Characterisation of the range of neutrophil stimulating mediators in Cystic Fibrosis sputum

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ABSTRACT

Background: Most cystic fibrosis (CF) patients die of respiratory failure due to chronic infection and destructive neutrophilic inflammation. Objective: To identify potential therapeutic targets by characterising the neutrophil stimulating mediators in the CF airway. Methods: Spontaneously expectorated CF sputum was extracted in phosphate buffered saline for assays of neutrophil chemotaxis, intracellular calcium mobilisation and cell shape change. Mediators were purified by ion exchange, C18 reversed phase and size exclusion chromatography. Results: A pool of CF sputum contained considerable neutrophil stimulating activity but neutralisation of interleukin (IL)-8/CXCL8 had little inhibitory effect on neutrophil chemotactic (10149 ± 2023 migrating cells vs. 8661 ± 2597 at 62 mg sputum/ml, NS) or shape change (% FSC increase 46 ± 8 vs. 38 ± 5 at 19 mg sputum/ml, p<0.05) responses. Further, the CF sputum pool induced an elevation of intracellular calcium ions even after desensitisation of the neutrophils to IL-8. Chromatography identified contributions to the neutrophil shape change inducing activity from IL-8, other CXC chemokines, leukotriene (LT) B4 and two formyl peptides. There was also suggestive evidence for contributions from platelet activating factor (PAF) and C5a. Using non-chromatographed individual sputum samples, anti-IL-8 alone did have an inhibitory effect on neutrophil chemotaxis (median inhibition 41%, p = 0.0002). However, even in this experiment, there were clearly significantly important, non-IL-8 mediated effects of CF sputum on neutrophils and an inhibitor cocktail of anti-IL-8 plus CXCR2, LTB4, formyl peptide, PAF and C5a receptor antagonists inhibited chemotaxis by a median of 97% (p = 0.0002). Conclusion: Many chemoattractants contribute to the neutrophil stimulating activity in CF sputum although the relative contribution of these mediators differs in different patients. The selective blockade of single mediators may not be sufficient to control neutrophil recruitment and activation in the CF airway.
INTRODUCTION

Cystic fibrosis (CF) is characterised by cycles of infection and predominantly neutrophilic inflammation. There is debate as to whether infection causes inflammation, or the CF airway is itself pro-inflammatory even in the absence of infection. Late on, both are consistently present, and it is generally believed that the major cause of the airway damage that ultimately leads to respiratory failure is the excessive host response. Modulation of this response by prednisone is beneficial, but with unacceptable side-effects, and inhaled corticosteroids are largely ineffective. There is a need for more specific immunomodulators and, in this context, understanding the neutrophil recruiting mediators is important.

The potent neutrophil chemoattractant interleukin (IL)-8/ CXCL8 is increased in CF samples, but there are many other potential candidate molecules besides IL-8. Several other CXC chemokines, containing the ELR motif immediately before the first of the conserved cysteines, are capable of stimulating neutrophils including growth-related oncogene-α (GRO-α/CXCL1), GRO-β/CXCL2, GRO-γ/CXCL3, epithelial-derived neutrophil activating protein-78 (ENA-78/CXCL5), granulocyte chemotactic protein-2 (GCP-2/CXCL6) and neutrophil activating protein-2 (NAP-2/CXCL7). IL-8 and GCP-2 can act via CXCR1 or CXCR2, both receptors being present on neutrophils, whereas the other ELR+ CXC chemokines only signal through CXCR2.

CXC chemokines are cationic and may bind to the anionic DNA that is released from necrotic cells in the CF lung, thus preventing the chemokines from binding to neutrophil receptors. For this reason, non-chemokine mediators may be of considerable importance in the excessive neutrophil recruitment in the CF lung. Potential non-chemokine neutrophil stimulating mediators include bacterial-derived peptides (such as N-formyl-L-methionyl-L-leucyl-L-phenyalanine, fMLP), host-derived peptides (such as N-acetyl-Pro-Gly-Pro, a degradation product of the extracellular matrix), complement activation products (such as C5a) and lipid mediators (such as leukotriene B4, LTB4, and platelet-activating factor, PAF). To our knowledge, only two of these mediators, LTB4 and C5a, have so far been identified in CF airway samples.

The relative importance of these mediators to CF pathology is not known. Since neutrophil stimulating mediators play such a vital role in the detrimental inflammatory response seen in CF patients, their characterisation may improve understanding of the pathogenesis of CF and enable potential therapeutic targets to be identified. We hypothesised that multiple neutrophil chemoattractants are present in CF sputum, many of which have been poorly studied. The aim of this study was therefore to characterise the range of mediators present in CF sputum, using a variety of neutrophil stimulating assays.
METHODS

Patients  We collected spontaneously expectorated sputum from 22 CF patients, diagnosed on conventional criteria and described in table 1, at the Royal Brompton Hospital (London, UK). Spirometry was performed to ATS standards.

Sputum supernatant  Sputum from CF patients was pooled (pool 1, online supplement table 1), homogenised in phosphate buffered saline (PBS, 300mg sputum/ml), centrifuged and the supernatant recovered was stored as aliquots at -20°C. Individual sputum samples were homogenised at 100mg/ml.

