Leukotriene B₄ synthesis and neutrophil chemotaxis in chronic granulocytic leukaemia

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SUMMARY A sensitive gas chromatography-mass spectrometric method was used to measure the generation in whole blood of leukotriene B₄ (LTB₄), a potent stimulator of neutrophil chemotaxis, in eight patients with chronic granulocytic leukaemia (CGL) and 12 healthy controls. LTB₄ was detectable in unstimulated samples from all the patients (mean 194 (70 SEM) pg/ml), and the capacity for LTB₄ production after stimulation with calcium ionophore (A23187) was similar in patients (32.1 (11) ng/10⁶ leucocytes) and controls (38.1 (4) ng/10⁶ leucocytes). In response to stimuli which induce neutrophil activation, LTB₄ production was significantly greater in the patients than in controls: 35.6 (13) v 13.0 (3) ng/ml, $p < 0.05$ (f-met-leu-phe); and 42.4 (16) v 14.7 (4) ng/ml, $p < 0.02$ (opsonised zymosan). Anti-IgE stimulated considerably more LTB₄ production in patients with CGL than in controls (3.86 (1.6) v 0.83 (0.43) ng/ml; $p < 0.005$) and this correlated significantly ($p < 0.05$) with the basophil count. Neutrophil chemotaxis to LTB₄, however, was significantly impaired in the patients with CGL even at the highest concentration of LTB₄ (10⁻⁵ M). Chemotaxis to f-met-leu-phe, phagocytosis, and bacterial killing were normal. Thus although LTB₄ synthesis is normal or even enhanced in patients with CGL, specific defects in LTB₄-mediated responses may contribute to neutrophil dysfunction in this disease.

Diverse functional and biochemical abnormalities have been described in neutrophils from patients with chronic granulocytic leukaemia (CGL). These include defects in phagocytosis and microbial killing,^{1,2} reduced adhesion and chemotaxis,³ impaired production of oxygen metabolites⁴ and changes in the neutrophil membrane.⁵ Selective deficiency of the 5-lipoxygenase products of arachidonic acid metabolism in CGL neutrophils has also been reported,⁶ leading to the suggestion that this may underlie or contribute to the neutrophil dysfunction in such patients. In neutrophils arachidonic acid is metabolised principally via the 5-lipoxygenase pathway to produce 5-hydroxy-eicosatetraenoic acid (5-HETE) and leukotriene B₄ (LTB₄).⁷ LTB₄ is the most potent endogenous inducer of chemotaxis and chemokinesis^{7,8} by human neutrophils and has also been shown to stimulate other aspects of phagocytic function, including neutrophil adhesion, oxygen metabolite production, and degranulation.^{9,10}

The purpose of this study was to investigate the production of LTB₄ in CGL and its association with abnormalities of neutrophil function. To address the

hypothesis that many of the reported defects in neutrophil function in such patients can be explained by impaired endogenous production of or responsiveness to LTB₄, or both, we developed a sensitive technique utilising gas chromatography-mass spectrometry to quantify LTB₄ generation in whole blood. We used this approach to measure LTB₄ synthesis in patients with CGL in response to a range of agonists known to induce neutrophil activation. To explore the association between changes in LTB₄ production and its functional consequences in vivo we also studied the ability of neutrophils from the same patients to respond to chemotactic stimuli in vitro.

Patients and methods

Patients were recruited from those attending the haematology clinics of Vanderbilt University Medical Centre and the Veterans' Administration Hospital, Nashville, Tennessee. We studied eight men (age 38-76 years) with chronic phase CGL and 12 healthy sex and age matched controls. CGL was diagnosed by characteristic peripheral blood and bone marrow appearances. Seven of the patients were Ph⁺ positive, six with the usual t (9;22) (q34; q11) translocation and

one with a t (17;22) (q25; q11) translocation. The remaining patient, who did not have any cytogenetic studies performed, had clinical and haematological features typical of Ph¹ positive CGL, including granulocytic hyperplasia, basophilia, eosinophilia and a neutrophil alkaline phosphatase score of 5. One patient had a history of pulmonary tuberculosis and recurrent chest infections and another, who was also diabetic, had recurrent cellulitis. The remaining patients had no clinically important history of infection. The study was approved by the committee for the protection of human subjects of both institutions.

