

Effects of glucocorticoids on lymphocyte activation in patients with steroid-sensitive and steroid-resistant asthma

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Background: Glucocorticoids are important medications used to control the airway inflammation associated with asthma. Synthetic glucocorticoids vary in their binding affinity for the glucocorticoid receptor (GCR).

Methods: We compared hydrocortisone, beclomethasone dipropionate, triamcinolone acetonide, flunisolide, and budesonide with regard to their capacity to inhibit phytohemagglutinin-induced peripheral blood mononuclear cell proliferation from six patients with steroid-sensitive asthma and seven patients with steroid-resistant asthma. Peripheral blood mononuclear cell GCR binding affinities for dexamethasone and budesonide were also determined for both patient groups by using a radioligand binding assay and Scatchard analysis.

Results: Dose-dependent inhibition was demonstrated for all glucocorticoids in both patient groups, with the steroid-resistant group requiring approximately 2 log-fold more glucocorticoids for an equivalent degree of inhibition. The mean concentrations necessary to cause 50% inhibition of lymphocyte proliferation (IC_{50} s) for the steroid-sensitive group ranged from 2×10^{-10} mol/L for budesonide to 7×10^{-8} mol/L for hydrocortisone, whereas the mean IC_{50} s for the steroid-resistant group ranged from approximately 2×10^{-8} mol/L for budesonide to greater than 10^{-6} mol/L for hydrocortisone. In addition, a significant correlation was noted between the degree of inhibition of lymphocyte proliferation (IC_{50}) and the binding affinity of dexamethasone to the GCR. Patients with steroid-resistant asthma have been shown to have a reduced GCR binding affinity. The GCR binding affinity for budesonide was significantly higher in both groups (i.e., lower dissociation constant) than that obtained for dexamethasone.

Conclusion: These data suggest that glucocorticoids such as budesonide, by virtue of their high GCR binding affinities and greater ability to suppress lymphocyte proliferation, may therefore be beneficial in the management of difficult-to-control asthma. (*J Allergy Clin Immunol* 1996;98:1073-9.)

Key words: Steroid-resistant asthma, pharmacotherapy, glucocorticoid receptor, T lymphocytes

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Inhaled glucocorticoids, by virtue of their ability to reduce bronchial hyperreactivity and reduce symptoms, have become the cornerstone of care for long-term prophylaxis in moderate and severe asthma. Several inhaled glucocorticoids are available for use in asthma including beclomethasone dipropionate (BDP), triamcinolone acetonide (TAA), flunisolide, and budesonide. Despite their widespread therapeutic applications, there are few data comparing the antiinflammatory effects of these drugs. A significant subset of patients have asthma that remains difficult to control despite aggressive pharmacologic therapy. Asthma that is clinically resistant to glucocorticoid therapy represents the most extreme example. Steroid resistance in asthma is distinguished by the failure of glu-

Abbreviations used

ANOVA:	Analysis of variance
BDP:	Beclomethasone dipropionate
BMP:	Beclomethasone monopropionate
GCR:	Glucocorticoid receptor
IC ₅₀ :	Concentration necessary to cause 50% inhibition of lymphocyte proliferation
K _d :	Dissociation constant
PBMCs:	Peripheral blood mononuclear cells
PHA:	Phytohemagglutinin
SR:	Steroid-resistant
SS:	Steroid-sensitive
TAA:	Triamcinolone acetonide

corticoids to inhibit T-cell proliferation *in vitro*.¹ Evidence for T-cell activation has also been demonstrated in severe asthma.² Furthermore, persistent T-cell activation associated with gene dysregulation appears to contribute to an acquired defect in glucocorticoid receptor (GCR) binding affinity in patients with steroid-resistant (SR) asthma.³⁻⁵ It was therefore of interest to analyze and compare the capacity of available topically active glucocorticoids to inhibit the proliferation of phytohemagglutinin (PHA)-induced peripheral blood T lymphocytes from patients with steroid-sensitive (SS) asthma and patients with SR asthma.

