Differential Activation of the Endogenous Leukotriene Biosynthesis by Two Different Preparations of Granulocyte-Macrophage Colony-Stimulating Factor in Healthy Volunteers


Results from in vitro investigations and recent data obtained in patients with drug-induced cytopenia or myelodysplasia suggest that leukotrienes may be involved in mediating some of the actions of granulocyte-macrophage colony-stimulating factor (GM-CSF). In the present study, the possible role of leukotrienes was further characterized in 21 healthy individuals to avoid modification of response to GM-CSF by disease-specific variables. The effects of two different preparations of human recombinant GM-CSF, i.e., glycosylated GM-CSF as expressed in a Chinese hamster ovary carcinoma (CHO) cell line and nonglycosylated GM-CSF obtained from Escherichia coli, were compared. GM-CSF was administered subcutaneously at a single dose of 0.7 nmol/kg body weight. Pharmacokinetic parameters and hematopoietic and adverse effects were monitored by blood analyses or physical examination, respectively. Leukotriene generation in vivo was evaluated by determination of leukotriene E₂ and N-acetyl-leukotriene E₄ in urine. After the injection of GM-CSF from E. coli, serum concentrations increased and decreased more rapidly and reached a 2.3-fold higher maximum compared with GM-CSF from CHO. GM-CSF induced a biphasic change in leukocyte counts that proceeded considerably faster after the E. coli preparation than after GM-CSF from CHO. The urinary leukotriene concentration increased 1.3- to 14-fold or 2.1- to 44-fold after the administration of GM-CSF from CHO or E. coli, respectively. Urinary leukotriene concentrations correlated significantly with the maximum of basophil counts and correlated with the occurrence of some adverse reactions, i.e., flu-like symptoms, bone pain, or dyspnoea. Our data confirm the conception that leukotrienes may play a significant role in GM-CSF action in vivo. They especially direct attention to the possible relevance of leukotrienes to untoward effects of GM-CSF treatment.

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MATERIALS AND METHODS

Volunteers. The study was conducted with 21 healthy male individuals without any evidence of disease from anamnestic exploration, physical, or laboratory examination. The age range was 20 to 38 years (median, 28 years). The weight range was 58.0 to 87.1 kg (median, 76.4 kg). Seven volunteers were randomly selected for treatment with GM-CSF from CHO; 14 individuals were treated with GM-CSF from E. coli.

GM-CSF. Recombinant human GM-CSF from CHO and recombinant human GM-CSF from E. coli was provided by Sandoz Ltd. (Basel, Switzerland). Whereas expression of GM-CSF in CHO cells results in glycosylation of the molecule and molecular masses of 14 to 32 Kd, GM-CSF from E. coli is devoid of glycosyl residues and has a molecular mass of 14 Kd. Quantitative specifications of GM-CSF dose or of GM-CSF serum concentrations will be given on request to the authors.

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a molar basis throughout this report. GM-CSF from CHO or E coli, respectively, was injected subcutaneously into the deltoid region of one upper arm at a dose of 0.7 nmol (corresponding to 10 μg pure protein) per kilogram of body weight.

Sample collection. Venous blood samples were taken twice before GM-CSF administration for determination of basal values of GM-CSF and blood cell counts. After the injection of GM-CSF, blood sampling was repeated at intervals of 10 minutes for the first 30 minutes. Thereafter, blood samples were collected every hour for 8 hours, followed by 2- to 4-hour intervals for another 8 hours and 8-hour intervals for 32 hours. Additional blood samples were collected at 96 and at 336 hours after GM-CSF administration. Urine was obtained from spontaneous micturition before GM-CSF and sampled 0 to 24 hours and 24 to 48 hours after administration of the cytokine. Urine was stored at -30°C until analysis. Urine samples were screened (Combur9Test; Boehringer, Mannheim, Germany) for pathologic constituents (leukocytes, erythrocytes, protein, etc). Creatinine concentrations in urine were determined by the Jaffe reaction.

