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Decreased polymorphonuclear leucocyte chemotactic response to leukotriene B_4 in cystic fibrosis

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SUMMARY

Evidence that leukotriene B_4 (LTB_4) is a significant inflammatory mediator in chronic pseudomonal respiratory disease was sought in adolescents and young adults with cystic fibrosis. Specific chemotaxis of peripheral blood polymorphonuclear leucocytes (PMN) was used as an indirect measure of remote *in vivo* exposure to LTB_4. PMN from 17 patients showed a significant decrease in chemotaxis to 10^{-7}–10^{-9} M LTB_4, but normal responses to 10^{-8} M *n*-formyl-methionyl-leucyl-phenylalanine and 4 mg/ml casein, when compared with 17 healthy age- and sex-matched controls. This result is consistent with chronic production of LTB_4, and specific deactivation of circulating PMN receptors for LTB_4 in patients with cystic fibrosis. Pharmacologic inhibition of LTB_4 production *in vivo* may help elucidate its role in the pathogenesis of lung damage in cystic fibrosis.

Keywords polymorphonuclear leucocyte chemotaxis leukotriene B_4 cystic fibrosis chronic infection

INTRODUCTION

Despite a significant increase in the life expectancy of patients with cystic fibrosis (CF) over the last 30 years, most die of progressive, destructive lung disease before the age of 40 [1,2]. The pathogenesis of lung disease in CF is complex and incompletely understood. Characteristics of severe disease include chronic colonization of the airways with *Pseudomonas aeruginosa*, with a marked local inflammatory response in which polymorphonuclear leucocytes (PMN) predominate. While the exuberant PMN response may prevent invasive disease, it does not eliminate *Ps. aeruginosa* from the lung, and PMN-derived mediators of inflammation may then contribute significantly to progressive lung damage [3–5].

Leukotriene B_4 (LTB_4) is an eicosanoid, produced from membrane-derived arachidonic acid by the 5-lipoxygenase pathway in human alveolar macrophages and PMN in response to various stimuli, including exposure to *Ps. aeruginosa* [6]. It is a potent stimulator of human PMN chemotaxis *in vitro* and has been proposed as a major mediator of pulmonary inflammation in response to infection [7–9]. LTB_4 has been found in significant quantities in the sputum of patients with cystic fibrosis [10–13]. Assessment of the role of LTB_4 in the human airway is complicated by the presence of a complex array of inflammatory mediators, enzymes, and cell types, in addition to the difficulty in reproducibly measuring nanomolar amounts of a locally metabolized substance *in situ*. Alternatively, the response of peripheral blood PMN can be used as an indirect measure of LTB_4 exposure *in vivo*.

Chemotaxis of peripheral blood-derived PMN in response to LTB_4 is mediated by specific receptors on the PMN membrane, which are deactivated by exposure to exogenous LTB_4 *in vitro* [14]. Changes in 5-lipoxygenase activity and hence LTB_4 production *in vivo* can be measured by bioassay, using chemotaxis of peripheral blood PMN towards LTB_4 [15]. The peripheral blood PMN chemotactic response to LTB_4 is decreased in patients with rheumatoid arthritis [16]. This hyporesponsiveness has been attributed to *in vivo* desensitization of LTB_4 receptors on circulating PMN [16], as a consequence of chronic local production of LTB_4 in inflamed joints [17]. Reduced chemotaxis of peripheral blood PMN to LTB_4 has also been reported in patients with chronic inflammatory bowel disease [18], and adult respiratory distress syndrome [19], conditions associated with local production of LTB_4. If excess LTB_4 is produced in the respiratory tract in patients with CF, there should be a similar, specific decrease in the peripheral PMN response to LTB_4.

We compared PMN chemotaxis to LTB_4, and to two unrelated chemotactic stimuli, *n*-formyl-methionyl-leucyl-phenylalanine (fMLP) and casein, in patients with cystic fibrosis and in age- and sex-matched normal controls.

PATIENTS AND METHODS

Patients

Seventeen patients with CF and *Ps. aeruginosa* isolated from sputum culture were recruited from out-patient clinics and

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during hospital admissions for treatment of exacerbations of their cystic fibrosis. All had a chronic productive cough, and had been admitted to hospital for treatment of an exacerbation of their CF symptoms within the previous 1–4 weeks. None of the patients had a fever at the time of sampling. All patients were on oral anti-staphylococcal and/or intravenous anti-pseudomonal antibiotics at the time of sampling, and all were taking pancreatic enzyme and vitamin supplements regularly. None of the patients was receiving corticosteroids. The median age of the group was 17 years (range 12–26 years); the mean age 17·1 years (s.d. 4 years). Eleven patients were male and six female.

