

Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor β -isoform

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Background: Glucocorticoid-resistant bronchial asthma is characterized by failure of corticosteroids to suppress key asthma-relevant, cell-mediated inflammatory responses in the airways.

Objective: The mechanism of this phenomenon is not clear but may involve aberrant expression of the β -isoform of the glucocorticoid receptor.

Methods: We have measured expression of the α - and β -glucocorticoid receptor isoforms in tuberculin-driven cutaneous cell-mediated inflammatory lesions in people with asthma who are glucocorticoid sensitive and resistant after 9 days of therapy with oral prednisolone (40 mg/day) or matching placebo in a random order, crossover design.

Results: After placebo therapy, the mean numbers of cells expressing glucocorticoid receptor α immunoreactivity in the lesions evoked in glucocorticoid-sensitive and -resistant patients with asthma were statistically equivalent. The numbers of cells expressing glucocorticoid receptor β were significantly elevated in the patients who were glucocorticoid resistant, resulting in an 8-fold higher ratio of expression of glucocorticoid receptor α /glucocorticoid receptor β in the patients who were glucocorticoid sensitive. Glucocorticoid receptor α /glucocorticoid receptors β were colocalized to the same cells. Oral prednisolone therapy was associated with a significant decrease in the numbers of cells expressing glucocorticoid receptor α but not glucocorticoid receptor β in the subjects who were glucocorticoid sensitive. No significant change was found in the numbers of cells expressing glucocorticoid receptor α and glucocorticoid receptor β in the patients who were glucocorticoid resistant. Prednisolone therapy reduced the ratio of glucocorticoid receptor α /glucocorticoid receptor β expression for the patients who were glucocorticoid sensitive to a level seen in the patients who were glucocorticoid resistant before therapy.

Conclusion: Because glucocorticoid receptor β inhibits α -glucocorticoid receptor-mediated transactivation of target genes, the increased expression of glucocorticoid receptor β in inflammatory cells might be a critical mechanism for conferring glucocorticoid resistance. (*J Allergy Clin Immunol* 2000;105:943-50.)

Key Words: Glucocorticoid receptor α and β , glucocorticoid-resistant asthma

Glucocorticoids are potent anti-inflammatory agents that are very effective in the treatment of most patients with bronchial asthma, but there is a group of subjects with asthma who do not benefit from glucocorticoid therapy—the patients who are glucocorticoid α resistant.^{1,2} These individuals fail to improve with systemic glucocorticoid therapy at high dosage, even though their airway obstruction is reversible in response to β_2 bronchodilators. This is associated with resistance of peripheral blood T cells and monocytes to glucocorticoid inhibition in vitro and failure of glucocorticoid therapy to reduce expression of key asthma-relevant cytokines in the airways in vivo. These patients show no abnormalities in glucocorticoid pharmacokinetics or in the hypothalamus/pituitary/adrenal axis and are susceptible to the development of Cushingoid side effects of glucocorticoid therapy.¹ These observations suggest that cellular resistance to glucocorticoids in these patients with asthma is not generalized as in rare cases of primary cortisol resistance, but it is compartmentalized to T cells and possibly other inflammatory cells. Although this phenomenon is relatively uncommon, it poses a difficult therapeutic problem because few alternative therapies are available. An understanding of the mechanisms of glucocorticoid insensitivity may pave the way to a rational approach to therapy for these patients whose disease tends to be severe. Furthermore, glucocorticoid insensitivity is not limited to asthma and is a feature of other inflammatory diseases, including rheumatoid arthritis and suppression of renal transplant rejection. Thus elucidation of the cause for the refractoriness of inflammatory cells to glucocorticoid inhibition in asthma may have important implications for other inflammatory disorders.

Glucocorticoids mediate their effects via the glucocorticoid receptor (GR) that belongs to the superfamily of steroid/thyroid/retinoic acid receptor proteins that function as ligand-dependent transcription factors.³⁻⁵ When

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TABLE I. Clinical characteristics of subjects with asthma who are glucocorticoid sensitive or glucocorticoid resistant

	Glucocorticoid sensitive	Glucocorticoid resistant
Number	9	6
Age (y)	43 ± 4	45 ± 8
Sex	5M	3M
Percent predicted FEV ₁	76 ± 4	71 ± 3
Percent salbutamol response*	37 ± 2	33 ± 3
Percent prednisolone response†	36 ± 2	3 ± 1
Atopy	6/9	4/6

Ranges are expressed as the mean and SE.

*Salbutamol response is the percent increase FEV₁ at 15 minutes after administration of 400 µg via a metered-dose inhaler.

†Prednisolone response is the percent increase in FEV₁ after a 2-week course of prednisolone 40 mg/day, orally.

