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Effects of steroid treatment on lung CC chemokines, apoptosis and transepithelial cell clearance during development and resolution of allergic airway inflammation

L. Uller^{*}, C. M. Lloyd[†], K. Rydell-Törmänen^{*}, C. G. A. Persson[‡], and J. S. Erjefält^{*}

^{*}Department of Experimental Medical Science, Lund University, Lund, Sweden

[†]Leukocyte Biology Section, Division of Biomedical Sciences, Faculty of Medicine, Imperial College, London, UK

[‡]Clinical Pharmacology, Lund University Hospital, Lund, Sweden

Summary

Background—Steroid treatment of allergic eosinophilic airway diseases is considered to attenuate cell recruitment by inhibiting several chemokines and to cause eosinophil clearance through inducement of apoptosis of these cells. However, roles of these mechanisms in the actions of steroids *in vivo* have not been fully established. Also, as regards clearance of tissue eosinophils other mechanisms than apoptosis may operate *in vivo*.

Objective—This study explores anti-inflammatory effects of steroids instituted during either development or resolution of airway allergic inflammation.

Methods—Immunized mice were subjected to week-long daily allergen challenges (ovalbumin). Steroid treatment was instituted either amidst the challenges or exclusively post-allergen challenge. CC chemokines, goblet cell hyperplasia, occurrence of eosinophil apoptosis, and airway tissue as well as lumen eosinophilia were examined at different time-points.

Results—Daily steroids instituted amid the allergen challenges non-selectively attenuated a range of chemokines, permitted egression of tissue eosinophils into airway lumen to increase, and reduced development of lung tissue eosinophilia. Steroid treatment instituted post-challenge selectively inhibited the CC-chemokine regulation upon activation, normal T cell expressed and secreted (RANTES), permitted continued egression of eosinophils into airway lumen, and resolved the tissue eosinophilia. Eosinophil apoptosis rarely occurred at development and resolution of the allergic eosinophilic inflammation whether the animals were steroid treated or not. However, anti-Fas monoclonal antibodies given to mice with established eosinophilia post-challenge produced apoptosis of the tissue eosinophils indicating that apoptotic eosinophils, if they occur, are well detectable *in vivo*.

Conclusion—Airway tissue eosinophils are likely eliminated through egression into airway lumen with little involvement of apoptosis and phagocytosis. Our data further suggest that therapeutic steroids may resolve airway inflammation by permitting clearance of tissue eosinophils through egression and inhibiting RANTES-dependent cell recruitment to lung tissues.

Keywords

apoptosis; asthma; chemokines; glucocorticoids

Introduction

The definition of asthma as an inflammatory eosinophilic airway disease in part originates from demonstrations of clinical efficacy of the anti-inflammatory and anti-eosinophilic glucocorticosteroid drugs [1, 2]. Steroids remain major controller drugs in asthma but their *modus operandi in vivo* has not been fully understood. Prophylactic steroids given prior to allergen exposure may have particularly broad effects inhibiting the generation of a wide range of cytokines and chemokines and inhibiting the development of airway eosinophilia [1, 3]. To explain such non-selective efficacy it has been proposed that steroids inhibit nuclear factor (NF- κ B) and its down stream gene targets involving many proteins implicated in airway eosinophilic inflammation [1]. However, the involvement of NF- κ B in effects of steroids in established asthma is unclear [4–6]. Also, airways where eosinophilic inflammation is already present would need treatment that mediates resolution, potentially involving other drug mechanisms than those operating prophylactically. As regards the resolution of airway inflammation much interest is now focused on apoptosis of inflammatory cells, and steroid-induced eosinophil apoptosis has been forwarded as an important drug action [7].

Actual data demonstrating occurrence of eosinophil apoptosis or supporting a pro-apoptotic effect of therapeutic steroids *in vivo* are scarce [8, 9]. In the search for alternative modes of cell elimination, which are independent of apoptosis, it has recently been observed that airway tissue eosinophils can be efficiently eliminated through egression into the airway lumen [10, 11]. Hence, studies comparing clearance through apoptosis followed by engulfment vs. loss of tissue eosinophils into the airway lumen are warranted. Furthermore, as cells harvested from the airway lumen have already been eliminated from the diseased tissue, cells present in the airway tissue must be examined to assess roles of leucocyte apoptosis. Tissue eosinophilia can further be expected to differ significantly from the eosinophilia that is detectable in the airway lumen. Recent demonstration of differing effects of anti-IL-5 treatment on airway tissue and lumen eosinophilia, respectively, has highlighted the limited conclusions that can be reached based merely on lumen cell data [12].

