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Detection of sputum eicosanoids in cystic fibrosis and in normal saliva by bioassay and radioimmunoassay

J. T. ZAKRZEWSKI, N. C. BARNES, P. J. PIPER & J. F. COSTELLO
Department of Thoracic Medicine, King's College School of Medicine and Dentistry, London SE5 8RX and Department of Pharmacology, Hunterian Institute, Royal College of Surgeons of England, London WC2A 3PN

1 We have measured arachidonic acid (AA) metabolites, leukotrienes (LTs) and prostanoids (Ps), in sputum of patients with cystic fibrosis (CF) and in normal saliva using bioassay and radioimmunoassay (RIA).
2 Almost three times as much LTB_4 is present in CF extracts compared with slow reacting substances (SRSs). Leukotrienes were not detected in normal saliva.
3 In CF sputum there is a three-fold increase in the level of the vasodilator prostanoid prostaglandin E_2 (PGE_2) and the stable metabolite of prostacyclin, 6-oxo $PGF_{1\alpha}$ compared with the vasoconstrictor prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane B_2 (TxB_2), a hydrolysis product of thromboxane A_2.
4 Experiments with BW755c (25 $\mu g\ ml^{-1}$, $n = 3$) indicated that the majority of this activity was not produced during the extraction procedure.
5 The detection of LTs and Ps in sputum of CF patients shows that these substances are present at biologically active concentrations and may contribute to the pathophysiology of this disease.

Keywords cystic fibrosis sputum leukotrienes prostanoids

Introduction

Cystic fibrosis is the commonest autosomal recessive disease in the Western world with an incidence in the UK of approximately one in 2,500 live births. The basic defect is still unknown but one characteristic feature of CF is the production of abnormally viscid mucus which predisposes to pulmonary infections, leading eventually to widespread bronchiectasis. The disease is also associated with pancreatic disorders, malabsorption and a high incidence of atopy shown by positive skin reactions and elevated serum immunoglobulin E and G_4 (Tobin *et al.*, 1980). Because of the bronchiectasis, most CF patients expectorate sputum which contains abnormal mucus together with neutrophils and macrophages.

Inflammatory cells are capable of synthesizing LTs and Ps from their common precursor AA (Moncada & Vane, 1979; Samuelsson, 1981). Leukotrienes C_4 and D_4 are a major component of human lung slow-reacting substance of anaphylaxis (Bach *et al.*, 1979; Lewis *et al.*, 1980; Samuelsson, 1981), LTB_4 is chemotactic/kinetic for polymorphonuclear leukocytes (PMNs) and monocytes (Smith *et al.*, 1980; Ford-Hutchinson *et al.*, 1980), and the eicosanoids (LTs and Ps) have potent actions on the airways and microvasculature (Kadowitz *et al.*, 1975; Williams, 1983; Piper, 1984). Thus these biologically active AA metabolites possess properties of relevance to the pathophysiology of CF. Although LTs have already been identified in CF sputum (Cromwell *et al.*, 1982), the detection of both 5-lipoxygenase and cyclo-oxygenase products (COPs) has not been previously undertaken using RIA and a superfusion bioassay which selectively discriminates between the cysteinyl-containing LTs and LTB_4.

The purpose of our investigation was to detect LTs and Ps in sputum of patients with CF in order to evaluate their possible importance as mediators in this disease.

Methods

Subjects

Eleven CF patients were studied. The group consisted of seven males and four females with a mean age of 21.7 years (range 9–38).

Diagnosis was based on a raised sweat sodium (> 70 mEq l^{-1}); all had evidence of widespread bronchiectasis and produced purulent sputum. Assessment of lung function by the forced expiratory volume in one second (FEV_1) and the forced vital capacity (FVC) indicated severely impaired lung function, FEV_1 (mean ± s.e. mean) = 1.71 ± 0.25, $n = 11$ (predicted = 3.21 ± 0.25); FVC = 2.151 ± 0.25, $n = 10$ (predicted = 3.91 ± 0.35).

Saliva was obtained from four normal subjects. This group consisted of three males and one female with a mean age of 29 years (range 22–31).

