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Induction of Leukotriene Production by Bleomycin and Asparaginase in Mast Cells *In Vitro* and in Patients *In Vivo*

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ABSTRACT. Bleomycin and asparaginase are widely used antineoplastic agents which may induce allergic or inflammatory side-effects. Mast cells are implicated as effector cells in allergic and inflammatory responses. The aim of this study was to establish whether bleomycin or asparaginase modulate leukotriene production *in vitro* and *in vivo*. Leukotriene C_4 (LTC_4) production by murine bone marrow-derived mast cells (BMMC) was determined by radioimmunoassay (RIA). Leukotriene production in patients was assessed by determining leukotriene E_4 and *N*-acetyl-leukotriene E_4 in urine by means of combined HPLC and RIA. Bleomycin induced an up to 2.1-fold increase in LTC_4 production both in unstimulated and in calcium ionophore-stimulated mast cells. In 3 of 7 patients treated with bleomycin, a greater than 2-fold increase in endogenous leukotriene production was observed. This effect was associated with febrile responses and was most pronounced in a patient who developed an Adult Respiratory Distress Syndrome (ARDS). Asparaginase increased leukotriene production up to 10-fold in stimulated but not in unstimulated BMMC. In a patient who developed an anaphylactic reaction after treatment with asparaginase, a pronounced increase in urinary leukotriene concentration was observed. In contrast to bleomycin or asparaginase, a number of other cytostatic agents did not significantly change leukotriene production by BMMC. Our data indicate that some of the inflammatory and allergic side-effects of bleomycin and asparaginase could be mediated by leukotrienes, a possible source of which may be mast cells. BIOCHEM PHARMACOL 55;4:447–453, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. leukotrienes; bleomycin; asparaginase; mast cells; side-effects; cytostatic agents

Bleomycin and asparaginase are widely used antineoplastic agents. Treatment with bleomycin is frequently associated with mild to moderate hypersensitivity reactions and inflammatory side-effects. Severe inflammatory reactions are less common. However, pneumonitis, which may proceed to interstitial pulmonary fibrosis, is the dose-limiting toxicity of bleomycin [1]. The pathogenesis of this toxicity is unclear [2]. Inflammatory reactions induced by bleomycin are characterized by edema and by increased microvascular leakage and subsequent infiltration by inflammatory cells, especially neutrophils [3, 4]. This process may be accompanied by proliferation of fibroblasts [5] and by an increase in collagen production, finally resulting in fibrosis [6]. Hypersensitivity reactions also represent major side-effects of asparaginase. They include erythema, exanthema, urticaria, dyspnoea, fever and anaphylactic shock. The pathophysiological basis of these reactions is also poorly understood.

Leukotrienes are potent mediators of allergic and inflammatory reactions. They induce plasma extravasation, smooth muscle contraction, mucus secretion, vaso- and bronchoconstriction, chemotaxis and chemokinesis of polymorphonuclear leukocytes [7]. They also stimulate proliferation of a wide range of cell types including fibroblasts [8]. Leukotrienes are produced via the 5-lipoxygenase pathway and mast cells are a rich source of these mediators [9]. Mast cells are ubiquitously distributed in tissues localized at exposed positions beneath epithelial surfaces, adjacent to blood and lymphatic vessels, and in bone marrow [10, 11]. They have been shown to be involved in allergic diseases, in host defense, tissue inflammation, growth and repair [11, 12]. In the present study, we investigated whether bleomycin or asparaginase modulate leukotriene production in mast cells *in vitro* and in patients *in vivo*.

MATERIALS AND METHODS

Drugs and Reagents

Asparaginase was purchased from Medac. Bleomycin was from Mack. Cyclophosphamide was from ASTA-Pharma AG. Acrolein was purchased from Aldrich. Cisplatin was from Bristol–Arzneimittel. Doxorubicin was from Farmita-

