on neutrophils (3–8). Neutrophils (PMN) are a potent major source of LTB_4 and other 5-lipoxygenase metabolites that are released in large amounts upon stimulation with calcium ionophore. Although PMN clearly possess the machinery to produce leukotrienes, little is known about the regulation of leukotriene synthesis through receptor-mediated processes (9, 10). In particular, the mechanism of activation of phospholipase A_2 in PMN is not known.

In previous studies (10), we found that the 5-lipoxygenase in resting PMN is an inactive enzyme. Upon stimulation with C5a or FMLP, however, PMN rapidly and transiently metabolize exogenous arachidonic acid (AA) into leukotrienes. Thus, the second messengers (i.e., high $[Ca^{2+}]_i$) generated through the interaction of C5a or FMLP with their respective receptors are sufficient to activate this enzyme. Indeed, more recent studies (11) on the purified enzyme show a requirement for high calcium concentration and ATP for activity. However, we did not find any measurable leukotriene generation from endogenous AA sources after stimulation with chemotactic factors, in marked contrast to Ca^{2+}-ionophore stimulation. This indicates that the regulation of phospholipase(s) activation is different from that of lipoxygenase. More recently we found (12) that the protein kinase C activator PMA rapidly primes PMN to respond to C5a and FMLP to generate leukotrienes from endogenous AA, albeit at very low concentration and only within a narrow transient time and concentration-dependent "window."

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[1] *Abbreviations used in this paper:* AA, arachidonic acid; $C5_a$, 74-amino acid glycopeptide from the 5th complement component; GM-CSF, granulocyte/macrophage colony-stimulating factor; 5-Hete, 5S-hydroxy eicosatetraenoic acid; 12-Hete, 12S-hydroxy eicosatetranenoic acid; PMN, polymorphonuclear leukocytes; RP, reverse phase; SP, straight phase.

Granulocyte/macrophage colony stimulating factor (GM-CSF) a 18–35-kD glycopeptide, is a growth factor for myeloid cells produced by antigen-stimulated PBL and stimulated macrophages, endothelial cells, and fibroblasts (reviewed in references 13, 14). Its RNA has recently been sequenced and GM-CSF has been cloned and expressed in monkey cells and *Escherichia coli* (15–18). Interestingly, GM-CSF is not only a growth factor for myeloid precursor cells, but it also stimulates the effector cell functions of mature monocytes, PMN, and eosinophils (14, 19–24). In the course of our investigations on the influence of products from stimulated PBL upon neutrophil function, we found that GM-CSF at physiological concentrations slowly primes PMN to respond to FMLP and C5a to generate relatively large amounts of 5-Hete, LTB_4, 20-OH LTB_4, and 20-COOH LTB_4. Neither C5a and FMLP nor GM-CSF alone induce leukotriene synthesis at any time point. The presence or absence of GM-CSF at inflammatory sites might therefore determine if additional effector cells are attracted by leukotrienes. In addition, these lipid mediators generated during cell activation by chemotactic factors might also have an intracellular role and be responsible for the enhancement of superoxide (O_2^-) generation by GM-CSF. Our study also shows that pre-exposure of PMN with GM-CSF not only enhances their response but qualitatively changes the secondary mediator profile of this effector cell when stimulated with C5a or FMLP.

Materials and Methods

Venous blood was collected from healthy adult donors and PMN were isolated and described (25). The cells were suspended at a density of 2–4 $\times$ 10^7/ml in Dulbecco's PBS, (Gibco Laboratories, Grand Island, NY) supplemented with 0.5 mg/ml fatty acid–free BSA (Boehringer, Mannheim, FRG) and 2 mM glucose (DPBS/A). These PMN preparations were essentially free of mononuclear cells (1%, by differential counts) and contained very few platelets, as judged by the fact that ionophore-reacted cells generated almost no 5S,12S-DiHETE. PMN suspensions (10^7 cells for RIA only or 5–10 $\times$ 10^7 cells/batch for HPLC and RIA in HPLC fractions) were incubated at 37°C in plastic tubes in a shaking waterbath. Before addition of GM-CSF, the cells were warmed up for 10 min. Incubation times were as indicated in Results, and the reaction was stopped at the indicated times by adding 2 vol of methanol.

Mediators. Lipoxygenase metabolite standards were generated, purified, and characterized exactly as reported in detail in (6, 26). Synthetic LTB_4, 20-OH LTB_4, and 20-COOH LTB_4 and LTA_4 were a generous gift from Dr. Rockach, Merk-Frosst, Pointe Claire, Quebec, Canada. FMLP was from Bachem AG (Bubendorf, Switzerland) and was dissolved in PBS. Human C5a was isolated from yeast-activated human serum as described previously (27, 28). GM-CSF was a generous gift from Dr. John F. DeLamarter, Biogen SA, Geneva, Switzerland. This recombinant *E.coli* product has the same biological profile as the glycosilated native factor (17). The activity of the human rGM-CSF (rhGM-CSF) was 10^8 U/mg protein. Units of colony-stimulating activity were calculated from the linear portion of the dose-response curve, assigning 50 U/ml to the concentration stimulating the formation of 50% maximal colony numbers (17). The GM-CSF used was endotoxin free (<0.0001 ng endotoxin/1,000 U GM-CSF). All the neutrophil stimuli were stored in small aliquots at −70°C and diluted in assay buffer just before use.