Extraction procedure for eicosanoids

During physiotherapy, sputum was collected directly into 80% ethanol on ice and after adjusting the final ethanol concentration to 80%, each sample was homogenized on ice for 2 min using an Ultra Turrax homogeniser (Model TP 18/10) and centrifuged (MSE: 12,000 *g* for 20 min at 4° C) to remove insoluble material. The supernatant was then decanted, evaporated to dryness under reduced pressure and extracts stored under nitrogen at −20° C for subsequent analysis.

Fresh normal saliva was obtained 10 min after rinsing the mouth with water. The sample was voluntarily produced, collected in ethanol (80%) on ice and extractions carried out in the same manner as for sputum.

Following the extraction procedure, samples were resuspended in 5 ml distilled water and partially purified using C_{18} Sep-Pak cartridges (Waters) and a millipore filter (0.45 μm) as previously described (Morris *et al.*, 1979). After evaporation to dryness, each extract was resuspended in 1 ml of distilled water and assayed biologically and/or immunologically.

Bioassay of leukotrienes

Leukotrienes were assayed biologically using a combination of guinea-pig lung parenchymal strip (GPP) and ileum smooth muscle (GPISM). The use of these two tissues allows discrimination between the cyteinyl-containing LTs and LTB_4 (Samhoun & Piper, 1984); furthermore only the cysteinyl-containing LTs are selectively antagonised by the SRS antagonist FPL-55712 1 μg/ml (Augstein *et al.*, 1973).

Assay tissues were prepared as previously described (Samhoun & Piper, 1984). Briefly, male guinea-pigs (Dunkin-Hartley strain 350–400 g) were sacrificed by cervical dislocation, lungs and ileum removed and the tissue strips superfused in series with warmed (37° C) oxygenated Tyrode's solution (5 ml min^{-1}) containing a mixture of antagonists: mepyramine (0.1 μg ml^{-1}), hyoscine (0.1 μg ml^{-1}), methysergide (0.2 μg ml^{-1}), phenoxybenzamine (0.1 μg ml^{-1}), and propranolol (2 μg ml^{-1}). Contractions were measured by auxotonic levers (Paton) connected to transducers and visualised on a six-channel pen recorder (Watanabe). The use of sensitive tissues, selective receptor antagonists and the characteristic profile of contractions makes this biological assay extremely selective for the detection and measurement of LTs.

Using the superfusion technique, the actions of both LTB_4 and cysteinyl-containing LTs are partially mediated via TxA_2 release in guinea-pig lung (Piper & Samhoun, 1982); thus tissue responses to injected samples were compared to standard LTD_4, B_4 (1–10 pmol) and a TxA_2 mimetic, U44069 (1–3 nmol). All extracts were assayed in terms of LTD_4 equivalents (pmol g^{-1}) since LTB_4 which only contracts GPP (whereas LTD_4 is also active on GPISM), can produce tachyphylaxis but is equipotent with LTD_4 on this tissue (Samhoun & Piper, 1984).

Radioimmunoassay for Leukotriene C_4

Leukotriene C_4 was measured in extracts by RIA using a double antibody technique and an antiserum which also cross-reacted to the extent of 50% with LTD_4 and 8.3% with LTE_4 (Hayes *et al.*, 1983). Cross-reactivity with other eicosanoids, e.g. sulphone derivatives, LTB_4 and its 20-OH or 20-COOH metabolites, primary PGs, 6-oxo-$PGF_{1\alpha}$, TxB_2 and AA are negligible.

Radioimmunoassay of prostanoids

Samples were assayed for PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2 according to the method described by Jose *et al.* (1976). Cross-reaction of antiserum to PGE_2 with PGE_1 was 26%; all other cross-reactivities of the four antisera with different COPs (PGE_2, $PGF_{2\alpha}$, PGB_2, PGD_2, 6-oxo-$PGF_{1\alpha}$ and TxB_2) were $< 3\%$ and with AA $< 0.06\%$.

Inhibition of cyclo-oxygenase and 5-lipoxygenase by BW755c

Sputum samples obtained from three patients (12.1 ± 0.7 g wet weight) were subdivided into

two equal fractions and extracted in either the absence or presence of BW755c (25 μg ml^{-1})—an inhibitor of both cyclo-oxygenase and 5-lipoxygenase at this concentration (Higgs & Flower, 1981). Each fraction was assayed for LTs and Ps in order to determine the possible generation of eicosanoids *in vitro* during the extraction procedure.