Cell preparation  Mixed granulocytes were isolated from the peripheral venous blood of healthy human volunteers and resuspended in assay buffer [AB; PBS without Ca²⁺ Mg²⁺ containing 0.1% BSA, 10mM glucose, 10mM HEPES (Invitrogen, Paisley, UK), pH 7.2-7.4]. For chemotaxis and CD11b assays, the cells were supplemented with Ca²⁺ and Mg²⁺ (0.9mM and 0.5mM respectively).

Chemotaxis  Chemotactic responses were examined using 96-well chemotaxis plates (ChemoTx) with 3µm pore size polycarbonate filters as described previously. Results are expressed as the mean number of migrated cells, as counted by flow cytometry.

Leukocyte shape change  Shape change responses of mixed granulocytes were analysed as previously described. Briefly, mixed granulocytes were stimulated at 37°C for 4 min, after which their cell shape was maintained by fixation and analysed by flow cytometry. A minimum of 1000 neutrophil events were acquired using the FL-2 fluorescence channel, allowing neutrophils to be distinguished from eosinophils by their lower autofluorescence. Results are expressed as percentage increase in forward scatter (FSC) compared to buffer treated cells.

CD11b upregulation  The upregulation of this integrin was measured by use of FITC-labeled anti-CD11b and flow cytometry as described previously.

Intracellular Calcium Ion Mobilisation  Intracellular calcium mobilisation responses were examined using the fluorescent indicator FURA-2 as previously described. Changes in fluorescence emitted at 510nm were measured after stimulation at 340 and 380nm. Results are expressed as the relative increase in fluorescence compared to baseline.

ELISA  Inflammatory mediators were measured by specific sandwich ELISAs.

Chromatography  To estimate the range of neutrophil stimulating mediators, sputum was pooled (n=4-6 CF patients), homogenised in appropriate buffer, centrifuged and the supernatant applied to combinations of ion exchange, C18 reversed phase and size exclusion chromatography protocols (see online supplement table 1). Fractions collected were analysed by the cell shape change bioassay which provides a rapid and sensitive indicator of potential chemoattractant mediators.

Statistical Analysis  Experimental data are presented as the mean ± the standard error of the mean (SEM), unless otherwise stated. Effects of inhibitors on concentration response curves were analysed using the repeated measures ANOVA with Bonferroni’s post test. Effects of inhibitors on single concentrations of mediators or chromatography fractions were analysed with the Student’s paired t test. The effects of inhibitors on sputum from 13 individual CF patients were analysed by non-parametric methods (Wilcoxon test). Statistical analyses were performed on raw data, using GraphPad Prism® Version 3.02. There were insufficient data in the literature to inform a prior power calculation.
RESULTS
We recruited 22 CF patients, their details are summarised in table 1.

Table 1 Characteristics of CF patients.

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<table>
<thead>
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<tr>
<td>Age, years</td>
<td>12.3 (0.6)</td>
</tr>
<tr>
<td>M/F</td>
<td>9/13</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>59 (4)</td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>77 (4)</td>
</tr>
<tr>
<td>Infected with P. aeruginosa</td>
<td>10</td>
</tr>
<tr>
<td>Undergoing exacerbation</td>
<td>17</td>
</tr>
<tr>
<td>Azithromycin treatment</td>
<td>16</td>
</tr>
<tr>
<td>rhDNase treatment</td>
<td>14</td>
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</tbody>
</table>

Values are mean (SEM), n=22. Exacerbation was defined as an independent decision by the centre physician to begin antibiotics.

M/F, Male/Female; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; rhDNase, recombinant human DNase.

Sputum from six CF patients was pooled (pool 1, online supplement table 1), homogenised in PBS, centrifuged, and the supernatant used in a variety of neutrophil stimulation assays. There was considerable neutrophil stimulating activity; for example, significant responses were detected at concentrations as low as 7.8 mg sputum per ml extract in chemotaxis assays and 1.2 mg/ml when cell shape change was measured. In the chemotaxis assay, cell migration was observed when the CF sputum supernatant was added to the lower chamber but not when added to the cells in the upper chamber, indicating that migration was a truly directional chemotactic response, rather than a more random chemokinetic response (data not shown). Chemotaxis to the CF sputum supernatant was much greater than could be explained by the IL-8 concentration and was not significantly inhibited by an excess of neutralising anti-IL-8 antibody (figure 1a). Indeed, CF sputum supernatant was more efficacious than IL-8, inducing a higher maximum neutrophil response. Similar discrepancies between CF sputum and IL-8 were found in assays of neutrophil cell shape change (figure 1b) and CD11b upregulation (online supplement figure 1). These results indicate that IL-8 is not the sole, and probably not the major, neutrophil stimulating mediator in the CF airway.

