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Inhibitory capacity of different steroids on neutrophil migration across a bilayer of endothelial and bronchial epithelial cells

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Abstract

Neutrophil infiltration to the airway lumen is a common feature of respiratory inflammatory processes. The aim of this study was to evaluate whether different corticosteroids exert any selective effect on the migration of isolated neutrophils. A bilayer of cultured human endothelial and bronchial epithelial cells was used as a model for neutrophil migration through the blood–air barrier. Low spontaneous migration of neutrophils (2.8 ± 0.9%, $n = 8$; mean ± S.E.M.) occurred, while in the absence of any steroid, a migration of 28.5 ± 7.6% could be induced by lipopolysaccharide. Pre-incubation during 1 h of epithelial cells with dexamethasone, budesonide, or prednisolone (10^{-10}–10^{-4} M) showed in all instances a concentration-dependent inhibition following a bell-shaped curve. At 10^{-7} M, both dexamethasone and budesonide were on the minimum effect peak of the bell-shaped curve. The peak for prednisolone was found at 10^{-8} M. However, when steroid pre-incubation was extended to 4 h, a sigmoid curve was observed, with significant inhibition of migration at concentrations $>10^{-7}$ M. Steroids can inhibit neutrophil recruitment through two different pathways with distinct result, depending on the length of incubation time.

Keywords: Neutrophil; Corticosteroid; Inhibition; Transmigration

1. Introduction

Chronic obstructive pulmonary disease is defined as a disease state characterised by poorly reversible airflow limitation that is usually both progressive and associated with an abnormal inflammatory response of the lung (Pauwels et al., 2001). In central airways, development of airflow limitation is associated with increase of macrophages and T lymphocytes in the airway wall (Di Stefano et al., 1996; Keatings et al., 1996) and of neutrophils in the airway lumen (O'Shaughnessy et al., 1997; Saetta et al., 2001), suggesting a selective passage of neutrophils across the epithelium into the airway lumen. The finding of increased numbers of neutrophils in the bronchial epithelium of patients with chronic obstructive pulmonary disease supports this hypothesis (Martin et al., 1985; Thompson et al., 1989). In addition, increased numbers of neutrophils are associated with a rapid decline in FEV_1, the forced expiratory volume in 1 s (Stanescu et al., 1996). Neutrophils are attracted to the lung because of chemotactic signals generated locally at the site of inflammation. Among these signals are microbial products, and induced mediators as complement factors (e.g. C5a), and chemokines as interleukin-8 and leukotriene B_4 (Stankova et al., 2002; Van Wetering et al., 2002). On their turn, neutrophils may contribute to recruitment of additional neutrophils by secretion of interleukin-8, tumour necrosis factor-α (TNF-α), interleukin-1β, and other factors like elastase and defensins, that induce chemokine expression in airway epithelial cells (Cassatella, 1995; Van Wetering et al., 2002).

Although smoking is the main risk factor of chronic obstructive pulmonary disease, smoking cessation does not appear to result in resolution of the inflammatory response in the airways (Barnes, 2000; Rutgers et al., 2000; Turato et al., 1995). The recognition that a chronic inflammatory condition is present in chronic obstructive pulmonary