Lipid Extraction. After the time indicated, 2 vol of methanol and a constant amount of internal standard (PGB_2, Sigma Chemical Co., St. Louis, MO) were added to the cell suspension. After 30–60 min at 4°C, aggregated albumin and cell debris were removed by centrifugation. To this supernatant 2.5 vol of diethyl-ether (Fluka) were added to form

a monophase. After acidification to pH 3 with 1 N HCl, a biphasic mixture was formed by the addition of 4 vol of H_2O. The organic phase was evaporated to dryness under a stream of N_2, taken up in methanol, and passed through a C18 Sepac extraction column equilibrated in methanol. The methanol eluent was dried under N_2 before dissolving it in HPLC buffer or RIA assay buffer for HPLC measurements or RIA, respectively. Recoveries of mono Hete, LTB_4, and other DiHete and PGB_2 were identical and ranged routinely between 80 and 95%. The results were corrected according to PGB_2 internal standard recoveries. 20-OH LTB_4 and 20-COOH LTB_4 could be reproducibly recovered within a range of 38–42% recoveries of that of PGB_2 internal standard over the whole concentration range analyzed in this study. Thus, values of these polar metabolites were corrected by a factor of 2.5.

HPLC Analysis. The HPLC system was composed of two Waters Associates (Milford, MA) model 510 pumps and a Waters Associates automatic gradient controller. Samples were injected with the use of a U6IK injector (Waters Associates) and the columns (25 × 0,4 cm) and precolumns (5 × 0,4 cm) were thermostated at 35°C with a Waters Associates column oven. The stationary phases were Nucleosil C18-100-5 μm for reverse-phase HPLC (RP-HPLC) and Nucleosil silica 50-5 μm for straight-phase HPLC (SP-HPLC). The flow rate was always 1 ml/min. The elution of lipids was monitored with a Spectroflow 783 absorbance detector (Kratos Analytical Instruments, Ramsey, NJ) at 270 nM for trienes and 238 nM for dienes and was programmed for baseline adjustments and wave length changes at appropriate time intervals. For some experiments, we also used a diode-array-detector connected to a NEC PC equipped with the appropriate software (System 990, all from Waters Associates), which allowed online spectral analysis of individual peaks of the chromatogram. Data derived from the Spectroflow detector were stored and analyzed with a Chromato-Integrator D2000 (Merck Hitachi, Tokyo, Japan). For routine quantitative analysis of lipoxygenase metabolites the following HPLC gradient system was used: Buffer A, water/acetonitrile/tetrahydrofuran/acetic acid (75:25:0.15:0.01); buffer B, acetonitrile/methanol/acetic acid (57:43:0.01). Gradient-program: 0 min initial 100% A, 0% B; 0.01 min 80% A, 20% B; isocratic until 7.5 min, 7.5–8.0 min linear 80% A, 20% B–45% A, 55% B; isocratic until 17.5 min; 17.5–18.0 min linear 45% A, 55% B–25% A, 75% B; isocratic until 27.7 min; 27.7–28.2 min linear 25% A, 75% B to initial. Retention times (in minutes) of a typical RP chromatogram using this program and solvents were as follows: 20-COOH LTB_4: 12.39; 20-OH LTB_4: 13.17; 20, 5S,12S-TriHete: 14.06; PGB_2: 18.93; 20-OH 12-Hete: 20.20; 6-trans LTB_4; 20.36; 12-epi-6-trans LTB_4: 20.66; LTB_4: 21.05; 5S,12S-DiHete: 21.55; HHT:23.71; 5, 6-DiHete: 25.42 and 25.76; 12-0-met: 26.54; 15-Hete: 27.09; 12-Hete: 27.71, 5-Hete: 28.43 min.

Calculation of Data and Identification of Metabolites. Approximately every sixth run the column was calibrated with a mixture of all the lipoxygenase metabolites listed above. Dose-response curves were established for PGB_2, LTB_4, and metabolites by doubling the concentration of compounds of each run. Over the concentration range analyzed in this study, there was a linear relationship between the amount of compounds injected and the integrated area of corresponding peaks. The amount of leukotriene was calculated from the integrated area over baseline, and is expressed in picomoles of lipoxygenase metabolites generated by 10^6 PMN. Values from RIA of HPLC fractions were converted to the same measures using a molecular weight of LTB_4 of 336. For RIA (Amersham Corp., Arlington Heights, IL) of LTB_4, 1-ml fractions of the chromatograms were collected, brought to dryness under N_2, and suspended in RIA assay buffer. The identity of PMN-derived lipoxygenase products separated by RP-HPLC was further confirmed by RP-HPLC and SP-HPLC, UV spectroscopy, and in some cases (20-COOH LTB_4, 20-OH LTB_4, LTB_4, and 5-Hete), by gas chromatography–mass spectroscopy exactly as described (6, 26). Also, lipoxygenase metabolites isolated from cells reacted with GM-CSF and chemotactic factors were physicochemically identical to synthetic material. The bioactivity determined by chemotaxis experiments as described (6) was identical for synthetic and purified neutrophil-derived material (20-OH LTB_4 and LTB_4).