This study involving allergic mice has explored modes of eosinophil elimination from airway tissues both when the tissue eosinophilia is developing and when it is resolving. Based on our previous observations [10] our hypothesis was that elimination of airway tissue eosinophils may occur without inducement of apoptosis. Moreover, daily steroid treatment is instituted both during development of inflammation, amid a series of daily allergen challenges, and exclusively post-challenge when airway inflammation had fully developed. As effects on cell elimination alone likely is insufficient to combat active inflammation this study also examined effects of steroids on several CC chemokines involved potentially in eosinophil recruitment to airway tissues [13]. Furthermore, as a positive control as regards detection of steroid-induced eosinophil apoptosis treatment with anti-Fas monoclonal antibody (mAb) to directly stimulate eosinophil death receptors was included in the study [14].

Material and methods

Allergen sensitization and challenge protocol

Eight to 10-week old male C57BL/6 mice (Bomholtgard, Copenhagen, Denmark) were kept in well-controlled animal housing facilities and fed *ad libitum*. The study was approved by the Regional Ethics Committee in Malmö–Lund, Sweden. The ovalbumin (OVA) sensitization and challenge protocol was as described previously [14]. Fourteen days after immunization mice were exposed daily for 7 days to aerosolized OVA (1% wt/vol). Control animals received saline challenge.

Drug treatments

Dry powder of budesonide (Astra Zeneca, Lund, Sweden) was dissolved and sonicated in ethanol and diluted in sterile saline (1 mg/kg given intraperitoneally (i.p.)). Control animals received vehicle (saline containing 1% ethanol).

Study design

Immunized mice were studied in 13 groups ($n = 8$); six groups during development and seven groups during resolution of the allergic inflammation (Fig. 1). Animals were sacrificed by pentobarbital i.p. followed by bronchoalveolar lavage (BAL) and lung tissue sampling. Outcome measurements were made twice during development and twice during resolution (Fig. 1). At each time-point vehicle-treated, allergen-challenged animals were compared with both saline-challenged animals and steroid-treated, allergen-challenged animals. One group of mice with established eosinophilic inflammation was treated with agonistic anti-Fas mAb (30 μ g/mouse, clone Jo2, Pharmingen, San Diego, CA, USA) by intranasal administration [15] followed by histological analysis of the lung tissue 24 h later.

Bronchoalveolar lavage and quantification of luminal cells

BAL was performed via a ligated tracheal cannula. One millilitre of PBS was allowed to passively enter the lungs at a pressure of 10 cm H₂O. This procedure was carried out twice. The obtained BAL fluid (BALF) from each animal was immediately centrifuged (700 g, 5 min) and the supernatant frozen for ELISA analysis. The cell pellet was washed and resuspended in 250 μ L PBS containing 10% fetal calf serum (FCS). The total number of cells was quantified using a haemocytometer and 5×10^5 cells cytocentrifuged to microscope slides. Differential cell counts were performed on May–Grünwald–Giemsa stained slides and percentage of eosinophils, lymphocytes, neutrophils, and macrophages determined by counting at least 200 cells in a blinded manner. This was considered a sufficient number of cells especially as already by day 3 (two allergen challenges) eosinophils constitute about 20% of the BALF cells (see also Fig. 2). To obtain the absolute number of each leucocyte subtype in each BALF, the percentage of cells was multiplied by the total number of cells recovered from the BALF.

Lung tissue processing for histology analysis

From each animal four tissue samples were taken from the superior lung lobes at the level just below the root of the lung. One tissue sample was immersed in Stefanini's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer pH 7.2) overnight, rinsed repeatedly in Tyrode buffer supplemented with 10% sucrose, and finally frozen in TissueTEK (Miles Inc., Elkhart, IN, USA). The frozen specimens were stored at -80°C until used for histochemistry. A separate sample was immersed overnight in buffered 4% paraformaldehyde (pH 7.2) and thereafter dehydrated and embedded in paraffin. An additional sample was placed in a fixative consisting of a mixture of 3% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 and used for transmission electron

microscopic (TEM) analysis. The rest of the lung tissue was immediately frozen for mRNA analysis.