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disease provides a rationale for the use of an anti-inflammatory treatment, i.e. corticosteroids, in this disease. Inhaled corticosteroids have a high level of topical anti-inflammatory activity and a low level of systemic activity (Johansson et al., 1982). The mechanisms of the anti-inflammatory effects of steroids have not been described satisfactorily. Although nuclear changes in cytokine gene transcription in leukocytes are a well-known effect (Munck et al., 1990; Schleimer, 1990), recent evidence suggests that steroids possess more rapid effects. One of the important immediate effects of steroids seems to be inhibition of neutrophil migration towards the inflamed areas, and a systemic evaluation of this function is needed. It has been reported that steroids reduce adhesion of polymorphonuclear cells (McGillen and Phair, 1979; van Overveld et al., 2000), and subsequent infiltration of inflammatory cells from the blood into the tissues (McGillen and Phair, 1979). In vitro studies have demonstrated that corticosteroids are able to inhibit both chemotaxis of polymorphonuclear cells (Lomas et al., 1991; Rivkin et al., 1976) and superoxide anion generation (Fuenfer et al., 1979), but other workers have failed to confirm these observations (Jayappa and Loken, 1983; Sheng et al., 1987). Although the efficacy of steroids in the treatment of chronic obstructive pulmonary disease is less clear than in asthma (Pauwels et al., 1999; Postma and Kerstjens, 1999), there is evidence that in chronic obstructive pulmonary disease, steroids could be expected to have anti-inflammatory actions (Llewellyn-Jones et al., 1996; Lomas et al., 1991; Maltais et al., 2002; Wiggins et al., 1982). Some studies using different markers have reported reductions in visible bronchial inflammation and epithelial lining fluid albumin, chemotaxis, and airway neutrophilia after inhaled corticosteroids (Confalonieri et al., 1998; Llewellyn-Jones et al., 1996; Thompson et al., 1992). However, some other studies demonstrated that no benefit was reached (Culpitt et al., 1999a,b; Keatings et al., 1997).

The aim of the present study was to evaluate in an in vitro model whether corticosteroids of different potency, but also different chemical structure, as dexamethasone, budesonide, and prednisolone, exert any selective effects on the migration of isolated neutrophils through a cultured bilayer of endothelial and epithelial cells.

2. Materials and methods

2.1. Reagents

Ficoll-Hypaque (Histopaque 1077), ethylenediaminetetra-acetic acid disodium salt (EDTA), phenolred, bovine serum albumin, dimethylsulfoxide (DMSO), dexamethasone 21-phosphate disodium salt, budesonide, prednisolone 21-acetate, and lipopolysaccharide from *Escherichia coli*, serotype O111:B4 were obtained from Sigma, St. Louis, MO, USA; tissue culture media, glutamine, foetal calf serum, trypsin/EDTA solution, Hanks' Balanced Salt Solution (HBSS) and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from InVitrogen, Paisley, UK; calcein-acetomethylester (calcein-AM) was purchased from Molecular Probes Europe, Leiden, The Netherlands; TNF-α and interleukin-1β from Peprotech, Rocky Hill, NJ, USA; transwell membranes and culture plates were purchased from Costar, Cambridge, MA, USA; and ECV-304, human endothelial cell line and H292, human bronchial epithelial cell line, were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. All other chemicals used were reagent grade and obtained from Merck, Darmstadt, Germany.

2.2. Isolation and loading of human granulocytes

For each experiment, granulocytes were isolated from heparinised venous blood of a healthy, non-smoking volunteer with no known existence of an inflammatory disease. All volunteers gave informed consent and the study was approved by the local Ethics Committee. Granulocytes were separated from mononuclear cells by density centrifugation over Ficoll-Hypaque, $d = 1.077$ g/ml for 25 min, $1000 \times g$ at room temperature. Contaminating erythrocytes were removed from the granulocyte suspension by isotonic ammonium chloride lysis at 0 °C (Roos and Loos, 1970) and subsequent centrifugation. Isotonic shock solution contained 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, and 10 mg/l of phenolred. The pH of the solution was adjusted to 7.40 at 0 °C. After centrifugation, the granulocytes were collected, washed twice in PBS, and suspended in PBS at a concentration of 10^7/ml.

To each millilitre of granulocyte suspension, a volume of 5 μl calcein-AM (5 μM in DMSO) was added, followed by incubation at 37 °C for 15 min. After uploading was stopped by adding excess PBS, not incorporated label was removed by washing the cells twice in HBSS ($400 \times g$, for 10 min at room temperature). Afterwards, the cells were resuspended in HBSS/0.1% bovine serum albumin at a concentration of 2×10^6 cells/ml and incubated for 1 h at 37 °C in humidified air with 5% CO_2 to activate the label.