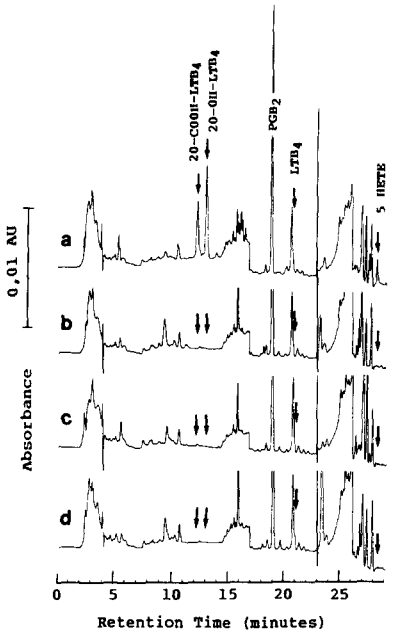

FIGURE 1. RP-HPLC of extracts from PMN. For each chromatogram extracts from 8 × 10^7 cells were analyzed, using the program indicated in Materials and Methods. The elution of the products was monitored at 270 nm from 0–23 min and at 238 nm from 23–30 min. (*a*) The cells were incubated for 2 h with 2 pM GM-CSF, then stimulated with 10^{-7} MC5a, and the reaction was stopped with methanol 5 min after C5a addition. (*b*) Neutrophils were preincubated in buffer alone for 2 h and then stimulated with C5a as in *A* for 5 min. (*c*) Neutrophils were incubated for 2 hours and 5 minutes with GM-CSF. (*d*) Cells were incubated in buffer alone for 2 h and 5 min. The elution position of purified lipoxygenase metabolites are indicated with arrows. 1-ml fractions of all chromatograms were analyzed by LTB_4-RIA and were negative except in *A*, where 922 pg LTB_4 was found at the elution position of LTB_4.

Results

Leukotriene Production of GM-CSF-primed PMN after Stimulation with C5a or FMLP. Neutrophils (80 × 10^6 cells/batch) were incubated in a shaking water bath at 37°C with (Fig. 1, *a* and *c*) or without (Fig. 1, *b* and *d*) 2 pM GM-CSF. After 2 h the cells were challenged with C5a (10^{-7} M; Fig. 1, *a* and *b*) or with control buffer (Fig. 1, *c* and *d*). 5 min after the addition of C5a, the cells and supernatants were extracted and the products formed were analyzed by RP-HPLC. Fig. 1 shows the typical chromatograms obtained in each experiment. Fig. 1 *d* represents the analysis of an extract of cells without any stimuli added. It can be seen that essentially no UV-absorbing material elutes at the retention times of 20-COOH LTB_4, 20-OH LTB_4, and 5-Hete. Unfortunately, at high detector sensitivity, UV-absorbing material was present just before and to a lesser degree after the elution position of LTB_4, rendering this region of the chromatogram more difficult to interpret. However, further experiments with RP-HPLC and SP-HPLC (6) and UV spectroscopy showed that none of these UV absorbing peaks were due to oxidized AA metabolites containing a conjugated diene or triene structure. Furthermore, LTB_4 RIAs performed from 1-ml fractions collected throughout the chromatogram were all negative (detection limit 17, 5 pg), even when extracts of up to 10^8 cells were analyzed. Thus, resting PMN do not produce detectable lipoxygenase metabolites even when incubated for prolonged periods. When PMN were incubated with GM-CSF or C5a alone, the HPLC profile was identical to the control (Fig. 1, *b* and *c*), and no LTB_4 was detected in the fractions of the chromatograms by RIA. These results indicate that none of the factors alone induces mobilization of endogenous AA and metabolism through the lipoxygenase pathway. However, when PMN were