METHODS

Subjects

Thirteen patients with a diagnosis of asthma, according to American Thoracic Society criteria, were selected for evaluation. Informed consent was obtained from all patients before entry into the study. Seven patients were classified as having SR asthma on the basis of their prebronchodilator morning FEV₁ of less than 70% of predicted value and failure to improve their morning prebronchodilator FEV₁ by more than 15% after a course of oral prednisone in doses equal to or exceeding 40 mg/day for 7 days, as previously reported.³ The patients with SR asthma were compared with a group of patients with mild to moderate asthma who either had responded to a prednisone burst in the past or had a GCR dissociation constant (K_d) of less than 25 nmol/L, a value previously identified to be associated with SS asthma.³ For the purposes of the study, these patients were classified as having SS asthma. Both groups of patients with asthma demonstrated significant bronchodilator responses. The mean prebronchodilator FEV₁ for the SR group was 1.79 L, which improved to 2.41 L after albuterol treatment (a 34.6% change), whereas the mean FEV₁ from the SS group was 3.32 L, which improved to 4.07 L after albuterol treatment (a

22.6% change). The demographics of the two study groups are shown in Table I. All the study subjects used inhaled β -agonists as needed for relief of symptoms. None of the study patients were receiving medications known to interfere with glucocorticoid metabolism.

Lymphocyte proliferation assay

Blood samples were obtained between 7:00 and 9:00 AM, before prednisone administration (when applicable), and peripheral blood mononuclear cells (PBMCs) were obtained by gradient separation and suspended in AIM-V serum-free lymphocyte media (Gibco, Grand Island, N.Y.) at a concentration of 1×10^6 cells/ml. Cells were plated in 96-well flat-bottom culture plates. PHA (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 10 μ g/ml. Cells were incubated for 72 hours at 37° C in 5% CO₂ with glucocorticoids at concentrations ranging from 10⁻⁶ to 10⁻¹⁰ mol/L. Glucocorticoid dilutions with media (AIM-V) were made each day from original stock steroid preparations. The stock steroids were dissolved at a concentration of 10⁻² to 10⁻³ mol/L in absolute ethanol. The ethanol concentration in the final incubation conditions never exceeded 0.1%. Cell viability was 95% or greater as measured by trypan blue exclusion. Diluent controls were used, and no effect on proliferation was noted. Four hours before harvesting, tritiated thymidine was added at a final concentration of 1 μ Ci per well. Cells were then harvested onto glass-fiber filter disks, and tritiated thymidine incorporation was assessed. The mean of the triplicate samples was expressed as a percent of the positive control (i.e., those cells stimulated with PHA in the absence of glucocorticoids).

GCR binding assay

Tritiated dexamethasone and tritiated budesonide radioligand binding assays and Scatchard analysis were performed on samples from four patients with SR asthma and four patients with SS asthma to determine GCR-binding affinity in PBMCs in both patient groups as previously described.^{3,4} Briefly, this assay is based on the method described by Crabtree et al.⁶ PBMCs were washed and incubated in RPMI-1640 medium (Gibco) for 30 minutes to remove any residual exogenous and endogenous glucocorticoids before exposure to increasing concentrations of tritiated dexamethasone (Amersham Corp., Arlington Heights, Ill.) or tritiated budesonide (gift from Astra/Draco, Lund, Sweden) from 0.8 to 400 nmol/L for 60 minutes at 37° C. For measurement of nonsaturable binding, a solution of 20 nmol/L tritiated dexamethasone and 2 mmol/L unlabeled dexamethasone or 20 nmol/L tritiated budesonide and 2 mmol/L unlabeled budesonide was also prepared. After incubation, the tubes were centrifuged, and 20 μ l of supernatant was counted in a beta spectrometer to measure the free dexamethasone concentration. The cell pellet, which measures whole cell dexamethasone concentra-

TABLE I. Patient characteristics

Parameter	Patients with SS asthma (n = 6)	Patients with SR asthma (n = 7)
Age (yr)	32.7 ± 2.2	26.6 ± 3.4
Baseline FEV ₁ (% predicted)	3.32 ± 0.5 L (80.2% ± 6.7%)	1.79 ± 0.1 L (51.6% ± 4.7%)
Prednisone dose (mg/day)	0	9.8 ± 2.0
Inhaled steroid dose (μg/day)	556.0 ± 246	1228.6 ± 601
GCR K _d (nmol/L)	19.8 ± 0.5	46.5 ± 2.3

tion, was next rinsed, dried, and counted in a beta spectrometer.