2.3. Migration assay

The human bronchial epithelial cell line H292 was grown to confluence in Ham's F12 medium, supplemented with 200 mM glutamine, 10% foetal calf serum, and 1% penicillin and streptomycin solution (100 U/ml, 10 μg/ml, respectively), on the laminin-coated basal side of transwell membranes (∅ 6.5 mm, 3-μm pore size, polycarbonate membrane). After overnight culture of H292 cells, the transwell filter inserts were suspended in 24-well culture plates. Then, on the apical side, the human endothelial cell line ECV-304 (Takahashi and Sawasaki, 1992) was also grown to confluence in the same type of medium. Con-

fluence was tested and confirmed by albumin permeability and transmission electron microscopy. To examine the influence of different steroids on neutrophil transmigration, the confluent epithelial cells were incubated with serial dilutions of dexamethasone, budesonide, or prednisolone in a concentration range of 10^{-10}–10^{-4} M during 1 h, and also dexamethasone (10^{-10}–10^{-4} M) during 4 h. Subsequently, the confluent bilayers were washed with pre-warmed medium and then pre-treated during 4 h with 10 ng/ml TNF-α and 10 ng/ml interleukin-1β in the lower compartment to induce adhesion molecule expression. After pre-incubation, media were replaced by 400 μl of 100 ng/ml lipopolysaccharide in HBSS in the lower compartment and 100 μl of the calcein loaded neutrophils in the upper compartment. After incubation for 2 h at 37 °C, the fluorescence in the entire well (F_{total}) was measured in a Cytofluor 2300 fluorometer (Millipore, Bedford, MA, USA) with an excitation wavelength of 485 nm and emission at 530 nm. After removal of the transwell insert, the remaining fluorescence (F_x) in the lower compartment was determined to assess the amount of migrated cells. The percentage of migrated leukocytes was calculated using the formula: $\%migration=(F_x/F_{total})\times 100$. The experiments were performed in duplicate for each experimental condition.

As a control for lipopolysaccharide-induced migration, a pre-incubation without any steroid was served. Spontaneous migration was obtained in the absence of any stimulus (only PBS), and in the absence of any steroid treatment, but the bilayers were treated with TNF-α and interleukin-1β as in the other wells. In addition, induction of migration by zymosan-activated serum was used as a positive control for migration in the absence of steroid treatment.

2.4. Statistical methods

All data are presented as mean ± S.E.M. (standard error of the mean), and as median ± interquartile ranges (25–75%) with the minimum and maximum in the figures. Treatments versus control effects were analysed for statistical difference by the non-parametric Wilcoxon test. Statistical significance was considered at $P<0.05$.

3. Results

Generally, the purity of the isolated neutrophils was 94–97%, and their viability, as assessed by trypan blue exclusion, always exceeded 95%.

The spontaneous migration of isolated neutrophils in the absence of any stimulus was low (2.8 ± 0.9%, geometric mean ± S.E.M.) while in the absence of any steroid, a migration of 28.5 ± 7.6% ($n=8$, $P<0.01$) could be induced with 100 ng/ml lipopolysaccharide (Fig. 1). Induction by zymosan-activated serum led to a migration of 69.4 ± 1.3%