To verify the presence of mediators other than IL-8, we used Fura-2 loaded neutrophils to measure kinetic changes in intracellular calcium ion concentrations in response to the successive administration of different test samples. Repeated administration of IL-8 induced a desensitisation of the calcium elevation response to this mediator but the cells still responded to the subsequent addition of CF sputum supernatant (figure 2a). In contrast, when the neutrophils were first desensitised to the CF sample they were unresponsive to subsequent IL-8 (figure 2b). To establish that the cells were still responsive to another stimulus, despite being desensitised to sputum components, we used PAF as the final addition. PAF always induced a calcium ion elevation response showing that the cells were still capable of
responding to appropriate stimuli (figure 2). However, this response (relative increase in fluorescence = 0.32 ± 0.12) was less than that seen when PAF was added as the first stimulus (data not shown, relative increase in fluorescence = 1.59, mean of two cell preparations). This may indicate an overall decline in responses with successive additions to the cells but it also raises the possibility of a partial desensitisation to PAF by the CF sputum. The results again suggest the presence of substantial neutrophil stimulating activity other than IL-8, possibly including PAF, in the CF sputum.

To examine the range of neutrophil stimulating mediators in CF sputum, we performed different chromatography protocols, each using a different pool of sputum (online supplement table 1). The fractions collected were analysed for their ability to induce neutrophil shape change in the absence and presence of a CXC chemokine blockade consisting of anti-IL-8 plus a CXCR2 antagonist to inhibit responses to other ELR+ CXC chemokines. Firstly, we verified that the bioactivity was unaffected by exposure of the sputum supernatant to trifluoroacetic acid (TFA) and acetonitrile (data not shown) which permitted the use of these reagents in the versatile and potentially highly-resolving C18 reversed phase HPLC.

Sputum (pool 2) was extracted in TFA containing 1.0 M NaCl and the soluble fraction applied to C18 HPLC (figure 3a). The salt-insoluble pellet was then re-extracted in TFA at a low salt concentration and applied to C18 HPLC (figure 3b). Both extracts contained neutrophil stimulating activity which separated into many fractions, indicating the range of mediators present. While the bioactivity in some fractions was inhibited by the chemokine blockade, indicating the presence of CXC chemokines, there were clear suggestions of the presence of non-chemokine mediators. Further, while the low salt re-extract of the pellet contained less bioactivity than the high salt extract, the low salt sample contained a higher proportion of more hydrophobic mediators which were unaffected by the chemokine blockade (figure 3), again indicating the range of different neutrophil stimulating mediators in CF sputum.

These high and low salt fractions contained a total of 8.7 pmol IL-8, 2.4 pmol GRO, 1.1 pmol ENA-78 and 0.2 pmol GCP-2 per gram of sputum but no detectable NAP-2. GRO (the ELISA does not distinguish between GRO α, β or γ) eluted before IL-8 and probably accounts for some of the activity in high salt fraction 4 (the proportion that was partially inhibited by the CXC chemokine blockade, figure 3a). GCP-2 can stimulate neutrophil CXCR1 and is the only ELR+ CXC chemokine that is not inhibited by the combination of anti-IL-8 and the CXCR2 antagonist. However, there was insufficient GCP-2 to have accounted for the neutrophil stimulating activity in the fractions at the dilutions used. We also measured a total of 7.6 pmol C5a, a complement-derived chemoattractant protein of similar molecular size to the chemokines, per gram sputum.

High salt fraction 4 and a pool of high salt fractions 13+14, which were largely resistant to inhibition by the chemokine blockade, were selected for further analysis using size exclusion chromatography. We detected neutrophil stimulating activities of approximately 25kD (unidentified), 8-12kD (likely to be the CXC chemokines previously detected) and two low molecular weight activities, differing in both hydrophobicity and molecular size, that were inhibited by a formyl peptide FPR receptor antagonist (online supplement figure 2).
The second chromatography protocol employed a combination of chemistries. The sputum extract (pool 3) was separated into three ion exchange fractions (cationic, anionic and the breakthrough from both cationic and anionic columns) which were then applied separately to C$_{18}$ reversed phase HPLC for subsequent bioassay. The cationic fraction contained neutrophil stimulating activity that eluted as a single peak from C$_{18}$ HPLC and was completely inhibited by the CXC chemokine blockade (online supplement figure 3a). The anionic fraction contained little detectable bioactivity (online supplement figure 3b). The breakthrough fraction separated into two clear C$_{18}$ peaks, the first of which may have contained a mixture of formyl peptide(s) plus some chemokine(s) (we found that approximately 28% of the sputum IL-8 unexpectedly broke through the cation exchange column, perhaps because of binding to DNA). More interestingly, the second peak was substantially inhibited by an LTB$_4$ BLT1 receptor antagonist (online supplement figure 3c).

We have thus identified a contribution of IL-8, other CXC chemokines such as GRO, formyl peptides and LTB$_4$, and a possible contribution from C5a and PAF, to the neutrophil stimulating activity in CF sputum. Next, we used a CF sputum extract in PBS (pool 1) and various inhibitor combinations to determine the relative contributions of these mediators to the overall bioactivity. The CXC chemokine blockade and the LTB$_4$ receptor antagonist each significantly inhibited the neutrophil shape change response, but only to a small extent (online supplement figure 4a,b), but there was no detectable inhibition by the formyl peptide receptor antagonist (online supplement Figure 4c). However, a combination of anti-IL-8 plus antagonists to CXCR2, BLT1 and FPR inhibited neutrophil responses to a much larger degree (64 $\pm$ 10%, p<0.001, online supplement figure 4d). This substantiates the HPLC data of a mixture of neutrophil stimulating mediators that contribute to the overall activity in CF sputum. To investigate the potential contribution of C5a or PAF we used antagonists to their receptors and found no significant inhibitory activity when the antagonists were tested individually (online supplement figure 4e,f). In contrast, addition of C5a and PAF receptor antagonists to the inhibitor combination described above produced a greater inhibitory effect (96 $\pm$ 4%, p<0.001, online supplement figure 4g). These data underline the complexity of the neutrophil stimulating mediators in CF sputum.