Detection of interleukin-5 and the CC-chemokines RANTES and eotaxin in bronchoalveolar lavage fluid

Regulation upon activation, normal T cell expressed and secreted (RANTES), eotaxin, and IL-5 were quantified in BALF by ELISA using three different ELISA kits according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

Staining and quantification of lung tissue eosinophils

Ten micrometre cryosections were stained for eosinophil peroxidase (EPO) and eosinophils identified by their dark brown reaction product and quantified as number of peribronchial eosinophils/0.1 mm² tissue area as previously described [10].

Periodic acid-Schiff reagent staining of mucus-containing cells and examination of epithelial integrity

Five micrometer paraffin sections were stained with periodic acid-Schiff (PAS) reagent as previously described [16]. Epithelial integrity was examined and specific signs of injury-repair processes [17] looked for.

Detection of apoptotic cells

Apoptotic cells in the lung tissue were detected using terminal deoxy RNase nick end labeling (TUNEL) technique. Combined staining with TUNEL and Chromotrope-2R identified apoptotic eosinophils as previously described [9, 10]. To assess an apoptotic morphology and detect engulfed eosinophils, ultrastructural analysis using TEM was performed as previously described [9].

Measurement of mRNA expression

Total RNA from the lungs was extracted with RNeasy RNeasy Lysis Buffer (Qiagen, Crawley, UK) according to the manufacturer's protocol. Chemokine mRNA expression was determined by multiprobe RNase protection assay (RPA) using the Riboquant RPA kit (mCK-5, Pharmingen, San Diego, CA, USA), according to supplier instructions and as previously described [18].

Data analysis

Histology analyses were performed and quantified in a blinded manner. For statistical analysis Wilcoxon's Rank sum test was performed using Analyze It™ (Analyse-it software Ltd., Leeds, UK). Data are expressed as mean - scanning electron microscopy (SEM). A value of $P < 0.05$ was considered statistically significant.

Results

Development of allergen challenge-induced eosinophilic inflammation

Eosinophilia in tissue and lumen—Two allergen challenges increased lung tissue eosinophils (Fig. 2a). After seven OVA challenges the increase in tissue eosinophilia was further pronounced (Fig. 2a). The development of lung tissue eosinophilia was followed by significant eosinophilia also in the airway lumen (Fig. 2b).

Goblet cell hyperplasia and epithelial integrity—Two allergen challenges with OVA-induced pronounced goblet cell hyper-plasia which was further increased after seven allergen challenges (Fig. 2c). There were no signs of epithelial injury-repair processes.

Chemokines—Up-regulation of mRNA for RANTES, eotaxin, MIP-1 α and MIP-1 β occurred after two allergen challenges (data not shown; $n = 2$) as well as after seven allergen challenges (Fig. 3). The occurrence of RANTES and eotaxin protein in the airway lumen was significantly increased 24 h after seven OVA challenges (Figs 4a and b). At this time-point BALF IL-5 was below detection limit; 15.6 pg/mL (data not shown).

Effects of budesonide treatment instituted during development of allergic inflammation

Eosinophilia of airway tissue and lumen—One day of treatment with budesonide did not acutely affect the ongoing development of airway eosinophilia (Figs 2a and b). However, by continued daily treatment with budesonide the further development of lung tissue eosinophilia was inhibited ($P < 0.01$; Fig. 2a). Contrasting the halted increase in airway tissue eosinophils, the number of eosinophils in the airway lumen was actually increased during steroid treatment (Fig. 2b). The fold increase in BALF eosinophils between days 3 and 8 was of a similar magnitude in steroid treated as in the control group (Fig. 2b).

Goblet cell hyperplasia—Budesonide treatment did not reduce the development of goblet cell hyperplasia (Fig. 2c).

Chemokines—Treatment with budesonide during development of allergic inflammation reduced expression of RANTES, eotaxin, MIP-1 α and MIP-1 β compared with saline-treated, allergen-challenged animals (Fig. 3). Budesonide also reduced secretion of both RANTES and eotaxin protein into the airway lumen (Figs 4a and b).

Effects of budesonide treatment instituted exclusively during the resolution phase

Eosinophilia of airway tissue and lumen—The allergen challenge-induced tissue eosinophilia exhibited a slow spontaneous decline during 6 days following the last allergen challenge (Fig. 5a). The decline was accelerated by budesonide (Fig. 5a). Yet the BALF eosinophilia was not reduced by budesonide (Fig. 5b).