GM-CSF serum concentrations. GM-CSF serum concentrations were determined using a two-antibody sandwich enzyme-linked immunossay developed at the Sandoz Pharma Laboratories (Basel, Switzerland). The assay system consisted of a murine monoclonal anti-GM-CSF antibody raised against GM-CSF from CHO and an anti-GM-CSF antiserum raised by injection of GM-CSF from E.coli in a mountain sheep. GM-CSF from the serum samples was fixed to solid phase by the monoclonal antibody and quantitated by binding of the biotinylated sheep antiserum and subsequent addition of alkaline phosphatase conjugated to streptavidin. The lower detection limit of the assay was at 10 fmol GM-CSF/mL serum both for GM-CSF from CHO and for GM-CSF from E.coli. No cross-reactivities were observed using up to 10 ng of granulocyte-CSF (G-CSF), Interleukin-3 (IL-3), IL-4, or IL-6, respectively.

LT analysis. LT analysis was performed by sequential use of high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) as recently described in detail,11 with a few minor modifications. Briefly, urine was deproteinized by storage in 80% methanolic solution at -40°C and subsequent centrifugation at 8,000g. HPLC of deproteinized urine samples was performed on a C18 Hypersil column (4.6 × 250 mm, 5 μm particles; Shandon, Runkorn, UK) with a C18 precolumn (Waters, Milford, MA). The mobile phase consisted of methanol, water, acetic acid (65:35:0.1 by volume). One nmol/L EDTA, pH 5.6, adjusted with ammonium hydroxide. The flow rate was 1 mL/min. The RIA was performed in the fractions coeluting with standard [³H]LTE4 using a rabbit cysteinyl LTE antiserum, kindly donated by Prof. B.A. Peskar (Ruhr-Universität, Bochum, Germany). Recovery of LTs from urine was tested routinely by adding defined amounts of standard LTE4 to urine samples followed by processing and analysis by HPLC and RIA as described above. Reproducibility was assessed by replicate determinations in urine samples.12 LTs and [³H]LTE4 were from commercial sources (Faasel, Frankfurt, Germany; Amersham, Buckinghamshire, UK; or DuPont, Boston, MA, respectively). N-acetyl LTE4 and N-acetyl [³H]LTE4 were synthesized from LTE4 and [³H]LTE4, as described.13

Statistics. Student's t-test for paired observations was used to analyze significance of differences between determinations before and after GM-CSF treatment. The t-test for unpaired observations was used to analyze significance of differences between values obtained with GM-CSF from CHO and from E.coli. Correlations were estimated by calculating product-moment correlation coefficients and rank correlation coefficients (Spearman).

Ethical approval. Ethical approval for the GM-CSF treatment studies was obtained from the Ethics Committee, München, incorporated.

RESULTS

GM-CSF pharmacokinetics and hematologic effects. Before GM-CSF administration, GM-CSF serum concentrations were below the detection limit of the assay system, ie, below 10 fmol/mL serum. Seven volunteers were treated with a subcutaneous injection of GM-CSF from CHO at a dose of 0.7 nmol/kg body weight; 14 volunteers were treated in the same way with GM-CSF from E.coli. The increase and the decrease in GM-CSF serum concentration were more rapid after GM-CSF from E.coli compared with GM-CSF from CHO (Fig 1). After GM-CSF from CHO, maximum GM-CSF serum concentrations were obtained at 7.6 ± 2.7 hours and resulted in 221 ± 100 pmol GM-CSF/L serum (mean ± SD, n = 7). After GM-CSF from E.coli, maximum GM-CSF serum concentrations were determined already at 3.9 ± 1.3 hours and resulted in 514 ± 136 pmol GM-CSF/L serum (n = 14). However, due to a more sustained increase in GM-CSF serum concentration, the area under the serum concentration-time curve obtained after GM-CSF from CHO (3.35 ± 1.03 nmol/L × hour, mean ± SD, n = 7) was not significantly different from the area determined after GM-CSF from E.coli (3.80 ± 0.72 nmol/L × hour, n = 14).

