Granulocyte-Macrophage Colony-stimulating Factor Is a Stimulant of Platelet-activating Factor and Superoxide Anion Generation by Human Neutrophils

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Human granulocyte-macrophage colony-stimulating factor (GM-CSF) was studied for its ability to stimulate the synthesis and release of the inflammatory mediator platelet-activating factor (PAF) from human neutrophils as measured by bioassay and incorporation of \[^{14}C\]arachidonic acid into PAF. GM-CSF stimulated the synthesis but not the release of PAF from neutrophils. PAF synthesis took place in a time- and concentration-dependent manner, was dependent on a pertussis toxin-sensitive G protein and could be inhibited by antibodies to GM-CSF. On the other hand, pre-incubation of neutrophils with GM-CSF followed by stimulation with the bacterial tripeptide formylmethionylleucylphenylalanine caused PAF synthesis and release. The effect of GM-CSF was qualitative and not simply the result of larger amounts of PAF being synthesized since similar amounts were generated in response to the calcium ionophore A23187 but no released PAF could be detected. In functional studies GM-CSF stimulated superoxide anion generation from neutrophils with a time- and dose relationship that paralleled PAF synthesis. In addition, the serine protease inhibitor L-1-tosylamide-2-phenylethyl chloromethyl ketone, which inhibits PAF synthesis, reduced PAF accumulation as well as superoxide generation, raising the possibility of a causal relationship between cell-associated PAF and cell activation. These results identify PAF as a direct product of GM-CSF stimulation in neutrophils where it may play a role in signal transduction and demonstrate that PAF is released only after subsequent neutrophil stimulation. The selective release of PAF may play a role in regulating and amplifying the inflammatory response.
Lymphoprep (Nycoderm, Oslo, Norway) followed by hypotonic  ysis of erythrocytes using 0.2% sodium chloride solution. This solution was then brought to the correct osmolality using a 1.6% sodium chloride solution. The cell preparations were resuspended to various concentrations ranging from 10^6 to 5 x 10^7/ml in RPMI 1640 (pH 7.2) plus 0.1% bovine serum albumin (BSA) Fraction V, fatty acid-free (Boehringer, Sydney, Australia) and 20 mM HEPES (Sigma). The assays were carried out in this medium unless otherwise stated. This method yielded cells which were >99% viable by trypan blue exclusion and >98% identifiable as neutrophils.

**Recombinant Human Granulocyte-macrophage Colony-stimulating Factor and Other Reagents**

Recombinant human GM-CSF was from lot no. 9A01N013 containing 0.3 x 10^9 units/ml and was 99% pure. This was generously provided by Dr. S. C. Clark (Genetics Institute, Cambridge, MA). The endotoxin content of this material and of GM-CSF diluted in medium was <0.002 ng/ml as measured by the limulus amebocyte lysate assay. rh tumor necrosis factor (TNF)-α was from lot no. 2905-55 containing 5 x 10^8 units/ml and was 99.8% pure (generous gift from Genentech, South San Francisco, CA). Anti-GM-CSF and anti-interleukin-3-specific sera were generous gifts from Genetics Institute. Purified pertussis toxin was a gift from Dr. G. Barrit (Flinders Medical Centre, Adelaide, Australia). The lipids were extracted from the stimulated neutrophils using the Bligh/Dyer extraction method. Initially, the reaction was terminated by the addition of 2 ml of methanol (1% acetic acid) to the neutrophil suspension (0.9 ml). The precipitate was removed by centrifugation at 4 °C. 1 ml of chloroform was added to the supernatant, and the tubes were vortexed to extract the PAF and other phospholipids. Phase separation was achieved by the addition of 2 ml of chloroform/H2O (90:10), and the lower phase was evaporated to dryness under reduced pressure. PAF in the chloroform phase was then separated from other lipids on TLC. The samples were visualized with iodine vapor and scraped from the TLC plates in a narrow zone based on their co-migration with authentic PAF. "RESULTS". Purified unlabeled PAF was used when separating PAF labeled by [3H]acetate incorporation. The bioactivity of the samples from all zones of the TLC plates was quantified by the platelet bioassay.