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lia Carlo Erba. Methotrexate was obtained from Lederle. Leukotriene C_4 (LTC_4)¶, D_4 (LTD_4) and E_4 (LTE_4) were from Paesel and Lorei. 14,15-[^{3}H]-LTC_4, D_4 and E_4 were obtained from Amersham International. Polyclonal rabbit cysteinyl leukotriene antibodies were kindly donated by Prof. Peskar, Graz, Austria. DMSO, calcium ionophore, dextran, gelatine, 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl and charcoal were from Sigma. Methanol HPLC-grade ROTISOLV® and Tuluol-scintillator ROTI-SZINT 22® were purchased from Carl Roth GmbH. Fetal calf serum (FCS) for mast cell cultures was purchased from GMB and from PAN-Systems. RPMI-1640 medium was from Gibco. Penicillin-streptomycin was bought from Gibco. L-Glutamine was from Biochrom/Seromed. α-Thioglycerol was from Ferwa. Recombinant IL-3 was from Bachem. Standard mixture of IL-3 plus IL-4 (IL-3/-4) was from Genenzyme. Spleen-conditioned medium was prepared as described [13]. Eosin was bought from Merck. LTE_4 and *N*-acetyl leukotriene E_4 (LTE_4NAc) were synthesized according to a method published previously [14].

Asparaginase was dissolved in 4 mL aqua bidest and diluted in phosphate-buffered saline (PBS) to final concentrations of 8–25000 U/L. Bleomycin was mixed with 5 mL saline and diluted in PBS to final concentrations of 0.16–100 mg/L (0.1–67 μmol/L). Cyclophosphamide was dissolved in 5 mL aqua bidest and diluted in PBS. Adriamycin was mixed with 5 mL saline and diluted in PBS.

Cell Cultivation and Sample Preparation

Murine mast cells were obtained from bone marrow of Balb/c mice at the age of 5 to 8 months. Bone marrow-derived mast cells (BMMC) were cultured for 2 weeks at 37° in an atmosphere of 10% CO_2 in RPMI-1640 medium supplemented with FCS (20%), L-glutamine (2 mmol/L), penicillin-streptomycin (100 U/L) α-thioglycerol (10^{-5} mol/L), spleen-conditioned medium as a source of mast cell growth factors (10%) or recombinant IL-3 (20 ng/mL) or IL-3/-4 (20 ng/mL). On day 14, the nonadherent BMMC were transferred into fresh RPMI-1640 medium with weekly refeedings for another 2 to 3 weeks. The seeding density was 1×10^5 viable cells/mL. BMMC (*in vitro* age: 4 to 5 weeks) were washed twice and resuspended in cytokine-free PBS supplemented with 0.5 mg/mL gelatine. Mast cell preparations were at least 97% pure as assessed by toluidine blue staining. The viability of the cells was assessed by eosin staining. Mast cell lines resembled mucosal type mast cells by functional and phenotypical criteria [15].

Purified mast cells in a concentration of 2×10^5 cells/500 μL PBS were preincubated with antineoplastic agents or their solvents in a water bath at 37° for 5 min prior to stimulation with 0.2 μmol/L calcium ionophore (A23187) or its solvent (DMSO). The incubation was terminated by centrifugation at 600 × *g* for 10 min at 4°. For deproteinization, a 300 μL aliquot of the supernatant of each sample was added to 2.4 mL 90% aqueous methanol containing 0.5 mmol/L EDTA and 1 mmol/L 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO), pH 7.4. The samples were stored for at least 12 hr at −40° followed by centrifugation at 9000 × *g* for 20 min at −10°. The supernatants were evaporated to dryness, resuspended in 140 μL 30% methanol and stored at −20° until analysis by radioimmunoassay (RIA).

Analysis of Endogenous Leukotriene Production

Endogenous leukotriene production in patients was evaluated by determination of leukotriene metabolites (LTE_4 plus LTE_4NAc) in urine. Urine was obtained from spontaneous micturition or via urinary catheter. Samples containing pathologic concentrations of erythrocytes, leukocytes or proteins were excluded from the study. Urine was stored in polypropylene tubes at −30° until assay. A trace amount of LTC_4 was added as an internal standard. This leukotriene metabolite is not detectable in normal urine [16, 17] and cannot be converted under the deproteinization conditions used. For deproteinization, urine was stored at −40° in 90% methanolic solution containing 0.5 mmol/L EDTA and 1 mmol/L TEMPO, pH 7.4, and subsequently centrifuged at 9800 × *g*. The supernatant was evaporated to dryness in a centrifuge under low pressure and redissolved in 30% aqueous methanol containing trace amounts of tritium-labelled leukotriene standards. The samples were kept at −20° until analysis by combined use of HPLC and RIA.

