

Corticosteroid-induced apoptosis in mouse airway epithelium: Effect in normal airways and after allergen-induced airway inflammation

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Background: Damage to the airway epithelium is one prominent feature of the damage seen in chronic asthma. Corticosteroids induce apoptosis in inflammatory cells, which in part explains their ability to suppress airway inflammation. However, corticosteroid therapy does not necessarily reverse the epithelial damage seen in asthmatic airways.

Objective: We hypothesized that corticosteroids might induce airway epithelial cell apoptosis as one potential explanation for this persistent damage.

Methods: BALB/c mice were treated with 1 mg/kg dexamethasone by means of intraperitoneal injection for 3 days to 4 weeks. Additional mice were sensitized and challenged with ovalbumin and then followed for 14 days with or without dexamethasone treatment starting on day 4 after challenge. Apoptosis was measured by using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling and immunohistochemistry for the p85 cleavage product of poly (adenosine diphosphate-ribose) polymerase. Shed epithelial cells were counted in bronchoalveolar lavage fluid.

Results: In a time-dependent manner dexamethasone treatment increased epithelial cell apoptosis and shedding into the airway lumen. This was not associated with any change in the abundance of the apoptotic regulator Bcl-x_L. In animals sensitized and challenged with ovalbumin, treatment with 1 mg/kg dexamethasone starting 4 days after challenge reversed the inflammatory changes but did not reverse either epithelial cell shedding or apoptosis seen after allergen challenge.

Conclusion: Corticosteroids might induce apoptotic cell death of airway epithelium in vivo and fail to mitigate epithelial cell shedding and apoptosis elicited by means of allergen challenge. This raises the possibility that at least one of the major components of chronic airway damage in asthma, epithelial shedding, might in part result from a major therapy used for disease control. (*J Allergy Clin Immunol* 2003;111:360-6.)

Key words: Airway epithelium, apoptosis, corticosteroids

Injury to the airway epithelium is a common finding in pathologic studies of patients with asthma, even when the clinical state is mild.^{1,2} Epithelial damage, as demonstrated on endobronchial biopsy, is seen in about half of subjects with mild asthma and in almost all subjects with persistent asthma.³ Although environmental factors,^{4,5} mediators from inflammatory cells (eg, eosinophils),^{6,7} and signals from other constitutive cells within the airway⁸ have been implicated in the genesis of epithelial cell loss, the precise mechanisms by which this occurs are unclear.

Corticosteroids elicit apoptosis in inflammatory cells, such as eosinophils^{9,10} and T lymphocytes.¹¹ This in part explains the potent anti-inflammatory effects of corticosteroids in asthma.¹² However, the effect of corticosteroids on airway epithelial injury and repair is less clear. Corticosteroids protect mammary gland epithelial cells from apoptotic stimuli^{13,14} and inhibit apoptosis induced by Fas ligation in alveolar epithelial cells.¹⁵ We have recently examined corticosteroid-induced apoptosis in cultured airway epithelium that was associated with mitochondrial depolarization and activation of caspase 9.¹⁶ These data suggest that in the in vitro setting corticosteroid effects on airway epithelium might differ substantially from those seen with other epithelial cells. These differing effects of corticosteroids make uncertain the prediction of the effects of these agents on central airway epithelium in the in vivo environment.

On the basis of our previous data, we hypothesized that corticosteroids could cause cell death of airway epithelium in vivo, and the resulting loss of epithelial cells might explain in part the damage to and denudation of the airway mucosa in chronic asthma, despite control of other markers of inflammation. To test this hypothesis, we examined whether corticosteroids could elicit apoptosis in mice under both homeostatic conditions and after allergen sensitization and challenge.

METHODS

Mice

BALB/c mice (Jackson Laboratory, Bar Harbor, Me) were used at 6 to 10 weeks of age. Mice were housed in sterile conditions and received a standard diet. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of the University of Chicago.