Liquid scintillation counting

Radioactivity was estimated in a Packard Tricarb liquid scintillation counter and counting efficiency calculated from a simple channels ratio. All results were converted to d min^{-1}. The scintillant used was Aquasol-2 (New England Nuclear).

Materials

The following were used: leukotriene B_4, C_4 and D_4 (Merck Frosst Laboratories), FPL-55712 (Fisons), prostaglandin E_2, $F_{2\alpha}$, 6-oxo-prostaglandin $F_{1\alpha}$, thromboxane B_2 and U-44069 (The Upjohn Company), mepyramine maleate (May & Baker), hyoscine hydrobromide (BDH), methysergide maleate (Sandoz), phenoxybenzamine hydrochloride and propranolol (ICI).

Calculations and statistical methods

Results are given as mean ± s.e. mean for the number of experiments indicated. The differences between means were considered significant if $P < 0.05$ as determined by the upaired Wilcoxon rank sum test (non-parametric).

Results

The average amount of sputum produced in CF patients was 5.3 ± 1.3 g wet weight, $n = 11$ and saliva from normal volunteers 1.9 ± 0.3 g wet weight, $n = 4$.

CF sputum extracts contracted GPP but not GPISM (Figure 1). This activity (26.5 ± 4.9 pmol Eq LTD_4 g^{-1}, $n = 11$) consisted mainly of LTB_4 and also a SRS-like component which could be antagonised by FPL-55712 (18.8 ± 3.4 and 7.7 ± 1.4 pmol Eq LTD_4 g^{-1} respectively $P < 0.05$, $n = 11$, Figure 3). The FPL-inhibitable contraction on GPP could not be measured on GPISM but was detected by RIA (LTC_4 = 5.7 ± 1.5 pmol g^{-1}, $n = 11$, Figure 3).

LTs were not detected in samples of normal saliva (Figure 2). Sputum and saliva extracts

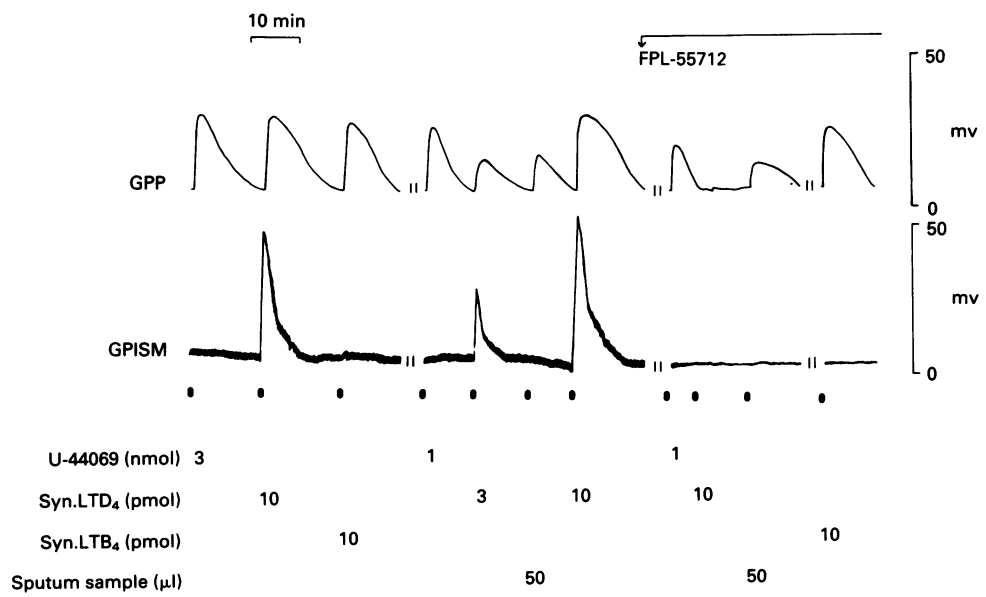

Figure 1 Bioassay of LT-like activity in sputum of a patient with CF. The trace illustrates the differential effect of LTs on GPP and GPISM. Synthetic LTB_4 and LTD_4 (10 pmol), U-44069 (3 nmol) produced contractions of similar height and duration of action (15 min) on GPP. LTB_4 and U-44069 have no effect on GPISM, unlike LTD_4. Injection of the sputum extract (50 μl) contracted GPP (3–10 pmol Eq LTD_4) but produced no effect on GPISM. In the presence of FPL-55712 (1 μg ml^{-1}), the GPP contraction was almost entirely maintained by the extract (50 μl) whereas the action of synthetic LTD_4 (10 pmol) was totally inhibited. Thus, LTB_4 accounts for most of the LT activity in CF sputum.