HPLC analysis was used to prepare urine samples for analysis by RIA. Isocratic HPLC was performed as described [16] on a C18 hypersil column (250 × 4.6 mm, 5 μm particle size, Shandon) guarded by a C18 precolumn (Waters). The mobile phase consisted of methanol, water, acetic acid (65:35:0.1 by volume), 1 mmol/L EDTA, adjusted to pH 5.6 with ammonium hydroxide. The flow rate was 1 mL/min. HPLC fractions were neutralized by K_2CO_3. Radioactivity of authentic [^{3}H]-leukotriene standards was determined by a radioactivity detector (HPLC Radioactivity Monitor LB 507 A). HPLC fractions corresponding to the retention time of authentic [^{3}H]-LTC_4, [^{3}H]-LTE_4 or [^{3}H]-LTE_4NAc were pooled for further analysis.

RIA analysis of endogenously produced leukotrienes in HPLC samples was performed as described in detail elsewhere [16]. Leukotriene production by mast cells was determined by RIA analysis in unseparated samples. Aliquots of deproteinized mast cell supernatants were evaporated to dryness and resuspended in assay buffer. Cysteinyl leukotriene antiserum was added and incubated together with samples and standards at room temperature for 30 min. [^{3}H]-LTC_4 (130 Bq) was added and incubation continued for 16–20 hr at 4°. Unbound [^{3}H]-LTC_4 was precipitated by

¶ *Abbreviations:* ARDS, Adult Respiratory Distress Syndrome; A23187, calcium ionophore; BMMC, bone marrow-derived mast cells; FCS, fetal calf serum; IL-3/-4, interleukin-3/-4; LTC_4, D_4, E_4, leukotriene C_4, D_4, E_4; LTE_4NAc, *N*-acetyl-leukotriene E_4; TEMPO, 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl; RIA, radioimmunoassay.

addition of charcoal suspension (0.5% charcoal, 0.5% dextran in 10 mmol/L phosphate buffer, pH 7.4) and subsequent centrifugation at 1400 × g for 15 min at 4°. The supernatant was added to 8 mL scintillation fluid. Leukotriene concentrations in samples were calculated from log-logit-transformed standard curves with the aid of a computer program. Urinary leukotriene concentrations in samples were corrected for recovery of internal standard (LTC_4). Recovery was ≥71%. The lower detection limit of the assay system was below 40 fmol LTC_4. Relative cross-reactivities for LTC_4, LTD_4, LTE_4 and LTE_4NAc were 100%, 68%, 53% and 82%, respectively [16]. The urinary leukotriene concentrations are expressed in relation to the creatinine concentrations, which were determined by the Jaffé reaction.

Statistics

LTC_4 production by mast cells is given as percent of LTC_4 generated during 30 min of incubation in the presence of 0.2 μmol/L A23187 to correct for variability of leukotriene production in different mast cell preparations. The Wilcoxon test for paired observations was used to analyze for significance of differences in leukotriene production in the presence and absence of drugs.

RESULTS

Bleomycin

As shown in Fig. 1, bleomycin stimulated LTC_4 production by mast cells in the presence and absence of calcium ionophore (A23187). The bleomycin dose-response curve was bell-shaped with a maximum LTC_4 production of 211 ± 32% ($n = 4$, $P < 0.05$) of the A23187 reference value at a bleomycin concentration of 2.7 μmol/L. In the absence of A23187, a smaller dose-dependent increase in LTC_4 production was observed up to a bleomycin concentration of 13 μmol/L, resulting in 159 ± 19% ($n = 4$, $P < 0.05$) of the A23187 reference value. The relative decrease in leukotriene generation at higher doses of bleomycin may be due to cytotoxic effects of the drug. This interpretation is supported by determinations of cell viability indicating 76% reduction of viable cells in the presence of bleomycin (13 μmol/L) and a 81% reduction in the presence of bleomycin (13 μmol/L) in combination with A23187 (0.2 μmol/L).

In the presence of A23187, bleomycin induced a rapid increase in LTC_4 production by mast cells (Fig. 2). Following the first 5 to 10 min, only a moderate LTC_4 production was observed. In the absence of A23187, bleomycin induced a moderate but sustained increase in leukotriene production.

In seven patients suffering from high-grade non-Hodgkin's lymphoma, 15 mg bleomycin was given as a bolus intravenously on day 14 of a polychemotherapy regimen (Fig. 3). Endogenous leukotriene production was determined before and after administration of bleomycin.