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Abbreviations used

BAL:	Bronchoalveolar lavage
OVA:	Ovalbumin
PARP:	Poly (adenosine diphosphate-ribose) polymerase
PAS:	Periodic acid-Schiff
TUNEL:	Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling

Corticosteroid treatment

At indicated time points, mice were treated with 1.0 mg/kg dexamethasone dissolved in 0.1 mL of ethyl alcohol by means of intraperitoneal injection. Control mice were injected with ethyl alcohol alone. Mice treated with dexamethasone for 2 to 4 weeks received an intraperitoneal injection 5 days per week.

Allergen sensitization and challenge

We used the method of Padrid et al.¹⁷ Animals were immunized on days 0 and 7 with 10 mg of ovalbumin (OVA; Grade V, Sigma Chemical Co) in 1 mg of precipitated alum by means of intraperitoneal injection. Control mice received alum only. Starting on day 14, mice were challenged in a ventilated chamber connected to a DeVilbiss model 920 nebulizer (particle size range, 1–4 μ m) containing 6 mL of 6% OVA in PBS for 3 days. Control mice received nebulized PBS alone. Mice were followed for 1 to 14 days after challenge and received dexamethasone or vehicle control on the indicated days.

Processing of mouse tissues and cells

Bronchoalveolar lavage (BAL) was performed through a tracheal catheter with 2 successive washes of 0.9 mL of PBS at 37°C. Cells were counted with a hemacytometer. Cytospin preparations were stained with Diff-Quik (Fisher, Inc). At least 500 cells per slide were counted. Lungs were fixed in 4% paraformaldehyde or 10% neutral buffered formalin for 1 hour. Lungs were not inflated before fixation to avoid potential artifacts, with epithelial shedding and damage caused by such inflation. Lungs from BAL experiments were never used in these histologic preparations.

Staining of tissue

Tissues sections on glass slides were stained by using either hematoxylin and eosin or periodic acid-Schiff (PAS) stain to examine morphology. Additional sections were preblocked in 1% BSA in PBS for 20 minutes and then incubated with primary antibody in PBS for 1 hour. A goat anti-mouse ZO-1 antibody (Santa Cruz Technologies) was used at 5 μ g/mL, a mouse anti-human Bcl-x_L antibody (Dako, Inc) was used at 20 μ g/mL, and a rabbit anti-human polyclonal antibody specific for the p85 cleavage product of poly (adenosine diphosphate-ribose) polymerase (PARP; Promega) was used at a 1:100 final concentration. Secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit IgG, goat anti-mouse IgG, and rabbit anti-goat IgG (Molecular Probes), as appropriate, and these antibodies were used for 1 hour in PBS. Slides were developed with 1:300 streptavidin-horseradish peroxidase (Dako) for 20 minutes, followed by diaminobenzidine augmented with nickel plus a 1:1000 dilution of 30% H₂O₂ for 5 minutes. Slides then were counterstained with hematoxylin. Specificity of staining was confirmed by substituting an irrelevant antibody of the appropriate type or by omitting the primary antibody.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling staining of tissue

This method permits evaluation of apoptosis in fixed, embedded tissue sections.¹⁸ Apoptosis in fixed lung sections was assessed by

labeling free 3'-hydroxyl groups of DNA by using a Trevigen terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) peroxidase assay kit according to kit directions and counterstaining with hematoxylin. Positive cells were referenced to mouse thymus stained and imaged at the same time, and specificity was confirmed in separate slides by omitting the terminal deoxynucleotidyl transferase from the reaction. Airway sections were photographed with a digital camera attached to a light microscope. Images were collated by using Photoshop 6.0 software (Adobe, Inc) and analyzed by using NIH Image software (Wayne Rasband, National Institutes of Health) to measure the circumference of the airway at the basement membrane. Measurements were made to ± 0.02 μ m at 200 $\times$ total magnification. A small intraparenchymal bronchus of 100 to 200 μ m in diameter and a large intraparenchymal bronchus of 250 to 500 μ m in diameter were scored. TUNEL-positive cells were counted within the epithelial layer of these airways and were normalized to the length of the basement membrane.

Data analysis

Data are expressed as the mean $\pm$ SEM. Because a normal distribution could not be ensured, data were compared by using the Kruskal-Wallis test, followed by the Mann-Whitney test when appropriate. Differences were considered significant at a *P* value of less than .05.