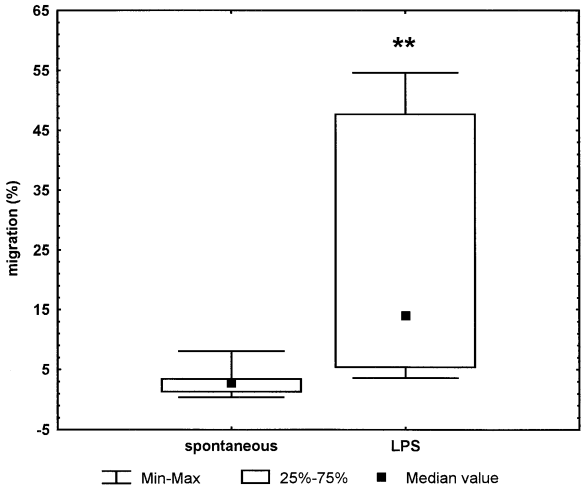

Fig. 1. Migration of isolated neutrophils through a bilayer of endothelial and epithelial cells induced by 100 ng/ml lipopolysaccharide compared to non-induced, spontaneous, migration. Results are expressed as median, interquartile range and minimum–maximum range of five experiments, each performed in duplicate. **$P<0.01$ as compared to spontaneous migration.

($n=5$). To examine the influence of different steroids on neutrophil migration, the epithelial cells of the bilayer were pre-treated during 1 h with serial dilutions of dexamethasone, budesonide, or prednisolone in the range of 10^{-10}–10^{-4} M. The steroid effects showed in all instances a concentration-dependent inhibition following a bell-shaped dose–response curve. As shown in Fig. 2A, treatment with dexamethasone at 10^{-7} M showed a migration of 19.4 ± 6.2% (median of 8.8%, $n=8$), which is the minimum effect peak of the bell-shaped curve. At this point, a mean inhibition of 20.6 ± 11.7% was observed, when compared with the lipopolysaccharide-induced migration in the absence of any steroid. Dexamethasone concentrations of 10^{-6} M and up inhibited significantly the neutrophil migration, but at 10^{-10} and 10^{-9} M also, significant inhibitory effects were noted, demonstrating clearly the bell-shaped nature of the curve. Budesonide showed a similar pattern with a migration of 15.8 ± 4.7% (median of 12.2%, $n=5$) at the minimum effect peak of the bell-shaped curve (at 10^{-7} M, inhibition of 11.0 ± 7.0%). Budesonide concentrations of 10^{-5} and up gave significant inhibition of migration, but also for 10^{-10} to 10^{-8} M, a significant inhibition was observed (Fig. 2B). The weakest steroid was prednisolone, with the minimum effect peak of the bell-shaped curve at 10^{-8} M, with a migration of 16.9 ± 4.8% (median of 13.8%, $n=5$), which stood for an inhibition of 22.5 ± 9.2% (Fig. 2C).

To prove our hypothesis that steroids may act through an acute pathway after a pre-incubation of 1 h, the epithelial cell layer was also incubated with dexamethasone (10^{-10} to 10^{-4} M) during 4 h before adhesion molecules were induced by TNF-α and interleukin-1β. This time the resulting migration followed a sigmoid dose–response curve instead of a bell-shaped curve, with significant inhibitory

