Impact of intranasal budesonide on immune inflammatory responses and epithelial remodeling in chronic upper airway inflammation

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Background: Histologic and immunohistologic features of nasal polyps (NP) are similar to those observed in asthma, thus suggesting a similar immunopathology.
Objective: The primary objective of this study was to further understand the anti-inflammatory and immunoregulatory effects of locally delivered corticosteroids. To this end, the effect of intranasal budesonide on the expression of specific cytokines, lymphocyte subsets, and epithelial remodeling in this model of airway tissue inflammation were studied.
Methods: We used immunohistochemical techniques to examine nasal mucosae (NM) from healthy individuals and nasal polyp (NP) tissues from patients with nasal polyposis obtained before and after intranasal budesonide treatment.
Results: First, the density of $CD8^+$ cells was markedly increased in NP tissues after intranasal budesonide treatment from 16.1 ± 8.4 (M ± SEM) per mm^2 to 39.9 ± 24.1. Second, the density of cells immunoreactive for IL-4, IL-5, IFN-γ, IL-12, and TGF-β in NP was significantly greater than in control NM tissues. The density of IL-4^+ and IL-5^+ cells in NP tissues significantly decreased after budesonide treatment from 40 ± 12 to 17.8 ± 8 and from 19.3 ± 11 to 10.4 ± 7, respectively. In contrast, the density of IFN-γ^+ and IL-12^+ cells remained unchanged. In addition, we found that the density of TGF-β^+ cells significantly increased after intranasal budesonide from 18 ± 5 to 41 ± 9. Third, damage to the entire length of the NP epithelium was quantified using a grading system. The epithelium of untreated NP was substantially damaged; remarkable epithelial restitution with no apparent changes in stromal collagen deposition was observed after intranasal budesonide treatment.
Conclusions: These findings demonstrate that intranasal budesonide induced an increase in CD8 population and a selective regulatory effect on tissue cytokine expression. Furthermore, intranasal budesonide promoted epithelial remodeling. We hypothesize that these immunoregulatory and remodeling effects elicited by steroids might be, at least in part, mediated by the induction of TGF-β. (J Allergy Clin Immunol 2003;112:37-44.)

Key words: *Nasal polyps, budesonide, TGF-β, CD4 T cells, CD8 T cells, IL-4, IL-5, epithelial remodeling*

Nasal polyps (NPs) commonly arise from the paranasal sinuses and are characterized by infiltration of the mucosa with eosinophils, lymphocytes and mast cells; epithelial damage; and expression of the T_H2–associated cytokines IL-4 and IL-5, a strikingly similar immunopathologic profile to that documented in asthma; moreover, the 2 conditions often coexist. Thus, from this perspective, NPs might be viewed as a paradigm of chronic and severe airway inflammation.[1,2]

$CD4^+$ T cells (T_H1 and T_H2) play a central role in the initiation of immune inflammatory responses; in particular, T_H2 cytokines induce a selective recruitment of eosinophils, the most salient inflammatory feature of inflammation in NPs. On the other hand, it is thought that regulatory suppressor $CD8^+$ T cells modulate CD4-driven inflammatory responses by providing negative feedback signals. Although the mechanisms involved in the generation of $CD8^+$ suppressor cells are poorly understood, TGF-β has been implicated as a key cytokine in the development of T cells with downregulatory activities.[3,4] Another important feature of mucosal inflammation, both in asthma and NPs, is epithelial cell damage followed by a re-epithelialization process. Under chronic inflammatory conditions, this process might become inadequate, resulting in abnormal tissue repair and remodeling.

In this context, the clinical efficacy of glucocorticoids in the treatment of inflammatory conditions such as asthma, allergic rhinitis, and nasal polyposis is well established. Particularly, topical intranasal budesonide has been shown to be an effective treatment of NPs in a number of placebo-controlled studies.[5,6] However, the mechanisms underlying the anti-inflammatory and, especially, immunoregulatory effects of these drugs in vivo remain to be fully explained. Moreover, it is not clear whether corticosteroid treatment might affect airway tissue repair. The objective of this study was to concurrently address these 2 issues. We used immunohistochemical tech-

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Abbreviations used
NM: Nasal mucosa
NP: Nasal polyp

niques to investigate the effects of a local steroid treatment on eosinophil and T cell infiltration, expression of prototypic T_H1 (IFN-γ and IL-12), T_H2 (IL-4 and IL-5), and regulatory (TGF-β) cytokines, and epithelial remodeling on NP tissues. We examined nasal mucosa (NM) tissues from healthy individuals and NP tissues obtained before and after 8 weeks of intranasal budesonide treatment. In essence, our study provides evidence that local treatment with budesonide redistributes the ratio of CD4/CD8 T cells, rebalances the T_H1/T_H2 cytokine expression, promotes an increase in the number of cells expressing TGF-β, and, last, mediates a rather remarkable epithelial reconstitution.