RESULTS

Airway epithelial shedding in normal mice after treatment with corticosteroids

BAL was done on 27 mice. Increased numbers of airway epithelial cells were found in mice treated with 1.0 mg/kg dexamethasone for 3 or 6 days compared with those in control mice (Table I). Increases were seen both in absolute numbers and in the relative proportion of epithelial cells in BAL fluid. These differences were sustained even after treatment was stopped: in mice in which dexamethasone treatment had been stopped for 4 days, the percentage of epithelial cells found in the BAL fluid was similar to that seen in fluid collected 1 day after dexamethasone treatment ended (Table I). BAL could not be performed successfully in mice treated beyond 6 days with dexamethasone because of loss of tissue integrity of the trachea and lungs during lavage. The numbers of neutrophils, lymphocytes, and eosinophils together in BAL fluid were less than 6% total in both treated and control mice.

Apoptosis in airway epithelium after treatment with corticosteroids

A total of 15 mice were used in histologic analysis of apoptosis after dexamethasone treatment. Small airways (433 ± 32 μ m in circumference) were lined with cuboidal cells and lacked smooth muscle. Large airways (1038 ± 146 μ m in circumference) were lined with cuboidal or columnar epithelium and had smooth muscle in the submucosa. In mice treated with 1.0 mg/kg dexamethasone for 5 days per week up to 4 weeks, there was significant apoptosis, as measured by means of TUNEL labeling of epithelial cells in both large and small airways (Table II). TUNEL-positive cells were seen in random distribution and were not found in greater abundance near areas of

TABLE I. Epithelial cells found in BAL fluid in mice treated with 1.0 mg/kg dexamethasone

Condition	No. of mice	Recovered airway epithelial cells	
		% Total cells	Cells/ μ L BAL fluid
Control mice	6	0.6 $\pm$ 0.2	1 $\pm$ 1
Mice treated for 3 d			
Collected on day 4	3	10.1 $\pm$ 0.5*	10 $\pm$ 3*
Collected on day 7	3	14.8 $\pm$ 3.7*	16 $\pm$ 9*
Mice treated for 6 d			
Collected on day 7	4	21.7 $\pm$ 4.4†	17 $\pm$ 3†
Collected on day 11	4	27.9 $\pm$ 7.2†	21 $\pm$ 3†

* $P < .02$ and † $P < .01$ versus the corresponding control value.

TABLE II. Number of TUNEL-positive airway epithelial cells in mouse airways treated with 1.0 mg/kg dexamethasone daily for 3 or 6 days or 5 days per week for 2 to 4 weeks

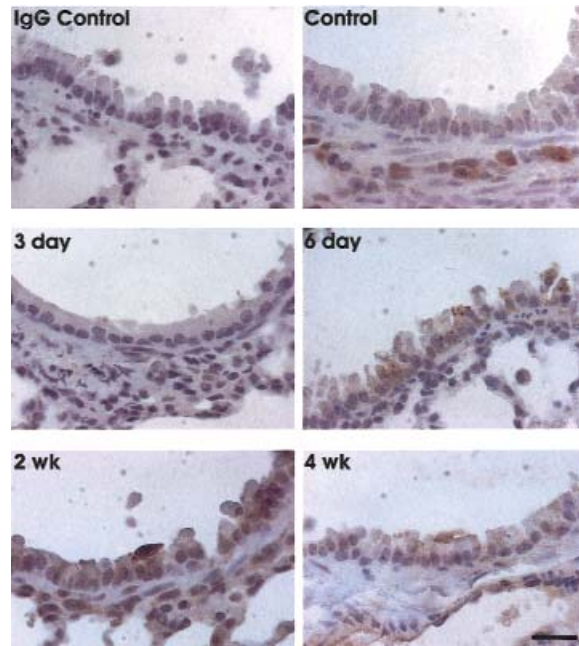
Condition	No. of mice	TUNEL-positive cells per mm of basement membrane	
		Small airway	Large airway
Control	6	4.3 $\pm$ 2.1	6.5 $\pm$ 1.9
3 d	3	6.1 $\pm$ 4.3	7.6 $\pm$ 2.2
6 d	3	6.0 $\pm$ 1.8	4.4 $\pm$ 2.7
2 wk	3	17.4 $\pm$ 9.0	39.5 $\pm$ 11.7*
3 wk	3	14.8 $\pm$ 3.6	16.2 $\pm$ 3.6†
4 wk	3	10.0 $\pm$ 1.9	18.5 $\pm$ 5.0†