Abbreviations used

FEV₁: Forced expiratory volume in 1 second
 GRα: Glucocorticoid receptor α
 GRβ: Glucocorticoid receptor β
 PPD: Purified protein derivative

hormones bind, the receptor undergoes a change in conformation, resulting in the dissociation of heat shock protein 90 and the other associated proteins. Liganded GRs translocate to the nucleus where they either activate or suppress gene transcription. Positive gene regulation by glucocorticoids occurs by binding of the GR to DNA motifs in the promoter regions of glucocorticoid-responsive genes known as glucocorticoid response elements.⁶ In contrast to this direct mechanism for positive gene regulation, glucocorticoids acting via the GR may repress gene activation by heterogeneous mechanisms. Ligand-bound GR may sequester proinflammatory transcriptional activating factors directly through protein/protein interaction, such as in the case of activating peptide-1 (AP-1),⁷ or indirectly through the induction of anti-inflammatory peptides, such as in the case of 1κB suppressing nuclear factor-κB (NFκB) activity.⁸ Alternatively, liganded GR may also bind to specific negative response elements although this mechanism is probably not widespread.

The human GR cDNA is expressed as 2 highly homologous isoforms, differing only at their carboxyl termini,⁹ resulting from alternative mRNA splicing.¹⁰ These 2 isoforms, termed GRα and GRβ, are identical through amino acid 727, but then diverge, with GRα having an additional 50 amino acids and GRβ having 15 additional, nonhomologous amino acids. GRα functions as a ligand-dependent transcription factor, whereas the GRβ isoform does not bind hormone and does not activate the promoter of glucocorticoid-responsive genes. GRβ inhibits GRα mediated transactivation of target genes^{2,10} in a concentration-dependent fashion. Transfection of HepG2 cell lines with the GRβ gene significantly reduced GRα DNA binding capacity.¹¹ The precise

mechanism is unclear, but it may involve competition between GRα and GRβ for glucocorticoid-response element binding, the formation of GRα/GRβ heterodimers that are transcriptionally inactive, and/or sequestration by GRβ of coactivators required by GRα for full transcriptional activity.

Glucocorticoid-resistant asthma is associated with enhanced mononuclear cell-mediated airway inflammation, which is unresponsive to the anti-inflammatory effects of glucocorticoids.¹ Previous work on peripheral blood T cells has demonstrated a decrease in the binding affinity of glucocorticoids to the GR in some people who are glucocorticoid resistant, which may be elicited partly through the action of cytokines, including IL-2 and IL-4.¹² Using a classical tuberculin-driven, cutaneous cell-mediated immune reaction as a well-characterized delayed hypersensitivity response that is mediated by macrophages and T lymphocytes, we have already shown that prednisolone inhibits the tuberculin reaction in patients with asthma who are glucocorticoid sensitive but not in those who are glucocorticoid resistant.¹³ These observations have now been extended to test the hypothesis that there may be an enhanced expression of the GRβ isoform in this model of mononuclear cell-mediated inflammatory lesion in patients who are glucocorticoid resistant, to account for the lack of responsiveness to the anti-inflammatory effects of glucocorticoids, because immunoreactive GRβ is increased in the PBMC of these patients.¹¹

MATERIALS AND METHODS

Subjects

The study was approved by the Guy's Hospital Ethical Committee. Nine subjects with asthma who were glucocorticoid sensitive and 6 who were glucocorticoid resistant, age and sex matched, entered into this study (Table I). All subjects had a history of episodic wheezing and were matched in terms of percent resting predicted forced expiratory volume in 1 second (FEV₁). All subjects demonstrated a greater than 15% reversibility in FEV₁ after inhalation of 400 µg salbutamol. Glucocorticoid-sensitive asthma was defined as an increase in FEV₁ of at least 30% after a 2-week course of oral prednisolone 40 mg daily, corrected for body surface area (41 ± 1.3 mg [mean ± SEM]). Glucocorticoid-resistant asthma was defined as a less than 15% improvement in FEV₁ after a similar course of corticosteroids. There was no difference between the 2 groups in the use of medication. All patients were receiving inhaled salbutamol (mean 3600 ± 900 µg [mean ± SEM], and 3520 ± 1000 µg [mean ± SEM] per day in the glucocorticoid-sensitive and glucocorticoid-resistant groups, respectively). Four out of 9 patients who were glucocorticoid sensitive were receiving inhaled beclomethasone at a mean dose per day of 900 ± 200 µg (mean ± SEM). Two out of 6 patients who were glucocorticoid resistant were receiving inhaled beclomethasone at a dose of 1000 µg and 800 µg per day, respectively. There was no difference in atopic status between the 2 groups of patients. None of the subjects had taken oral corticosteroids for at least 1 month before the study.