**Superoxide Anion Generation**

Superoxide anion release was measured in a colorimetric assay based on the reduction of ferrocytochrome c. Briefly, 100 μl of purified neutrophils (10^6 cells) was added to 900 μl of freshly prepared cytochrome c (Sigma, type VI; 12.4 mg/ml). Various concentrations of cytokines were added in volumes of less than 10 μl and the reaction mixtures were made up to 1 ml with medium and incubated in polypropylene tubes that were continuously shaken. The mixtures were then incubated at 37 °C for various periods of time up to 60 min (see "RESULTS"). Under these conditions, >98% neutrophils could be recovered indicating that little or no adherence to the surface of the tubes had occurred. Cells were incubated with FMLP (Sigma) or with PMA (Sigma) for a further 15 min. After incubation, the cells were rapidly cooled and pelleted at 4 °C, and supernatants were transferred to plastic disposable cuvettes (Kartelle Plastics, Adelaide, Australia). Superoxide production was measured by the reduction of cytochrome c at 550 nm using a DU-50 spectrophotometer (Beckman Instruments, Palo Alto, CA). Levels of superoxide were quantitated as described previously (9).

**Statistical Analysis**

All experiments were performed 2-6 times. Within one representative experiment, means of values were compared by the Student's t test. When evaluating data from several donors, the Wilcoxon's matched pairs test was used.

**RESULTS**

**Characterization of GM-CSF Stimulation of PAF Synthesis in Human Neutrophils—GM-CSF stimulated PAF synthesis in human neutrophils. In experiments using neutrophils from 16 different individuals, GM-CSF consistently increased the levels of cell-associated PAF (Fig. 1). FMLP also stimulated PAF synthesis; however, most individuals responded more to GM-CSF than to FMLP. In these experiments no PAF was detected in the cell supernatants (limit of detection, 100 fmol/ml). The stimulation of PAF synthesis by GM-CSF was characterized by carrying out time course and dose-response experiments. A time course experiment with 3 x 10^-11 M GM-CSF representative of four others showed that the optimal time of GM-CSF stimulation was 45 min with a clearly detectable effect at 10 min (Fig. 2A). During this time, the levels of PAF in neutrophils incubated with medium alone remained low and relatively constant. A dose-response experiment with GM-CSF representative of six others showed that the maximal stimulation of PAF accumulation could be achieved at a concentration of 3 x 10^-10 M, with a 50%
maximal stimulation at approximately $3 \times 10^{-11}$ M (Fig. 2B). While GM-CSF or FMLP, used separately, directly stimulated PAF synthesis, GM-CSF and FMLP showed a synergistic effect when used sequentially. In this case, the amount of cell-associated PAF was 5,945 fmol/10^7 cells (mean of 14 experiments), a value significantly higher than those obtained with GM-CSF (1,086 fmol/10^7 cells) or FMLP (935 fmol/10^7 cells) alone ($p < 0.001$ by the Wilcoxon's matched pairs test).

To establish that PAF accumulation in neutrophils was stimulated by GM-CSF and not a contaminant, two types of controls were carried out. First, endotoxin levels were measured in the medium containing GM-CSF. By the limulus amebocyte assay this preparation contained <0.002 ng/ml endotoxin. Second, the use of a polyclonal anti-GM-CSF antibody inhibited the ability of GM-CSF to directly stimulate PAF synthesis and to prime neutrophils for an enhanced response to FMLP (Table I).

The involvement of G proteins in the stimulation of PAF synthesis by GM-CSF was examined by pre-incubating neutrophils with 500 ng/ml pertussis toxin, a concentration previously shown to be optimal in inhibiting FMLP stimulation (24). Pertussis toxin inhibited PAF accumulation in neutrophils stimulated with GM-CSF, TNF-α, or FMLP (Fig. 3). This inhibition was due to a direct effect on G proteins and not to a toxic effect on neutrophils since pertussis toxin did not inhibit stimulation of PAF accumulation by PMA, an agonist that directly interacts with protein kinase C.

In order to seek further evidence that GM-CSF stimulates PAF synthesis, a second technique was used. Neutrophils were pre-incubated with [³H]acetate and examined for the amount of [³H]acetate incorporated into a polar lipid that comigrated with authentic PAF on TLC following stimulation. GM-CSF was found to stimulate the incorporation of [³H]acetate into PAF (Table II).