* $P < .02$ and † $P < .05$ versus the corresponding control value.

damage. To confirm that TUNEL labeling was not caused by nonspecific labeling of RNA,¹⁹ we examined p85-PARP labeling in airways. The abundance of p85-PARP was minimal in airways of control animals and animals treated with dexamethasone for 3 days. However, longer treatment with dexamethasone elicited a significant increase in p85-PARP abundance (Fig 1).

One potential explanation for increased apoptosis is decreased expression of antiapoptotic regulators, such as Bcl-x_L. To examine this, we localized Bcl-x_L protein abundance in small and large mouse airways after dexamethasone treatment. No significant change in Bcl-x_L abundance was seen, regardless of the length of dexamethasone treatment (data not shown).

Airway epithelial cells bind together at their apical surface through tight junctions.^{20,21} Corticosteroid therapy does not alter tight-junction structure in cultured mammary epithelial cells²² and actually increases tight-junction formation in rat intestinal epithelial cells.²³ However, it is possible that the effect in vivo on airway epithelium might be different. To test this, we examined the abundance of the tight-junction protein ZO-1 in control and dexamethasone-treated mice. This protein was abundant in airway epithelial cells at all levels of the bronchi in both control and dexamethasone-treated mice (data not shown).

**FIG 1.** Abundance of p85-PARP in airways of mice treated with dexamethasone. Mice were treated daily for 3 or 6 days or 5 days a week for 2 to 4 weeks. The negative control, from a mouse treated for 2 weeks, omitted the primary antibody (bar = 20 μ m).

Airway epithelial shedding in allergen-challenged mice without treatment with corticosteroids

Challenge with PBS alone of mice previously sensitized to OVA did not elicit significant shedding of epithelial cells into BAL fluid (Table III). In contrast, significant increases, as both absolute numbers and as a proportion of total cells, were seen 7 days after challenge and were still present at 14 days (Table III and Fig 2). Coordinated with this was a substantial influx of eosinophils that occurred within 2 days after OVA challenge, which was still present at 14 days (Fig 3).

Histology of selected mouse airways after allergen challenge

In 24 mice the circumference of the small airways examined was 498 $\pm$ 48 μ m, and the circumference of the large airways was 1020 $\pm$ 57 μ m. In mice 2 or more days after OVA challenge, there were many PAS-staining secretory epithelial cells present (data not shown). The abundance of ZO-1 in OVA-challenged mice was not different from that seen in sham-challenged mice (data not shown). There was significant apoptosis, as measured by means of TUNEL labeling, of epithelial cells in large airways (Table IV). This was confirmed by increased abundance of p85-PARP in epithelial cells in large airways (Fig 3). Neither TUNEL labeling nor p85-PARP abundance in small airways was significantly dif-