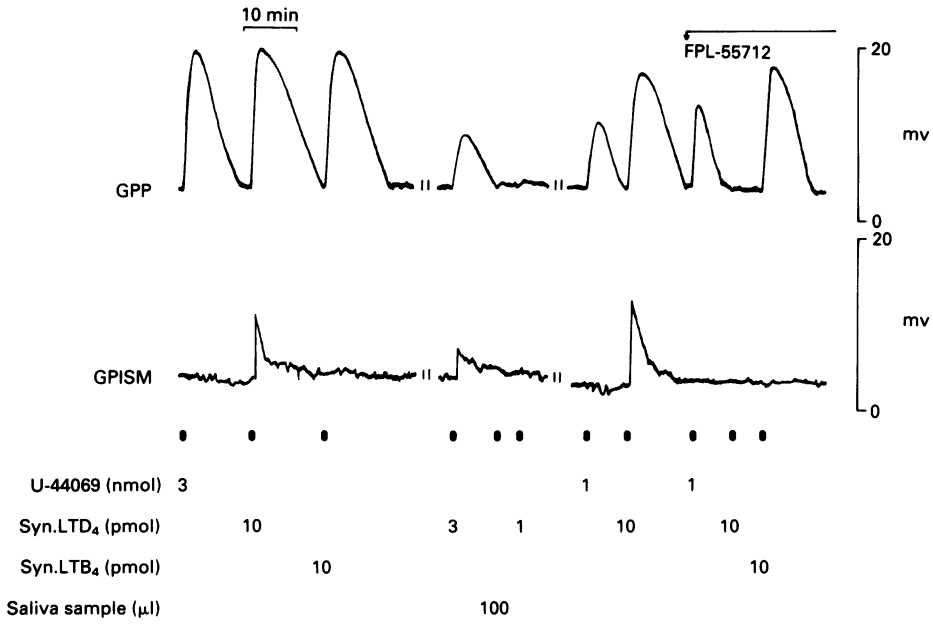

Figure 2 Bioassay for LT-like activity in normal saliva. The trace illustrates the differential effect of LTs on GPP and GPISM. Synthetic LTB_4 and LTD_4 (10 pmol) and U-44069 (3 nmol) produced contractions of similar height and duration of action (15 min) on GPP. LTB_4 and U-44069 have no effect on GPISM, unlike LTD_4. The actions of LTD_4 are selectively inhibited by the SRS antagonist FPL 55712 (1 μg ml^{-1}). Injection of the saliva extract (100 μl) did not contract either tissue.

contained PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2 (CF: 45.7 ± 11.2, 10.4 ± 1.9, 18.0 ± 5.3 and 10.5 ± 2.7 pmol g^{-1}, n = 11; Saliva: 12.6 ± 2.2, 10.2 ± 3.7, 2.4 ± 0.5 and 2.5 ± 2.1 pmol g^{-1}, n = 4, respectively;Figure 4). In CF sputum, prostanoid production is directed in the ratio 3:1 toward the vasodilator (PGE_2, prostacyclin) rather than vasoconstrictor ($PGF_{2\alpha}$, thromboxane A_2) agents, unlike saliva which has a more even distribution (1:1).

Experiments with BW755c (25 μg ml^{-1}, n = 3) indicated that approximately 50% of LTB_4 (22.5 ± 3.8 pmol Eq LTD_4 g^{-1}, n = 3) may be formed during homogenization. No effects were observed on the levels of other eicosanoids measured (Figure 5).

Discussion

Leukotriene B_4 and cysteinyl-containing LTs were detected in CF sputum by bioassay and RIA, confirming a previous report in which RP-h.p.l.c. was used in conjunction with biological methods (activity on guinea-pig ileum and chemotaxis) to identify these compounds (Cromwell *et al.*, 1982). RP-h.p.l.c. analysis of sample extracts produces good resolution and a high level of purity (Cromwell *et al.*, 1982) but significant loss of material leads to quantitative problems despite the fact that both RIA (Hayes *et al.*, 1983) and bioassay (Samhoun & Piper, 1984) techniques are capable of detecting LTs below or at the 1 pmol level respectively.