Goblet cell hyperplasia—There was little spontaneous restoration of a normal goblet cell-poor epithelium during the study period post-challenge (Fig. 5c). However, the goblet cell hyperplasia was reduced by steroid treatment (Fig. 5c).

Chemokines selective inhibition of RANTES—During the spontaneous resolution of inflammation the mRNA expression of RANTES, eotaxin, MIP-1 α and MIP-1 β remained up-regulated compared with saline-treated animals (Fig. 6). Notably, only mRNA expression for RANTES was now significantly reduced by budesonide (Fig. 6). Also, budesonide treatment inhibited secretion of RANTES but not eotaxin protein into the airway lumen during the resolution of inflammation (Figs 7a and b).

Apoptosis

Along with the development of eosinophilic inflammation the allergen challenges increased the total number of apoptotic TUNEL-positive cells lying scattered in the lung tissue (Fig. 8, Table 1). In agreement with previous observations [10] this aspect of airway inflammation (the total number of apoptotic cells in inflamed lung tissue) was reduced by budesonide treatment ($P < 0.01$, Table 1). Apoptotic eosinophils were rarely observed at any time-point, irrespective of steroid treatment (Fig. 9, Table 1) nor were apoptotic epithelial cells detected.

Thus only a few cells exhibited dual TUNEL and chromotrope-2R staining. Indeed, merely one eosinophil out of all eosinophils examined by TUNEL and TEM together exhibited apoptosis in the steroid-treated animals (Table 1).

By contrast, in the group of animals treated with agonistic anti-Fas mAb pronounced apoptosis of tissue eosinophils in the lung-airway tissues was induced (Figs 8 and 9, Table 1).

Discussion

The present series of allergen challenges of sensitized mice evoked increased lung tissue expression of five CC chemokines along with development of airway tissue and lumen eosinophilia and goblet cell hyperplasia. Steroid treatment once daily, instituted amidst the allergen challenges, non-selectively reduced the CC chemokines and disrupted further development of tissue eosinophilia. When steroid treatment was instituted exclusively post-challenge only the CC-chemokine RANTES was inhibited along with steroid-induced resolution of the established eosinophilic inflammation. Apoptotic eosinophils, engulfed or unengulfed, rarely occurred during turnover and resolution of the airway eosinophilic inflammation. Instead lung tissue eosinophils apparently were lost through migration into the airway lumen and this mode of cell clearance was promoted or permitted by the steroid treatment. The present data provide novel aspects on modes of action of airway steroids and mechanisms of resolution of airway eosinophilic inflammation *in vivo*.

CC chemokines, recruitment of eosinophils and effects of steroids at development of airway inflammation

The present model of week-long allergen challenges exhibit important characteristics of airway-allergic inflammation including eosinophilia and several features of remodelling [19, 20]. Although the mouse models suffer from distinct shortcomings regarding the mimicking of asthmatic airway pathology [21] these specific features also apply to human asthma [22]. RANTES and eotaxin, both of which were increased in BALF by the present allergen challenges, have previously been shown to attract eosinophils to the lung in a coordinated manner at development of airway inflammation in allergic mice [18]. Involvement of these two proteins and possibly several more CC chemokines in the present development of lung eosinophilia is consistent with their increased expression at allergen exposure and with the broad inhibitory effects of budesonide on CC-chemokine expression during development of inflammation in this study. As shown previously budesonide may also attenuate the development of airway eosinophilia through effects on generation of eosinophils in the bone marrow [23].

Selective inhibition of RANTES at resolution of airway inflammation

One of the novel and intriguing aspects of this study was the demonstration that the steroid-induced resolution post-challenge, when all the determined CC chemokines remained elevated, was associated with selective attenuation of RANTES expression in the lung tissue. This selectivity was not a chance observation because one advantage with the present mRNA analysis is that it generates reliable comparisons within the series of proteins that are expressed [18]. Furthermore, selectivity was confirmed in this study because RANTES protein levels in BALF, but not eotaxin, was also inhibited by steroid treatment post-challenge. IL-5 is important for development of lung tissue eosinophilia in allergic mice [24] and contribution by airway IL-5 to the present maintained eosinophilia post-challenge cannot be excluded. However, a major role of IL-5 seems less likely because this cytokine was below detection limit already 24 h after the allergen challenges in this study. The present selective anti-RANTES action is a rare finding as regards the anti-inflammatory

steroids and it contrasts the pan-inhibitory effects on CC chemokines expression produced by the same daily dose of budesonide during development of the allergic inflammation (this study).