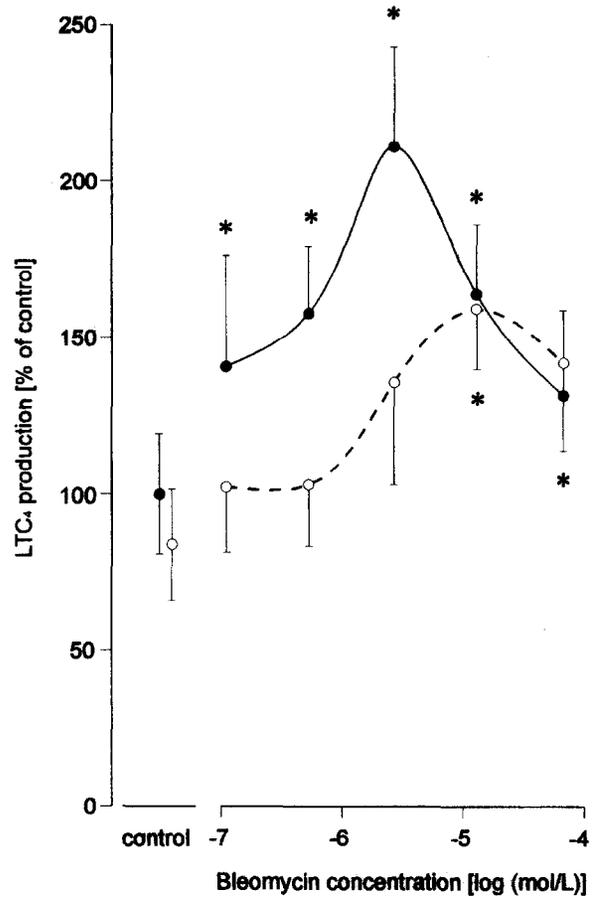

FIG. 1. Dependence of leukotriene production by murine mast cells on bleomycin concentration. LTC_4 production is shown as percent of the LTC_4 production of calcium ionophore (A23187)-stimulated cells, 100% corresponding to 45 pmol $LTC_4/10^6$ cells. Mast cells were incubated for 30 min in the presence (closed circles) or absence (opened circles) of A23187 (0.2 μmol/L). Data represent means ± SD from 4 independent experiments. *, Indicates a significant difference from control by $P < 0.05$.

In 3 of the 7 patients, bleomycin induced a greater than 2-fold increase in endogenous leukotriene production, an effect which appeared to increase with repeated administration (Fig. 3). Increased concentrations of urinary LTE_4 and LTE_4NAc were associated with febrile reactions (body temperature >38.5°). The most dramatic increase in leukotriene production (17.9-fold) was observed in a patient who developed an Adult Respiratory Distress Syndrome (ARDS).

Asparaginase

Asparaginase stimulated leukotriene production during 30 min of incubation in the presence of A23187 in a bell-shaped way (Fig. 4). Maximum leukotriene production (430 ± 75.5% of the A23187 reference) was detected at an asparaginase concentration of 200 U/L. Similar to that observed with bleomycin, the stimulatory effect of aspara-

