Effect of fluticasone propionate on neutrophil chemotaxis, superoxide generation, and extracellular proteolytic activity in vitro

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Abstract

Background — Corticosteroids are widely used in the treatment of many inflammatory conditions but the exact mode of action on neutrophil function is uncertain. Fluticasone propionate is a new topically active synthetic steroid which can be measured in body fluids and which undergoes first pass metabolism.

Methods — The effects of fluticasone propionate on the function of neutrophils isolated from normal, healthy control subjects and on the chemotactic activity of sputum sol phase were assessed.

Results — Preincubation of neutrophils with fluticasone propionate reduced the chemotactic response to 10⁻⁴ mol/l F-Met-Leu-Phe (FMLP) and to a 1:5 dilution of sputum sol phase in a dose dependent manner. Furthermore, when fluticasone propionate was added to sputum from eight patients with stable chronic obstructive bronchitis the chemotactic activity of a 1:5 dilution of the sol phase fell from a mean (SE) value of 22.2 (1.21) cells/field to 19.6 (0.89), 17.1 (0.74), and 11.9 (0.6) cells field at 1 μmol/l, 10 μmol/l, and 100 μmol/l, respectively. In further experiments fluticasone propionate preincubated with neutrophils inhibited fibronectin degradation by resting cells and by cells stimulated by FMLP (15:2% inhibition of resting cells, 5:1% inhibition of stimulated cells with 1 μmol/l fluticasone propionate, 24% and 18:7% inhibition respectively at 100 μmol/l fluticasone propionate. Fluticasone propionate had no effect on generation of superoxide anion by resting or stimulated cells.

Conclusions — These results indicate that fluticasone propionate has a direct suppressive effect on several aspects of neutrophil function and may suggest a role for this agent in the modulation of neutrophil mediated damage to connective tissue.

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Neutrophils have an important role in protecting the host against invasion by microorganisms. Neutrophil activation, recruitment to tissues, phagocytosis, release of proteinases, and generation of oxygen metabolites results in killing and elimination of the pathogen or antigen. Although this process is usually self limiting it may, in some circumstances, become continuous or excessive, thus contributing to inflammation and tissue damage. For this reason neutrophils are thought to be involved in the pathogenesis of a wide range of inflammatory diseases including chronic destructive lung disease.¹² Neutrophils isolated from the peripheral blood of patients with emphysema show an enhanced response to chemotactic signals, and are able to degrade more connective tissue than normal.¹ The implication of these findings is that, for a given chemotactic signal, neutrophils from these patients would be recruited in larger numbers than normal and cause more tissue damage. The lungs of patients with bronchitis contain increased numbers of neutrophils, suggesting continued recruitment. This indicates the presence of a chemotactic signal and lung secretions have been shown to possess chemotactic activity.¹ Since the recruitment of neutrophils to the lung probably plays a major part in the pathogenesis of bronchitis and emphysema, factors which modulate this process will be of major importance in controlling disease progression.⁶

Corticosteroids have an important role in the treatment of inflammatory conditions, although their precise mode of action is not fully understood. In vitro studies have shown that corticosteroids are able to inhibit neutrophil chemotaxis,⁷⁻⁹ extracellular degranulation¹⁰ and, in some studies, superoxide anion generation,¹¹ although the latter effect has not been confirmed by other workers.¹² In vivo studies with oral corticosteroids in animals and man have confirmed an inhibition of the neutrophil chemotactic response, but have shown no effect on superoxide anion release.¹³⁻¹⁴ In a further preliminary study inhaled beclomethasone dipropionate had an inhibitory effect on peripheral neutrophil chemotactic response and degranulation, as well as reducing sputum chemotactic activity and albumin concentrations, suggesting a beneficial effect on lung inflammation.¹⁵

Fluticasone propionate is a new, inhaled, topically active synthetic glucocorticosteroid which undergoes first pass metabolism in the liver, is quantifiable in body fluids, and has greater anti-inflammatory activity than beclomethasone dipropionate in animal studies. As a preliminary study before clinical evaluation of this inhaled steroid we have investigated the effects of fluticasone propionate on normal neutrophils in vitro. The aims of the present study were therefore, firstly, to assess the effect of fluticasone propionate on neutrophil chemotaxis, secondly to assess the in vitro...
effect of fluticasone propionate on neutrophil degranulation as measured by the degradation of fibronectin, and thirdly to assess the effect of fluticasone propionate on superoxide anion generation by neutrophils.