METHODS

Subjects and study design

NM tissues were obtained from 10 healthy volunteers with no nasal disease. NPs were obtained from 10 subjects with nasal polyposis. This study was not carried out to evaluate the clinical efficacy of topical budesonide in airway disease, which has been abundantly demonstrated by a number of studies in asthma, allergic rhinitis, and nasal polyposis. Rather, the objective was to primarily investigate the immunoregulatory impact of this drug on chronically inflamed airway mucosa using objective measurements in tissue samples. Hence, we elected to use a constituent-free and preservative-free budesonide powder and to use an open-label design without a placebo treatment group (considering it would not be ethical to include a placebo treatment group). The evaluation of immunoreactivity for positive cells in NM and NP tissues before and after steroid treatment was done blindly by 2 independent observers. Patients with a history of cystic fibrosis, infectious sinusitis, and patients with allergic seasonal symptoms were excluded. All patients had taken no steroids or antibiotics for at least 4 weeks before entering the study. At the initial visit, a full clinical evaluation and skin prick tests were performed. Then, a first biopsy of the polyp was obtained. All intranasal medications were stopped, and none were allowed throughout the study. A week later, patients received budesonide (Rhinocort) through a device for powder insufflation. They were instructed to take 1 insufflation (100 μg) in each nostril twice daily. After 8 weeks, subjects were re-evaluated, and a polypectomy was performed. Clinical features of all subjects included in the study are presented in Table I. The study was approved by the local ethics committee, and all subjects provided written informed consent.

Biopsies and immunohistochemistry

Biopsy samples (2-4 mm) from the healthy subjects were obtained with a 5-mm biopsy forceps from the middle turbinate. In patients with nasal polyps we first performed a wire loop biopsy on the polyp tissues. After 8 weeks, a polypectomy was performed to remove the remaining polyp.

Tissues for immunohistochemistry were processed as previously described.[2] Staining was performed with the following antibodies: anti EG2 (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden), anti-CD4, anti-CD8 (Dako S.p.A., Milan, Italy), anti-CD3, anti-IL-4, anti-IL-5 (BD PharMingen Intern, San Diego, Calif), anti-IFN-γ anti-IL-12 (Bender MedSystems Diagnostics, Wien, Austria), anti-TGF-β1, β2, β3 (R&D Systems Europe Ltd, Abingdon, UK). Eosinophil counts were performed on sections stained with Congo Red. In addition, sections from each specimen were stained with conventional Masson's trichrome, which stains collagen blue and cells red. Quantification of immunoreactive cells was performed as previously described.[2] The mean number of stained cells was calculated from a total of at least 20 fields. The number of positive cells were counted, and the results were expressed as the number of cells per square millimeter of stroma. All slides were coded before evaluation, and positive cells were counted blindly by 2 independent observers.

Quantification of epithelial damage

We scored epithelial damage into four grades following a previously described classification.[7] Grade 0 indicates that all layers of epithelium are intact; grade 1 indicates that 2 or more layers of cells are present; grade 2 indicates that only 1 layer of cells remains; grade 3 indicates that occasional or no epithelial cells (naked basement membrane) are present. In addition, the presence or absence of ciliated epithelial cells was carefully noted in areas graded 0 and 1. Pictures were taken of the entire epithelium with a magnification of ×100. Then, the length of the epithelium was measured and the damage assessed. Thus, data shown indicate the percentage of epithelium with a given degree of damage.

Statistical analysis

Results are shown as mean ± SEM. The coefficient of variance for 3 repeat counts of a single section was less than 5%. A P value less than .05 was regarded as statistically significant in all comparisons. For statistical analysis of cytokine expression and T cell subset infiltration on NM and NP tissues, Student t test for unpaired data was applied. Comparisons of NP tissues before budesonide and after budesonide were performed with Student t test for paired data. Linear regression analysis was performed to assess the relationship between the budesonide-induced percent increase of TGF-β and the percent changes of cytokine expression and T cell subset infiltration.