Since the analytical methods employed in this study can be used for the selective detection of eicosanoids (see **Methods**) we have analysed partially purified extracts using Sep-Pak cartridges in which sample recovery of corresponding [^{3}H]-eicosanoids is high (85–95%).

Bioassay of 5-lipoxygenase products showed LTB_4 to be a major metabolite and SRSs present in smaller amounts. Values for LTC_4 measured by RIA were in broad agreement with the FPL 55712-inhibitable activity observed on GPP. Since bioassay of LTC_4 and D_4 produces contractions of similar height/duration in this tissue (Samhoun & Piper, 1984) and the antiserum to LTC_4 also recognizes LTD_4 (Hayes *et al.*, 1983), it seems reasonable to compare the results obtained by both techniques. CF sputum collected during expectoration may have been contaminated with small quantities of saliva. Although LTs (mainly LTE_4) have been measured by RIA

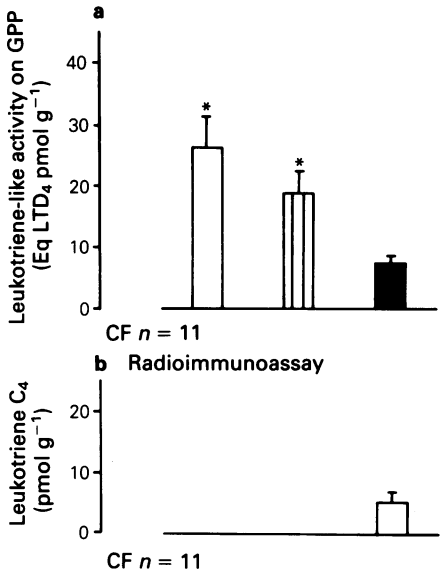

Figure 3 Concentrations of LTs in sputum of patients with CF (n = 11). (a) Bioassay. The histogram shows LT-like activity on GPP (□) which mainly consists of LTB_4 (▥) and an SRS-like component (FPL-55712 inhibitible contraction) (■). Results are expressed in terms of LTD_4 Eq pmol g^{-1} (LTB_4 > SRSs by three-fold). (b) Radioimmunoassay. The histogram represents the LTC_4 concentration (pmol g^{-1}) which is in broad agreement with the FPL 55712-inhibitable activity measured on GPP. All values are the mean (± s.e. mean) for the number of experiments indicated. *$P < 0.05$ compared with SRS.

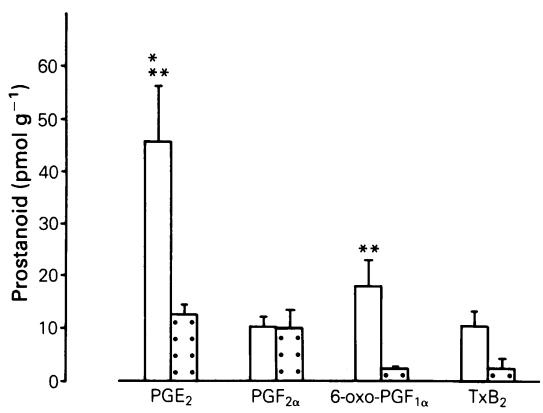

Figure 4 Radioimmunoassay of prostanoids in sputum of patients with CF (□, n = 11) and in normal saliva (▦, n = 4). The histogram represents prostanoid concentrations for PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2 (pmol g^{-1}). In CF sputum metabolism favours vasodilator (PGE_2, 6-oxo-$PGF_{1\alpha}$) rather than vasoconstrictor ($PGF_{2\alpha}$, TxB_2) prostanoids, whereas saliva contains a more even distribution. All values are the mean (± s.e. mean). *$P < 0.05$ compared with sputum $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2, **$P < 0.05$ compared with equivalent saliva prostanoid.