All values obtained were corrected for nonsaturable binding for each respective concentration. Saturation binding analysis was performed, assuming a linear binding plot of the bound divided by the free tritiated dexamethasone concentration versus the amount bound and extrapolating to the amount bound at an infinite free hormone concentration. A least-squares linear regression fit was used to define both receptor sites per cell and binding affinity. When repeated on samples from several different individuals who maintain similar disease activity, the GCR binding affinity has been shown to be highly reproducible with intraclass correlation coefficients of 0.95 for GCR number and 0.999 for the GCR K_d, with 1.00 indicating perfect agreement between assays.

Data analysis

For studies comparing the differences between glucocorticoid potency within each group and comparing single glucocorticoids between groups, an analysis of variance (ANOVA) with repeated measures was used. ANOVA with repeated measures was also used in determining differences between GCR binding affinities for dexamethasone and budesonide among the patients with SS and SR asthma. When calculating IC₅₀ values, a value of 5×10^{-5} mol/L was used when an IC₅₀ value exceeded the highest concentration of the glucocorticoids studied, and a value of 5×10^{-11} mol/L was used when an IC₅₀ concentration was less than the most dilute concentration of glucocorticoids studied. These values were designated because the range of glucocorticoid concentrations used did not always encompass the IC₅₀ value because of solubility or extremely low concentrations.

RESULTS

Clinical characteristics and the effect of glucocorticoids on lymphocyte proliferation

The mean ages of the study groups were similar, whereas the majority of other characteristics were significantly different. As expected, patients with SR asthma had poorer pulmonary function and were receiving higher doses of inhaled steroids; and six of the seven were receiving maintenance oral steroid therapy (Table I). Patients with SS

asthma were significantly more sensitive to the suppressive effects of all glucocorticoids studied compared with those with SR asthma. Fig. 1 compares the differences between the glucocorticoids within each group. Hydrocortisone was the least potent glucocorticoid, whereas budesonide was the glucocorticoid most potent in the suppression of PHA-induced lymphocyte proliferation in both groups of patients with asthma. Fig. 2 compares the difference in response between patients with SS asthma and those with SR asthma for each glucocorticoid studied. Despite the fact that six of the seven patients with SR asthma were receiving oral glucocorticoid therapy, lymphocytes from this group required greater concentrations of each glucocorticoid to suppress PHA-induced proliferation compared with the lymphocytes from the patients with SS asthma (Fig. 2). To quantify the difference in responsiveness between the patients with SS asthma and those with SR asthma, IC₅₀ values were determined for each glucocorticoid (Table II). The IC₅₀ is the concentration of the glucocorticoid that results in a 50% suppression of proliferation when PHA-stimulated cells are used, in the absence of glucocorticoids, as the positive control. Within each group, budesonide was approximately 2 log-fold more potent than hydrocortisone and 1 log-fold more potent than the other inhaled glucocorticoids in the suppression of PHA-induced proliferation. The subjects with SR asthma required approximately 2 log-fold more glucocorticoids to achieve the same degree of inhibition of proliferation. Significant differences between SS and SR IC₅₀ values were noted with hydrocortisone, the least potent glucocorticoid, and budesonide, the most potent glucocorticoid studied.