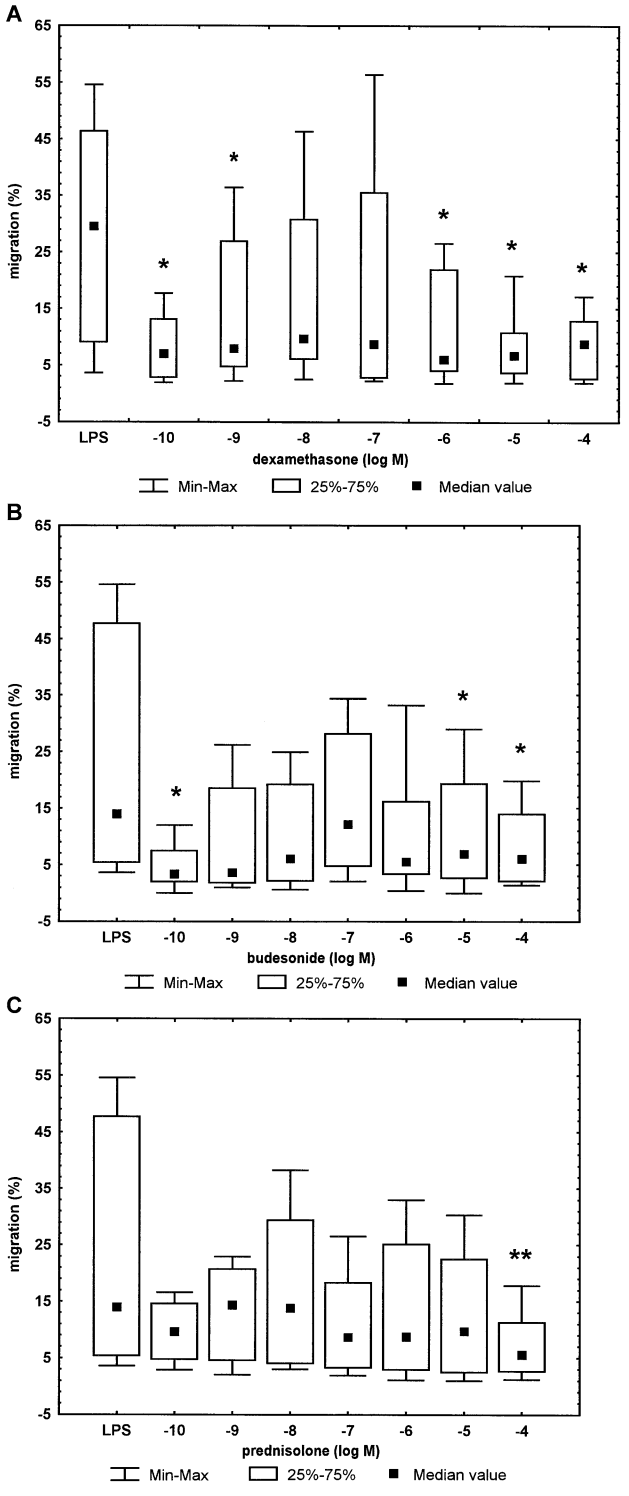

Fig. 2. The effect of 1-h incubation of epithelial cells with different corticosteroids on neutrophil migration to 100 ng/ml lipopolysaccharide in vitro. The *x*-axis represents increasing concentrations of steroids, and the *y*-axis the percentage migration of neutrophils through a bilayer of endothelial and epithelial cells. Bars represent median, interquartile ranges and minimum–maximum range of five experiments, each performed in duplicate. The significance of each difference from the lipopolysaccharide-induced control migration in the absence of any steroid is indicated. $*P<0.05$, $**P<0.01$. (A) Dexamethasone, (B) budesonide, and (C) prednisolone.

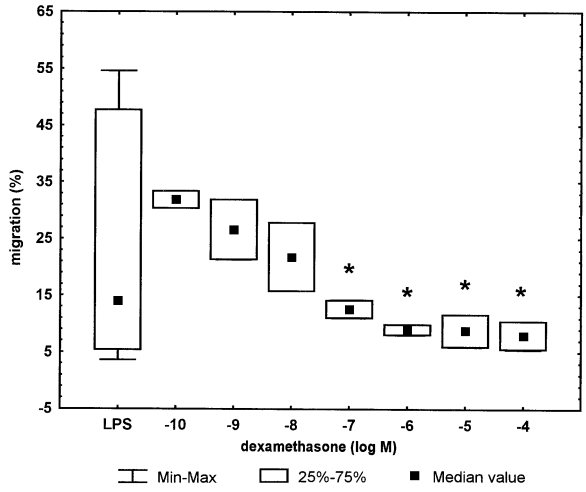

Fig. 3. The effect of 4-h incubation of epithelial cells with dexamethasone on neutrophil migration to 100 ng/ml lipopolysaccharide in vitro. The *x*-axis represents increasing concentrations of dexamethasone, and the *y*-axis the percentage migration of neutrophils through a bilayer of endothelial and epithelial cells. Bars represent median, interquartile ranges and minimum–maximum range of three experiments, each performed in duplicate. The significance of each difference from the lipopolysaccharide-induced control migration in the absence of any steroid is indicated. $*P<0.05$.

effects for concentrations of 10^{-7} M and up (Fig. 3), clearly demonstrating the distinctive pathways steroids follow when incubation times are different.