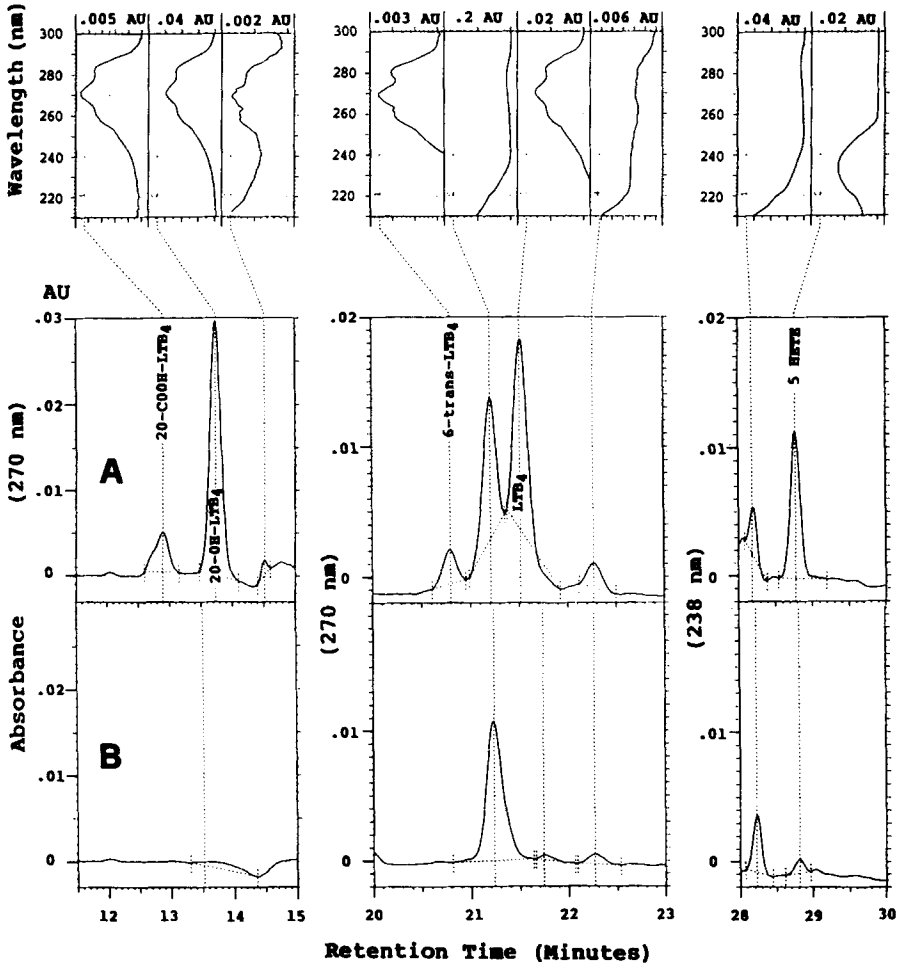

FIGURE 2. Detailed analysis of HPLC chromatograms at elution position for polar LTB_4 metabolites, LTB_4, and 5-Hete, using a Waters Associates photo-diode-array detector and the spectrum index program. The lower chromatogram (*B*) shows the extract of 8×10^7 cells preincubated in absence of GM-CSF during 2 h and then stimulated with FMLP (10^{-6} M) for 2.5 min. No lipoxygenase metabolites were detected and none of the UV-absorbing materials showed any indication of a spectrum typical of dienes or trienes. The upper chromatogram (*A*) shows a chromatogram of cells preincubated with 4 pM GM-CSF for 2 h and treated with FMLP in an identical fashion. Of each peak the UV spectrum is shown using the spectrum index program and shows for the corresponding fractions the typical spectra of LTB_4 metabolites, LTB_4, and 5-Hete.

first primed with GM-CSF and then stimulated with C5a for 5 min, relatively large amounts of 20-COOH LTB_4, 20-OH LTB_4 and 5-Hete were formed by the PMN (Fig. 1 *a*). No LTB_4 could be detected by UV spectroscopy above background, but when the more sensitive RIA for LTB_4 was performed, immunoreactive LTB_4 was detected at the elution position of LTB_4 (922 pg LTB_4). Fig. 2 shows an example of an analysis of the most important regions of two chromatograms performed with the aid of a Waters Associates photo-diode-array

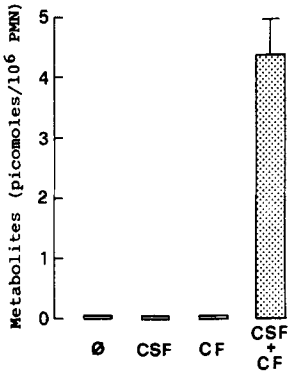

FIGURE 3. Total of AA-derived trienes formed by PMN. Columns show the sum of amounts of LTB_4 (determined by RIA in the corresponding HPLC fraction), 20-OH LTB_4 and 20-COOH LTB_4 (both determined by integration values of HPLC chromatograms), and represent the mean ± SEM of 21 experiments for each condition. Cells were incubated for 2 h in buffer alone (Ø), or with 2-10 pM GM-CSF without addition of chemotactic factors *(CSF)*, or incubated with buffer for 2 h and then stimulated with chemotactic factor, C5a (10^{-7} M) or FMLP (10^{-6} M) *(CF)*. Under these conditions amounts of trienes formed were below the detection limit, which is shown in these columns. *(CSF + CF)* Cells were preincubated for 2 h with GM-CSF and then stimulated with either C5a or FMLP for 2.5–5min.