It is of interest that RANTES expression is increased in human allergic airways in asthma [25, 26] and rhinitis [27]. RANTES is also selectively increased at exacerbations of human bronchial eosinophilic diseases [4, 28]. In allergic individuals local administration of RANTES together with allergen evokes desquamative nasal inflammation rich in eosinophils, basophils, and lymphocytes [29]. At budesonide-induced resolution of allergic rhinitis we have preliminarily observed that RANTES is inhibited along with the decline in symptoms and nasal mucosal eosinophilia [30]. These human airway *in vivo* data, including the demonstration by Castro et al. [4] of increased RANTES expression at exacerbation of asthma following glucocorticoid withdrawal, support the possibility that the present selective inhibition of RANTES is an important aspect of the therapeutic pharmacology of airway steroids.

Apoptosis of airway tissue eosinophils

To explain spontaneous or drug-induced decline in tissue eosinophilia a paradigm involving eosinophil apoptosis followed by phagocytosis of apoptotic eosinophils has become widely accepted [7]. However, except in anti-Fas mAb-treated animals apoptosis of lung tissue eosinophils was a rare event in this study. During development of airway inflammation eosinophil apoptosis was not detected at all in the steroid-treated animals (Table 1). Yet, there were clear signs of turnover of the newly arrived lung tissue eosinophils because eosinophilia was also developing in the airway lumen. Similarly, whether eosinophilia was declining spontaneously or by steroid treatment, only one or two occasional apoptotic eosinophils were detected in the tissue in this study. These data actually agree with prior reports that fail to demonstrate significant eosinophil apoptosis in airway tissues *in vivo* [8–10].

The lack of *in vivo* support for eosinophil apoptosis as a major mechanism has, however, not been regarded a significant weakness of the hypothesis that eosinophils are eliminated through apoptosis followed by engulfment. It has rather been reasoned that apoptotic cells are engulfed so rapidly that they may not be detected in a tissue at any one time [31]. Importantly, therefore, in the present allergen-challenged mouse lungs receiving anti-Fas mAb treatment we could demonstrate for the first time a significant (although not overwhelming) occurrence of tissue eosinophils with clear ultrastructural features of apoptosis. In a separate study we have confirmed and extended this observation [14]. As apoptosis basically is defined through morphologic criteria [32] the present TEM analysis was essential for demonstrating occurrence and absence of apoptotic eosinophils. Anti-Fas-induced eosinophilic apoptosis in this study, together with the present occurrence of single apoptotic eosinophils, equally in control and steroid-treated animals, thus provide essential positive control data regarding the detection of apoptotic eosinophils in lung tissues *in vivo*.

Clearance of airway tissue eosinophils and effects of therapeutic steroids during development and resolution of airway inflammation

It is of note that the daily treatment with budesonide, that reduced the increase in tissue eosinophilia during development of allergic inflammation, still allowed the number of BALF eosinophils to increase. The relative changes of tissue and BALF eosinophilia are also of interest. After two allergen challenges (Fig. 2b) the tissue eosinophilia had already risen to one third of that recorded after seven challenges. By contrast, the corresponding ratio of BALF eosinophils was only about 1/20 indicating that tissue eosinophilia was allowed to build up for a few days without great loss of cells into the airway lumen.

However, after 1 week of allergen challenges, a substantial number of eosinophils was retrieved by BAL suggesting that entry into the airway lumen had become a major route of migration for the lung tissue eosinophils.