GCR binding affinity

In an attempt to determine whether diminished GCR binding affinity could in part explain the difference in glucocorticoid responsiveness between patients with SS asthma and those with SR

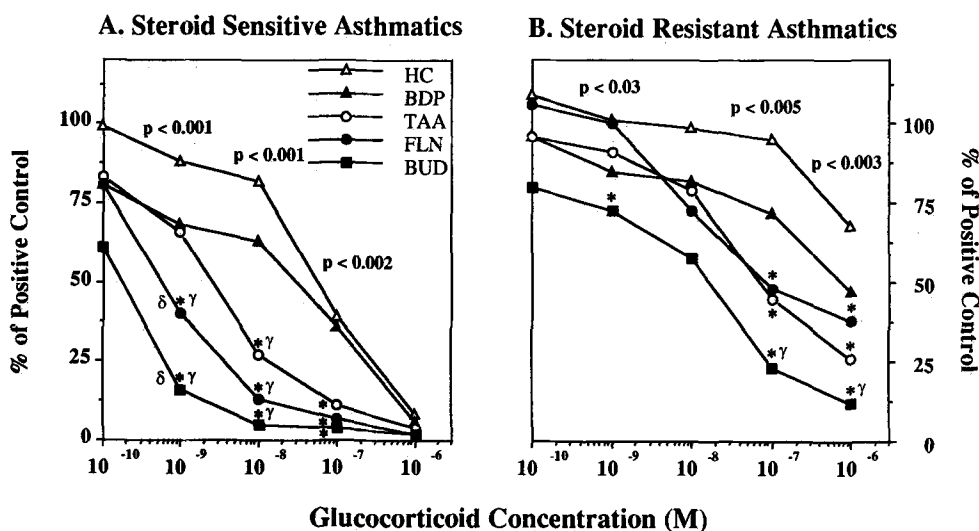


FIG. 1. Effects of hydrocortisone (HC), BDP, TAA, flunisolide (FLN), and budesonide (BUD) on PHA-induced lymphocyte proliferation in patients with SS asthma (A) and patients with SR asthma (B). Shown are mean values ($n = 6$ in A, $n = 7$ in B). Data were analyzed by using ANOVA with statistically significant values at each concentration studied noted on the figure. Asterisk indicates significant difference from hydrocortisone, γ indicates significant difference from BDP, and δ indicates significant difference from TAA.

asthma, GCR binding affinities for dexamethasone and budesonide were determined in each group. As seen in Fig. 3, there was a significant difference in dexamethasone GCR binding affinity between patients with SS asthma (K_d , 19.8 ± 0.5 nmol/L) and those with SR asthma (K_d , 46.5 ± 2.3 nmol/L, $p = 0.003$). Because budesonide was most effective in inhibiting lymphocyte proliferation in both patients with SS asthma and those with SR asthma, we determined whether this was related to enhanced GCR binding affinity. The GCR binding affinity of budesonide was significantly greater than that of dexamethasone in each group with the K_d for budesonide 0.87 ± 0.05 nmol/L versus 19.8 ± 0.5 nmol/L for dexamethasone among patients with SS asthma ($p < 0.005$), whereas the K_d for budesonide was 2.1 ± 0.95 nmol/L versus 46.5 ± 2.3 nmol/L for dexamethasone among patients with SR asthma ($p < 0.001$). Using linear regression analysis, we compared the GCR binding affinity of dexamethasone with its ability to suppress lymphocyte proliferation using the IC_{50} as a measure of suppression, from three patients with SS asthma and three patients with SR asthma. The mean dexamethasone IC_{50} value for the patients with SS asthma was $2.5 \times 10^{-9} \pm 2.9 \times 10^{-10}$ mol/L compared with $1 \times 10^{-7} \pm 5.6 \times 10^{-8}$ mol/L for the patients with SR asthma. A significant correlation between the dexamethasone IC_{50} value

and dexamethasone GCR binding affinity was found with an r^2 value of 0.67 and a p value of 0.046 (data not shown). Dexamethasone was chosen in place of the other synthetic glucocorticoids because it is the glucocorticoid routinely used for GCR assay and because tritiated forms of the other glucocorticoids, except for budesonide, were not available.