detector and software (Spectrum index program). The lower chromatogram (Fig. 2 *B*) shows the analysis of extracts of 8×10^7 cells, stimulated with FMLP (10^{-6}M for 2.5 min). None of the peaks obtained contained a spectrum representing conjugated trienes or dienes and no LTB_4 was detected by RIA performed in HPLC fractions. The upper part (*A*) shows details of the chromatogram and corresponding spectra of the UV peaks of the same cells, primed for 2 h with a supramaximal concentration of GM-CSF and subsequently stimulated with FMLP (10^{-6} M). Note the appearance of metabolites with a typical spectrum of the corresponding lipoxygenase metabolites eluting at the expected retention times. When the reaction was stopped already 2.5 min after the addition of the chemotactic factor, as done in this example of an experiment, LTB_4 was also demonstrable by UV absorbance in addition to RIA. The identity of the

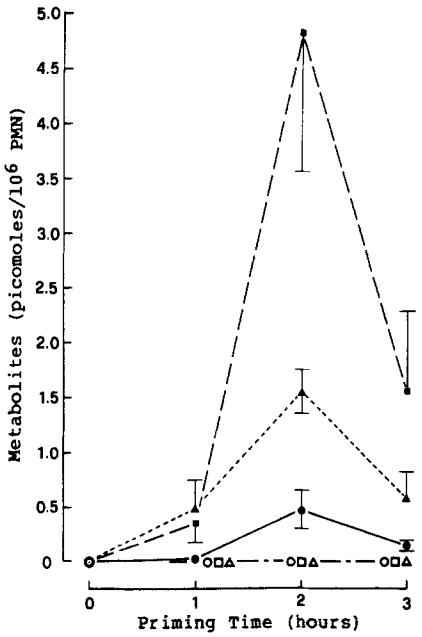

FIGURE 4. Influence of the priming time with GM-CSF upon the stimulation of chemotactic factor–induced leukotriene formation: The data represent the mean and ± SD of three experiments. The cells (8×10^7/batch) were incubated with 4 pM GM-CSF for the time indicated and then stimulated with FMLP (10^{-6} M) for 2.5 min. 20-OH LTB_4 (–■–) and 20-COOH LTB_4 (–▲–) were measured from integration values of HPLC experiments. LTB_4 (—●—) was measured with RIA from HPLC fractions at the elution position of LTB_4. The open symbols illustrate the absence of the different lipoxygenase metabolites in extracts of cells without addition of chemotactic factor. Identical results are obtained with C5a as the second signal.

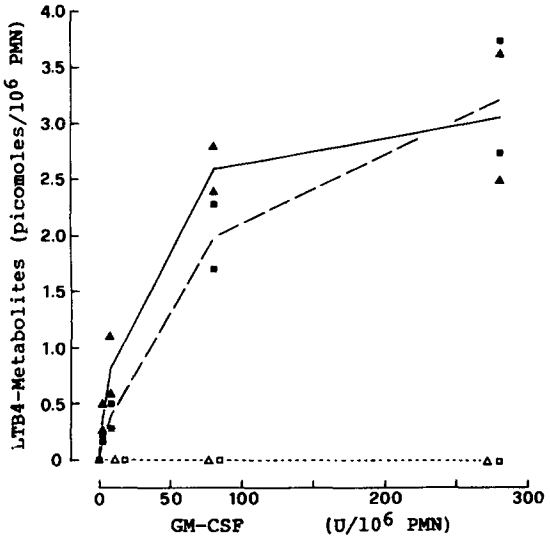

FIGURE 5. Dose-response relationship of the priming effect of GM-CSF upon the chemotactic factor induced leukotriene release: Each data point represents the values from a HPLC experiment using 8×10^7 cells/batch. The closed symbols represent data from cells stimulated with 10^{-6} M FMLP for 5 min after 2 h of preincubation with different concentrations of GM-CSF and the open symbols indicate the absence of metabolites formed when cells were incubated with GM-CSF alone. (-■-) 20-OH LTB_4, (—▲—) 20-COOH LTB_4.

different lipid metabolites as shown in Figs. 1 and 2, was also further confirmed as indicated in Materials and Methods.

In a total of 21 HPLC experiments, each performed with extracts of $6–8 \times 10^7$ PMN, we found that PMN primed with GM-CSF and stimulated with chemotactic factors produced 4.36 ± 0.95 (SEM) pM LTB_4 and LTB_4 metabolites/10^6 PMN (Fig. 3). When LTB_4 alone was measured by a combination of HPLC and RIA, it was found that 2.5 min after stimulation with C5a or FMLP of primed PMN, 0.351 ± 0.065 (SEM) pM $LTB_4/10^6$ cells (n = 13) was present in such extracts, and 5 min after stimulation the level of LTB_4 was 0.025 ± 0.004 (SEM) pM $LTB_4/10^6$ cells (n = 8). There was no statistically significant difference in the amount of products formed from primed cells subsequently stimulated with either C5a or FMLP. No LTB_4 metabolites (detection limit 0.056 ± 0.014 picomoles) could be detected in control incubations in the absence of any stimuli or with GM-CSF or C5a and FMLP alone (Fig. 3), and no immunoreactive LTB_4 was detected in the HPLC fractions (detection limit of HPLC + RIA 0.008 pM/10^6 cells).