*GM-CSF Primes Neutrophils for PAF Release*—Although GM-CSF stimulated PAF synthesis in human neutrophils this remained cell-associated and no PAF could be detected in the cell supernatants under these conditions (Table III) or using a range of cell numbers (10^6 to 5 x 10^6 neutrophils/ml) or of BSA concentrations (0.01-1%) in the medium (data not shown). In addition, we examined the possibility that endogenous inhibitors of PAF-induced aggregation were masking the release of PAF. Supernatants from FMLP-stimulated neutrophils had no effect on the ability of synthetic PAF (6-600 fmol) to induce platelet aggregation.

However, PAF release was detected in neutrophils primed with GM-CSF and stimulated with FMLP (Table III). As a control, neutrophils were stimulated with the calcium ionophore A23187. In this case equally large amounts of PAF were cell-associated but no PAF release could be detected, indicating that the PAF detected in the supernatants of neutrophils treated with GM-CSF and FMLP is not simply a result of the increased levels of cell-associated PAF but of a qualitative
TABLE I
Specificity of GM-CSF-mediated stimulation of PAF production in human neutrophils

| Treatment                          | f mol PAF/10^7 neutrophils |
|-----------------------------------|-----------------------------|
| Medium                            | 262 ± 39*                   |
| GM-CSF                            | 678 ± 27                    |
| GM-CSF + anti-GM-CSF              | 178 ± 37                    |
| FMLP                              | 636 ± 42                    |
| GM-CSF + FMLP                     | 9515 ± 3626                 |
| GM-CSF -anti-GM-CSF + FMLP        | 804 ± 423                   |

*Mean ± S.D. of an experiment performed in triplicate and representative of two others. GM-CSF was used at 10^{-10} M and FMLP at 10^{-7} M. Anti-GM-CSF was used at a 1/1000 dilution.

DISCUSSION

This study shows that human GM-CSF stimulates the synthesis of PAF in human neutrophils, and that PAF is released upon subsequent stimulation with FMLP. The release of PAF from human neutrophils in vivo may amplify the inflammatory response by increasing vasopermeability, enhancing the recruitment of leukocytes, and directly activating and priming granulocyte function.

Our results disagree with a previous report in which GM-CSF was shown not to stimulate PAF formation in neutrophils (26). This discrepancy may be explained by the use of a very sensitive bioassay in the present studies. However, we also detected PAF formation by measuring the incorporation of [3H]acetate into PAF which was the method used in the previous study. Neutrophils were incubated with [3H]acetate throughout the exposure to GM-CSF, whereas in the previous report (26) [3H]acetate was added to the cells only for the last 10 min of GM-CSF incubation. The latter approach may be suboptimal for detection of GM-CSF effects. In addition, the [3H]acetate-labeling technique appears to be a less sensitive index of PAF formation than measuring endogenous PAF levels since in our experiments GM-CSF increased the latter by 2.8-fold, whereas acetate incorporation increased by 1.8-fold. This may be related to the bioassay measuring net amounts of PAF regardless of the pathways involved, whereas in the [3H]acetate method the label is incorporated into several pools including a PAF precursor pool, and assumes the specific activity of [3H]acetyl-CoA remains unchanged following stimulation.

Specificity controls demonstrated that the stimulation of PAF synthesis was due to GM-CSF and not to lipopolysaccharide, a bacterial product that induces PAF production in neutrophils at concentrations above 10 ng/ml (27). First, the experiments described here used highly purified GM-CSF which contained undetectable levels (<0.002 ng/ml) of lipopolysaccharide at the highest concentration used. Second, a polyclonal antibody specific for GM-CSF significantly re-
TABLE III

| Experiment | Treatment | Cell-associated | Cell supernatant |
|------------|-----------|-----------------|------------------|
| I          | Medium    | 0               | 0                |
|            | GM-CSF (10^{-9} M) | 194.2 ± 15.7*   | 0                |
|            | FMLP (10^{-7} M) | 95.4 ± 6.6      | 0                |
|            | GM-CSF (10^{-9} M) + FMLP (10^{-7} M) | 2,168.9 ± 669.0 | 4,080.4 ± 988.9 |
|            | A23187 (10^{-5} M) | 3,231.2 ± 555.2 | 0                |
| II         | Medium    | 58.8 ± 4.2      | 0                |
|            | GM-CSF (10^{-9} M) | 174.2 ± 23.8    | 0                |
|            | FMLP (10^{-7} M) | 943.0 ± 214.2   | 0                |
|            | GM-CSF (10^{-9} M) + FMLP (10^{-7} M) | 18,145.3 ± 3,507.0 | 468.0 ± 39.4 |
|            | A23187 (10^{-5} M) | 13,472.0 ± 2,088.9 | 0                |
| III        | Medium    | 0               | 0                |
|            | GM-CSF (10^{-9} M) | 65.5 ± 3.1      | 0                |
|            | FMLP (10^{-7} M) | 100.0 ± 3.3     | 0                |
|            | GM-CSF (10^{-9} M) + FMLP (10^{-7} M) | 8,086.2 ± 2,944.0 | 172.0 ± 20.8 |
|            | A23187 (10^{-5} M) | 7,383.7 ± 871.8 | 0                |