RESULTS

Tissue density of total cells and eosinophils

The overall cellular density of control NM tissues was 1224 ± 265.3 cells/mm^2 (M ± SEM, n = 10). The cellular density of NP tissues obtained before steroid treatment was significantly smaller, 482.3 ± 137.6 ($P < .01$), likely as a consequence of the edematous component of the chronic inflammation existing in the NP tissues. The cellular density of NP tissues obtained after steroid treatment did not change significantly (423.1 ± 171).

To verify the anti-inflammatory effect of the treatment, the tissue density of eosinophils and EG2$^+$ cells was examined. The density of eosinophils and EG2$^+$ cells in NM tissues was 3.1 ± 1.6 and 1.9 ± 1.3 cells/mm^2, respectively, and it was significantly increased in NP tissues before steroid treatment (94 ± 17.1 and 68.5 ± 8.4, respectively; $P < .01$). After intranasal budesonide, the density of eosinophils and EG2$^+$ cells was considerably lower, 26 ± 7.7 and 21 ± 6.8, respectively ($P < .01$). In terms of percentages, eosinophils represented 0.3% ± 0.2%, 20.3% ± 3.9%, and 6.6% ± 1.6% of all cells in NM tissues and in NP tissues before and after steroid treatment, respectively. EG2$^+$ eosinophils represented 0.2% ±

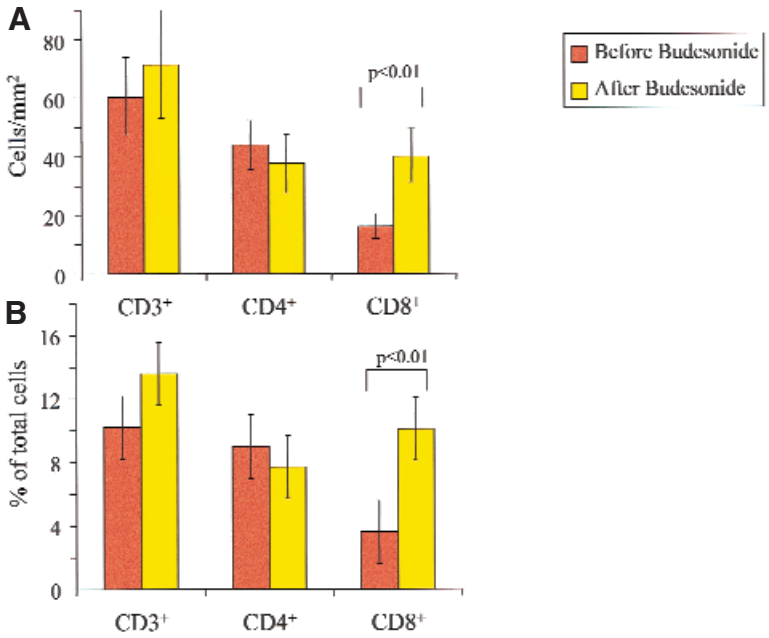

FIG 1. CD3+, CD4+, and CD8+ cell density **(A)** and cell percentage **(B)** in nasal polyp tissues obtained before *(red bars)* and after *(yellow bars)* intranasal budesonide treatment. Data represent mean ± SEM (n = 10).

TABLE I. Characteristics of patients

Subject	Age (y)	Sex	No. of positive skin tests*	Serum IgE† (U/mL)	Prior polypectomies	Other diagnosis
NP1	53	M	2	40	2	AR
NP2	35	M	0	60	1	None
NP3	42	M	0	64	1	None
NP4	61	F	13	855	0	AS + AR
NP5	62	F	0	52	4	None
NP6	56	M	5	48	5	None
NP7	52	M	4	116	2	None
NP8	22	F	4	202	0	AS
NP9	31	F	0	40	1	AS
NP10	20	M	3	100	0	AR
C1	34	M	0	ND	0	None
C2	25	F	0	ND	0	None
C3	24	F	0	ND	0	None
C4	56	F	0	ND	0	None
C5	30	M	0	ND	0	None
C6	42	M	0	ND	0	None
C7	20	F	0	ND	0	None
C8	27	F	0	ND	0	None
C9	39	M	0	ND	0	None
C10	48	M	0	ND	0	None

NP, Nasal polyposis patients; *C,* control subjects; *ND,* not done; *AR,* allergic rhinitis; *AS,* asthma.
*Greater than 3 mm.
†Normal range is less than 120 U/mL.

0.1%, 14.9% ± 2.9%, and 5.4% ± 1.8% of all cells in NM tissues and in NP tissues before and after steroid treatment, respectively.