from normal and CF salivary secretions in similar amounts (Rigas & Levine, 1984), we were unable to detect this compound since the LTC_4 antiserum used in our study does not significantly cross-react with this ligand. Furthermore, bioassay of saliva extracts indicated no biological activity corresponding to these compounds. We have, however, shown normal saliva to contain various cyclo-oxygenase products which agrees with earlier reports describing salivary prostanoids and hydroxyeicosatetraenoic acids (HETEs) in many species, including man (Rigas *et al.*, 1983). PGE_2 and 6-oxo-$PGF_{1\alpha}$ are present in higher levels from CF sputum compared with normal saliva, but significant differences have been reported for $PGF_{2\alpha}$ as well as PGE_2 in salivary secretions from both groups (Rigas & Levine, 1984). In our experiments, concentrations of Ps found in CF sputum extracts suggest that the pathway of AA metabolism via cyclo-oxygenase is directed towards vasodilator (PGE_2, PGI_2) rather than vasoconstrictor ($PGF_{2\alpha}$, TxA_2) products unlike normal saliva, which has a more even distribution. Vasodilator Ps play a key role in inflammation in potentiating the actions of other agonists (Williams, 1983).

Sputum is a complex mixture of mucus secretions and different cell types, including PMNs, mainly neutrophils and macrophages. PMNs have the necessary enzyme systems for the synthesis of LTs (Ford-Hutchinson *et al.*, 1980; Samuelsson, 1981; Salmon *et al.*, 1982) and Ps (Higgs *et al.*, 1975; Zurier, 1976). Macrophages also possess this capability (Humes *et al.*, 1977; Wei Hsueh, 1979; MacDermott *et al.*, 1984). It seems likely, therefore, that these inflammatory cells are a major source of 5-lipoxygenase and cyclo-oxygenase products in CF. All CF patients suffer from bacterial colonization of the airways but antibiotic treatment during which the patient improved clinically and sputum purulence was diminished, had no effect on eicosanoid production (unpublished observations) which suggests that bacteria are not a direct source of AA metabolites even though phagocytic cells are attracted to the site of infection. Neutrophils mainly synthesize LTB_4 which, unlike HETEs, is not incorporated into cellular phospholipids (Salmon *et al.*, 1982), whereas macrophage activation leads primarily to the formation of vasodilator PGE_2 and PGI_2 (Humes *et al.*, 1977).

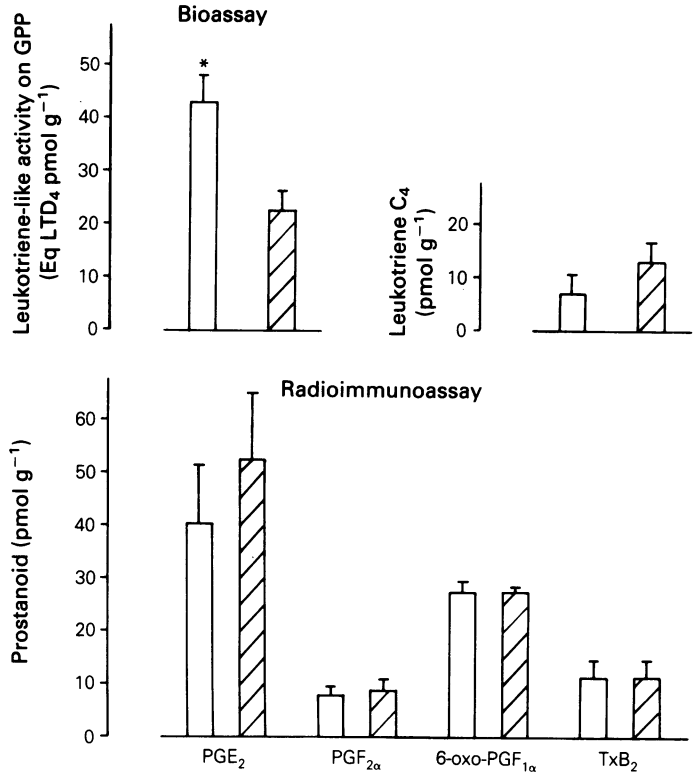

Figure 5 Effect of BW-755c (25 μg ml^{-1}) on LT and P concentrations in sputum of patients with CF (n = 3). Samples were subdivided and extracted for eicosanoids in the absence (□) and presence (▨) of inhibitor (BW-755c 25 μg ml^{-1}) which reduces only LTB_4 levels. All values are mean (± s.e. mean) of three experiments. *$P < 0.05$ compared to presence of BW-755c

Sputum also contains phospho- and neutral lipid and large amounts of lipid are associated with respiratory glycoproteins in mucus (Woodward *et al.*, 1983). Eicosanoid levels in CF sputum are not only related to the presence of inflammatory cells but may also be the consequence of disturbed Ca^{2+} homeostasis in polymorphs leading to increased phospholipase activity (Banschbach *et al.*, 1978; Case, 1984) or increased lipolysis in response to malnutrition and pulmonary sepsis (Chase *et al.*, 1979). There could be a relationship, therefore, between observed abnormalities in fatty acid metabolism, particularly in relation to LT/P synthesis and the pathophysiology of CF. This relationship is strengthened when considering the pharmacological actions and interactions of these compounds with respect to abnormal mucus secretion, bronchoconstriction and oedema formation.