Methods
PREPARATION OF SPUTUM SOL PHASE
Sputum samples were collected from eight patients with stable chronic obstructive bronchitis receiving only inhaled bronchodilator treatment. The samples were ultracentrifuged at 50 000 g for 90 minutes at 4°C, the supernatant collected, aliquoted and stored separately. In addition purulent sputum was collected from several patients with bronchiectasis, the sol phase was obtained, and the samples were pooled and stored in separate aliquots. All samples were stored at −70°C until required.

ISOLATION OF BLOOD NEUTROPHILS
Neutrophils were isolated by the method of Jepsen and Skottun. Briefly, venous blood from normal healthy controls was collected between 9:00 and 10:00 hours into lithium heparin tubes. Each sample was diluted with an equal volume of 0-15 mol/l sodium chloride and layered onto a Percoll gradient (Sigma Chemicals, Dorset, UK). The top layer consisted of 2 ml 54% Percoll (density 1-075 g/ml) and the lower layer of 3 ml 78% Percoll (density 1-096 g/ml). The tubes were centrifuged for 25 minutes at 200 g at room temperature. The neutrophils (>90% pure, >98% viable as assessed by exclusion of trypan blue) were harvested from the interface of the 54% and 78% layers, washed twice in 0-15 mol/l saline solution, counted, and resuspended at the required concentration in relevant assay medium; RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) for the fibronectin degradation assay, RPMI solution containing 2 mg/ml bovine serum albumin for the chemotaxis assay, or phosphate buffered saline (0-15 mol/l, pH 7-2) containing 1 mol/l calcium chloride and 1 mol/l magnesium chloride for the superoxide assay. All reagents were confirmed to contain less than 20 ng/ml endotoxin activity using the KablVitrum Coat test (Flow Laboratories, Rickmansworth, UK).

NEUTROPHIL CHEMOTAXIS
The chemotaxis assay was based on the method described by Falk et al. using the 48-well micro chemotaxis chamber. The lower wells contained 27 μl chemotaxant (10−4 mol/l F-Met-Leu-Phe (FMLP) or 1:5 dilution of the sputum sol phase in RPMI with 2 mg/ml bovine serum albumin) and the upper wells contained 50 μl cells at 1×10⁶ cells/ml. Fluticasone propionate at varying concentrations (0-01 μmol/l to 100 μmol/l) was either preincubated with neutrophils for 30 minutes at 37°C before performing the assay and then added with neutrophils to the upper wells, or placed in the lower wells of the chamber with the chemotaxant. The upper and lower wells were separated by a 2 μm pore PVP-free polycarbonate filter (Costar Nucleopore, Costar UK, High Wycombe, UK). The chemotaxis chamber was incubated at 37°C for 20 minutes, the filter was then removed, and the upper surface wiped across a wiper blade (to remove any cells that had not migrated through the pores) followed by fixing and staining with Diff-quick (Baxter, UK). The cells adherent to the lower surface were counted at ×400 magnification from five random fields for each of three replicate wells. A mean value was obtained for each well and the average value for the replicates was taken as the result for that sample.

FIBRONECTIN DEGRADATION
Degradation of fibronectin was assessed by the method of Campbell et al modified by Burnett et al. Purified human fibronectin was obtained from the Sigma Chemical Co. (Poole, Dorset, UK) and iodinated by the chloramine-T method with radiolabelled sodium 125-i-iodide (ICN Flow, High Wycombe, UK). The radiolabelled fibronectin was diluted with unlabelled fibronectin in 0-05 mol/l carbonate/bicarbonate buffer, pH 9-6, to give 2000 cmap/μg fibronectin, and dispensed into the wells of microtitre plates at 30 μg/well. The plates were allowed to dry at 37°C and then washed three times with phosphate buffered saline (pH 7-2). The isolated neutrophils suspended in RPMI medium (3×10⁶ cells/well) were dispensed into the wells and the plates incubated at 37°C in a humidified atmosphere of 5% carbon dioxide/95% air for three hours. After incubation the supernatant was collected from the wells and the proteolysed fibronectin was measured by counting with an LKB Multigamma II gamma counter. The iodine-125 counts in the supernatants of wells containing RPMI medium alone (blank) were deducted from those containing neutrophils. All experiments were performed in the absence (resting) and presence (stimulated cells) of 1 μmol/l FMLP and the result for each assay was determined from the mean value for three replicate wells. The experiments were performed in two ways: (1) cells were preincubated with fluticasone propionate at varying concentrations (0-01 μmol/l to 100 μmol/l) for 30 minutes followed by gentle mixing, before being added together onto the fibronectin plate (preincubation), or (2) the cells were allowed to adhere onto the fibronectin for 30 minutes followed by fluticasone propionate (adherent cells). The values obtained for the adherent cells were corrected for the 30 minutes before the addition of fluticasone propionate.