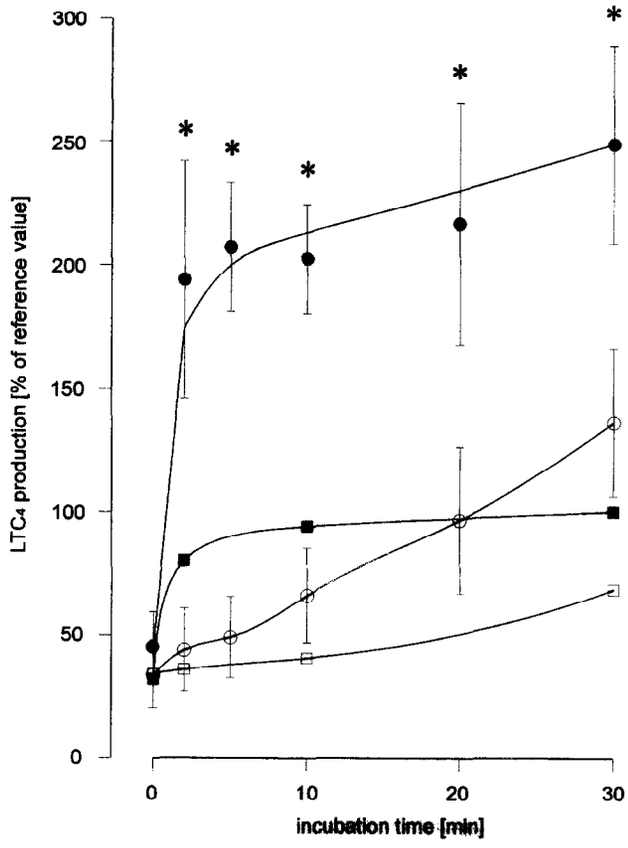

FIG. 2. Time-course of leukotriene production by murine mast cells in the presence (circles) or absence (squares) of bleomycin (2.7 μmol/L). Open and closed symbols represent data obtained in the absence and presence of A23187 (0.2 μmol/L), respectively. Data were calculated as percent of the A23817 reference value (100% corresponds to 6 pmol/10^6 cells). Data represent means ± SD from 3 independent experiments. *, Indicates a significant difference ($P < 0.05$) between data obtained with bleomycin as compared to data without bleomycin.

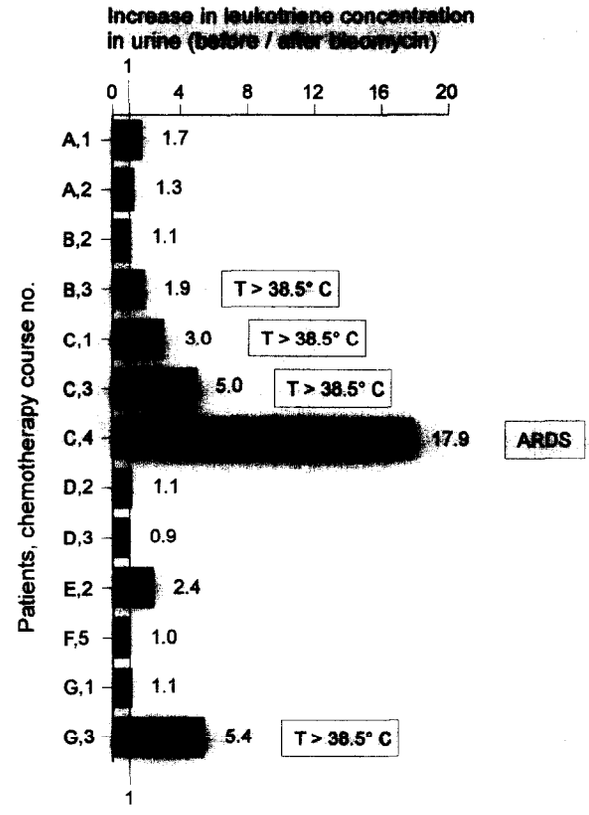

FIG. 3. Leukotriene production in patients after treatment with bleomycin. Seven patients (A to G) from the age of 19 to 45 years suffering from high-grade non-Hodgkin's lymphoma were treated with a single dose of 15 mg bleomycin on day 14 of a polychemotherapy regimen. Measurements from different courses of chemotherapy are shown as indicated in arabic numbers. The ratios of the urinary leukotriene concentration (nmol LTE_4 plus LTE_4NAc per mol creatinine) after/before intravenous application of bleomycin are shown together with observed side-effects. T > 38.5° indicates a febrile reaction (body temperature above 38.5°). ARDS indicates manifestation of an Adult Respiratory Distress Syndrome.

ginase on LTC_4 production was most pronounced during the first 10 min of incubation (data not shown). However, in contrast to bleomycin, asparaginase did not stimulate LTC_4 production by BMMC in the absence of A23187 (Fig. 4). The relative decrease in leukotriene production at asparaginase concentrations above 200 U/L may be due to toxic effects of the enzyme in combination with A23187. Asparaginase (200 U/L) in combination with A23187 (0.2 μmol/L) reduced the number of viable cells below 8% after 30 min of incubation.