4. Discussion

Corticosteroids are potent inhibitors of inflammation, although their effects on migration of polymorphonuclear cells are not clearly defined. In this study, we evaluated the effects of steroids with different anti-inflammatory potencies on the migration of isolated neutrophils through bilayers of endothelial and epithelial cells in an in vitro model, after pre-treatment of the epithelial cell layer with the steroid for both short (1 h) and longer (4 h) incubation times. We selected epithelial cells for the site of pre-incubation, because this resembles the in vivo situation as found in chronic obstructive pulmonary disease patients, who are generally treated with inhaled corticosteroids. As in some other studies, we found that corticosteroids successfully inhibited the migration of neutrophils in a dose-related manner (Davenpeck et al., 1998; Lomas et al., 1991). Not only suprapharmacological concentrations of steroids are required to produce a significant reduction in migration, also for concentrations as low as 10^{-10} to 10^{-9} M, significant decrease in migration was observed. Thus, in all instances, the dose–response curve of steroids followed a bell-shaped curve when epithelial cells were pre-incubated during 1 h with the steroids investigated. The bell-shaped form of a dose–response curve is not unusual for pharmacological processes. This may indicate that more than one mecha-

nism of inhibition by steroids may exist and this effect may be even cell-specific.

Effects observed in vitro at dexamethasone concentrations of 10^{-3} M are likely to be nonspecific as receptor mediated events are usually observed at concentrations of 10^{-7} M (Petroni et al., 1988). However, in this study, we did not use concentrations higher than 10^{-4} M.

We are aware that in our complex model, different mechanisms may play a role in the transmigration of neutrophils. We have chosen for this type of in vitro model since it resembles the existing blood–air barrier in the airways. The use of calcein-AM in DMSO for labelling of neutrophils has no effects on cell viability, on chemotactic response and on adhesion of neutrophils to endothelial cells (De Clerck et al., 1994). Mechanisms that may play a role in this model are the expression of adhesion molecules induced by a pre-incubation with TNF-α and interleukin-1β, or induction of mediator release by these cytokines. It is not excluded that these two mechanisms of action may also be linked together. On the other hand, lipopolysaccharide challenge may also lead and add to these effects. Previous studies have reported that steroids reduced L-selectin expression on neutrophils in circulation without inhibiting the upregulation of CD11b which occurs during migration in the airways (O'Leary and Zuckerman, 1997). Another study has also shown that dexamethasone reduces the binding of P-selectin to circulating leukocytes (Yamaki et al., 1998), and it reduces the expression of CD18 on systemic leukocytes (Burton et al., 2002). In vitro studies examining the direct effects of steroids on adhesion molecule expression still do not have yielded definitive data. It was found that budesonide did not inhibit interleukin-1β or TNF-α-induced expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), or vascular cell adhesion molecule-1 (VCAM-1) on human umbilical cord vascular endothelial cells (Kaiser et al., 1993), but it was demonstrated that dexamethasone was effective in inhibiting both lipopolysaccharide- and interleukin-1β-induced synthesis and expression of E-selectin and ICAM-1 (Cronstein et al., 1992). Pre-treatment of rats with dexamethasone before a stimulation with lipopolysaccharide inhibited the induced leukocyte rolling and adhesion in mesenteric postcapillary venules (Davenpeck et al., 1998). These effects of dexamethasone may be due to inhibition of interleukin-1, TNF-α, and cytokine-induced neutrophil chemoattractant-1 (CINC-1) generation, since agonists to these mediators were able to mimic dexamethasone effects on lipopolysaccharide-induced leukocyte–endothelial interactions and circulating leukocyte phenotype (Davenpeck et al., 1998). Therefore, the effects of steroids on adhesion molecules are complex and may contribute to the reduction in neutrophil migration by promoting shedding of adhesion molecules. In addition, by permitting CD11b upregulation, they may enable a significant neutrophil migration following lipopolysaccharide challenge.