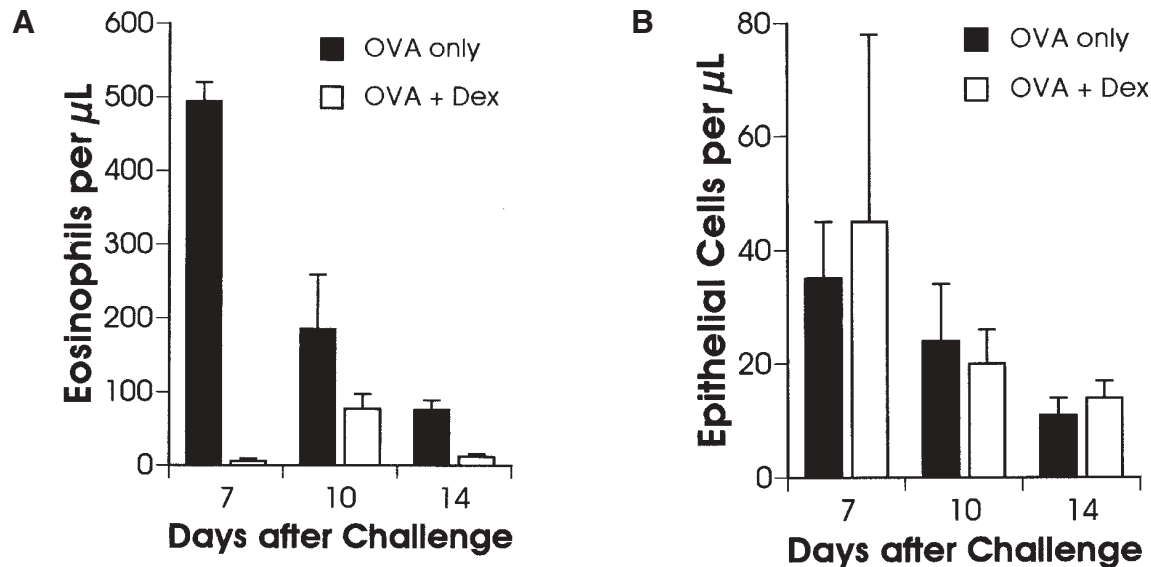


FIG 2. Presence of eosinophils (**A**) and epithelial cells (**B**) in BAL fluid of mice after sensitization and challenge with OVA. Some mice were treated with 1 mg/kg dexamethasone daily starting on day 4 after OVA challenge. *Dex*, Dexamethasone.

ferent from that seen in control, nonchallenged mouse small airways. TUNEL-positive cells in large airways were seen in random distribution around the airway perimeter and were not found in greater abundance near areas of damage or near junctions of airways. Bcl-x_L abundance was unchanged after treatment with OVA (data not shown).

Effect of dexamethasone on airway epithelial cell shedding and apoptosis in allergen-challenged mice

We tested the hypothesis that high-dose corticosteroids given after the establishment of allergen-induced airway inflammation in mice would suppress airway inflammation but would not resolve epithelial cell shedding and apoptosis. A high concentration of dexamethasone (1 mg/kg) was chosen so as to provide maximum suppression of inflammation. Dexamethasone was given daily starting on day 4 after OVA challenge. There were fewer eosinophils in BAL fluid compared with in OVA-challenged mice not treated with dexamethasone (Fig 2). Despite dexamethasone therapy, however, the proportion of epithelial cells in BAL fluid of these mice was similar to that seen in non-corticosteroid-treated mice at the same time points after OVA challenge (Table III). Treatment with dexamethasone decreased the number of PAS-positive cells found in large airways. However, no significant change in either epithelial cell TUNEL labeling or p85-PARP abundance was seen compared with that seen in untreated, OVA-challenged mice (Table IV and Fig 3). As with mice treated with dexamethasone alone, Bcl-x_L abundance was unchanged after treatment with both OVA and dexamethasone (data not shown).