All experiments so far have been performed on pools of sputum to investigate the range of neutrophil stimulating mediators that are likely to be present in at least some CF patients. To test our findings in a cross section of patients, sputum samples were homogenised individually and the supernatants assayed for neutrophil chemotaxis at a single concentration of 50 mg sputum/ml (figure 4). There was a wide range in responses, indicating different overall activity in samples from different patients, and a partial inhibition in the presence of anti-IL-8 (median inhibition = 41%, range -10 to +73%, p = 0.0002). However, inhibition of the wider range of mediators that we have found to contribute to activity in CF sputum resulted in a much more effective inhibition (median inhibition = 97%, range 90 to 99%, p = 0.0002). These results confirm the presence of multiple neutrophil stimulating mediators in the sputum of the majority of CF patients.
DISCUSSION

Neutrophils are important cells in the inflammatory pathogenesis of CF and knowledge of the mediators that recruit neutrophils to the CF airway has been sought by many investigators. However, many of these studies have focused on specific, often single, candidate mediators without regard to the complex nature of the inflammatory response. This study has characterised the range of neutrophil stimulating mediators in CF sputum by use of specific inhibitors and a variety of chromatographic methods. We have found evidence for a contribution of IL-8, the related CXC chemokine GRO, LTB₄ and two distinct formyl peptides to the neutrophil stimulating activity of CF sputum. In addition, there was suggestive evidence that C5a and PAF might also play a role.

IL-8 has been the most widely implicated neutrophil chemoattractant in CF disease. However, we found that inhibition of IL-8 with a monoclonal antibody had only a partial effect on the neutrophil stimulating activity of CF sputum in a variety of assays when pooled sputum was used. When looking at individual sputum samples there was a significant effect of anti-IL-8 suggesting that IL-8 may play a more important role in some CF patients than others; further studies in patients with CF lung disease of different severities are needed to explore this further. Furthermore, blockade of responses to other CXC chemokines also had little effect. Since IL-8, and possibly other CXC chemokines, may be inhibited by the large amount of DNA in the CF lung, the non-chemokine mediators described in this article may be more important in CF airway disease.

We have corroborated results from other studies that LTB₄ is indeed present and active in CF samples. However, blockade of the BLT1 receptor had little effect on neutrophil shape change induced by CF sputum. The presence of formyl peptides in CF sputum is not surprising based on the high degree of bacterial infection seen in these patients and the report of a size exclusion fraction that acted on the formyl peptide receptor. Indeed, we found evidence for at least two formyl peptides of different size and hydrophobicity that appear to act on the same neutrophil receptor. Blockade of this receptor had no detectable effect on the activity in CF sputum. In contrast, the combined blockade of responses to IL-8, other CXC chemokines, LTB₄ and formyl peptides produced a 64 ± 10% inhibition of the neutrophil response to CF sputum.

The above mentioned mediators were clearly defined by use of specific inhibitors of the bioactivity in chromatography fractions. There was also suggestive evidence that C5a and PAF might contribute to the neutrophil stimulating activity in CF sputum. C5a has been suggested to contribute to CF disease and the levels of immunoreactive C5a that we found in reversed phase chromatography fractions of CF sputum would be expected to contribute to the neutrophil stimulating activity detected. Despite this fact, none of the activities that we isolated from CF sputum could be ascribed to this mediator. PAF is a phospholipid mediator that is usually rapidly metabolised to the inactive lyso-PAF metabolite although airway epithelium has the potential to reverse this process, at least transiently. Thus, it is often difficult to detect bioactive PAF and we are not aware of any studies demonstrating PAF in CF sputum. However, the PAF receptor has a rather broad specificity and recognises a number of bacterial lipids. Thus, the suggestion of an activity in CF sputum that partially de-sensitised Fura-2 loaded neutrophils to PAF was sufficient to
warrant the use of a PAF antagonist in later studies. C5a and PAF receptor antagonists, used separately, did not inhibit neutrophil shape change responses to CF sputum. However, the addition of these antagonists to the combined blockade of CXC chemokines, LTB4 and formyl peptides mentioned above resulted in a 96 ± 4% inhibition of the neutrophil response to CF sputum. Similarly, in cross-sectional studies this complex mixture inhibited neutrophil chemotaxis by a median of 97% (range 90 to 99%), whereas the inhibitory effect of anti-IL-8 alone was a more variable median 43% (range -10 to 73%).

Thus, CXC chemokines, LTB4, formyl peptides and one or both of C5a and PAF account for a substantial proportion of the neutrophil stimulating activity in CF sputum. We also found evidence for two mediators that were only partially characterised owing to their minor contribution. One of these has an apparent molecular weight of 25000 but was detected in only one pooled sputum sample. The second unidentified mediator (data not shown) had molecular weight and hydrophobicity characteristics of a lipid mediator. Lipids such as hepoxilin A3 have been shown to promote the migration of neutrophils through epithelial cell monolayers but a role for this lipid in CF has not been defined.