Tissue density of T cell subsets

With respect to T cells, the density of CD3+ cells in NM tissues was 101 ± 39 cells/mm^2. The density of these cells in NP tissues before steroid treatment was significantly lower, 60.2 ± 27 ($P < .05$) and did not change significantly after budesonide treatment, 71.4 ± 37 (Fig 1, *A*). The smaller number of CD3+ cells/mm^2 in NP tissues compared with NM tissues is also likely the consequence of the edematous component of NP tissues and, thus, might be misleading. In fact, the percentage of CD3+ lymphocytes was 8.5% ± 3% in normal NM, which was not significantly different than that in NP tissues either before or after steroid treatment, 10.2% ± 3% and 13.6% ± 6%, respectively (Fig 1, *B*).

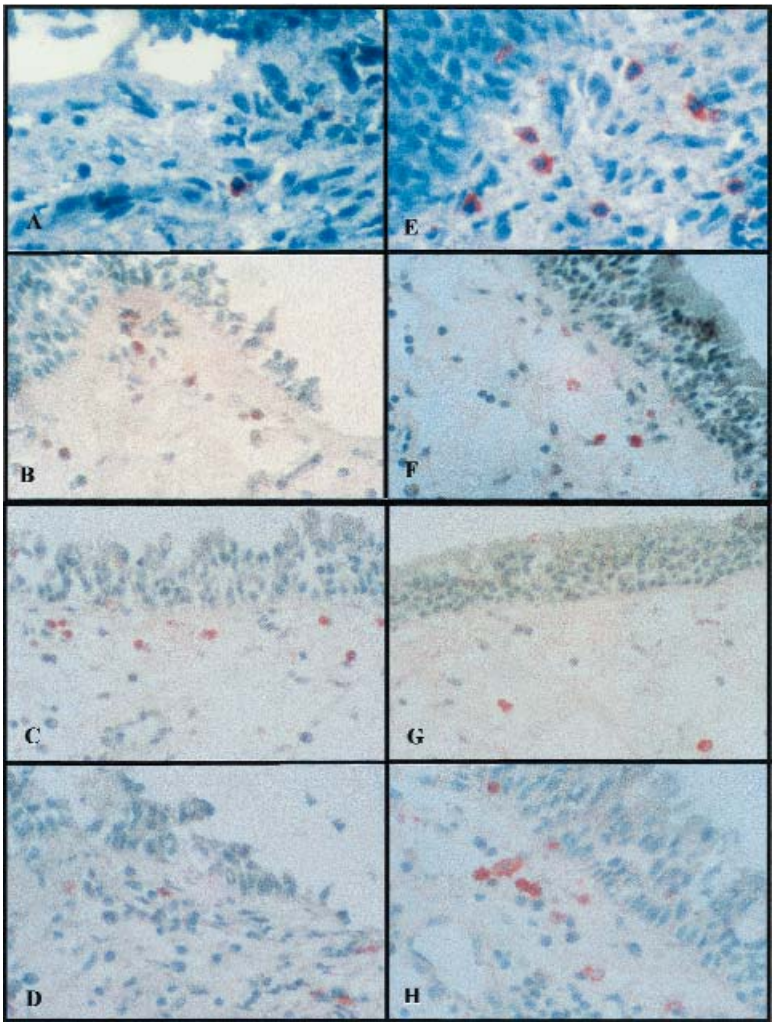

FIG 2. Immunohistochemistry of nasal polyp tissues from the same patient obtained before **(A-D)** and after **(E-H)** intranasal budesonide treatment. **A** and **E,** $CD8^+$ cells. **B** and **F,** IL-4^+ cells. **C** and **G,** IL-5^+ cells. **D** and **H,** TGF-β^+ cells. Development of red color indicates positive immunoreactivity.

The tissue density and the percentage of $CD4^+$ cells in NM tissues and in NP tissues before and after budesonide treatment were not significantly different (Fig 1).

In NP tissues, the tissue density and the percentage of $CD8^+$ cells were significantly lower than in NM and significantly increased after intranasal budesonide (Fig 1). Fig 2, *A* and *E*, shows an illustrative example of CD8 immunohistochemistry in NP tissues.

The CD4/CD8 ratio in NM tissues was 1.6 ± 0.4. In NP tissues before steroid treatment, the CD4/CD8 ratio was significantly higher, 3.5 ± 1 ($P < .05$) and significantly decreased to 1.1 ± 0.5 in NP tissues after steroid treatment ($P < .05$).