Normal controls were recruited from local high schools, hospital staff, and from otherwise well patients admitted for minor elective orthopaedic procedures. They were matched for sex and age (within 1 year) with the CF patients. The control patients were all well, with no history of recent respiratory tract infection, injury, allergy, asthma, or medication. They were all non-smokers. Informed consent for the assay was obtained from all the participants, and, in the cases of adolescents younger than 18 years, from parents.

Isolation of human polymorphonuclear leucocytes

A 10 ml sample of venous blood was collected into 2 ml of acid citrate dextrose (ACD) from each patient on the morning of the assay. PMN were isolated by a modification of the method of Boyum [20,21]. Platelet-rich plasma was separated from ACD anti-coagulated whole blood by centrifugation, and the majority of the erythrocytes separated from the remaining cell pellet by sedimentation through 3·5% dextran (Dextran T500, Pharmacia, North Ryde, Australia) in normal saline. Leucocyte-rich supernatant was removed and centrifuged at 250 ***g*** for 10 min at 4°C. The resultant cell pellet was then resuspended in 5 ml 0·9% sodium chloride at 4°C, layered over 5 ml Ficoll–Paque (Ficoll 400, 5·7 g/100 ml, diatrizoate sodium 9·0 g/100 ml, Pharmacia) in a 15 × 80 mm polystyrene tube and centrifuged at 600 ***g*** for 30 min at 4°C. PMN and residual erythrocytes sedimented to the bottom of the tube. Monocytes and lymphocytes present in the overlying Ficoll layer were removed by aspiration. Erythrocytes remaining in the pellet were lysed by exposure to distilled water at 4°C for 30 s, followed by restoration of osmolality by the addition of 2 ml of cold 3·5% sodium chloride solution. PMN were resuspended in PBS saline and counted in a Technicon H2 system cell counter. Purification was complete within 2 h, and resulted in a pellet comprising 99% PMN. Trypan blue exclusion demonstrated 98% cell viability.

Chemotaxis assay

PMN ($2{\cdot}5 \times 10^6$) in 100 μl in Hank's buffered saline solution (ICN Biomedicals, Seven Hills, Australia) supplemented with 0·1% bovine serum albumin (Sigma A7030, St Louis, MO) (HBSS/BSA) were placed in the upper chamber of a modified Boyden apparatus. The Boyden apparatus was made by the method of Kemp *et al.* [22], with the upper chamber formed by the barrel of 1 ml tuberculin syringes (Terumo, Melbourne, Australia) cut off 2 cm below the hilt, to which 6 mm diameter, 100 μm thick discs cut from a single 3 μ pore-size nitrocellulose filter (SM11302, Sartorius GmbH, Göttingen, Germany) were glued with MF cement (Waters Millipore, Lane Cove, Australia). The lower chambers consisted of 2 ml autoanalyser sample cups (Bacto Laboratories, Liverpool, Australia).

The chemotactic factors to be tested were freshly made up in HBSS/BSA with 1% methanol, and 400 μl placed in the lower chamber. The factors tested consisted of a negative control (HBSS/BSA with 1% methanol), four concentrations of LTB_4 (Amersham, North Ryde, Australia) in HBSS/BSA/1% methanol (10^{-10}–10^{-7} M), 10^{-8} M fMLP (Sigma) in HBSS/BSA/1% methanol, and 4 mg/ml casein ('Hammarsten' Merck, Darmstadt, Germany) in HBSS/BSA/1% methanol. These concentrations of fMLP and casein were shown, in preliminary studies, to elicit maximal chemotactic responses in PMN from normal subjects. Each stimulus was tested in triplicate.

The upper chambers, containing PMN in HBSS/BSA, were suspended in the lower so that the fluid levels in each were equal, and incubated at 37°C in a humidified air/5% CO_2 mixture for 30 min, after which the filters were removed, fixed with ethanol, stained with haematoxylin, decolourized by methanol/butanol, and immersed in xylol to render the filter transparent. Chemotaxis was assessed by a leading front technique [22]. The maximum distance of penetration of PMN from the upper surface of the filter was assessed by microscopy of five high power fields per filter (magnification × 400) using a calibrated vernier scale. For each stimulus, the results were expressed as relative migration – the mean distance migrated, in micrometres, minus the negative control.