* Mean femtomoles of PAF/10^7 cells ± S.E. of triplicate determinations. Incubations with medium and with GM-CSF were for 45 min at 37 °C, with FMLP for 15 min at 37 °C and with A23187 for 5 min at 37 °C. 0 = less than 100 fmol/ml. Some WEB 2086-resistant platelet aggregation was observed in supernatants of A23187-treated neutrophils probably resulting from a direct effect of A23187 on the platelets.

![Fig. 4. Stimulation of superoxide anion production by GM-CSF. A, time course of superoxide anion production in neutrophils stimulated with 10^{-9} M GM-CSF (●) or incubated in medium (■) at 37 °C. B, dose response of GM-CSF. Each value is the mean of triplicate determinations and the bars span the S.E.](image)

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duced GM-CSF-stimulated PAF synthesis and superoxide anion generation.

PAF production required a relatively prolonged exposure of neutrophils to GM-CSF. PAF was detectable by 10 min and plateau levels were reached by 45 min. In contrast, FMLP has been shown to maximally stimulate PAF formation at 5 min (28). In other experiments not shown here, 300 pM GM-CSF and 45 min preincubation were also optimal in priming neutrophils for enhanced PAF production in response to subsequent stimulation with FMLP.

The synergistic effect of GM-CSF and FMLP in PAF formation may be related to the combined effect of two second messenger pathways involving phospholipase A\(_2\) and phospholipase C. Thus both GM-CSF and FMLP liberate arachidonic acid (16, 19) probably by activation of phospholipase A\(_2\), which may lead to the accumulation of lyso-PAF and conversion to PAF by the rate-limiting enzyme acetyltransferase. Both phospholipase A\(_2\) and acetyltransferase can be activated by phosphorylation and calcium (29).

Stimulation of PAF biosynthesis by GM-CSF was dependent on a pertussis toxin-sensitive G protein. Pre-incubation of neutrophils with pertussis toxin did not significantly reduce either the synthesis of PAF or superoxide anion generation in response to PMA, indicating that the inhibition of GM-CSF-induced PAF synthesis was specific. These results are consistent with the GM-CSF receptor being coupled to a pertussis toxin-sensitive G protein for the stimulation of some neutrophil functions, the others being induction of c-fos mRNA (30), and the uptake of phosphate (31).

An important finding was the demonstration that the synthesis of PAF stimulated by either GM-CSF or FMLP remained cell-associated. PAF, however, could be readily detected in the cell supernatants after sequential stimulation with GM-CSF and FMLP. This did not appear to be simply due to an overspill of the now much increased cell-associated PAF into the supernatants since the calcium ionophore A23187 stimulated similar cell-associated levels of PAF but no detectable release, suggesting that GM-CSF induces qualitative changes in human neutrophils leading to PAF release. This was a consistent finding observed in 10 of 11 individuals examined. Neutrophil heterogeneity in the amount of PAF produced and in the relative amounts secreted were also...
Fig. 5. GM-CSF-mediated stimulation of O$_2^-$ production in human neutrophils. A, specificity of GM-CSF stimulation. Anti-GM-CSF and anti-interleukin-3 (αIL-3) sera were used at 1:1,000. The bars span the S.E. The values obtained in the presence of anti-GM-CSF serum were significantly different (p < 0.05) from those obtained in the presence of GM-CSF alone. B, inhibition by pertussis toxin. Neutrophils were pretreated with medium (open bars) or with 500 ng/ml pertussis toxin (hatched bars) for 90 min before incubation with 3 × 10$^{-8}$ M GM-CSF, 10$^{-7}$ M TNF-α, 10$^{-7}$ M FMLP, or 1.6 × 10$^{-8}$ M PMA. The bars span the S.E. The values obtained with pertussis toxin were significantly different (p < 0.05) in neutrophils treated with GM-CSF, TNF-α, and FMLP.