Tissue density of IFN-γ, IL-12, IL-4, IL-5, and TGF-β immunoreactive cells

The cellular density and the percentage of IL-4^+ and IL-5^+ cells were significantly increased in NP tissues compared with NM, and both significantly decreased after steroid treatment (Fig 3). Fig 2, *B, F, C,* and *G,* shows illustrative examples of IL-4 and IL-5 immunohistochemistry, respectively.

The tissue density of IL-12^+ and IFN-γ^+ cells, as well as their percentage, was lower in NM tissues than in NP tissues. No differences were noted between NP tissues before and after treatment (Fig 3).

The cell density of TGF-β^+ cells in NM tissues was 5 ± 3 cells/mm^2. In NP tissues before steroid treatment, the cell density of TGF-β^+ cells was significantly greater, 18 ± 5 ($P < .01$), and, interestingly, doubled the increase in NP tissues after steroid treatment, 41 ± 9 ($P < .01$; (Fig 3, *A*). In terms of percentages, TGF-β^+ cells represented 0.5% ± 0.3%, 3.8% ± 1.1%, and 11.9% ± 6% of all cells in NM and NP tissues before and after budesonide treatment (Fig 3, *B*). Fig 2, *D* and *H*, shows an illustrative example of TGF-β immunohistochemistry in NP tissues. Of note, there was an inverse correlation between the percentage increase of TGF-β^+ cells and the percentage

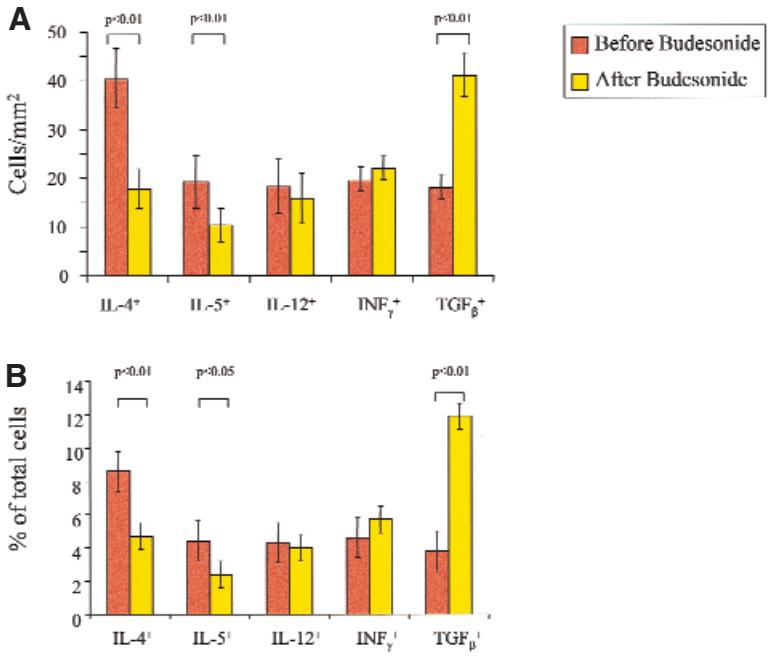

FIG 3. IL-4⁺, IL-5⁺, IL-12⁺, IFN-γ⁺, and TGF-β⁺ cell density **(A)** and cell percentage **(B)** in nasal polyp tissues obtained before *(red bars)* and after *(yellow bars)* intranasal budesonide treatment. Data represent mean ± SEM (n = 10).

decrease of IL-4⁺ cells in NP tissues obtained after budesonide treatment (Fig 4, *A*). Moreover, in the same tissues, a direct correlation was detected between the percentage increase in TGF-β⁺ cells and that of CD8⁺ cells (Fig 4, *B*).

Structural changes before and after intranasal steroid treatment

Extensive evidence of epithelial damage to the point of an almost complete exposure of the basement membrane in some areas was readily observed in untreated NP tissues. In tissues obtained after intranasal budesonide treatment, there was overt evidence of major changes in epithelial integrity, with clear evidence of multilayered epithelium and re-emergence of ciliated cells (Fig 5). All tissues were stained with Masson's trichrome to examine collagen deposition. It was apparent that the intensity of staining was greater in control NM tissues than NP tissues. In NP tissues, most of the staining was observed beneath the epithelium, with relatively little staining noted in the stromal area. Although we did not attempt to quantify the area or the intensity of the staining, all NP tissues were carefully examined. Microscopically, we could not observe appreciable differences in collagen staining or distribution between tissues obtained before and after budesonide treatment.