While we have identified a complex mixture of neutrophil chemoattractants in sputum, it is difficult to predict their relative importance in the lung of CF patients. In vitro studies have demonstrated an intracellular signalling hierarchy that may determine the chemoattractant to which the neutrophil will respond. For example, there is a predominant attraction by even low concentrations of end stage chemoattractants, such as formyl peptides and C5a, over intermediary chemoattractants such as IL-8 and LTB4.

We used pools of sputum, for the most part from patients in whom an infective exacerbation had been diagnosed by an independent physician, because we wanted to maximise our chances of capturing as many mediators as possible. While we have shown that IL-8 has a partial contribution to neutrophil stimulating activity in CF, we have not demonstrated the full range of mediators in individual patients. Pooling sputum, and the cross-sectional design, means that we cannot assess the pathophysiological significance of the individual mediators we found. We also cannot say whether these mediators are important in very early stage disease, since all our patients were spontaneously expectorating sputum, and thus by definition had disease of at least moderate severity. Further cross-sectional studies looking at patients with a range of disease severities, and longitudinal studies, observing the changes in individual mediators over time and with treatment, are needed to see which have significance at particular stages of the disease. It is probable that the CF airway contains a mixture of neutrophil stimulating mediators from different sources in response to variations in disease severity in the patient.

It is also possible that treatment for CF with, for example, macrolide antibiotics may mean that some mediators are not detected. We accept that ideally we would have studied patients who were not taking azithromycin, but the benefits of this antibiotic are well known, and so its use is so widespread that it is difficult to find many patients with moderate lung disease who are not currently taking macrolides.
In conclusion, this study has demonstrated the presence of several neutrophil stimulating mediators in CF sputum. The inhibition of neutrophil responses achieved by a combination of inhibitors of CXC chemokines, LTB4 and formyl peptides suggests that these mediators, probably in combination with lipids acting on the PAF receptor and/or C5a, are the key players in neutrophil recruitment in the CF airway. Selective blockade of single mediators was usually insufficient to markedly reduce the neutrophil stimulating activity. These findings suggest a broader targeting of neutrophil stimulating mediators may be required to reduce the accumulation of neutrophils in the CF airway and result in beneficial treatment for patients.

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COMPETING INTERESTS None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Figure 1 Neutrophil chemotaxis (A) and shape change (B) in response to CF sputum supernatant (squares) and IL-8 (circles) in the absence (closed symbols) or presence of anti-IL-8 MAB208 antibody (1.7µg/ml, open symbols). The highest concentration of sputum, 250mg sputum/ml, contained 7.5nM IL-8 by ELISA. Results are expressed as number of cells migrating to the test sample (A) and percentage increase in FSC over buffer treated cells (B). The dotted line represents the baseline (to assay buffer alone) and the shaded area represents the standard deviation of the baseline. Data are mean ± SEM for n=3 (A) or 4 (B) experiments each using cells from a different healthy donor. Concentration response curves were analysed using the repeated measures ANOVA with Bonferroni’s post test; results from the single concentration of IL-8 (1nM, in B) were analysed with the Student’s paired t test. A significant difference compared to absence of anti-IL-8 is indicated by * (p<0.05) and *** (p<0.001).

Figure 2 Neutrophil intracellular calcium mobilisation in response to successive additions of IL-8, CF sputum supernatant and PAF (A) and CF sputum supernatant, IL-8 and PAF (B). The concentration of CF sputum supernatant used, 20mg/ml, contained 0.6nM IL-8 by ELISA. Results are expressed as relative intensity of fluorescence for one experiment (trace) and increase in relative intensity of fluorescence, mean ± SEM (below the trace) for n=3 experiments each using cells from a different healthy donor.

Figure 3 Neutrophil shape change in response to C18 HPLC fractions of CF sputum high salt extract assayed at 1/300 (A) and low salt extract assayed at 1/60 (B). CF sputum fractions (squares) and 1nM IL-8 (circles), were assayed alone (closed symbols) and in the presence of anti-IL-8 MAB208 antibody (1.7µg/ml) + CXCR2 antagonist (0.3mM SB225002) (open symbols). Results are expressed as percentage increase in FSC over buffer treated cells. The dotted line represents the baseline (to assay buffer alone) and the shaded area represents the standard deviation of the baseline. Data are mean ± SEM for n=2-3 experiments each using
cells from a different healthy donor. All results were analysed using the Student's paired t test. A significant difference compared to absence of the CXC chemokine blockade is indicated by * (p<0.05), and ** (p<0.01).