DISCUSSION

The vast majority of patients with asthma respond favorably to glucocorticoid therapy with relief of symptoms, improvement in baseline pulmonary function, and reductions in nonspecific bronchial hyperresponsiveness. Unfortunately, a small subset of patients with severe asthma that fails to adequately respond to high-dose inhaled and long-term oral glucocorticoid therapy exists. These "steroid resistant" asthmatic patients are often the most difficult group of patients with asthma to treat. Alternative therapies such as methotrexate, cyclosporine, dapsone, and gold are sometimes used in an attempt to improve asthma control. These alternative therapies are often associated with potentially harmful adverse effects, and none have been proven to be universally successful in this group of patients with severe asthma.⁷ Given these limitations, the introduction of new potent inhaled glucocorticoids with im-

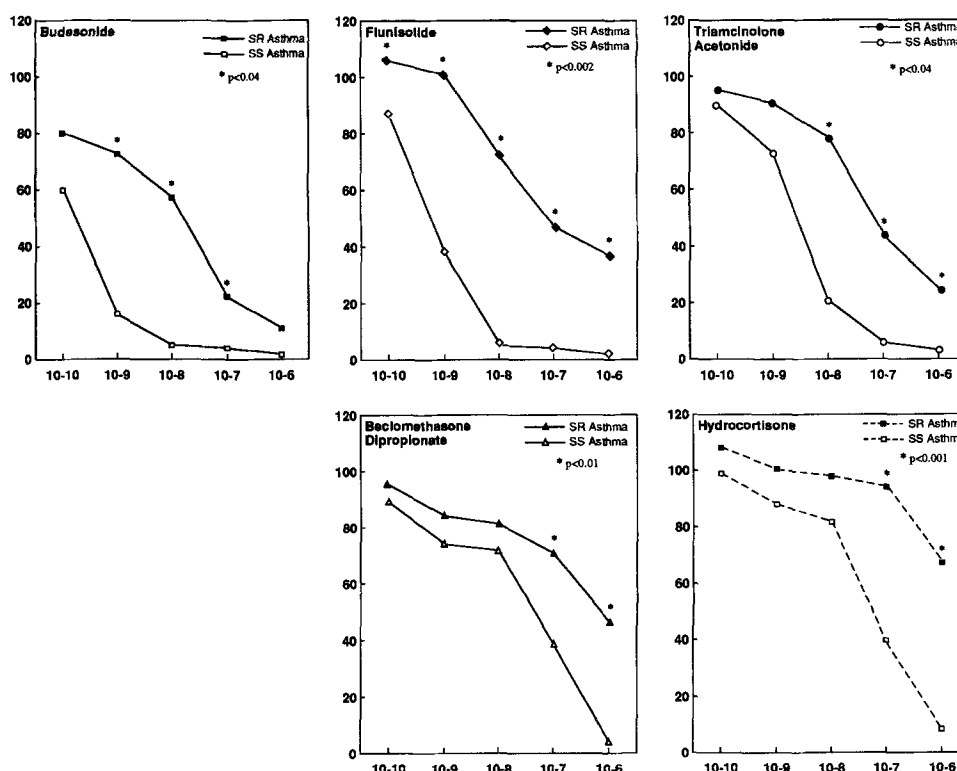


FIG. 2. Difference in responsiveness of each glucocorticoid between patients with SS asthma (open symbols) and patients with SR asthma (filled symbols). Shown are means, with asterisk indicating significant differences between patients with SS asthma and those with SR asthma at specified concentrations with significance determined by using ANOVA.

proved delivery devices offers a new approach to treatment.

Several abnormalities in T-cell responsiveness to glucocorticoids have been described in SR asthma including: a reduced ability of methylprednisolone to inhibit PHA-stimulated PBMC proliferation, elevated percentages of T lymphocytes bearing the activation markers IL-2R and HLA-DR, and inability of glucocorticoids to suppress IL-2 and interferon- γ production in mitogen-stimulated T lymphocytes from patients with SR asthma compared with patients with SS asthma and normal control subjects.^{1, 8-10} GCR abnormalities may also contribute to steroid resistance in asthma,³ and at least two types of GCR abnormalities have been identified to date in PBMCs. The vast majority of patients with SR asthma have displayed significant reductions in GCR binding affinity (termed type 1). This diminished ligand binding affinity is confined to the T-cell subset and is thought to be an acquired, reversible, and inflammation-driven phenomenon.⁴ Individuals with the type 1 defect will often manifest many of the steroid-induced adverse effects of long-term oral steroid use while