MEASUREMENT OF LEUKOTRIENE B₄

Leukotriene B₄ was measured in venous blood collected into heparin and incubated at 30°C for 15 minutes in the absence and presence of various stimuli. The stimuli used were anti-IgE 1 and 2 µg/ml, f-met-leu-phe (fMLP) 1 and 2 µM, opsonised zymosan 100 and 250 µg/ml, and calcium ionophore (A 23187) 50 µM. After the addition of a deuterated internal standard (kindly supplied by Dr J Rokach, Merck Frosst Research Laboratories) the samples were acidified, applied to a C-18 Sep-Pak (Waters), and eluted with ethyl acetate. After further purification by thin layer chromatography derivatisation of the sample was carried out as previously described for other eicosanoids.¹² Thus the pentafluorobenzyl derivative was formed by addition of 12.5% pentafluorobenzyl bromide in acetonitrile and di-isopropylethylamine, and following a second thin layer chromatography step derivatisation was completed by formation of the trimethylsilyl ether using bis(trimethylsilyl)-trifluoroacetamide and pyridine. Quantitation was accomplished by negative ion chemical ionisation gas chromatography-mass spectrometry utilising the Nermag R10-10C instrument monitoring m/z 479 for endogenous leukotriene B₄ and m/z 481 for the deuterated internal standard.

NEUTROPHIL FUNCTION TESTS

Neutrophils were prepared from heparinised venous blood separated by Ficoll-Hypaque gradient centrifugation. Purity and viability of the neutrophil suspension (assessed by Trypan blue exclusion) was consistently more than 95%. Chemotaxis was measured by Chenoweth's modification of the underagarose method originally described by Nelson.¹² After two and a half hours' incubation with LTB₄, fMLP, or buffer at 37°C the cells were examined by inverted microscopy, fixed, and stained with Wright's solution. The distances migrated towards (directional-chemotaxis) and away from (non-directional, chemokinesis) were measured using a micrometer eyepiece attachment and the results expressed as the distance to the leading edge of neutrophils in mm and

as the chemotactic index (ratio of directional:non-directional migration). The ability of neutrophils from patients with CGL and healthy controls to phagocytose and kill micro-organisms was also assessed as an index of neutrophil function largely independent of LTB₄ using the Giorgio strain of *Staphylococcus aureus* as described by Schaffner *et al.*¹³

Data were analysed by non-parametric methods, thereby avoiding assumptions as to the distribution of the variables studied. Analysis of variance was done by the method of Friedman and subsequent comparisons by the Mann Whitney U test. All values are given as mean SEM.

Results

LEUKOTRIENE B₄ PRODUCTION IN WHOLE BLOOD LTB₄ was detectable in unstimulated blood samples from all the patients and healthy controls. The mean concentration of LTB₄ was slightly higher in the patients with CGL than in controls (194 (70) v 100 (39) pg/ml; p = NS) (table 1). When corrected for the total leucocyte or neutrophil count, however, LTB₄ generation was significantly reduced in the patients with CGL compared with the healthy subjects: 6.9 (3) v 39 (14) pg/10⁶ leucocytes (p = 0.02) and 9.7 (5) v 45.0 (14) pg/10⁶ neutrophils (p = 0.02).

While in normal subjects anti-IgE, as expected, was a very weak stimulus (table 2), in the patients with CGL a pronounced increase in LTB₄ generation was observed from 0.19 (0.07) to 2.29 (0.89) ng/ml (1 µg anti-IgE) and 3.86 (1.6) ng/ml (2 µg anti-IgE). The

Table 1 Mean (SEM) leukotriene B₄ synthesis in whole blood in patients with CGL and healthy subjects

	Patients with CGL	Controls
pg/ml	194 (70)	111 (39)
Neutrophils (pg/10 ⁶)	9.7 (5)	45 (14)
Leucocytes (pg/10 ⁶)	6.9 (3)	39 (14)

Table 2 Mean (SEM) leukotriene B₄ generation in whole blood (ng/ml) in patients with CGL and healthy subjects: effect of neutrophil agonists

	Patients	Controls
Buffer	0.194 (0.07)	0.111 (0.04)
Anti-IgE		
(1 µg/ml)	2.29 (0.89)†	0.146 (0.05)
(2 µg/ml)	3.86 (1.6)††	0.827 (0.43)
fMLP		
(1 µM)	41.9 (16)*	6.08 (1.7)
(2 µM)	35.6 (13.0)*	13.0 (3.0)
Zymosan		
(100 µg/ml)	17.6 (8.5)	5.35 (1.3)
(250 µg/ml)	42.4 (15.9)†	14.7 (3.6)
A 23187		
(50 µM)	642.8 (90)†††	152.9 (16)

*p < 0.05; †p < 0.02; ‡p < 0.005; ††p < 0.001.