To exclude the possibility that sufficient LTB_4 to occupy and deactivate receptors is produced, *in vitro*, from PMN during isolation and incubation, PMN from CF patients were assessed for LTB_4 production. PMN (1×10^7) from CF patients and from normal controls were incubated in HBSS/0·1% BSA at 37°C, with and without 5 μM of the calcium ionophore A23187 (Boehringer Mannheim, Castle Hill, Australia) for 5 min, subjected to a total lipid extraction, and the resultant lipid extract analysed for the presence of LTB_4 and its metabolites by HPLC and u.v. spectroscopy. The method used can detect as little as 10 pmol of LTB_4. A23187-exposed cells from controls and CF patients produced similar amounts of LTB_4 (100–400 pmol) and significant amounts of its metabolites. Unexposed cells produced no detectable LTB_4 or metabolites, indicating that these cells have the capacity to generate LTB_4, but do not do so as a result of the isolation technique or under the conditions of the chemotaxis assay used.

Statistical analysis

Relative migration for each stimulus was compared in CF patients and age- and sex-matched controls by paired *t*-test (two-tailed) after determination that the distribution of results approximated normality. The effect of age on relative migration to each stimulus, in both CF and control groups, was assessed by regression analysis. The effect of sex was assessed by two-way analysis of variance (ANOVA) adjusted for disease status.

RESULTS

The maximum chemotactic response to LTB_4 occurred at a concentration of 10^{-8} M in both CF and control groups (Fig. 1). Comparative analyses were performed on PMN exposed to optimal concentrations of chemoattractants, i.e. 10^{-8} M LTB_4, 10^{-8} M fMLP and 4 mg/ml casein (see Fig. 2). In normal controls the greatest chemotactic response was to 4 mg/ml casein, followed by 10^{-8} M LTB_4, and 10^{-8} M fMLP. In the patients with CF, the response to 10^{-8} M LTB_4 was significantly depressed,

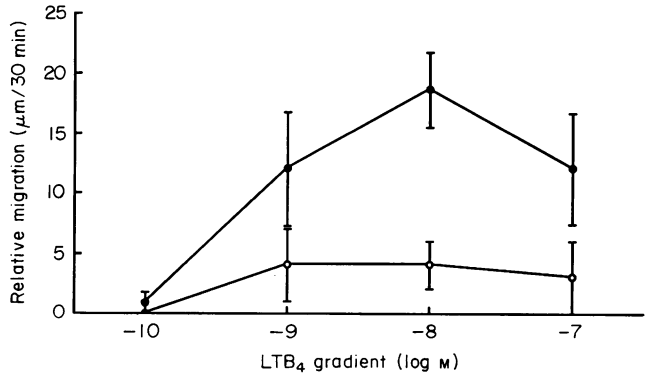

Fig. 1. Dose response of polymorphonuclear leucocyte (PMN) chemotaxis to leukotriene B_4 (LTB_4) gradient. Mean and s.d. for each concentration. ●, Normal ($n=17$); ○, cystic fibrosis ($n=17$).

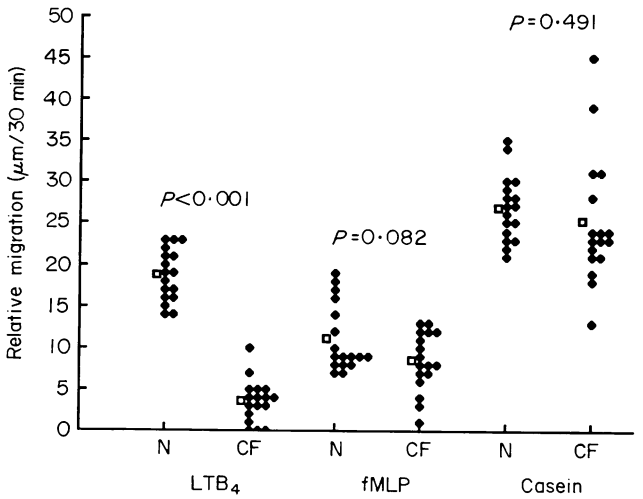

Fig. 2. Polymorphonuclear leucocyte (PMN) chemotaxis of individual patients to specific stimuli. For each stimulus, N represents chemotaxis in normal controls and CF those of patients with cystic fibrosis. ◆, Individual measurements; □, mean value for each group. *P* values (two-tailed *t*-test) are given for comparison of CF and N for each stimulus. LTB_4, leukotriene B_4; fMLP, *n*-formyl-methionyl-leucyl-phenylalanine.

while responses to 4 mg/ml casein and 10^{-8} M fMLP were not significantly different from those in the control group. Regression analysis showed no significant effect of age on chemotaxis to any of the stimuli in either CF patients or controls, and two-way ANOVA demonstrated no significant effect of sex on the response in either group.