Figure 4 Neutrophil chemotaxis in response to CF sputum supernatant (50mg sputum/ml) from 13 CF patients. Responses were measured in the absence of inhibitors (alone), in the presence of anti-IL-8 MAB208 antibody (1.7µg/ml) and in the presence of a combination of anti-IL-8 + CXCR2 antagonist (0.3mM SB225002) + LTB4 receptor antagonist (100nM CP-105,696) + formyl peptide receptor antagonist (10µM Boc-Met-Leu-Phe) + PAF receptor antagonist (1µM UK-74,505) + C5a receptor antagonist (1µM W-54011). Results are expressed as number of cells migrating to the test sample. Each point is the mean of three assays, each using cells from a different healthy donor. Results were analysed using a non-parametric paired t test (Wilcoxon). Significant differences between responses in the different inhibitor conditions are indicated by *** (p<0.001). The anti-IL-8 ± the receptor antagonist mixture did not induce neutrophil migration in the absence of CF sputum. The dotted line represents the baseline (to assay buffer alone) and the shaded area represents the standard deviation of the baseline.

REFERENCES


Alone + anti-IL-8 + anti-IL-8 antagonists for CXCR2, LTB4, formyl peptide, PAF & C5a receptors

No. of migrating cells

0

10000

20000

30000

***

****

****
ONLINE DATA SUPPLEMENT

All reagents were purchased from Sigma-Aldrich, Dorset, UK, unless otherwise stated.

**Cell preparation** Mixed granulocytes were isolated from the peripheral venous blood of healthy human volunteers according to the method of Haslett *et al.* 1. Platelet-rich plasma was removed by centrifugation of citrated whole blood, followed by dextran® sedimentation of erythrocytes and subsequent separation of granulocytes from other leukocytes over a Histopaque® gradient. Any erythrocyte contamination of the granulocyte pellet was removed by hypotonic shock lysis 2. Differential counts were provided by cytospin analysis for chemotaxis, CD11b and intracellular calcium assays (>95% neutrophils) or flow cytometric analysis for cell shape change assays (62.0-99.8% neutrophils).

**Chemotaxis** Agonist or assay buffer (30µl) was added to the lower wells of 96-well chemotaxis plates (ChemoTx, Receptor Technology Ltd., Adderbury, UK) and neutrophils [20µl, 12.5 x 10^6 cells/ml AB supplemented with Ca^{2+} and Mg^{2+} (0.9mM and 0.5mM respectively)] were added to the top wells. After 1 hour incubation at 37°C, migrated cells in the lower wells were removed, with two washes to ensure good recovery. Assays were performed in triplicate. Results are expressed as the migrated cells, as counted by flow cytometry.

**Leukocyte shape change** Mixed granulocytes (20µl, 12.5x10^6 cells/ml) were added to agonist or assay buffer [AB; PBS without Ca^{2+} Mg^{2+} containing 0.1% BSA, 10mM glucose, 10mM HEPES (Invitrogen, Paisley, UK), pH 7.2-7.4], with or without inhibitors, in polypropylene tubes to a final volume of 50µl. Tubes were placed in a 37°C shaking water bath for 4 min, after which they were transferred to ice and 100µl of ice-cold fixative (Cellfix® diluted ¼ in FACSFlow) was added to terminate the reaction and maintain cell shape until analysis by flow cytometry using a Becton Dickinson FACSCalibur. A minimum of 1000 neutrophil events were acquired using the FL-2 fluorescence channel, allowing neutrophils to be distinguished from eosinophils by their lower autofluorescence. Results are expressed as percentage increase in forward scatter (FSC) compared to buffer treated cells.

**CD11b upregulation** Neutrophils (50µl, 10x10^6 cells/ml) in AB supplemented with Ca^{2+} and Mg^{2+} (0.9mM and 0.5mM respectively) were added to agonist or AB, with or without inhibitors, in polypropylene tubes to a final volume of 100µl. Tubes were placed in a 37°C shaking water bath for 1 hour, after which they were transferred to ice and 500µl of ice cold staining buffer (SB; PBS without Ca^{2+} Mg^{2+} containing 0.25% BSA, 10mM HEPES, pH 7.2-7.4) was added. These samples were centrifuged at 130 g for 4 minutes at 4°C and the cells were resuspended in 50µl FITC-labeled anti-CD11b antibody (diluted 1/20 in SB). Samples were incubated at 4°C for 20 minutes in the dark. Cells were diluted in 500µl SB and centrifuged at 130 g for 4 minutes at 4°C. The supernatant was discarded and the cells washed in 500µl ice cold FACSflow and resuspended in 200µl ice cold Cellfix®. The samples were then analysed by flow cytometry. Viable cells were gated on forward and side scatter plots and the amount of CD11b upregulation was measured as an increase in FL-1. 10,000 events were acquired and results are expressed as percentage increase in FL-1 compared to buffer treated cells. No staining was observed when the anti-CD11b antibody was replaced with a FITC-labeled isotype control antibody.

**Intracellular Calcium Mobilisation** Neutrophils were loaded with FURA-2 (1µM, conjugated with acetoxymethyl) in assay buffer for 30 min at 37°C. Cells were washed and resuspended in assay buffer. Aliquots of cells (3 x 10^6/1.5ml) were
dispensed into cuvettes and equilibrated with 1mM CaCl$_2$ at 37°C for 2 min prior to use. Changes in fluorescence emitted at 510nm were measured after stimulation at 340 and 380nm. Results are expressed as the relative increase in fluorescence compared to baseline.