Time Course of the Priming Effect of GM-CSF. Fig. 4 shows that a relatively prolonged incubation of PMN with GM-CSF is necessary to produce the observed change in the lipid metabolism of cells stimulated with chemotactic factors. LTB_4 and LTB_4 metabolites are reproducibly detected after a 1-h incubation of PMN with GM-CSF but a maximal enhancement occurs only after 2 h. At 3 h the effect tends to decline again. An identical time course is obtained regardless of whether C5a or FMLP is used as the second signal (data not shown). In two of four experiments we observed a small production of LTB_4 measured by RIA already after a priming period of 30 min, but LTB_4 metabolites could not be identified with certainty by HPLC. No lipoxygenase metabolites were found in the absence of a second trigger with chemotactic factors over the whole incubation period (Fig. 4, *open symbols*). Thus, the time required to

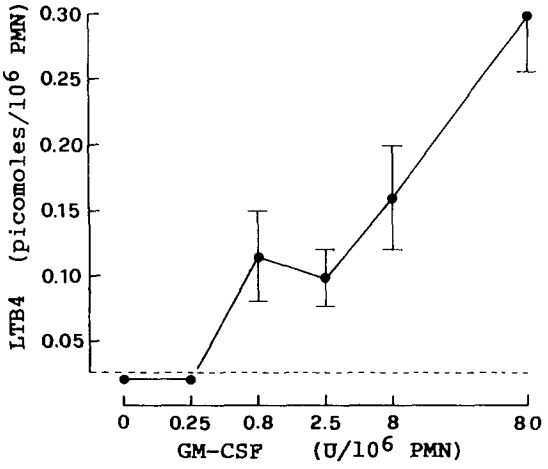

FIGURE 6. LTB_4 formation measured by RIA from cells stimulated with 10^{-6} M FMLP for 2.5 min after preincubation with various concentrations of GM-CSF during 2 h. Data shown represent mean ± SEM of two experiments performed in triplicates. No further increase of LTB_4 was observed with higher amounts of GM-CSF. The interrupted line represents the detection limit of the RIA assay.

optimally induce LTB_4 synthesis in PMN is similar to the time course for the enhancement of FMLP induced superoxide (0_2-) production (20).

Dose-Response of GM-CSF–induced Priming for Leukotriene Synthesis. PMN were incubated with different amounts of GM-CSF for an optimal time period of 2 h and were then stimulated for 5 min with 10^{-6} M FMLP before extraction and HPLC analysis. The results of such experiments shown in Fig. 5 indicated that already at 2.5 U/10^6 PMN GM-CSF (3×10^{-11} M), significant amounts of 20-COOH LTB_4 and 20-OH LTB_4 could be identified by HPLC. The effect was optimal with 80 U/10^6 PMN (10^{-9} M) and the half-maximal response was between 20 and 40 U/10^6 PMN ($2.5–5 \times 10^{-10}$ M). LTB_4 metabolites seemed even to be produced below 2.5 U/10^6 PMN, but could not be unequivocally

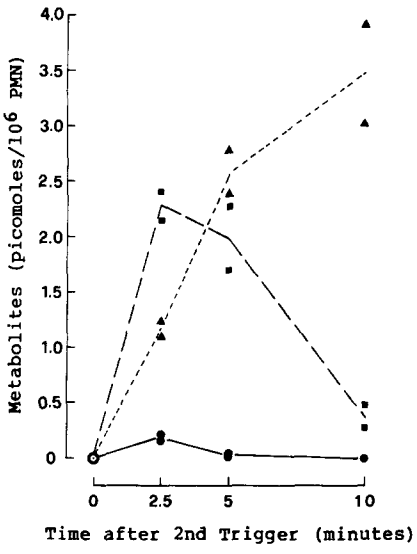

FIGURE 7. The time course of lipoxygenase metabolite formation after stimulation with 10^{-6} M FMLP. The cells were first incubated with 2 pM GM-CSF for 2 h. Each data point represents the results from a HPLC experiment using extracts from 6×10^7 cells per experiment. (—●—) LTB_4; (-■-) 20-OH LTB_4; (-▲-) 20-COOH LTB_4. 20-OH LTB_4 and 20-COOH LTB_4 were measured with HPLC and UV spectroscopy and LTB_4 was measured with RIA of corresponding HPLC fractions.