Concerning mediator release from endothelial and epithelial cells, it was shown that neutrophil products stimulated epithelial cells to increased production of interleukin-8 and interleukin-6 protein, but in contrast to TNF-α and interleukin-1β induced cytokine secretion, this result is depending on the cell type used (Cromwell et al., 1992; Van Wetering et al., 2002). In our model, lipopolysaccharide may have induced interleukin-8 release leading to migration and activation of neutrophils, since lipopolysaccharide is a potent stimulus for cytokine and chemokine release from several cell types. In vivo, cytokines such as interleukin-1β and TNF-α are rapidly released in response to lipopolysaccharide (Chensue et al., 1991; Martich et al., 1991) and both of these cytokines induce endothelial adhesion molecule expression (Kaiser et al., 1993). The migrated neutrophils on their turn may have increased the ongoing cytokine production by their released products. However, the studies of Van Wetering et al. (2002) indicate that neutrophil products selectively stimulate the secretion of different sets of cytokines. Also, with respect to expression of adhesion molecules between different airway epithelial cells, marked differences were observed by others (Wang et al., 1996). In addition, the induced cytokine secretion is sensitive to inhibition by glucocorticoids (Barnes and Adcock, 1993). Perhaps this is more likely the mechanism by which glucocorticoids may inhibit leukocyte recruitment in response to lipopolysaccharide. Thus, glucocorticoids may inhibit the mediators that induce adhesion molecule-mediated leukocyte endothelial interactions and leukocyte migration. Since dexamethasone did not affect neutrophil chemotaxis towards *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or recombinant interleukin-8 in vitro (Van Wetering et al., 2002), this effect is most likely explained by the fact that dexamethasone reduces interleukin-8 levels in supernatants of stimulated airway epithelial cells, resulting in a reduced neutrophil chemotactic activity.

The primary means by which glucocorticoids mediate their actions is through regulation of gene expression (Munck et al., 1990; Schleimer, 1990), and numerous genes involved in metabolism, immunological responses, and inflammation are known to be glucocorticoid sensitive. If gene transcription is the primary mechanism of action for steroids, the time course for steroid effects has been felt to be over the course of several hours. Thus, the majority of in vivo and in vitro studies have looked at steroid actions after prolonged treatment (>4 h). However, our study and some other recent studies indicate that steroids are also extremely effective in inhibiting lipopolysaccharide-induced leukocyte adhesion and migration after short incubation times (Davenpeck et al., 1998; O'Leary and Zuckerman, 1997; Vainer and Nielsen, 2000).

When the epithelial cell layer was pre-incubated with dexamethasone for 4 h, a different dose–response curve was observed. The bell-shape, which was found after 1-h steroid pre-incubation, was replaced by a sigmoid curve. This means that steroid action can follow at least two different pathways. In this case, steroids may act via their receptors that interact directly with the transcription factors activator

protein-1 (AP-1) and nuclear factor-kappa B (NF-κB), counteracting the activation of these transcription factors by TNF-α. In case of short incubation times, steroids do not inhibit the rolling and adhesion phase, and the passage through the endothelial cells, whereas they inhibit migration from the venular wall into the interstitial space. As a result, the neutrophils remain in the venular wall, between the endothelial layer and the basement membrane (Davenpeck et al., 1998; Tailor et al., 1997). Furthermore, the secretion of granular content by the neutrophils during extravasation is inhibited by steroids (Gallin et al., 1975; Wright and Gallin, 1979).

Our data indicate that in an in vitro model with endothelial and epithelial cells, the glucocorticoids dexamethasone, budesonide and prednisolone inhibit lipopolysaccharide-induced leukocyte transmigration in a dose-dependent manner. However, acute or long-term administration of the steroid led to distinct dose–response curves, reflecting different pathways through which steroids may act. The complex concerted action of the responsible mechanisms still needs to be elucidated in future studies.

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