**Inhibition of bioassay responses** The inhibitors and receptor antagonists used in neutrophil stimulation assays were various combinations of: anti-IL-8 MAB208 (R&D Systems, 1.7µg/ml); CXCR2 antagonist SB225002 (Merck Biosciences, 0.3mM); LTB$_4$ receptor BLT1 antagonist CP-105,696 (Pfizer, 100nM); formyl peptide receptor FPR antagonist, Boc-Met-Leu-Phe (Bachem, 10µM); C5a receptor antagonist, W-54011 (Merck Biosciences, 1µM); PAF receptor antagonist, UK-74,505 (Pfizer, 1µM). None of these inhibitors, either singly or in combination, had any effect on baseline responses.

**ELISA** Immunoreactive IL-8, GRO, ENA-78, GCP-2, NAP-2 and C5a were measured by specific sandwich ELISAs. 96-well flat bottomed plates were coated with 100µl per well of coating antibody diluted in 10mM carbonate/bicarbonate buffer pH 9.6, 0.004% tartrazine. They were left at 22°C for 6 hours and then washed 4 times in coat block wash buffer (0.01M potassium phosphate pH 7.5, 0.02% thimerosal and 0.05% Tween® 20). 250µl of blocking buffer (1% BSA, 0.02% sodium azide in PBS) was added to each well and the plate was incubated for 16 hours at 4°C. The plate was washed 4 times in coat block wash and 100µl of sample or standard, diluted in AB, was added to each well. This was incubated at 22°C for 2 hours and the plate washed 4 times with assay wash buffer (PBS with 0.02% thimerosal and 0.2% Tween® 20). 100µl of detector antibody (diluted in ELISA buffer EB; 0.1% BSA, 0.05% thimerosal in PBS) was added to each well and the plate incubated at 22°C for 1.5 hours. The plate was washed 4 times in assay wash buffer and 100µl of neutravidin-horse radish peroxidase conjugate (Pierce, Cheshire, UK), diluted in EB, was added to each well. The plate was incubated at 22°C for 1 hour and washed 4 times in assay wash buffer. 100µl of substrate (Enhanced K-Blue® TMB Substrate, Skybio, Bedford, UK) was added to each well and the plate incubated at 22°C for 30 minutes. The reaction was terminated by addition of 100µl of 0.19M H$_2$SO$_4$ to each well and the absorbance read at 450nm. The results were read from a standard curve of best fit.

**Chromatography** The Smart System HPLC, with an incorporated fraction collector, and columns were from Amersham Biosciences (Buckinghamshire, UK). All chromatography was run at 10°C.

Protocol 1: Sputum from CF patients (pool 2, online supplement table 1) was homogenised at 100mg/ml in TFA (VWR International, Dorset, UK) /1M NaCl, centrifuged, and the high salt supernatant recovered. The pellet was re-extracted with 0.08% TFA without added salt, centrifuged to obtain a much smaller pellet and the low salt supernatant recovered. Samples were adjusted to pH 2 (with 20% TFA) and 0.5M NaCl and then de-salted and concentrated by use of C$_{18}$ SepPaks (Waters, Watford, UK) in 0.08% TFA, eluted with acetonitrile [ACN (VWR International), 100%] and lyophilised. Samples were then dissolved in 0.08% TFA and applied to a µRPC C$_2$/C$_{18}$ PC 3.2/3 reversed phase HPLC column at one column volume (240µl/min). Bound materials were eluted with an acetonitrile gradient (0-100% ACN at 3.3% ACN/min). Fractions were collected (2 min fractions from the start of the gradient for 0-8min, 0.5 min fractions for 8-17.5min and 2 min fractions for the remainder of the gradient) and aliquots were lyophilised in the presence of carrier protein (20µg BSA). Lyophilised aliquots were dissolved in AB for bioassay using the cell shape change
assay. Aliquots of selected C₁₈ HPLC fractions were lyophilised for size exclusion chromatography.

Protocol 2: Sputum from CF patients (pool 3, online supplement table 1) was homogenised in 10 mM sodium phosphate pH 7.4 (50 mg/ml) and centrifuged. The supernatant was applied to a carboxymethyl (CM)-Sepharose cation exchange column mounted above a diethylamino ethyl (DEAE)-Sepharose anion exchange column (each column was 12 x 15 mm, Amersham Biosciences). The cation and anion eluates as well as the breakthrough (which did not bind to either column) were collected and adjusted to pH 2 (with 20% TFA) and 0.5 M NaCl. These were then applied separately to C₁₈ SepPaks before reversed phase HPLC as described above except that the fractions collected were 2 min fractions for 0-6 min, 0.5 min fractions for 6-15.5 min and 2 min fractions for the remainder of the gradient.

Size exclusion Aliquots of selected C₁₈ HPLC fractions were lyophilised with carrier BSA, dissolved in 60 µl PBS and applied to a Superdex® 75 PC 3.2/30 gel filtration column, previously calibrated with a range of protein molecular weight standards. The flow of PBS was 40 µl/min and 60 µl fractions were collected into PBS/BSA, such that the final BSA concentration was 0.1%, for bioassay.
Online supplement Table 1  Characteristics of CF patients and the mediator extraction conditions used for pooled and individual sputum samples.