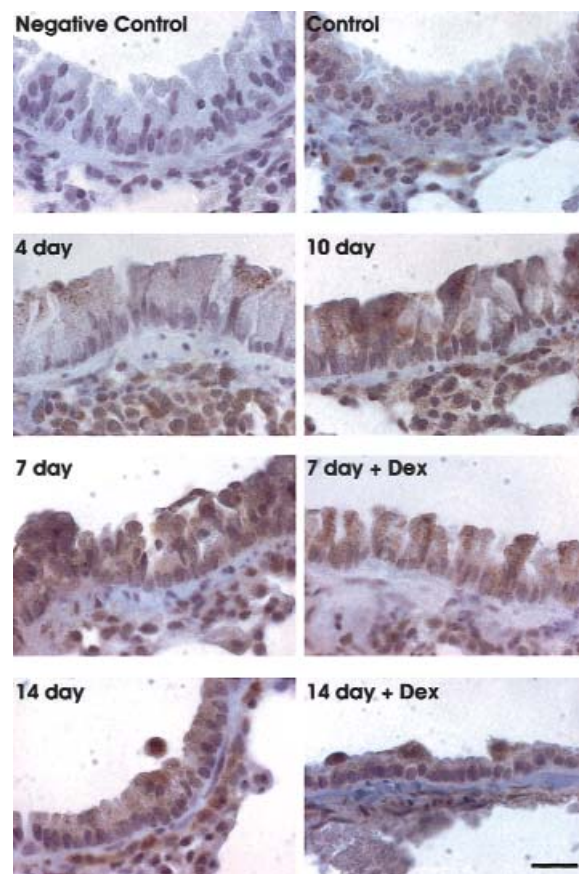


FIG 3. Abundance of p85-PARP in large intraparenchymal airways 7 to 14 days after sensitization and challenge with OVA. The control mouse was sham sensitized, challenged with OVA, and examined 7 days later. The negative control, from a mouse 7 days after OVA challenge, omitted the primary antibody (*bar* = 20 μm). *Dex*, Dexamethasone.

TABLE III. Epithelial cells found in BAL fluid in days after OVA sensitization and challenge

Condition	No. of mice	Recovered airway epithelial cells	
		% Total cells	Cells/ μ L BAL fluid
Control mice	6	2.4 $\pm$ 1.7	1 $\pm$ 0
Mice not treated with dexamethasone			
Day 2	4	0.5 $\pm$ 0.5	1 $\pm$ 1
Day 4	5	0.0 $\pm$ 0.0	0 $\pm$ 0
Day 7	4	4.4 $\pm$ 1.3	35 $\pm$ 10*
Day 10	7	6.5 $\pm$ 1.5 [†]	24 $\pm$ 10*
Day 14	7	5.5 $\pm$ 1.0*	11 $\pm$ 3*
Mice treated with 1 mg/kg dexamethasone starting on day 4 after OVA challenge			
Day 7	4	5.0 $\pm$ 1.6	45 $\pm$ 33*
Day 10	7	10.4 $\pm$ 3.3 [†]	20 $\pm$ 6*
Day 14	7	8.0 $\pm$ 1.0*	14 $\pm$ 3*

* $P < .01$ and [†] $P < .05$ versus the corresponding control value.**TABLE IV.** Number of TUNEL-positive airway epithelial cells in airways after OVA sensitization and challenge

Condition	No. of mice	TUNEL-positive cells per mm of basement membrane	
		Small airway	Large airway
Control	8	4.2 $\pm$ 1.4	3.3 $\pm$ 0.8
Mice not treated with dexamethasone			
Day 2	5	4.5 $\pm$ 2.1	25.3 $\pm$ 10.2
Day 4	5	5.4 $\pm$ 1.6	15.0 $\pm$ 7.0
Day 7	5	6.9 $\pm$ 2.7	28.0 $\pm$ 4.3*
Day 10	5	14.9 $\pm$ 7.0	27.6 $\pm$ 12.1 [†]
Day 14	4	3.2 $\pm$ 1.6	12.5 $\pm$ 5.4
Mice treated with 1 mg/kg dexamethasone starting on day 4 after OVA challenge			
Day 7	7	9.3 $\pm$ 3.1	19.3 $\pm$ 4.8 [‡]
Day 10	3	10.0 $\pm$ 4.1	20.1 $\pm$ 8.5 [‡]
Day 14	6	8.5 $\pm$ 2.5	15.9 $\pm$ 3.3*

* $P < .01$, [†] $P < .05$, and [‡] $P < .03$ versus the corresponding control value.

DISCUSSION

Corticosteroids are the mainstay of controller therapy for asthma. Corticosteroids elicit apoptosis of eosinophils^{9,10} and lymphocytes¹¹ and suppress production and release of both inflammatory eicosanoids and cytokines.^{24,25} However, adequate treatment with corticosteroids might not completely prevent airway inflammation, and some patients might continue to have asthma symptoms and a long-term decrease in lung function, despite their use. Although corticosteroids are recognized to elicit deleterious effects in other organ systems, no report to date has suggested that their use in airway disease might be associated with in vivo damage to constitutive cells of the airway. In this article we present the first such report by demonstrating that dexamethasone elicits apoptosis in mouse airway epithelial cells. This is not accompanied by changes in structural markers, such as loss of tight-junction proteins. Furthermore, although dexamethasone suppresses allergen-induced airway inflammation, as noted by the presence of eosinophils and lymphocytes in the airways, allergen-induced epithelial cell apoptosis and shedding are not corrected with corticosteroid treatment.