stimulation of LTB₄ by anti IgE in the blood of patients with CGL was significantly correlated ($p < 0.5$, $r = 0.94$) with their blood basophil counts, and in one patient with a basophil count of $8.2 \times 10^9/l$. LTB₄ generation in whole blood increased more than 40-fold from 0.29 to 12.2 ng/ml in response to 2 μ g anti-IgE. Opsonised zymosan and fMLP were more potent stimuli to LTB₄ production both in healthy controls and in the patients (table 2). The total amount of LTB₄ generated was significantly greater in the patients with CGL: 42.4 (16) ν 14.7 (3.6) (patients ν controls, opsonised zymosan 250 μ g/ml; $p < 0.02$) and 35.6 (13) ν 13.0 (3) ng/ml (patients ν controls, 2 μ M fMLP; $p < 0.05$). In contrast to these "physiological" stimuli, the most vigorous stimulus to LTB₄ release was the calcium ionophore, A23187, which increased LTB₄ production in whole blood by a thousand-fold to 643 (90) ng/ml and 153 (16) ng/ml in the patients with CGL and controls, respectively. The high concentration of A23187 tested (50 μ M) was chosen to achieve maximal stimulation and thus provide an index of the capacity of leucocytes for LTB₄ formation in whole blood. Although the absolute increment in LTB₄ formation was significantly greater in the patients ($p < 0.001$), the capacity of CGL leucocytes to generate LTB₄ in response to A23187 was the same as normal leucocytes: 32.1 (11) ν 38.1 (4) ng/ 10^6 leucocytes neutrophils (patients ν controls).

NEUTROPHIL FUNCTION

There was no significant difference in the chemotactic response of neutrophils from patients with CGL and those of control subjects to a range of concentrations (10^{-8} - 10^{-5} M) of fMLP (figure). The peak response was seen at a concentration of fMLP of 10^{-6} to 10^{-7} M, which was comparable with the concentrations used to stimulate LTB₄ production in whole blood. LTB₄ was less potent as a stimulator of chemotaxis in this system, the peak response in the healthy controls being observed at a concentration of 10^{-5} M: distance to leading edge was 1.42 (0.16) ν 0.92 (0.09) mm (LTB₄,

Figure 2 Chemotaxis under agarose in response to fMLP and LTB₄.

10^{-5} ν buffer; $p = 0.02$), but a significant effect was also apparent at lower concentrations (figure). Neutrophils from patients with CGL exhibited a reduced response to LTB₄ at every concentration tested (figure), although the difference reached significance only at the highest concentration: distance to leading edge was 1.11 (0.18) ν 1.42 (0.16) mm (patients ν controls; $p < 0.05$). The chemotactic index for LTB₄ was also reduced at each concentration tested: 1.24 (0.1) CGL ν 1.51 (0.12) in controls; $p < 0.005$ (10^{-5} M). Phagocytic ingestion and killing of *S aureus* by neutrophils from the patients with CGL was normal and not significantly different from the healthy controls (table 3).

Discussion

LTB₄ is the principal product of arachidonic acid metabolism in human neutrophils.⁷ Studies in vitro have shown that it is an extremely potent stimulator of chemotaxis, chemokinesis, aggregation and adhesion of neutrophils and that in higher doses it induces lysosomal enzyme release and oxygen metabolite

Table 3 Phagocytosis and killing of *S aureus* by neutrophils from patients with CGL and healthy subjects

	Patients	Controls
Phagocytosis:		
No of intracellular bacteria/100 neutrophils	252 (39)	298 (44)
Percentage of neutrophils with > ingested organism	88 (4)	89 (3)
Killing:		
Colony forming units of <i>S aureus</i> (log CFU) before and after 90 min-utes incubation of 10^7 organisms with 10^7 neutrophils		
Before	7.14 (0.17)	7.14 (0.16)
After	5.79 (0.22)	6.04 (0.43)
With lysostaphin (intracellular killing)	5.40 (0.27)	5.50 (0.14)

production.^{9,10} The reported abnormalities in neutrophil function and in arachidonate metabolism in CGL led us to investigate LTB₄ synthesis in this condition and to explore the association between changes in LTB₄ production and its possible functional effects in vivo. We developed a sensitive technique utilising negative ion-chemical ionisation gas chromatography-mass spectrometry which allowed us to measure LTB₄ generation in whole blood with a limit of sensitivity of 2 pg/ml.

Using this technique we have shown that small amounts of LTB₄ are synthesised in whole blood, both in normal subjects and in patients with CGL. Despite previous work suggesting a deficiency of 5-lipoxygenase products in such patients,⁶ we found no evidence of reduced LTB₄ generation in any of the patients with CGL studied, either in unstimulated whole blood or in response to several physiological stimuli. The content of arachidonate substrate in neutrophil membranes obtained from patients with CGL was also normal (data not shown). In addition, as shown by the effect of calcium ionophore, the capacity of CGL leucocytes to synthesise LTB₄ was no less than that of normal leucocytes. The amounts of LTB₄ produced depended on the nature of the stimulus. Anti-IgE was a very weak agonist in healthy subjects, presumably reflecting the low numbers of circulating basophils in normal subjects. While human mast cells are known to release LTB₄ after IgE-dependent activation,¹⁴ the extent of LTB₄ generation by peripheral blood basophils has not previously been determined in man. It is therefore interesting that in those patients with CGL, who had a mean basophil count of $2.5 \times 10^9/l$, the amount of LTB₄ in whole blood increased more than 20-fold in response to anti-IgE, reaching concentrations which are biologically active in vitro. This increase was significantly correlated with the patients' basophil counts, strongly suggesting the basophil as the source of anti IgE-induced LTB₄ production. In response to a phagocytic stimulus opsonised zymosan, and to concentrations of the synthetic peptide fMLP which induce chemotaxis in vitro, LTB₄ generation increased more than 100-fold. This resulted in LTB₄ concentrations in whole blood in the patients with CGL which were considerably higher than those measured in healthy subjects and probably reflected the higher leucocyte counts in these patients.