The ability of LTs and Ps to stimulate mucus formation using radiolabelled glycoprotein as a model for release has been well documented *in vitro* (Shelhamer *et al.*, 1982; Coles *et al.*, 1983) and *in vivo* (Peatfield *et al.*, 1981), although direct effects of bacteria on mucus secretion should not be overlooked. LTs contract isolated airway preparations (Dahlén *et al.*, 1980; Piper & Tippins, 1982; Samhoun & Piper, 1983) and when administered by aerosol to humans, cause bronchoconstriction which is at least 1000 times more potent than that caused by histamine (Barnes *et al.*, 1984a). Inhaled $PGF_{2\alpha}$ and PGD_2 also produce bronchoconstriction (Smith *et al.*, 1975; Hardy *et al.*, 1984) and a sensitizing effect of inhaled LTD_4 to $PGF_{2\alpha}$ has been demonstrated in man (Barnes *et al.*, 1984b) suggesting an interaction between these bronchospastic agents. Interactions of 5-lipoxygenase and cyclooxygenase products have already been reported in the isolated guinea pig trachea (Piper & Tippins, 1982).

It is interesting that CF patients have a high incidence of atopy as measured by skin tests and serum IgE and IgG_4 concentrations, and show bronchial hyperactivity (Tobin *et al.*, 1980) which is also a characteristic of CF carriers (Davies,

1984), especially since LTs have been suggested as a cause of airway hyperreactivity (Weissman, 1983). Moreover, there may be a degree of protection against enzymatic degradation of SRSs by serum-type glycoproteins e.g. protease-inhibitors present in mucus of CF sputum (Roussel, 1984).

A consequence of pulmonary infection in CF is inflammation and in this respect LTB_4 and vasodilator Ps may be candidates as mediators. This is supported by the knowledge that LTB_4 is a potent chemotactic, chemokinetic and aggregating agent for PMNs (Ford-Hutchinson *et al.*, 1980) which have specific 'LTB_4 recognition receptors' (Goetzl, 1983). Leukotrienes injected intradermally can induce oedema formation as measured by extravasation of ^{125}I-albumin which is considerably potentiated by PGE_2 or 6-oxo-$PGF_{1\alpha}$ (Bray *et al.*, 1981; Peck *et al.*, 1981) and LTB_4-induced plasma exudation is dependent on circulating neutrophils (Wedmore & Williams, 1981; Williams, 1983). LTs also cause vascular permeability changes in the lung (Woodward *et al.*, 1983) as well as weal and flare responses in human skin (Camp *et al.*, 1983). It seems possible, therefore, that in CF, LTB_4 and vasodilator Ps interact to stimulate oedema formation.

Experiments with BW755c (25 $\mu g\ ml^{-1}$), an inhibitor of both cyclo-oxygenase and 5-lipoxygenase pathways at this concentration (Higgs & Flower, 1981) indicate that 50% of LTB_4 may be formed during homogenization, whereas the concentrations of SRSs and Ps were unaffected. This suggests some degree of selectivity and experiments are in progress to confirm this.

For accurate quantitation of eicosanoids pure material must be available and a measure of recovery obtained by the use of corresponding radiolabelled compounds taken through the same extraction and analytical procedures. It is essential that the presence of ^{3}H-markers of known specific activity do not affect the viability of assay methods, in particular, when biological systems are used.

Although problems associated with the quantitation of 5-lipoxygenase and cyclo-oxygenase products from pure extracts have not been overcome, our results show that LTs and Ps are present in sputum of CF patients in amounts likely to contribute to mucus secretions, bronchoconstriction and inflammation. Evidence for the presence of eicosanoids in CF secretions produced in the airways may be important in future approaches to the treatment of pulmonary problems in this disease.

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