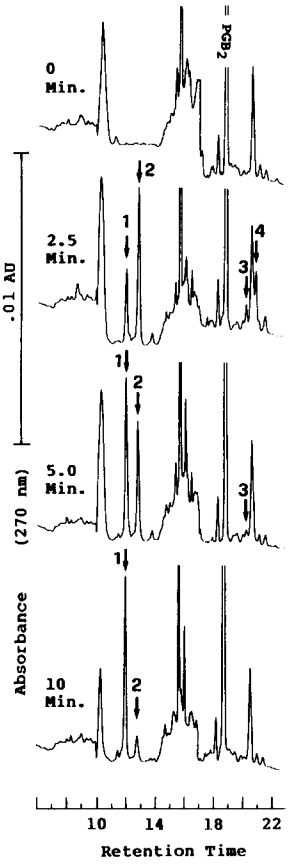

FIGURE 8. HPLC-analysis of LTB_4 and LTB_4 metabolites formed after stimulation with C5a and preincubation for 2 h with GM-CSF. The data represent examples of a typical experiment using extracts from 6×10^7 cells for each chromatogram. Arrows indicate the elution position of leukotriene standards: *1*, 20-COOH LTB_4; *2*, 20-OH LTB_4; *3*, 6-trans LTB_4; *4*, LTB_4. The reaction was stopped after C5a addition at 0, 2.5, 5, and 10 min using 2 vol of methanol. PGB_2 was used as an internal standard (PGB_2).

identified with UV spectroscopy. When LTB_4 was measured with RIA, however, significant production of LTB_4 could even be found at 0.8 $U/10^6$ PMN (10 pM), as shown in Fig. 6.

Time Course of the Mediator Profile from GM-CSF–primed PMN Stimulated with Chemotactic Factors. The induction of the synthesis of lipoxygenase products in GM-CSF–preincubated PMN occurs very rapidly upon stimulation with C5a or FMLP. Figs. 7 and 8 show that LTB_4 formed under these conditions is metabolized almost as rapidly as it is formed into 20-OH LTB_4 and more slowly into 20-COOH LTB_4. Only within 2.5 min after the addition of FMLP (Fig. 7) or C5a (Fig. 8) is it possible to detect LTB_4 by UV absorbance in HPLC chromatograms and already at this early time point the major metabolites are 20-OH LTB_4 and 20-COOH LTB_4. These results are in contrast to the mediator profiles found shortly after calcium ionophore stimulation, where LTB_4 is formed in such large amounts as to exceed the capacity of the Ω-oxidizing enzymes within the cells. Already 5 min after the addition of chemotactic factors, LTB_4 can only be demonstrated when extracts of very large numbers of cells are analyzed by a combination of HPLC and RIA.

Discussion

Our data demonstrate a dramatic effect of GM-CSF upon chemotactic factor–induced lipid metabolism in human PMN. Neither GM-CSF nor C5a or FMLP alone seems to induce a measurable generation of leukotrienes in PMN tested at any time point after the addition of the factor, under incubation conditions used in this study. PMN preincubated under optimal conditions with GM-CSF, however, produce substantial amounts of these important lipid mediators after stimulation with C5a or FMLP, albeit to a lesser extent than ionophore-stimulated PMN (9, 10, 26). Our data indicate that the synthesis of lipoxygenase metabolites from endogenous AA pools can be initiated through receptor-mediated processes by the appropriately timed combination of biological soluble inflammatory mediator peptides. In our hands, this is the first example of leukotriene synthesis in PMN induced exclusively with peptidic biomolecules. The only other stimuli found so far to induce the synthesis of lipoxygenase metabolites from endogenous AA in PMN were calcium ionophore (9, 10), a combination of phorbol esters with calcium ionophore or chemotactic factors (12, 29, 30), and phagocytosis (31). Claims that chemotactic factors (32) induce leukotriene synthesis directly and in the absence of exogenous AA could so far not be reproduced when highly purified neutrophil preparations were used, in agreement with more recent reports of other investigators (33).

Our results may be related to the recently reported effect of GM-CSF on LTC_4 synthesis and cytotoxicity in eosinophils (23). These authors observed a significant, but variable, enhancement by GM-CSF of leukotriene C_4 synthesis in eosinophils stimulated with a suboptimal dose of calcium ionophore A 12387. Although these results cannot be compared directly with our findings, since calcium ionophores are by themselves very potent stimuli for leukotriene synthesis, it seems likely to us that the underlying mechanism of this enhancement by GM-CSF is identical to the priming effect in PMN shown here. Our findings confirm and extend the recently proposed important role of GM-CSF as an inflammatory mediator and modulator of PMN and other inflammatory effector cells (19–24, 34–36), in addition to its well-recognized function as a growth factor of myeloid precursor cells (13). Other cytokines (TNF-α, TNF-β and IFN-γ) also enhance PMN functions (24, 37). The enhancement of antibody-mediated cytotoxicity is even stronger for TNF-α than for GM-CSF (24, 37) and TNFs seem to directly stimulate a respiratory burst of PMN (38). However, despite extensive experiments, we did not find leukotriene production in PMN stimulated with TNF-α alone or in a combination with chemotactic factors over a wide range of concentrations and incubation times (our unpublished results).