<table>
<thead>
<tr>
<th></th>
<th>Pool 1</th>
<th>Pool 2</th>
<th>Pool 3</th>
<th>Individual</th>
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</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>10.0 (1.1)</td>
<td>12.3 (1.0)</td>
<td>13.4 (0.8)</td>
<td>13.0 (0.6)</td>
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<tr>
<td>M/F</td>
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<td>2/2</td>
<td>2/2</td>
<td>6/7</td>
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<tr>
<td>FEV1, % predicted</td>
<td>55 (9)</td>
<td>55 (11)</td>
<td>65 (6)</td>
<td>61 (5)</td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>66 (8)</td>
<td>76 (14)</td>
<td>84 (3)</td>
<td>81 (5)</td>
</tr>
<tr>
<td>Infected with P. aeruginosa</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Undergoing exacerbation</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Azithromycin treatment</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>rhDNase treatment</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>g sputum used in pool</td>
<td>7.1</td>
<td>5.4</td>
<td>3.0</td>
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<td>10mM Phosphate</td>
<td>PBS</td>
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<tr>
<td>Concentration, mg/ml</td>
<td>300</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are mean (SEM).
M/F, Male/Female; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; rhDNase, recombinant human DNase; PBS, phosphate buffered saline; TFA, trifluoroacetic acid.

Online supplement Figure 1  Neutrophil CD11b upregulation in response to CF sputum supernatant (squares) and IL-8 (circles) in the absence (closed symbols) or presence of anti-IL-8 MAB208 antibody (open symbols, 1.7 µg/ml). The highest concentration of sputum, 250mg sputum/ml, contained 7.5nM IL-8 by ELISA. Results are expressed as percentage increase in FL-1 over buffer treated cells. No significant difference compared to absence of anti-IL-8 was found. The dotted line represents the baseline (to assay buffer alone) and the shaded area represents the standard deviation of the baseline. Data are mean ± SEM for n=3 experiments each using cells from a different healthy donor.

Online supplement Figure 2  Neutrophil shape change in response to gel filtration HPLC fractions of CF sputum high salt supernatant C18 HPLC fraction 4 (A) and a pool of fractions 13 + 14 (B) assayed at 1/20 alone (closed squares) and in the presence of a formyl peptide receptor antagonist (10µM Boc-Met-Leu-Phe, in fractions 23-29, open squares). Results are expressed as percentage increase in FSC over buffer treated cells. The dotted line represents the baseline (to assay buffer alone) and the shaded area represents the standard deviation of the baseline. Data are mean ± SEM for n=3 experiments each using cells from a different healthy donor. All results were analysed using the Student’s paired t test. A significant change in response compared to absence of antagonist is indicated by ** (p<0.01).

Online supplement Figure 3  Neutrophil shape change in response to C18 HPLC fractions of CF sputum previously separated by ion exchange. C18 fractions of the cationic extract were assayed at 1/100 (A), C18 fractions of the anions were assayed...
at 1/20 (B) and C18 fractions of the ion exchange breakthrough were assayed at 1/20 (C), alone (closed squares) and in the presence of an inhibitor (open squares). Inhibitor in (A) = anti-IL-8 MAB208 antibody (1.7µg/ml) + CXCR2 antagonist (0.3mM SB225002); in (C) = LTB4 receptor antagonist (100nM CP-105,696). Results are expressed as percentage increase in FSC over buffer treated cells. The dotted line represents the baseline (to assay buffer alone) and the shaded area represents the standard deviation of the baseline. Data are mean ± SEM for n=2-4 experiments each using cells from a different healthy donor.

**Online supplement Figure 4** Neutrophil shape change in response to CF sputum supernatant (squares) and standards (circles) in the absence (closed symbols) or presence (open symbols) of inhibitors. Inhibitor in A = anti-IL-8 MAB208 antibody (1.7µg/ml) + CXCR2 antagonist (0.3mM SB225002); B = LTB4 receptor antagonist (100nM CP-105,696); C = formyl peptide receptor antagonist (10µM Boc-Met-Leu-Phe); D = an inhibitor cocktail of anti-IL-8 + SB225002 + CP-105,696 + Boc-Met-Leu-Phe; E = C5a receptor antagonist (1µM W-54011); F = PAF receptor antagonist (1µM UK-74,505); G = an inhibitor cocktail of anti-IL-8 + SB225002 + CP-105,696 + Boc-Met-Leu-Phe + W-54011 + UK-74,505. Results are expressed as percentage increase in FSC over buffer treated cells. The dotted line represents the baseline (to assay buffer alone) and the shaded area represents the standard deviation of the baseline. Data are mean ± SEM for n=3-4 experiments each using cells from a different healthy donor. Concentration response curves were analysed using the repeated measures ANOVA with Bonferroni’s post test; results from single concentrations of agonists were analysed with the Student’s paired t test. A significant difference compared to absence of inhibitor is indicated by * (p<0.05), ** (p<0.01), and *** (p<0.001). The anti-IL-8 ± the receptor antagonist mixture did not induce neutrophil migration in the absence of CF sputum.

**REFERENCES**


A  
Cations

B  
Anions

C  
Breakthrough

$C_{18}$ fraction number

% FSC Increase
Characterisation of the range of neutrophil stimulating mediators in Cystic Fibrosis sputum

Kathryn J Mackerness, Gavin R Jenkins, Andrew Bush and Peter J Jose

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