SUPEROXIDE ASSAY
Superoxide release from neutrophils in suspension was determined by measuring the superoxide dismutase inhibitable reduction of ferricytochrome C. Neutrophils were preincubated with fluticasone propionate at varying concentrations (1 μmol/l to 100 μmol/l) for 30 minutes at 37°C and then dispensed (10⁶ cells
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**Figure 1** Inhibition of neutrophil chemotaxis to 10⁻⁴ mol/l FMLP following preincubation with varying concentrations of fluticasone propionate. Each histogram is the mean (SE) of four experiments. A significant dose response is seen (p<0.0001).

in 100 µl phosphate buffered saline 0·15 mol/l, pH 7·2, containing 1 mmol/l calcium chloride and 1 mmol/l magnesium chloride into the wells of a limbro plate (24 well, flat bottomed, Flow Laboratories). To reaction wells either 10 µl (5 mg/ml) superoxide dismutase (from bovine erythrocytes, Sigma) to mop up released superoxide, or 10 µl reaction buffer, was added. Horse heart ferriyctochrome C (Sigma) was added to all wells (to a volume of 100 µl) to make a final concentration of 10 mmol/l. Fluticasone propionate (100 µl) at varying concentrations was added to test wells to achieve the same concentration incubated previously with neutrophils (1 µmol/l to 100 µmol/l). All experiments were performed in the presence (stimulated cells) and absence (resting cells) of 1 µmol/l FMLP. The total volume of each well was made up to 1 ml with reaction buffer, with blank wells containing ferriyctochrome C and reaction buffer alone. After incubation for one hour at 37°C in 5% carbon dioxide/95% air, the supernatant from each well was recovered, centrifuged at 1000 g to remove any cells, and the absorbance of each sample was measured at 550 nm with a spectrophotometer. The amount of reduced cytochrome C was calculated using an extinction coefficient of 21·1 nmol⁻¹. Neutrophil superoxide generation was calculated as the difference in absorbance between reaction wells with and without superoxide dismutase, and the results expressed as nmol/l superoxide released per hour per 10⁶ cells.

**DATA ANALYSIS**
Statistical analyses of the dose effect of fluticasone propionate on neutrophil chemotaxis, degradation of fibronectin, and superoxide anion release were performed by analysis of variance.

**Results**

**CELL VIABILITY**
The viability of the neutrophils after incubation of the cells with up to 100 µmol/l fluticasone propionate remained >98%, as assessed by exclusion of trypan blue.

**CHEMOTAXIS**
When fluticasone propionate was preincubated with neutrophils inhibition of chemotaxis to FMLP occurred in a dose dependent manner (fig 1) from a mean (SE) control value of 34·2 (2·0) cells/field to 28·6 (3·5), 28·8 (3·2), 26·6 (3·2), 25·6 (3·0) to 20·1 (2·3) at 0·01 µmol/l, 0·1 µmol/l, 1 µmol/l, 10 µmol/l, and 100 µmol/l fluticasone propionate respectively (p<0.0001). When sputum sol phase collected from patients with bronchiectasis was used as the chemoattractant the results were similar whether fluticasone propionate was preincubated with neutrophils or whether it was added to the sputum (table 1). Again a dose related reduction in chemotactic response was seen (p<0.0001).

The mean (SE) chemotactic activity of a 1:5 dilution of the sputum sol phase collected from the eight patients with clinically stable chronic bronchitis was 22·2 (1·2), range 16·9·27·2 cells/field. When fluticasone propionate was added to the sputum sol phase the chemotactic activity was again reduced in a dose dependent manner to 19·6 (0·89), range 15·7·23·4 at 1 µmol/l; 17·1 (0·74), range 14·3·20·2 at 10 µmol/l; 11·9 (0·61), range 9·8·15·1 at 100 µmol/l, as summarised in fig 2 (p<0.0001). In all experiments the back-
Treatment of adherent neutrophils with fluticasone propionate

When fluticasone propionate was added to neutrophils that had been allowed to adhere to fibronectin, it was less effective at inhibiting fibronectin degradation than after preincubation with neutrophils as shown in table 2. Values fell from a control of 1.63 (0.32) μg for resting cells to 1.43 (0.24) μg at both 1 μmol/l and 100 μmol/l (p < 0.05). When the adherent cells were stimulated with 1 μmol/l FMLP, fluticasone propionate reduced fibronectin degradation from 2.13 (0.29) μg to 1.78 (0.2) μg and 1.75 (0.2) μg at 1 μmol/l and 100 μmol/l respectively (p < 0.05).