TABLE II. IC₅₀ values of various glucocorticoids from PHA-stimulated PBMCs from patients with SS and SR asthma

Glucocorticoid	IC ₅₀ (nmol/L)		ANOVA <i>p</i> Value
	SS group (<i>n</i> = 6)	SR group (<i>n</i> = 7)	
Hydrocortisone	73 ± 16	3670 ± 858	0.0027
BDP	50 ± 22	1710 ± 857	0.108
TAA	3.7 ± 1.4	243 ± 108	0.066
Flunisolide	0.80 ± 0.2	900 ± 691	0.139
Budesonide	0.19 ± 0.06	21.8 ± 7.5	0.0165

deriving little, if any, therapeutic benefit from glucocorticoid treatment.

This *in vitro* study was undertaken to answer the following question. Are there differences in potency between the various inhaled glucocorticoids compared with hydrocortisone both within each group (i.e., within the SS or SR group) and between groups (i.e., how effective is each inhaled glucocorticoid in the SS compared with the SR group)? This study is among the first to document

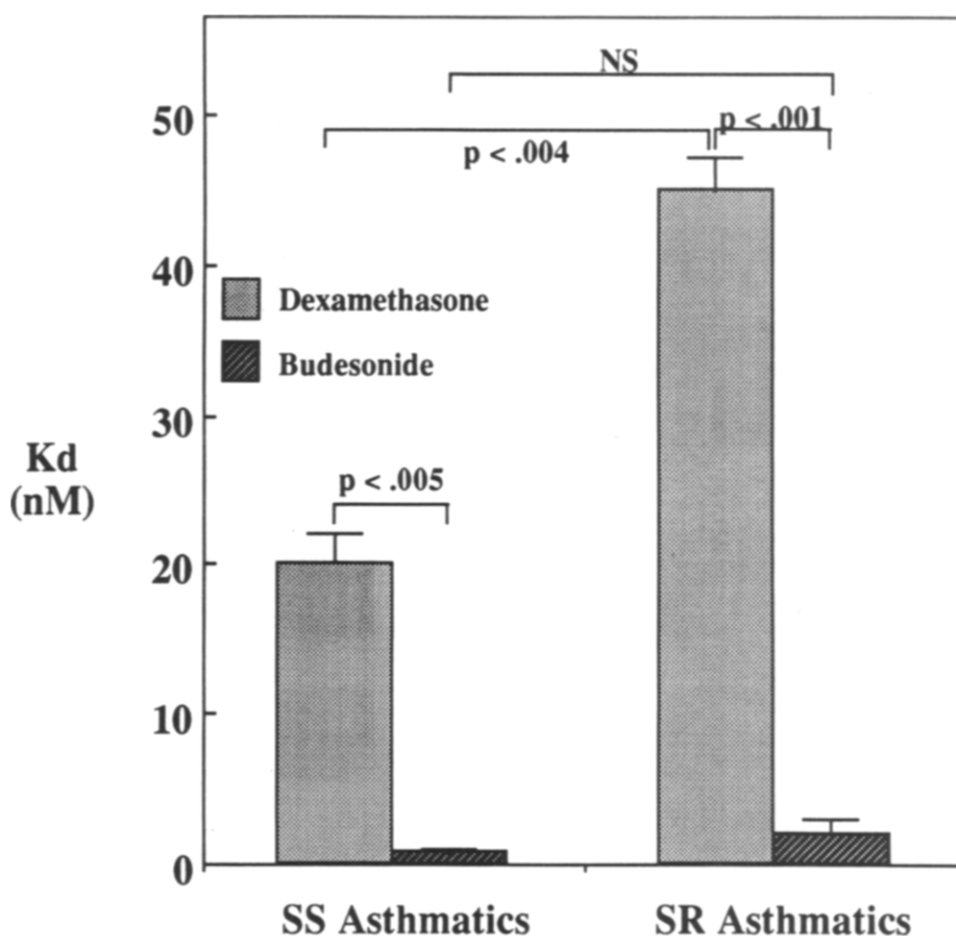


FIG. 3. GCR binding affinities for dexamethasone and budesonide in patients with SS asthma and patients with SR asthma. Shown are means $\pm$ SEM ($n = 4$ in each group). Significance was determined by using ANOVA.

significant differences in the ability of various inhaled glucocorticoid preparations to suppress PHA-induced lymphocyte proliferation.