Anaphylactic reactions are major side-effects of asparaginase. An anaphylactic reaction presenting with hypotension, angioedema, asthma and shock was observed in a patient treated with 10,000 U asparaginase for acute lymphoblastic leukemia. This anaphylactic reaction was associated with strongly enhanced urinary leukotriene concentrations (Fig. 5). The maximum concentration was 305 nmol LTE_4 plus LTE_4NAc per mol creatinine representing at least 8-fold excess as compared to 46 healthy controls (7 to 35, mean 21 nmol LTE_4 plus LTE_4NAc per mol creatinine) [18]. In this patient, the urinary leukotriene concentration returned to normal levels following 4 days of symptomatic treatment using H_1 receptor blocking agents, suprarenine and corticosteroids. No other cytostatic agent was given on the day of asparaginase administration and no anaphylactic reaction was observed with any other component of the polychemotherapy regimen that was administered to the patient. Only a minimal increase in urinary leukotrienes was observed in three patients who were treated with the same dose of asparaginase but did not exhibit any clinical signs of anaphylaxis (maximum concentrations 54, 49 and 30 nmol LTE_4 plus LTE_4NAc per mol creatinine). Besides bleomycin and asparaginase, a number of other antineoplastic agents were tested for their effects on leukotriene production by mast cells. Acrolein, an active metabolite of cyclophosphamide, cisplatin, doxorubicin and methotrexate, induced slight to moderate inhibition of leukotriene production. These effects are most likely explained by the cytotoxic activity of these agents.

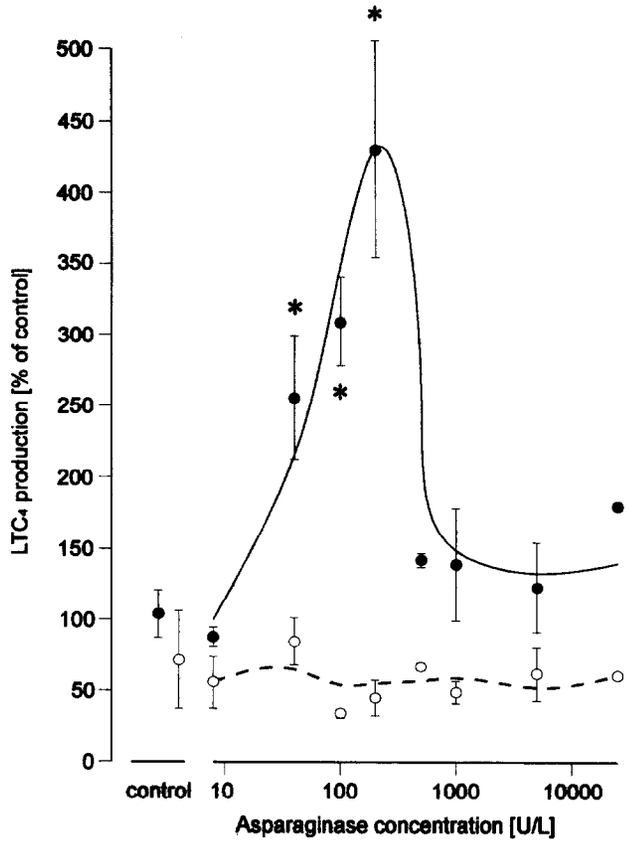

FIG. 4. Dependence of leukotriene production by murine mast cells on asparaginase concentration. LTC_4 production is shown as percent of the LTC_4 production of A23187 (0.2 μmol/L) stimulated cells, 100% corresponding to 64 pmol $LTC_4/10^6$ cells. Mast cells were incubated for 30 min in the presence (closed circles) or absence (open circles) of A23187 (0.2 μmol/L). Data represent means ± SD of 5 independent experiments. *, Indicates a significant difference from control by $P < 0.05$.