The hematologic effects of GM-CSF were characterized by biphasic changes in leukocyte counts. During the first hour after injection of GM-CSF, leukocyte counts declined to about one-third of the counts before treatment (Fig 2). Values returned to the original level within 3 or 4 hours after GM-CSF from E.coli or CHO, respectively. Maximum leukocyte concentrations were determined 11 or 27 hours after the respective GM-CSF preparation. They were about 2.4-fold higher than pretreatment values. The changes in leukocyte counts occurred significantly faster after GM-CSF from E.coli. Leukocyte counts remained elevated for a significantly longer period of time after GM-CSF from CHO (Fig 2).

The biphasic shape of leukocyte concentrations was determined mainly by changes in the number of neutrophils, which almost disappeared in the first phase after GM-CSF treatment and which strongly increased in the second phase together with a shift to the left. However, monocyte and eosinophil counts also declined initially and markedly increased together.

Fig 1. Time course of GM-CSF serum concentrations in volunteers treated subcutaneously with 0.7 nmol/kg body weight of GM-CSF from CHO (n = 7) or from E.coli (n = 14). Mean values ± SD are indicated.
with basophil counts during the second day after GM-CSF. Monocytes increased to 328% ± 259% (mean ± SD, n = 7) or 243% ± 177% (n = 14) of pretreatment values after GM-CSF from CHO or E coli, respectively. Maximum eosinophil counts were 477% ± 177% (n = 7) or 320% ± 156% (n = 14) of pretreatment values; maximum basophil counts were 143 ± 83 M/L (n = 7) or 91 ± 79 M/L (n = 14) after GM-CSF from CHO or E coli, respectively.

Effect of GM-CSF on urinary cysteinyl LTs. Before treatment with GM-CSF, healthy volunteers secreted 1.1 to 28 (mean, 9.9) nmol LTE4 plus LTE4N-ac/mol creatinine (Fig 3), as determined by combined use of HPLC and RIA. LTE4N-ac constituted a minor, yet significant component amounting up to 36% (mean, 13%) of LTE4. The concentrations in urine of LTC4 and LTD4 were below the detection limit of our assay system, ie, below 50 pmol/L. There was a close relation between the urinary LTE4 plus LTE4N-ac concentration corrected for the urinary creatinine concentration and the amount of LTE4 plus LTE4N-ac secreted per day. On the average, 1 nmol LTE4 plus LTE4N-ac in urine per mole creatinine corresponded to 14 (range, 12 to 18) pmol LTE4 plus LTE4N-ac secreted per 24 hours. The respective product moment correlation coefficient ranged from 0.91 to 0.99 in the healthy volunteers.

During the first 24 hours after the injection of GM-CSF, urinary cysteinyl LTs increased 1.3- to 14-fold or 2.1- to 44-fold in volunteers treated with GM-CSF from CHO or E coli, respectively (Fig 3), resulting in urinary LT concentrations of 32 ± 26 or 161 ± 124 nmol LTE4 plus LTE4N-ac/mol creatinine (mean ± SD). Enhancement of urinary cysteinyl LTs was statistically significant (P < .001) with both GM-CSF preparations, but GM-CSF from E coli was significantly (P < .01) more effective in this respect. Twenty-four hours after administration of GM-CSF from CHO, urinary cysteinyl LTs returned to pretreatment values (10 ± 9 nmol LTE4 plus LTE4N-ac/mol creatinine, mean ± SD), whereas they were still significantly elevated after GM-CSF from E coli (52 ± 35 nmol LTE4 plus LTE4N-ac/mol creatinine; P < .01). There were no significant changes in the proportions of urinary LTE4 and LTE4N-ac after GM-CSF administration.