SUPEROXIDE ASSAY

When fluticasone propionate was preincubated with neutrophils for 30 minutes there was no change in resting or stimulated neutrophil superoxide anion generation: mean (SE) control value for resting cells of 2.5 (0.50) nmol/10 6 cells/hour and 2.52 (0.58) after incubation with fluticasone propionate at 1 μmol/l, 2.55 (0.74) at 10 μmol/l fluticasone propionate, and 2.53 (0.69) at 100 μmol/l fluticasone propionate; when stimulated with 1 μmol/l FMLP the mean control was 3.78 (0.63) nmol/10 6 cells/hour and 3.96 (1.0), 3.48 (0.58), and 3.67 (0.63) with 1 μmol/l, 10 μmol/l, and 100 μmol/l fluticasone propionate respectively (n = 3).

Discussion

The results show that fluticasone propionate has a direct suppressive effect on several neutrophil functions in vitro. It inhibited chemotaxis to both FMLP and sputum sol phase when preincubated with neutrophils, although high levels of fluticasone propionate were required with a 22-2% reduction in response at 1 μmol/l and 41-2% at 100 μmol/l. This is comparable with, although slightly greater than, values obtained in a previous study from our group using the same chemotaxis assay system to assess the effects of another glucocorticosteroid (dexamethasone) in vitro, and when administered systemically in vivo to normal healthy control subjects. The authors reported an 11% inhibition of neutrophil chemotaxis with 1 μmol/l dexamethasone, 29% at 100 μmol/l, and 54% inhibition at 1 mmol/l dexamethasone in vitro. However, inhibition was achieved with much lower levels in vivo (54% inhibition at 0.126 μmol/l), indicating greater efficacy when given as a treatment. This latter value is also similar to observations in patients with rheumatoid arthritis where there was a 60% reduction of neutrophil migration into the knee joint following the administration of intra-articular steroid.

Sputum from patients with chronic destructive lung diseases has been shown to contain a number of unknown chemotactic factors, with greater chemotactic activity of purulent than mucoid sputum. We have confirmed this with purulent sputum collected from patients with bronchiectasis and from

Table 2 Effect of fluticasone propionate on fibronectin (FN) degradation by resting and stimulated neutrophils added to cells tightly adherent to fibronectin (p < 0.05).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Resting neutrophils (μg FN)</th>
<th>Stimulated neutrophils (μg FN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.63 (0.32)</td>
<td>2.13 (0.29)</td>
</tr>
<tr>
<td>0.01 μmol/l</td>
<td>1.40 (0.26)</td>
<td>1.99 (0.32)</td>
</tr>
<tr>
<td>0.1 μmol/l</td>
<td>1.43 (0.26)</td>
<td>1.81 (0.24)</td>
</tr>
<tr>
<td>1.0 μmol/l</td>
<td>1.43 (0.24)</td>
<td>1.78 (0.20)</td>
</tr>
<tr>
<td>10 μmol/l</td>
<td>1.43 (0.28)</td>
<td>1.79 (0.22)</td>
</tr>
<tr>
<td>100 μmol/l</td>
<td>1.43 (0.28)</td>
<td>1.75 (0.20)</td>
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those with smoking related bronchitis and emphysema who produce mucoid sputum. We have assessed the chemotactic activity of each type of sputum using a 1:5 dilution of the sputum as this was found to be the optimum concentration.7 The chemotactic response of neutrophils to both types of sputum was reduced when fluticasone propionate was present in the sol phase with 22%, 26%, and 44% inhibition at concentrations of 1, 10, and 100 μmol/l fluticasone propionate respectively for bronchietatic sputum, and 12%, 23%, and 46% inhibition respectively for bronchitic sputum. The presence of fluticasone propionate in lung secretions following inhalation treatment may therefore have a beneficial effect on lung inflammation by inhibiting neutrophil migration to the lung and thus reducing further tissue damage. However, as fluticasone propionate and neutrophils were added together into the upper wells of the chemotaxis chamber in the earlier experiment, it is difficult to separate the effects of the drug on the cells and on the sputum as some fluticasone propionate may have diffused through the pores into the lower wells during the assay.