Epithelial damage plus changes, including smooth muscle hypertrophy, basement membrane thickening, and submucosal fibrosis, are cardinal features of long-standing, persistent asthma. Incomplete restitution might lead to continued production of inflammatory cytokines and excessive allergen passage. Subjects with severe asthma receiving regular inhaled corticosteroid therapy might still have substantial epithelial destruction at several levels in histologic studies,²⁶ and epithelial cells are seen in the BAL fluid of patients with asthma of varying severity, regardless of corticosteroid use.²⁷ Other reports suggest that glucocorticoid therapy can lead to epithelial healing,²⁸ as evidenced by changes in epithelial cell height after treatment with inhaled corticosteroids.²⁹ One potential explanation for the apparent contradiction in data is that corticosteroids might modulate selected reparative processes in addition to suppressing the release of inflammatory mediators. These events then might lead to healing of a damaged epithelium. A second potential explanation is that surviving epithelial cells in the setting of inflammation are resistant to deleterious effects of both corticosteroids and different inflammatory mediators. A third potential explanation is that apoptosis is occurring in cells that need to be cleared for

repair to occur: in this light corticosteroid-induced apoptosis might be of benefit and not harmful to the airway.

Recent studies suggest that corticosteroid treatment might induce expression of apoptosis regulators in human airway epithelium. Bcl-2 expression is decreased in untreated asthmatic subjects but is increased in those treated with inhaled corticosteroids.³⁰ It is not clear whether this increase represents a compensatory rescue mechanism by which epithelial cells survive exposure to corticosteroids or whether the epithelial cells that remain represent a different population of more resistant cells. In the present study abundance of the related Bcl family regulator Bcl-x_L was not appreciably changed in epithelial cells after dexamethasone treatment.

Glucocorticoids are known to induce apoptosis of lymphocytes and eosinophils⁹⁻¹¹ but have differing effects on epithelial cells. Mammary luminal cells treated with corticosteroids are resistant to apoptotic stimuli,^{13,14} and removal of the steroid promotes cell death by apoptosis. Dexamethasone treatment of corneal epithelial cells induces both apoptosis and proliferation,³¹ whereas corticosteroid treatment of young rats leads both to suppression of gastric epithelial proliferation and increased gastric epithelial cell apoptosis.³² Our present data suggest that the response to glucocorticoids in other epithelial cell types cannot be generalized as a model for central airway epithelium.

A potential concern is the use of the TUNEL assay to denote apoptosis in mouse airways. Electron microscopic observation of TUNEL-positive cells in fixed tissue has demonstrated a high correlation between labeling and ultrastructural markers of cell death.³³ We confirm the findings of the TUNEL assay in our study by demonstrating the presence of the p85 cleavage product of PARP. Nevertheless, TUNEL labeling *in vivo* should be interpreted with caution.

Considerable caution should be used in extrapolating these data on the basis of a mouse model of airway inflammation to the situation of human asthma. Data generated in the mouse might or might not mimic events in human asthma, and additional studies to address the potential for apoptosis caused by corticosteroids versus potential benefits of these agents on epithelial repair and differentiation will be necessary. Especially in clinical situations of persistent asthma, glucocorticoids remain an agent of choice for controller therapy.

In summary, we demonstrate that systemic treatment with high doses of dexamethasone elicits apoptosis in mouse airway epithelial cells in normal and inflamed airways. Apoptosis was elicited in a concentration range similar to that attainable *in vivo*, was not associated with changes in the abundance of tight-junction proteins, and was not associated with changes in the antiapoptotic regulator Bcl-x_L. Epithelial cell apoptosis also occurs in allergen-induced airway inflammation and is not corrected by means of subsequent treatment with corticosteroids in concentrations that suppress inflammation. Our data suggest the possibility that systemic application of high doses of corticosteroids might contribute to the airway

remodeling and epithelial damage seen in some patients with chronic, persistent asthma.

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