Association of urinary cysteinyl LTs with pharmacokinetic parameters of GM-CSF. The higher concentrations of urinary cysteinyl LTs obtained after GM-CSF from E coli (Fig 3) were associated with earlier and higher peak serum concentrations of GM-CSF as compared with GM-CSF from CHO (Fig 1). However, there were no statistically significant correlations between individual pharmacokinetic parameters (GM-CSF maximum serum concentration, serum half life, area under the serum concentration-time curve) and individual changes in urinary cysteinyl LTs in the volunteers treated with GM-CSF from CHO or E coli, respectively (the product moment correlation coefficients ranged from −0.2 to 0.3).

Association of urinary cysteinyl LTs with hematologic effects of GM-CSF. Urinary cysteinyl LT concentrations and their relative changes after GM-CSF administration were tested for correlation with the cytokine's effects on total leukocytes, neutrophils, monocytes, eosinophils, basophils, bands, and youngs. There was a significant positive correlation between the LT concentrations in the urine sampled during the first 24 hours after GM-CSF administration and the maximum basophil counts with both the CHO and the E coli
GM-CSF preparations. The corresponding product moment correlation coefficients were 0.85 and 0.70, respectively (Fig 4). Correlations of urinary cysteinyl LTs and maximum basophil counts differed with respect to their slopes in the groups of volunteers treated with GM-CSF from CHO or E coli. A similar increase in basophil counts in volunteers treated with either GM-CSF preparation was paralleled by significantly higher urinary LTs in the group of individuals treated with GM-CSF from E coli (Fig 4).

No consistent correlations between urinary cysteinyl LTs and hematologic parameters other than basophils were found in the healthy volunteers. Product moment correlation coefficients for urinary cysteinyl LTs with maximum leukocyte, neutrophil, monocyte, or eosinophil counts were 0.15, 0.28, 0.37, and 0.38 after GM-CSF from CHO and 0.42, 0.46, 0.0, and 0.41 after GM-CSF from E coli, respectively.

Association of urinary cysteinyl LTs with adverse effects of GM-CSF. Adverse effects of GM-CSF were reported in 17 of the 21 (81%) healthy volunteers. The severity of adverse effects was WHO grade 1 to 2 with symptoms abating without pharmacologic intervention. Figure 5 illustrates associations of adverse effects of GM-CSF with different ranges of urinary cysteinyl LTs. Low, intermediate, or high LT producers were classified according to their urinary cysteinyl LTs in the 0- to 24-hour fraction after GM-CSF administration. The respective ranges of urinary LT concentrations and of LTs secreted per day are given in Fig 5. The lowest range of urinary leukotrienes (<30 nmol LTE₄ plus LTE₄NAc/mol creatinine, equivalent to <450 pmol LTE₄ plus LTE₄NAc per day) overlaps with the pretreatment values.

Absence of adverse effects was associated with low urinary cysteinyl LTs. Flu-like symptoms, including a transient increase in body temperature, nasal congestion and catarrh, pharyngeal irritation, conjunctival redness and congestion were observed in 10 of the 21 (48%) volunteers. They were virtually absent in the low LT producers and increased in frequency with increasing urinary cysteinyl LTs. Bone pain was felt in the area of the lumbar and/or thoracic spine and/or in the sternum. It was characterized as tenderness, as a feeling of pressure, or as pulsating pain. Eight of the 21 (38%) volunteers complained of bone pain. It was most frequent in the high LT producers. Dyspnoea was reported as difficulty of breathing, mainly expiratory. Wheezes and coarse rhonchi were heard during auscultation. Dyspnoea may thus be classified as an asthmatic response. The two volunteers suffering from this adverse effect had been treated with GM-CSF from E coli and had high urinary cysteinyl LT concentrations. Gastrointestinal symptoms included nausea, vomiting, ab-

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**Fig 4.** Correlations between the LT concentrations in urine sampled 0 to 24 hours after GM-CSF administration and the maximum of basophil counts. (a) Data from individuals treated with GM-CSF from E coli; (b) data from those treated with GM-CSF from CHO. The continuous and the broken lines represent the linear regression curves of the data obtained with GM-CSF from E coli or CHO, respectively. Product moment correlation coefficients were 0.70 or 0.85 and rank correlation coefficients were 0.70 or 0.50 for the data obtained with GM-CSF from E coli or from CHO, respectively.