The cellular source of the LTB₄ measured in whole blood was not directly addressed in this study. Previous work with purified cell preparations has shown that monocytes are able to synthesise similar quantities of LTB₄ to neutrophils in response to calcium ionophore and opsonised zymosan,¹⁵ and in this study, may have contributed to the LTB₄ generated in whole blood in response to these stimuli. While eosinophils synthesise little LTB₄,¹⁴ our results

suggest that basophils produce substantial quantities of LTB₄ when stimulated by a specific agonist. The ability of granulocyte precursors to synthesise LTB₄ is unknown, although studies in HL-60s and other promyelocytic cell lines have shown that these cells have receptors for LTB₄.¹⁶ It seems likely that LTB₄ in whole blood is principally derived from neutrophils, but that other leucocytes with a role in the inflammatory response may synthesise high amounts of LTB₄ in response to specific stimuli. Measurement of LTB₄ in whole blood thus has the advantage of taking into account LTB₄ generation from all circulating leucocytes and might therefore be expected to reflect more accurately LTB₄ synthesis in vivo during the inflammatory response, particularly when the leucocyte profile is abnormal as in CGL.

The most potent biological activity of LTB₄ is its ability to induce neutrophil chemotaxis and chemokinesis. The results of this study showed that despite enhanced production of LTB₄ in whole blood in response to stimuli known to induce neutrophil activation, neutrophils from patients with CGL were unable to respond normally to a range of concentrations of LTB₄. Nevertheless, their capacity for directed movement towards fMLP was unimpaired. Thus the neutrophils from these patients exhibited a specific defect in chemotaxis of LTB₄. There are several possible reasons for this. A recent study found that chemotaxis is the last of the functional properties acquired by neutrophils during normal differentiation.¹⁷ Our results may therefore reflect an increased proportion of more immature neutrophils in the cell suspension derived from the patients with CGL which are less well able to respond to chemotactic stimuli. This seems unlikely, however, as the cells responded entirely normally to fMLP, and morphologically over 95% of the cells studied were mature neutrophils. The results could also be explained by an abnormality of the interaction between LTB₄ and its specific receptor. The chemotactic responses of normal neutrophils to fMLP and LTB₄ are mediated via distinct receptors on the neutrophil surface.¹⁸ A recent study showed reduced binding of fMLP to CGL neutrophils, but the corresponding chemotactic response of these cells to fMLP was not tested.¹⁹ Although there are no available data on the LTB₄ receptor on CGL neutrophils, preliminary evidence suggests that at least in HL-60s the number and affinity of LTB₄ receptors on the cell surface is governed by the stimulus to differentiation.¹⁶ The impaired response to LTB₄ in the patients with CGL may therefore be the result of reduced numbers of receptors on CGL neutrophils which, though morphologically mature, are functionally immature. This remains to be investigated. Alternatively, the poor chemotactic response may be due to down-regulation of the LTB₄ receptor to its low affinity state

as a consequence of exposure to higher concentrations of LTB₄ in the patients with CGL. There is evidence that while chemotaxis is mediated by the LTB₄ receptor when it is in a high affinity state, a low affinity state is associated with transduction of superoxide generation and degranulation.^{20,21} That the CGL neutrophils which we studied had no major abnormality of oxidative metabolism and degranulation can be inferred to some extent from their normal microbicidal capacity.

In conclusion, we have shown that the capacity for LTB₄ generation in whole blood is normal in patients with CGL and that in response to a range of stimuli known to induce neutrophil activation, LTB₄ synthesis was actually enhanced. Nevertheless, neutrophil chemotaxis in response to LTB₄ was selectively impaired in these patients even when high concentrations of LTB₄ were used. Thus defects in LTB₄-mediated neutrophil responses may contribute to neutrophil dysfunction in CGL. Whether this reflects an abnormality of the numbers or affinity of LTB₄ receptors, and is therefore another example of the cell membrane abnormalities described in CGL neutrophils, remains to be investigated.

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