DISCUSSION

LTB_4-induced chemotaxis of peripheral blood PMN from patients with CF who were colonized with *Ps. aeruginosa* was significantly depressed compared with that of age- and sex-matched normal controls. This abnormality was not demonstrated with two unrelated chemotactic stimuli, which is consistent with a specific decrease of receptor-mediated response to LTB_4 rather than a generalized abnormality in PMN chemotactic function.

Hyporesponsiveness of peripheral blood PMN to specific chemotaxins, such as LTB_4 or C5a, without evidence of a generalized abnormality of PMN locomotion (assessed by fMLP- or casein-induced chemotaxis) has been reported in rheumatoid arthritis [16,23], chronic inflammatory bowel disease [18], chronic granulomatous leukaemia [24] and adult respiratory distress syndrome [19]. The rationale for these findings is that local production of mediators causes not only migration, localization and activation of PMN at the site of production, but also deactivation (or change to a lower affinity state) of mediator-specific receptors on the majority of blood PMN, which are exposed at the site but not recruited from the circulation.

Based on our findings we propose that LTB_4 is generated within the lungs of patients with CF, by resident alveolar macrophages and by PMN recruited from peripheral blood, in response to chronic exposure to *Ps. aeruginosa* and endogenous inflammatory mediators such as tumour necrosis factor (TNF). Circulating PMN are thus exposed to local high concentrations of LTB_4 as they pass through the pulmonary circulation, but not all are recruited. The resultant LTB_4-induced deactivation of specific surface receptors *in vivo* is indicated by chemotactic hyporesponsiveness to LTB_4, but not to other chemotaxins, *in vitro*.

Previous studies of PMN chemotaxis in CF are few and somewhat conflicting. Hill *et al.* [25] examined chemotaxis of PMN in response to a filtrate of *Escherichia coli* (in which the chemotaxin was not defined, but may have included fMLP) from 25 patients with cystic fibrosis, and 25 age-matched controls, using a modified Boyden chamber assay. A significant increase in chemotaxis was noted in those patients with 'active infection', compared with both normals and patients who were not 'actively infected'. Church *et al.* [26,27] demonstrated no significant difference in PMN or monocyte chemotaxis in response to zymosan-activated serum (the active component of which is C5a) between normal adults and 'infected' and 'non-infected' patients with CF, using an under-agarose assay. Zielinski *et al.* [28], in a conflicting report, compared PMN chemotaxis to 1 mg/ml casein and zymosan-activated serum in 27 patients with CF and 15 normal controls, using a modified Boyden chamber technique. These authors noted significant depression of the chemotactic response to both stimuli in the patients with CF, and suggested that the chemotactic abnormality in CF is one of generalized PMN hyporesponsiveness. Clinical details of these patients, particularly nutritional status and use of corticosteroids, were not provided. The use of a lower concentration of casein in this study makes direct comparison with our data difficult.

None of the four studies addressed the possibility of LTB_4-mediated inflammation in CF. Only the last of these reports is consistent with stimulus-specific desensitization to C5a in CF [28]. If substantiated, the finding of an impaired response to zymosan-activated serum would support the concept of excessive local C5a production in a manner analogous to that of LTB_4. The two hypotheses are not incompatible—there is other evidence that complement activation occurs in the airways of patients with CF and chronic *Ps. aeruginosa* infection [3–5,7,29]. However, the relative importance of C5a- and LTB_4-induced PMN responses in the airways of patients with CF is uncertain.

Significant quantities of LTB_4 have been measured in sputum of patients with CF [10–13] and the clinical picture of massive PMN infiltration into the airways can be elicited by local instillation of LTB_4 *in vivo* in humans [9]. There is evidence that C5a is degraded rapidly in sputum by proteases of bacterial and/or host cell origin [5,29]. Local eicosanoid production is therefore likely to be important in the disease process *in vivo*.

LTB_4 production can be safely inhibited, *in vivo*, by dietary supplementation with eicosapentaenoic acid [15,16]. A study of the effects of eicosapentaenoic acid, or of other 5-lipoxygenase inhibitors, in CF may help clarify the role of LTB_4 in lung infections.

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