**Fig 5.** Frequency of adverse reactions of GM-CSF correlated with the endogenous LT production as assessed by determination of LTs in urine. (a) Data obtained with GM-CSF from CHO; (b) data obtained with GM-CSF from E coli. Volunteers were ranked as low (I), intermediate (II), or high (III) LT producers according to the LT concentrations in their 0- to 24-hour urine fraction after GM-CSF administration. The ranges for the respective urinary LT concentrations as well as the corresponding absolute amounts of cysteinyl LTs secreted per day are given (n = number of individuals).
dominal pain, and diarrhea. Thirty-eight percent of the volunteers complained of these symptoms. There was no obvious relation of gastrointestinal symptoms with urinary cysteinyl LTs. Skin reactions included erythema, scattered pustulation, or pustular eruption at the injection site. These symptoms were observed in 48% of the volunteers. They also do not seem to be related to increased urinary LTs. Arthralgia in the olecranon and knee joints combined with pain in neighboring muscles occurred in one volunteer treated with GM-CSF from *E. coli*. The LT generation was ranked intermediate in this individual.

Some volunteers suffered from more than one adverse effect. Combinations of up to four untoward effects were observed without a detectable interrelation. Some participants had additional complaints of mild to moderate headache, tiredness, heat sensations, or feeling of cold extremities. However, the relation of these latter symptoms to GM-CSF treatment was uncertain.

**DISCUSSION**

In the present study, we demonstrated that GM-CSF activates the endogenous LT production in healthy volunteers. Urinary LTE₄ plus LTE₄NAC corrected for urinary creatinine was used as a measure for the endogenous LT biosynthesis. Because in some individuals a significant portion of LTE₄ may be acetylated to LTE₄NAC, determination of both LTE₄ plus LTE₄NAC appears to be superior to determination of LTE₄ alone for a survey of the generation of these lipid mediators. LTE₄ in urine is by now widely accepted as a parameter for the endogenous LT production. The close correlation between urinary LT concentrations corrected for urinary creatinine and the renal LT elimination rate confirms earlier observations, suggesting that the renal elimination of LTs proceeds via glomerular filtration without appreciable secretion or reabsorption.

Data obtained in vitro suggest that GM-CSF is not a direct stimulator of LT production, but rather primes leukocytes for enhanced LT production elicited by succeeding stimuli. If this is the case, our results suggest that such stimuli are constitutively present or concomitantly induced by GM-CSF administration, not only in cytopenic or myelodysplastic patients, but also in healthy volunteers (Fig 3).

Our results encourage speculations on causal relations between hematologic effects, adverse reactions, and the elevation of endogenous LTs after GM-CSF administration. Indeed, LTs may play a role in both aspects of GM-CSF action.

LTs may be involved in the proliferative response to GM-CSF, as LTs and LT inhibitors have been shown to modulate the proliferation of myeloic progenitor cells and malignant blasts in vitro. In cytopenic patients, a linear correlation was found between the increase in total leukocyte counts and the increase in urinary LTs after GM-CSF administration. The corresponding correlation was not statistically significant in the present study in healthy volunteers who had normal blood cell counts before treatment with the cytokine. However, a more rapid increase in leukocyte counts (Fig 2) was associated with a higher LT production (Fig 3) in the volunteers treated with GM-CSF from *E. coli* as compared with those treated with GM-CSF from CHO. This may be interpreted as an argument for a role of LTs in the proliferative response after GM-CSF administration in vivo.

LTs may also be involved in the GM-CSF-induced stimulation of leukocytes. The initial decrease in leukocyte counts to about one-third of pretreatment values (Fig 2) is most likely caused by activation of leukocytes. This effect was found to be associated with an upregulation of adhesion molecules, promoting adherence of leukocytes to the blood vessel wall. LTs induce leukocyte adhesion to endothelial cells via the same type of adhesion molecules (CD11/CD18) and leukocyte adhesion can be antagonized by LT biosynthesis inhibitors as demonstrated in a model of reperfusion injury. It seems possible, therefore, that the GM-CSF–induced initial decrease in leukocyte counts is mediated by LTs.