During the resolution phase BALF eosinophils were equally numerous in untreated allergic mice and in those receiving daily steroid treatment. As the number of tissue eosinophils (amenable for egression) was more reduced in the steroid-treated animals than in untreated controls this latter finding suggests that steroids may even facilitate egression of eosinophils. Although a similar tendency was recorded in a study of steroid-induced resolution of rat lung eosinophilia [10] further work is clearly warranted to elucidate this possibility. The present BAL would not have retrieved all eosinophils that entered the airway lumen and no attempt was made at quantifying actual numbers of cells that were cleared from lung tissues. Thus, we only sampled at a few time-points between which many lumen cells would have been eliminated by mucociliary transport and other physiological processes. However, in recent work involving guinea-pigs *in vivo* [11], where several factors of importance for assessment of cell clearance across the epithelial lining were controlled, we demonstrated that about 35 000 airway tissue eosinophils per minute and per cm² mucosal surface may readily be cleared into the airway lumen. Furthermore, the epithelial integrity was not compromised by this acute and intense cell traffic [11] nor could we detect any morphological signs of epithelial injury-repair processes [17] in the present study. Hence, transepithelial migration of eosinophils occurring in the present steroid-treated and untreated animals is likely a major mode of non-injurious clearance of these cells thus complementing a prior concept stating that the epithelial passage is a pathogenic event in airway disease [33]. The molecular and cellular mechanisms involved in clearance of tissue eosinophils through transepithelial egression will have to be explored in future studies. It is possible that lumen-tissue gradients of select chemokines potentially including eotaxin (that was not reduced in the lumen at resolution; this study), may contribute to this mode of clearance of eosinophils [34]. Interestingly, eosinophil-active cytokines such as GM-CSF and IL-5 may exhibit increased lumen to tissue ratio post-allergen challenge in asthmatic individuals [35, 36]. The present hypothesis regarding clearance of airway tissue eosinophils agree with actual findings in airway tissues and airway lumen in asthma including the centennium-old observation that resolution of asthma exacerbations is followed by profuse sputum eosinophilia [2, 8].

In conclusion, we propose that resolution of airway tissue eosinophilia *in vivo* involves non-injurious clearance of these cells through transepithelial egression. Furthermore, our data indicate that transepithelial cell clearance together with inhibition of RANTES-dependent cell recruitment is involved in the present steroid-mediated resolution of allergic inflammation. Considering the possibility that steroid-induced effects *in vivo* may reveal important pathogenetic processes the present data strongly support the view that RANTES, a chemoattractant for eosinophils and T cells, is an important regulator protein in allergic airway diseases.

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Fig. 1.

Study design and treatment schedule. Two weeks after immunization with ovalbumin (OVA) mice were exposed to daily OVA challenges for 1 week (days 1–7). Mice were then treated either during the development or during the resolution of airway inflammation. During the development of inflammation daily steroid, or vehicle, treatment was instituted day 2 and animals were euthanized at days 3 and 8. During resolution of the established allergic inflammation separate groups of animals received daily steroid, or vehicle, treatment starting day 9 and animals euthanized days 10 and 13. Treatment day 9 with a single dose of anti-Fas monoclonal antibody (mAb) in a group of allergen-challenged animals was attempted as a positive control regarding the possibility of detecting apoptotic eosinophils in the airway tissue.

Fig. 2.

Development of airway inflammation and effects of steroids. Seven daily allergen challenges induced eosinophilia in airway tissue (a) and lumen (b). Treatment with budesonide during the developing inflammation (see Fig. 1) apparently inhibited recruitment of tissue eosinophilia occurring between days 3 and 8 (a) but permitted the bronchoalveolar lavage fluid eosinophils to increase significantly (although the increase was less than in vehicle-treated animals). (b) The allergen challenges gradually increased goblet cell hyperplasia that, however, remained unaffected by the present steroid treatment regimen (c). Data are mean $\pm$ SEM; $n = 8$. ** $P < 0.01$, * $P < 0.05$ indicates differences between ovalbumin (OVA) and saline treatments. §§ $P < 0.01$ indicates difference between budesonide- and placebo-treated OVA-challenged animals. # $P < 0.05$ indicate difference between budesonide-treated animals at days 3 and 8.

Fig. 3.

Chemokine expression day 8 (24 h after seven allergen challenges with ovalbumin (OVA) or saline) in control and steroid-treated animals. Total lung RNA was extracted and mRNA expression for Ltn (Lymphotactin), RANTES, eotaxin, MIP-1 α and MIP-1 β was determined. The intensity of the band for each chemokine was determined and expressed as arbitrary units (AU). All chemokines were up-regulated by the allergen exposure and reduced by the budesonide treatment. Data are mean - SEM; $n = 8$. ** $P < 0.01$ indicates difference between placebo-treated OVA-challenged animals and saline-challenged animals. §§ $P < 0.01$ and § $P < 0.05$ indicates difference between budesonide treated and placebo-treated OVA-challenged animals.