Preincubation of neutrophils with fluticasone propionate for 30 minutes resulted in inhibition of fibronectin degradation by resting and stimulated cells in a dose dependent manner (15% and 24% inhibition for resting cells at 1 μmol/l and 100 μmol/l respectively). When compared with previous work from our group using the same fibronectin degradation assay system to assess the effect of dexamethasone on neutrophil proteolysis,10 fluticasone propionate appears to be less effective. The authors reported an 11% inhibition of resting neutrophil fibronectin degradation by dexamethasone at 10−10 mol/l, 19% at 10−8 mol/l, 27% at 10−6 mol/l, and 42% at 10−4 mol/l.10 In our assay, however, fluticasone propionate was even less effective when the cells were stimulated with 1 μmol/l FMLP with only a 5-1% inhibition at 1 μmol/l fluticasone propionate and 18-7% inhibition when the concentration was increased to 100 μmol/l.

Allowing the cells to adhere to the fibronectin before the addition of fluticasone propionate resulted in only 12% inhibition with resting cells (1 μmol/l and 100 μmol/l), and 16% and 18% inhibition respectively when the cells were stimulated. This activation of neutrophils by adherence or by FMLP appears to have reduced the effect of fluticasone propionate on fibronectin degradation. This may be because the activation of the cells by either mechanism is able to overcome the suppressive effects of fluticasone propionate, or that the activation process uses a different cell pathway which is not inhibited by fluticasone propionate.

Fluticasone propionate had no effect on neutrophil superoxide anion generation by resting or stimulated cells even at high concentrations. Several other workers have assessed the effects of steroids on superoxide production with variable results. There are reports of suppression of superoxide generation by steroids in vitro,11 with others failing to confirm this observation.12 In addition there are contradictory reports about the effects of steroids in vivo.13,22 The results presented here suggest that steroids are unable to influence superoxide generation by neutrophils.

However, corticosteroids have a suppressive effect on other neutrophil functions in vitro and in vivo, although the mechanisms are not clearly understood. It is thought that the in vivo effects of steroids are caused by the synthesis of a phospholipase A2 (PLA2) inhibitory peptide which inhibits the release of arachidonic acid, thereby limiting arachidonic acid metabolism and hence suppressing cell functions.23 However, this mechanism could not explain the rapid effects of steroids in vitro as the effect would require the synthesis of the inhibitory peptide which would take longer than the time scale of our in vitro experiments. More recently it has been suggested that glucocorticoids may inhibit PLA2 directly by inducing dephosphorylation of the active form of the enzyme and thus would not involve the synthesis of PLA2 inhibitory peptide.24 The latter mechanism would induce a more rapid response and may account for the suppression of neutrophil functions by glucocorticoids in vitro as shown here for fluticasone propionate. However, previous studies with indomethacin, which is known to inhibit PLA2,25 have not shown any effect of this agent on the chemotactic response of mature neutrophils in vitro despite significant inhibition of neutrophil chemotaxis when given in vivo,26 suggesting that inhibition of PLA2 is not an important factor in the regulation of neutrophil function in vitro.

The results presented show that fluticasone propionate suppresses neutrophil function in vitro and that it also has an effect on the chemotactic activity of lung secretions. Fluticasone propionate has been formulated for inhalation in the treatment of steroid responsive obstructive lung diseases, and its ability to reduce the chemotactic response to sputum may be an important therapeutic factor in the treatment of chronic destructive lung diseases through inhibition of neutrophil recruitment and hence further tissue damage. In vivo studies using inhaled beclomethasone dipropionate in chronic bronchitis have shown a reduction in parameters of lung inflammation and an improvement in spirometric indices over a six week treatment period.27 In a preliminary study of beclometasone dipropionate in similar patients the authors found a reduction in the chemotactic activity of sputum, inhibition of neutrophil chemotaxis to FMLP and degradation, as well as a reduction in markers of lung inflammation.19 These findings support the concept of an overall benefit of such treatment in chronic lung disease. However, as beclometasone dipropionate is absorbed into the blood stream it is difficult to distinguish its effects on circulating neutrophils and on lung inflammation. If the observations were due to a direct influence of the drug in the sputum with a beneficial effect on lung inflammation, this would reduce the release of chemotactic signals and hence neutrophil recruitment and
activation. However, the observations may be due to a direct effect of the circulating drug on peripheral neutrophils with inhibition of neutrophil function and a secondary effect on sputum. Clinical studies with fluticasone propionate may provide a means of separating the effects of inhaled steroids on secretions from those on circulating cells because it undergoes first pass metabolism, and thus any effect of this inhaled drug in vivo will reflect only changes occurring in the lung. Furthermore, as fluticasone propionate is measurable in body fluids the results presented here can be compared directly with results obtained with this drug in future in vivo studies. Our results suggest that inhaled treatment with fluticasone propionate may reduce the chemotactic activity of lung secretions, reduce neutrophil recruitment to the lung and cell activation in lung tissues, while maintaining superoxide anion generation.

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