DISCUSSION

A number of studies have already evaluated and demonstrated the clinical efficacy and the anti-inflammatory prowess of topically delivered budesonide in asthma, allergic rhinitis, and nasal polyposis.[5,6,8,9] Less information is available with respect to the immunoregulatory properties of glucocorticosteroids, particularly in vivo, in the context of severe airway inflammation. Similarly, whether locally delivered glucocorticosteroids affect airway remodeling is currently a subject of intensive investigation. In this study, we investigated these 2 aspects concurrently in nasal polyposis, a condition that clearly shares many important immunopathologic features with both allergic rhinitis and asthma.

To ascertain the effectiveness of the 8-week treatment with budesonide, we first verified the anti-inflammatory effects of the intervention by evaluating the number of eosinophils and EG2⁺ cells before and after steroid treatment. The decreases in eosinophil density and in the number of EG2⁺ cells after budesonide were considerable, confirming the expected anti-inflammatory effects. Eosinophilia, a major feature of type 2 immune responses, is inextricably linked with elaboration of cytokines produced by T_H2 cells, such as IL-5 and IL-4. IL-5 participates in the generation, priming, activation, and increased survival of eosinophils, whereas IL-4 can enhance eosinophil migration.[10,11] Thus, we set out to examine in detail the impact of local steroid treatment on T cell and type 1 and type 2 archetypic cytokine subsets.

In NP tissues before treatment we observed an elevation in the percentage of CD3 and CD4 lymphocytes and an increase in the CD4⁺/CD8⁺ cell ratio, thus suggesting that CD4⁺ T cells might function to initiate and sustain the inflammation associated with NP. In addition, after

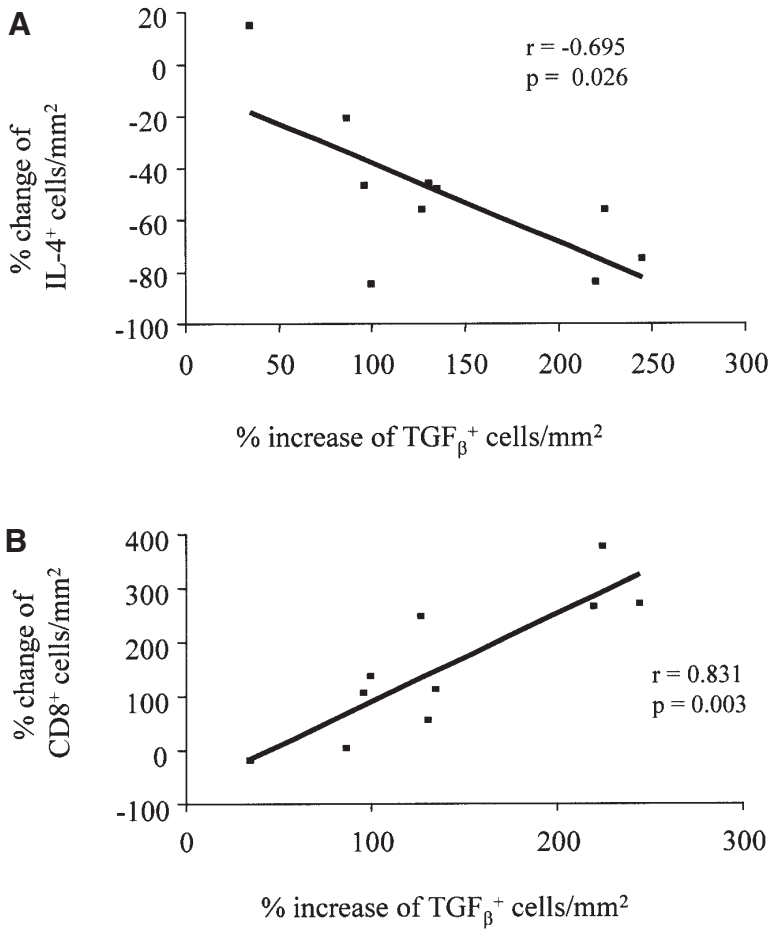

FIG 4. Correlation between budesonide-induced percent increase of TGF-β+ cells and percent decrease of IL-4+ cells **(A)** and between budesonide-induced percent increase of TGF-β+ cells and percent increase of CD8+ cells **(B)**.

steroid treatment we observed an increase in both the absolute number and the percentage of CD8+ T cells and a significant decrease in the CD4/CD8 ratio, which is believed to be a good indicator of therapeutic efficacy in chronic inflammatory diseases.[12] Mechanistically, regulatory-suppressor CD8 T cells are able to switch off established immune responses by means of numerous pathways, including apoptosis induction of specific CD4 T cell targets and secretion of cytokines, such as TGF-β, IL-10, and IFN-γ, thus influencing the development and function of other effector cells. CD8 T cell–mediated immune suppression has been demonstrated in a number of clinical conditions, including the immune inflammatory response occurring in asthma.[13]