Fig. 4. RANTES and eotaxin levels in the airway lumen at development of allergic inflammation. Along with the increasing lung eosinophilia the daily allergen challenges increased bronchoalveolar lavage fluid (BALF) levels of RANTES and eotaxin (a, b). Institution of daily budesonide treatment during the development phase reduced the levels of both proteins. Data are mean $\pm$ SEM; $n = 8$. ** $P < 0.01$ indicate difference between placebo-treated OVA-challenged animals and saline-challenged animals. §§ $P < 0.01$ indicates difference between budesonide treated and placebo-treated ovalbumin (OVA)-challenged animals.

Fig. 5. Steroid-induced effects on established airway eosinophilia and goblet cell hyperplasia. Budesonide treatment instituted post-challenge resolved the tissue eosinophilia (a) While permitting luminal entry of tissue eosinophils (b). Budesonide also accelerated the return of epithelial changes towards a normal non-secretory epithelium (c). Data are mean - SEM; $n = 8$. §§ $P < 0.05$ indicates difference between budesonide- and placebo-treated OVA-challenged animals.

Fig. 6. Chemokine expression in the lung at resolution of allergic inflammation day 13. The CC-chemokines remained up-regulated compared with immunized controls several days post-challenge. Treatment with budesonide exclusively during the resolution phase selectively reduced the mRNA expression of RANTES. Data are mean $\pm$ SEM; $n = 8$. ** $P < 0.01$ indicate difference between placebo-treated ovalbumin (OVA)-challenged animals and saline-challenged animals. §§ $P < 0.01$ indicates difference between budesonide- and placebo-treated OVA-challenged animals.

Fig. 7. Chemokine levels in the airway lumen at resolution of allergic inflammation. Daily budesonide treatment instituted during the resolution phase reduced bronchoalveolar lavage fluid (BALF) levels of RANTES (a) but did not affect the eotaxin levels (b). Data are mean - SEM; $n = 8$. § $P < 0.05$ indicates difference between budesonide and placebo-treated OVA-challenged animals.

Fig. 8. Terminal deoxy RNase nick end labeling (TUNEL) staining of mouse lungs. Very few TUNEL-positive cells were present in immunized non-challenges animals (a). In immunized animals challenged with ovalbumin (OVA) and increased number of TUNEL-positive cells were observed (green fluorescence) (b). In animals treated with anti-Fas monoclonal antibody pronounced apoptosis was induced in areas around bronchi and vessels (c). In allergen challenged and steroid treated animals a reduced number of TUNEL-positive cells were present in the lung (D).

Fig. 9.

Transmission electron micrographs of lung eosinophilic inflammation. Numerous eosinophils with a normal ultrastructural morphology are present in the airway mucosa after the final ovalbumin (OVA) challenge (a). The lung in (a) also received budesonide treatment days 9–10. Treatment with anti-Fas monoclonal antibody (days 9–10) in a separate group of allergen-exposed animals induced a pronounced occurrence of apoptotic eosinophils in the airway mucosa. The apoptotic eosinophils exhibited different signs of apoptosis including a dark condensed cell nucleus and reduced cell volume (b).

Table 1

Apoptotic cells in lung tissues

	Treatment	Total TUNEL-positive cells*	TUNEL-positive eosinophils*	Apoptotic eosinophils by TEM [†]
Day 3	Saline	0.1±0.04	0	0
	OVA/vehicle	0.5±0.04	0	0
	OVA/budesonide	0.5±0.03	0	0
Day 8	Saline	0.2±0.04	0	0
	OVA/vehicle	1.8±0.06 ^a	0.5±0.04	0
	OVA/budesonide	0.6±0.05 ^b	0	0
Day 10	OVA/vehicle	1.6±0.07	0.3±0.02	2 (300)
	OVA/budesonide	0.4±0.03 ^b	0	1 (350)
	OVA/anti-Fas mab	4.8±1.2 ^b	3.5±0.3 ^b	65 (300) ^b
Day 13	OVA/vehicle	0.3±0.04	0	0
	OVA/budesonide	0.1±0.05	0	0

* Number of cells/0.1mm² peribronchially.[†] The total number of tissue eosinophils examined by TEM is given within parentheses.^a $P<0.05$ compared with saline.^b $P<0.01$ compared with OVA/Vehicle.

Data expressed as mean value±SEM.

TUNEL, terminal deoxy RNase nick end labelling; TEM, transmission electron microscopy; OVA, ovalbumin; SEM, scanning electron microscopy.