Functional activation of leukocytes, including increased generation of LTs, may also explain some of the adverse effects caused by GM-CSF. After GM-CSF treatment, a significant positive correlation was found between the urinary cysteinyl LTs and the basophils (Fig 4), which are known to be central components in hypersensitivity reactions. A positive relation was also found between enhanced LT production and flu-like symptoms, bone pain, or asthmatic reactions (Fig 5). Flu-like symptoms associated with enhanced vascular permeability and inflammatory reactions in mucous membranes might well be explained by known actions of LTs. Involvement of LTs in asthmatic responses is widely accepted. No obvious correlation was observed between GM-CSF–induced LT production and gastrointestinal symptoms or skin reactions (Fig 5).

Comparison of the effects of the two different GM-CSF preparations showed several differences. Structurally, GM-CSF from *E. coli* differs from GM-CSF from CHO in the absence of glycosyl residues. Pharmacokinetic consequences of this difference were a more rapid increase and decrease in serum concentration and a higher maximum serum concentration of GM-CSF from *E. coli* (Fig 1). The data demonstrated are in line with results derived from a larger number of healthy volunteers (R. Pokorny et al, unpublished observations). Differences in the pharmacokinetics between glycosylated and nonglycosylated GM-CSF also appear likely when results from recent studies using either preparation are compared. Pharmacodynamic consequences of the differences between the two GM-CSF preparations were a faster increase and a faster decrease in leukocyte counts (Fig 2), higher urinary cysteinyl LTs (Fig 3), and a different pattern of side effects (Fig 5) after administration of GM-CSF from *E. coli* compared with GM-CSF from CHO. GM-CSF from CHO caused a number of pathologic skin reactions, whereas the other side effects (flu-like symptoms, bone pain, dyspnoea, gastrointestinal symptoms) clearly predominated in the volunteers treated with GM-CSF from *E. coli* (Fig 5). Our results suggest that differences in pharmacokinetics between the two GM-CSF preparations may explain some of the differences in their pharmacodynamics: a more sustained increase in serum GM-CSF after GM-CSF from CHO (Fig 1) corresponded to a more sustained increase in leukocyte counts (Fig 2); a higher maximum GM-CSF serum concentration after GM-CSF from *E. coli* (Fig 1) corresponded to a higher LT production (Fig 3) and to more severe side effects.

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(Fig 5). The GM-CSF dose, route, and velocity of administration are empirical determinants of its toxicity,3,4,14 and the better tolerated GM-CSF from CHO behaves like a prolonged-release form compared with GM-CSF from E coli (Fig 1).

GM-CSF–induced side effects are usually mild to moderate. However, a case of a capillary leak syndrome and a case of an adult respiratory distress syndrome (ARDS), probably triggered by GM-CSF, have been presented recently.23,24 Involvement of LTs in these pathophysiologic situations is likely. Enhanced vascular permeability resulting in macrovascular leakage is a well-characterized feature of LT action.25,26 Recent studies on endogenous LT production in ARDS provide additional evidence for a role of leukotrienes in this disease.20-22

Increased leukocyte activity that is associated with enhanced LT biosynthesis and adverse effects is clearly undesirable in healthy individuals and may be dangerous in certain clinical situations. However, a limited increase in LT biosynthesis may be beneficial in immunocompromised patients. Besides a possible involvement in the proliferative response to GM-CSF, enhanced LT biosynthesis may be involved in the upregulation of host defense, including upregulation of immune functions, enhanced antimicrobial activity, and enhanced antitumor cytostasis.25-27 The more pronounced increase in the endogenous LT production after GM-CSF from E coli is, therefore, not necessarily disadvantageous in a therapeutic situation.

Future trials will show whether drugs affecting LT biosynthesis or action can be used to optimize GM-CSF treatment.

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