Our data show that before budesonide treatment there was a significantly greater number of cells expressing not only IL-4 and IL-5 but also IFN-γ and IL-12 in NP tissues, suggesting that the nature of immune upregulation present in chronically inflamed NP tissues is pervasive although ultimately privileged toward T_H2. Our data also show that budesonide treatment selectively affected cytokine expression. Indeed, only the density of type 2 cytokine–positive cells (IL-4+ and IL-5+ cells) but not type 1 cytokine–positive cells (IL-12+ and IFN-γ cells) significantly decreased after treatment. These observations indicate a steroid-mediated redirection of the cytokine balance in vivo that results in a reversal of an exaggerated T_H2 cytokine expression.

In this study we have shown that very few cells expressed immunoreactive TGF-β in the control NM, whereas the density of such cells was significantly greater in untreated NP tissues and further increased in NP tissues obtained after intranasal budesonide. The increased tissue expression of TGF-β after the course of topical budesonide is of great interest, considering the emerging role of TGF-β, as a pivotal immune-regulatory cytokine showing a wide range of biologic activities.[14] Fargeas et al[15] demonstrated that TGF-β is able to downregulate the synthesis of IL-4 from human lymphocytes in vitro. Furthermore, a number of experimental findings strongly support the notion that TGF-β plays an important role in inhibiting ongoing inflammatory-immune responses selectively costimulating CD8+ cell proliferation[16] and leading CD8+ cells to develop suppressor

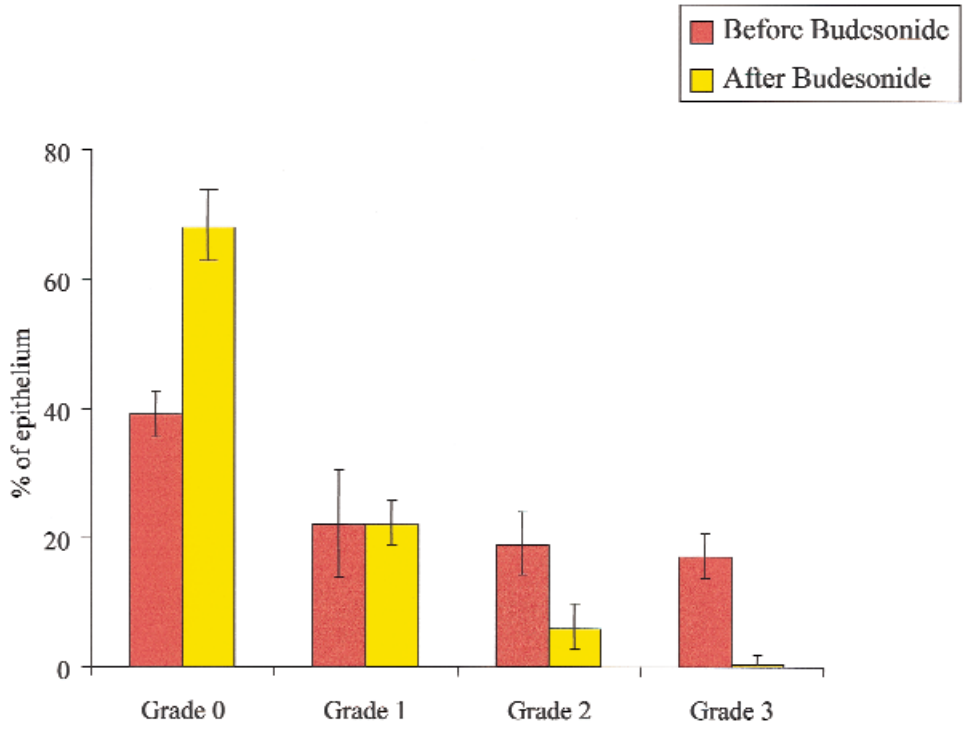

FIG 5. Quantification of epithelial damage in nasal polyp tissues obtained before *(red bars)* and after *(yellow bars)* intranasal budesonide treatment. Data represent M ± SEM (n = 10). The scoring system goes from grade 0 (normal or near normal epithelium) to grade 3 (severely damaged epithelium). See text for details.