First, budesonide was the glucocorticoid most effective at inhibiting lymphocyte proliferation in patients with SS asthma, followed by flunisolide, TAA, BDP, and hydrocortisone. Similar results were obtained among the patients with SR asthma, and budesonide again was the most effective at suppressing proliferation followed by TAA, flunisolide, BDP, and hydrocortisone. In both SS and SR groups, budesonide was 10 to 20 times more potent in suppressing lymphocyte proliferation than the next most potent glucocorticoid and 150 to 300 times more potent than the endogenous glucocorticoid, hydrocortisone. Of note, BDP was observed to be the least potent of the inhaled glucocorticoids studied. Clinical studies that have compared the efficacy of BDP to budesonide have shown BDP to be nearly as effective as budes-

onide.¹¹ This apparent discrepancy in potency is observed most likely because after inhalation, BDP is rapidly hydrolyzed by lung tissue to a more active metabolite, beclomethasone monopropionate (BMP). The binding affinity of BMP to the GCR is approximately 10-fold greater than that for BDP.¹² It is unclear whether PBMCs have the enzymatic ability to hydrolyze BDP to BMP. Our data would suggest that BDP is not converted to BMP by PBMCs, at least in vitro.

Second, lymphocytes from patients with SR asthma were less sensitive to the suppressive effects of all glucocorticoids. As seen in Table II, the mean IC_{50} values for the SR group were approximately 100-fold greater for each glucocorticoid studied than those seen among the patients with SS asthma, and statistically significant differences were noted for hydrocortisone and budesonide. This observation further supports the premise that T lymphocytes from patients with SR asthma are at

baseline in an activated state compared with those from patients with SS asthma and nonasthmatic control subjects, which renders them less susceptible to the suppressive effects of glucocorticoids (Table II).

Third, physiologic concentrations of hydrocortisone (i.e., 10^{-7} to 10^{-8} mol/L) were ineffective in inhibiting PHA-induced lymphocyte proliferation in cells from patients with SR asthma, whereas these concentrations resulted in significant suppression among lymphocytes obtained from patients with SS asthma, suggesting that endogenous hydrocortisone may be ineffective in suppressing airway inflammation in these patients with difficult-to-control asthma.

Fourth, a significant correlation between the GCR binding affinity (K_d) to dexamethasone and IC_{50} value for dexamethasone was observed, suggesting that GCR binding affinity is involved in steroid responsiveness to PHA-induced lymphocyte proliferation.

Finally, not only was budesonide the glucocorticoid most effective at inhibiting proliferation, it also bound to the GCR with a significantly higher affinity than dexamethasone in both patient populations.

The development of glucocorticoids with high topical to systemic potency, such as budesonide, may play a role in the management of severe asthma. Budesonide offers several theoretical advantages over the currently available inhaled glucocorticoids (in the United States). Budesonide binds to the GCR with high affinity,¹² and as documented in this study, was more potent at suppressing lymphocyte proliferation than the other glucocorticoid preparations. This increase in antiinflammatory effect coupled with its high first-pass metabolism greatly increase its topical to systemic potency.¹¹ This is an important consideration, especially in children in whom the potential for adverse effects of long-term inhaled glucocorticoid therapy remains uncertain. Another advantage to budesonide comes from its delivery device, which has been shown to deliver approximately twice as much drug to the lower airway as traditional pressurized metered-dose inhalers.^{13,14} The development of inhaled steroids with high GCR binding affinity and extensive first-pass hepatic metabolism may offer therapeutic advantages over the currently available inhaled glucocorticoid

preparations (in the United States) in the management of severe SR asthma.

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