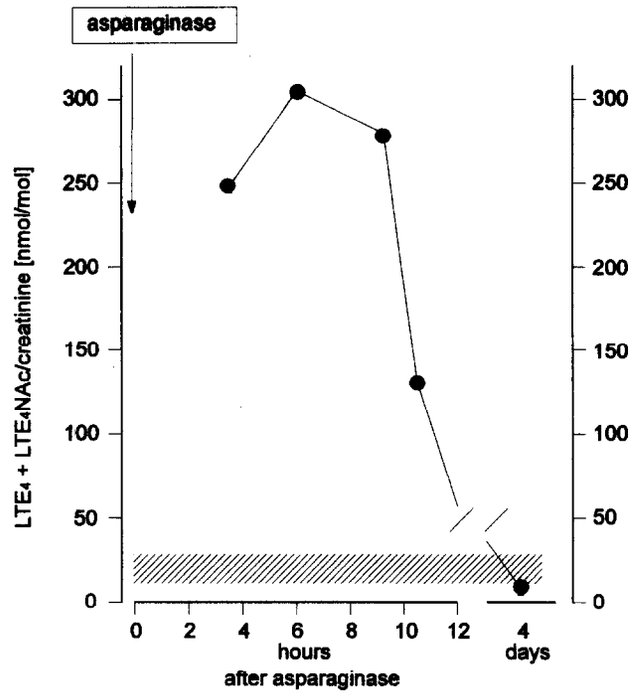

FIG. 5. Leukotriene production in a patient who developed an anaphylactic reaction after treatment with 10,000 U asparaginase. The anaphylactic reaction presented with hypertension, angioedema and bronchoconstriction and necessitated antihistamines, suprarenine, corticosteroids and assisted ventilation. This reaction was associated with enhanced urinary leukotriene concentrations. The increased LTE_4 plus LTE_4NAc levels decreased to normal values after 4 days of treatment. The range of leukotriene production in healthy controls (7–35 nmol LTE_4 plus LTE_4NAc per mol creatinine, $n = 46$) [18] is indicated by the hatched area.

DISCUSSION

The present study demonstrates that bleomycin and asparaginase enhance leukotriene production in murine bone marrow-derived mast cells *in vitro* and in some clinical situations in patients *in vivo*. In contrast to bleomycin (Figs. 1 and 3), the stimulatory effect of asparaginase in mast cell suspensions was only observed in the presence of calcium ionophore (Fig. 4), suggesting a regulative role of the intracellular calcium concentration. High concentrations of both antineoplastic agents induce a decrease in leukotriene biosynthesis, probably due to cytotoxic effects, especially in combination with A23187. Enhanced leukotriene production in the presence of bleomycin may be explained by a stimulation of phospholipase A_2, an effect that has been demonstrated both *in vitro* and *in vivo* [19]. This calcium-dependent enzyme liberates arachidonate from membrane phospholipids, which is the initial step of leukotriene biosynthesis.

Bleomycin can also enhance endogenous leukotriene production, as demonstrated in some patients suffering from high-grade non-Hodgkin's lymphoma who were treated with bleomycin on day 14 of a polychemotherapy regimen (Fig. 3). The drug was given as a single agent at least 6 days apart from the administration of other antineoplastic substances, thus precluding a contribution of other components of the treatment protocol. Enhanced endogenous leukotriene production was associated with febrile reactions and was most pronounced in a patient who developed an ARDS after treatment with bleomycin. This is in accordance with data from other authors demonstrating enhanced endogenous leukotriene production in ARDS after polytrauma [20, 21] or during sepsis [22]. Our data suggest that the bleomycin-induced increase in leukotriene production may play a role in the pathogenesis of inflammatory and pulmonary side-effects of the drug. Mast cells may contribute significantly to bleomycin-induced leukotriene production as indicated by our *in vitro* results. Accumulation of mast cells is frequently observed in inflammatory parenchymal lung diseases [12]. Since LTC_4 has been shown to enhance collagen synthesis in fibroblasts [8], elevated concentrations of this mediator may also be

involved in lung fibrosis presenting a fatal complication of bleomycin-containing regimens.

The increase in leukotriene production induced by asparaginase in A23187-stimulated mast cells (Fig. 4) and the association of increased endogenous leukotriene production with a serious anaphylactic reaction (Fig. 5) is in line with a role for mast cell-derived leukotrienes in this untoward effect of the antineoplastic enzyme. Enhanced endogenous leukotriene production has previously been shown to be associated with anaphylaxis induced by a number of other triggers [18]. The close association between the administration of asparaginase and the anaphylactic reaction on the one hand and between the anaphylactic reaction and the increase in urinary leukotrienes on the other (Fig. 5) strongly suggests a causal connection.

In contrast to bleomycin and asparaginase, several other cytostatic agents had only moderate inhibitory effects on leukotriene production by BMMC. These effects are likely to be explained by cytotoxic effects. However, different observations have been made by other authors in other experimental situations. Peritoneal macrophages from mice produce increased amounts of LTC_4 after pretreatment with cyclophosphamide [23]. Following treatment of patients with methotrexate, leukotriene production in leukocytes from peripheral blood was found to be inhibited [24–26] or enhanced [27].

In summary, our *in vitro* and *in vivo* results suggest that leukotrienes may play a role in allergic and inflammatory reactions observed after treatment with bleomycin or asparaginase. Mast cells represent a rich source of these mediators and are likely to be involved in the mediation of these reactions. The forthcoming availability of potent and selective leukotriene biosynthesis inhibitors and receptor antagonists for clinical use opens a novel perspective to counteract adverse effects of antineoplastic regimens containing asparaginase or bleomycin.

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