effector functions.[5,6] In fact, it has been observed that the exposure of $CD8^+$ T cells to TGF-β might induce these cells to produce immunosuppressive amounts of this and other inhibitory cytokines, generating an inhibitory loop, acting not only as a paracrine but also as an autocrine regulator of T lymphocyte function. Interestingly, a recent study reported that TGF-β–producing mediastinal lymph node cells downregulated allergen-specific eosinophil-mediated airway inflammation,[17] and several experimental studies have attempted to manipulate TGF-β to obtain therapeutic benefit in experimental animal models[18] and in human chronic diseases.[19]

As far as we know, this is the first in vivo study showing that topical steroid treatment produces a local increase in TGF-β expression, suggesting that glucocorticoids might, at least in part, mediate their anti-inflammatory effects by inducing TGF-β expression. Several in vitro studies have shown that glucocorticoids are able to induce the expression of TGF-β in different experimental conditions in a concentration-dependent and time-dependent manner.[20,21] However, because data on the effect of corticosteroids on TGF-β expression are largely based on in vitro studies, the in vivo physiologic role of TGF-β in mediating glucocorticoid effects is still debated.

The findings from this study suggest that the steroid-induced increased expression of TGF-β might have a critical role in immunosuppression of airway inflammatory responses. It is not possible to establish causality on the basis of studies such as ours, because the evidence thus obtained represents only a snapshot of a surely dynamic process. However, the observation of a significant relationship between the percentage increase of $TGF\text{-}\beta^+$ cells and the subsequent reduction of $IL\text{-}4^+$ and increase of $CD8^+$ cells induces us to speculate that the upregulation of TGF-β, the change in IL-4, and the immune modulation of suppressor activity of CD8 might be mechanistically related.

TGF-β has also been shown to upregulate collagen synthesis in fibroblasts in vitro and has been associated with lung fibrosis in human and experimental animal models.[22,23] This is a particular relevant issue in light of our finding of an increased density of cells expressing TGF-β immunoreactivity after steroid treatment. Although we did not carry out quantitative analysis in the case of collagen staining, most of the staining in NP tissues was localized beneath the epithelium in the region including the basement membrane, with only weak staining in the stromal area, likely a reflection of the highly edematous nature of NP tissues. Microscopically, we were not able to appreciate differences between tissues obtained before and after intranasal budesonide. The examination of collagen deposition that we carried out at this time is admittedly limited, and additional studies to detail immunostaining of specific collagen types and other extracellular matrix proteins are currently ongoing. However, an indication of fibrosis, or of increased collagen deposition, was not reported in previous studies examining the effect of a high dosage of inhaled steroids

on the airway wall of patients with asthma.[24,25] Thus, we can reasonably speculate that the level of TGF-β present in NP tissues after intranasal budesonide treatment has, primarily, an immunoregulatory and not a fibrogenic biologic effect.

In this study we also investigated the ability of steroids to induce epithelial remodeling of inflamed airways tissue. Our study demonstrated a quite remarkable epithelial restitution in chronically and severely inflamed airways tissues after only 8 weeks of 400 μg of intranasal budesonide daily. On the basis of the grading system that we used to quantify epithelial damage, there was an actual increase in the number of epithelial cells with the evidence of multilayering and reappearance of ciliation. This indicates active epithelial proliferation and, possibly, phenotypic differentiation. Our findings are consistent with those of Laitinen et al,[24] demonstrating an improvement in the structure of bronchial epithelium and predominance of ciliated over goblet cells after 3 months of 1200 μg of budesonide daily. Epithelial cell repair is not a simple process, because it likely involves a number of growth factors and complex epithelial–mesenchymal interactions.[26] Thus, at this point, whether the marked epithelial restitution that we observed after intranasal budesonide is the consequence of a direct effect of steroids on epithelial cells or involves intermediate growth factor signals remains to be investigated.

In conclusion, results from this study demonstrated that budesonide is able to induce selective changes in cytokine immunoreactivity in NP tissues, an increase in the CD8 population, and a remarkable epithelial restitution with no apparent evidence of fibrosis. The observed effect of budesonide on increasing the expression of TGF-β$^+$ cells in the mucosa could be related to the change in IL-4$^+$ cell expression and T cell suppressor activity, a hypothesis that is in agreement with previously reported in vitro findings. It is tempting to speculate that corticosteroid treatment acts, at least in part, by upregulating induction of TGF-β, thus affecting T_H2/T_H1 cytokine balance and modulating CD8 suppressor activity. Although the mechanisms by which TGF-β exerts its immunoregulatory effects remain elusive, data from this study insinuate that a better knowledge of this potent cytokine could perhaps lead to the development of structural analogs with therapeutic benefit in chronic airway diseases.

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