Table IV

| Stimuli | PAF | O$_2^-$ | LDH release |
|---------|-----|---------|-------------|
| None    | 0   | 0       | 0           |
| GM-CSF  | 10  | 10      | 10          |
| GM-CSF + TPCK | 20 | 20      | 20          |

*Femtomoles of PAF/10$^7$ neutrophils. Mean ± S.E. of one experiment performed in triplicate and representative of two others.

**Nanomoles of O$_2^-$/10$^6$ neutrophils. Mean ± S.E.

Units/liter. The release of lactate dehydrogenase in sonicated neutrophils was 992 units/liter. GM-CSF was used at 3 × 10$^{-8}$ M.

noticed, consistent with several reports showing individual variability in the neutrophil response to GM-CSF (1, 2, 9, 32, 33).

The release of PAF has important implications since PAF is a powerful modulator of inflammation, increasing vascular permeability (34), chemotaxis of leukocytes (35), and the release of cytokines from monocytes and macrophages (29). In addition, local levels of neutrophil-derived PAF may act in an autocrine manner to directly stimulate granulocyte function as shown in vitro with high doses of PAF or to "prime" neutrophils as shown with lower doses of PAF (36). In this respect, PAF would operate as an autocrine factor capable of feeding back on the same cells that release it.

The observation that GM-CSF induced PAF biosynthesis is intriguing and raises the possibility that PAF functions as a second messenger in neutrophils (23, 37, 38). In an effort to relate PAF generation to neutrophil function we investigated the effect of GM-CSF in stimulating the neutrophil respiratory burst. We found that GM-CSF caused significant O$_2^-$ production which was specific, since it could be inhibited by antibodies to GM-CSF, and was mediated through a pertussis toxin-sensitive G protein. Previous experiments by us and others (9, 39, 40) showed that GM-CSF enhanced O$_2^-$ production in response to a second stimulus while by itself having little or no effect. The inability to detect stimulation of O$_2^-$ generation by GM-CSF alone was probably due to the fact that cytochrome c was present only during the second incubation (5 min) with FMLP but was not included in the first incubation with GM-CSF. Superoxide anion, having a half life of the order of milliseconds, would have dissipated spon-
taneously before being able to reduce cytochrome c. Similar results have been recently reported with adherent neutrophils where O$_2^-$ (41) and H$_2$O$_2$ (42) were measured. In these cases GM-CSF stimulated O$_2^-$ and H$_2$O$_2$ production from adherent neutrophils with a similarly prolonged time course. Although the neutrophils used in the present study were not adherent to the incubation tubes we cannot rule out some transient cell-cell or cell-tube contact nor some activation during the purification procedure (43). Thus these experiments cannot unequivocally distinguish between a direct effect of GM-CSF and an enhancing effect on preactivated cells.

The stimulation of O$_2^-$ production by GM-CSF provides an important functional correlate to PAF production. The temporal correlation between PAF generation and O$_2^-$ release can also be extended to other functions including surface receptor upregulation (9), adherence (33), and degranulation (44). Inhibition experiments also seem to support the notion of PAF playing a role in signal transduction. Thus, inhibition of GM-CSF stimulated PAF synthesis by the serine protease inhibitor TPCK, a compound previously used to inhibit TNF-α-stimulated PAF production (25), caused a parallel decrease in superoxide anion release (Table IV) and in adherence to human endothelial cells,2 showing that H$_2$O$_2$ may be bound immediately to specific surface receptors or with the release of the vital enzyme lactate dehydrogenase (Table IV). However, TPCK could affect other pathways and its inhibitory effect suggests but does not prove a causal relationship between PAF synthesis and superoxide production. In a study using rabbit neutrophils, the specific PAF receptor antagonists WEB2086 and CV6209 inhibited FMLP-induced superoxide anion production and leukotriene B$_4$ formation (38), suggesting a role for PAF in the signal transduction process.

The accumulation of GM-CSF-stimulated PAF may be also related to the priming effect of this cytokine. Small amounts of PAF may be bound immediately to specific surface or intramembranous receptors causing priming of the cells. The use of established PAF antagonists as well as developing antagonists that cross the cell membrane should facilitate the testing of these hypotheses.

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