The biochemical mechanism by which LTB_4 is formed in PMN stimulated with GM-CSF and chemotactic factor is not known. Our previous studies (10) have shown that the second messengers formed after the interaction of C5a with its neutrophil receptors are sufficient to activate the lipoxygenase, but not enough endogenous AA is liberated and/or available at the same time the lipoxygenase is transiently active. The activation of the lipoxygenase in PMN by chemotactic factors occurs rapidly and parallels the transient elevation in $[Ca^{2+}]_i$ levels (10). Interestingly, leukotriene generation of GM-CSF primed PMN proceeds equally rapidly and transiently after stimulation with chemotactic factors as previously

shown for metabolism of exogenous AA. These observations are consistent with the hypothesis that GM-CSF does not modulate the activation of the lipoxygenase, but rather enhances the rapid release and availability of AA through the activation of phospholipase A_2 or phospholipase C/diacylglycerol lipase.

Lipoxygenase metabolites are formed very rapidly after stimulation of GM-CSF–primed PMN with chemotactic factors. LTB_4 is metabolized by the Ω oxidation pathway almost as rapidly as it is formed, and already 2.5 min after stimulation with FMLP or C5a the major products present were 20-OH LTB_4 and 20-COOH LTB_4. Obviously, Ω oxidation is a very efficient metabolic pathway in human PMN; and LTB_4 is only the major product when very large amounts of LTB_4 are formed, exceeding the capacity of the Ω-oxidizing enzymes, as for example in the case of calcium ionophore–stimulated cells. The mediator profile of lipoxygenase products shown in this study might in fact more closely resemble the conditions found in vivo than results obtained with artificial stimuli. We previously found that 20-OH LTB_4 is a potent chemotactic factor with similar activity as LTB_4 itself and proposed that this more hydrophilic mediator could play an important role in inflammation (6, 39). Our results in this study further support this hypothesis. The predominance of polar LTB_4 metabolites also clearly shows the importance of an analysis of these products in order to estimate more accurately the activity of the lipoxygenase pathway.

The priming effect upon lipid metabolism occurs at physiologically relevant concentration, commonly found in supernatants of stimulated mononuclear cells. Both the concentration dependence and the time required for optimal priming are very similar to the reported enhancement of FMLP-induced superoxide production (20), which is consistent with the hypothesis that an identical mechanism underlies both effects. It has been recently shown (34) that GM-CSF results in an increased number of low-affinity FMLP receptors, and the authors proposed that this could be responsible for the enhancement of O_2^- production. The possibility exists that this change in receptor affinity might also be responsible for the priming of leukotriene synthesis. However, GM-CSF also primes C5a responses and enhances O_2^- production in an identical manner (Dahinden, C. A. unpublished data), and up to now only a single class of high-affinity C5a receptors has been defined in PMN. Thus, the lipid mediators formed might also have an intracellular or autocoid role and in fact be responsible for the enhancement of O_2^- production. Indeed, the lipid mediators are formed at the same time O_2^- is generated after FMLP stimulation, and several lipids like 5-Hete, LTB_4, and platelet-activating factor enhance effects of FMLP and C5a even at low concentrations, which are by themselves not stimulatory (40, 41, and our unpublished results). Clearly, more work is required to establish the relationship between the enhancement of O_2^- production and leukotriene release.

In summary, we find that GM-CSF qualitatively changes the lipid mediator profile of PMN stimulated with chemotactic factors. These lipid mediators formed might be important in amplifying and modulating inflammatory reactions or might also result in a change of effector cell functions. Our study further documents the possible central role of GM-CSF as an inflammatory mediator.

Summary

Neutrophils (PMN) preincubated with recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF) for 2 h and then stimulated with the chemotactic factors, C5a or FMLP, produce substantial amounts of the lipoxygenase products 5-Hete, LTB_4, and Ω-oxidised LTB_4 metabolites (4.36 ± 0.95 (SEM) pM ($n = 21$) LTB_4 and LTB_4 metabolites/10^6 PMN). No lipoxygenase metabolites are detected by HPLC and RIA if purified PMN are stimulated by either GM-CSF or chemotactic factors in the absence of exogenous arachidonate. The priming effect of GM-CSF upon chemotactic factor induced generation of lipid mediators is a relatively slow process, clearly evident after 1 h and optimal after 2 h. Leukotriene generation is measurable with 0.8 U GM-CSF/10^6 PMN and is maximal with 80 U (10^{-11}–10^{-9} M). Upon activation of primed PMN with chemotactic factors, leukotriene synthesis is induced very rapidly. Already 2.5 min after activation the major lipoxygenase metabolites present are 20-OH LTB_4 and 20-COOH LTB_4. Our study shows that the synthesis of lipoxygenase metabolites from endogeneous AA can be initiated in PMN through receptor mediated processes by the appropriately timed combination of biological soluble inflammatory mediator peptides. Furthermore, these results indicate that GM-CSF not only enhances effector cell functions but can qualitatively change the mediator profile formed after activation with a second triggering signal. Such a mechanism might be important in amplifying inflammatory responses. Alternatively, lipid mediators formed might also have an intracellular or autocoid role and be responsible for the enhancement of other PMN functions like oxygen radical release.

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