Trial protocol

Subjects with asthma entered into a double-blind study on the basis of demonstrating tuberculin skin positivity to 10 or 100 units of purified protein derivative (PPD) of *Mycobacterium tuberculosis* injected intradermally as previously described.¹³ A positive skin reaction was determined as greater than or equal to an average of 6

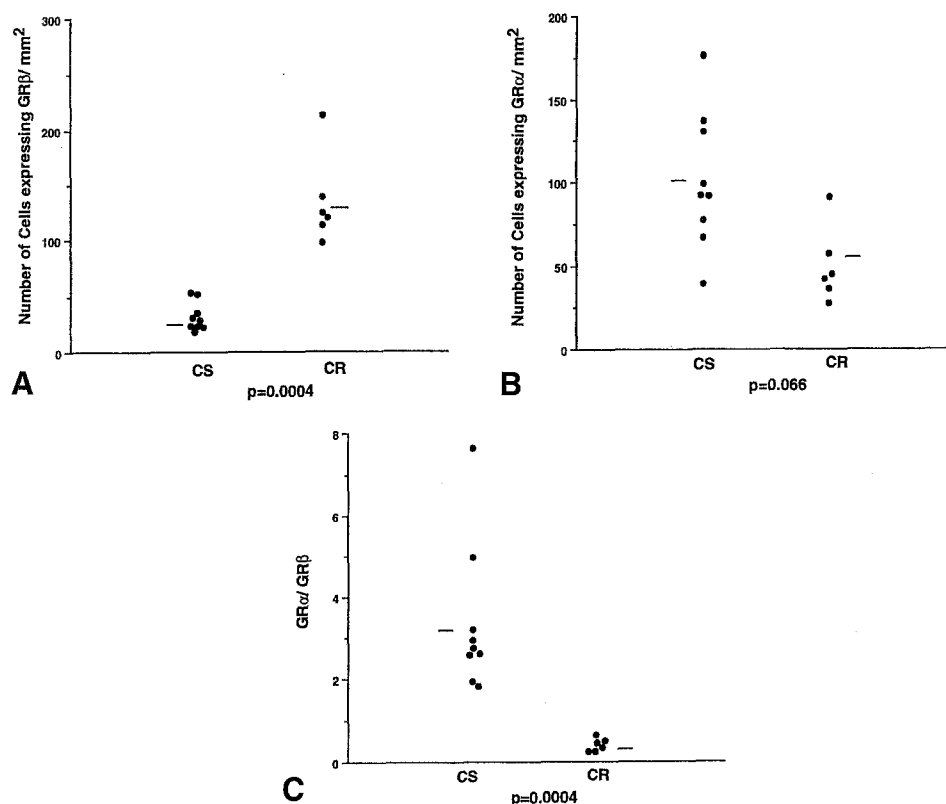


FIG 1. Number of cells expressing GRβ (A); GRα (B); and the ratio of GRα/GRβ (C) in skin biopsy specimens of the tuberculin response from patients with asthma who are glucocorticoid sensitive (CS) and glucocorticoid resistant (CR). Each point represents data from an individual patient; bars, medians.

mm induration in 2 perpendicular diameters at 48 hours after intra-dermal testing. After a washout period of at least 1 month after initial testing, a 40 mg oral dose of prednisolone (corrected for body surface area, ie, calculated from height, surface area, and weight by using a nomogram) or placebo daily was started on day 1. On day 7 the previously determined dose of PPD antigen was injected intra-dermally in the right forearm. On day 9 the prednisolone or placebo was stopped, and the indurated reaction site was biopsied for immunohistochemical analysis. After a washout period of 6 weeks, the procedure was repeated and PPD was injected into the other forearm after the subject had received the alternative treatment.

Immunohistochemical analysis

Four millimeter punch skin biopsy specimens were immediately immersed in 10% formalin and paraffin processed. Five-micron sections were cut and placed on slides coated with APES. One of the primary antibodies used was polyclonal rabbit antihuman GRβ from Dr J.A. Cidlowski (National Institutes of Health, North Carolina).¹⁰ This antibody was raised against a peptide corresponding to the 15 additional nonhomologous amino acids. The second primary antibody was polyclonal rabbit antihuman GRα from Dr J.A. Cidlowski (National Institutes of Health, North Carolina). This antibody is specific to the 50 additional amino acids. Specificity of the above primary antibodies was tested by means of Western analysis, immunoprecipitation, and immunohistochemistry. The avidin-biotin-complex technique was used as previously described.¹³ Polyclonals were developed with a goat antirabbit biotinylated secondary antibody

(Dako). Immunoperoxidase brown color reaction was developed with diaminobenzidine (Sigma). Endogenous biotin reactivity and endogenous peroxidase activity were abolished as previously described.¹³

Double staining for GRα and T cells, GRα and macrophages, GRβ and T cells, and GRβ and macrophages was performed to assess whether T cells and macrophages are capable of expressing both GR isotypes. The mAbs UCHL1 (Dako) and PGM1 (Dako) were used to identify T cells and macrophages, respectively. Alkaline phosphatase conjugated swine antimouse secondary antibody (Seralab) was used to detect the monoclonals. A blue color reaction was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Double-stained cells were identified positively for both blue and brown precipitate.

Data analysis

Sections were coded and read by an investigator without prior knowledge of the protocol by using an Olympus microscope connected to a television screen through an image analyzer (Zeiss KSS300 3.0, Imaging associates, Oxfordshire, UK). Positive staining cells were counted, and the total area was measured by use of the image analyzer. All cell counts per square millimeter of tissue were expressed as median (range). Statistical analysis of the data was performed by Mann-Whitney *U* test for between-group comparisons. Wilcoxon signed-rank test for matched pairs was used for within-group comparisons. Spearman rank correlation was used when analyzing relationships between data. Differences were taken as significant when $P < .05$.

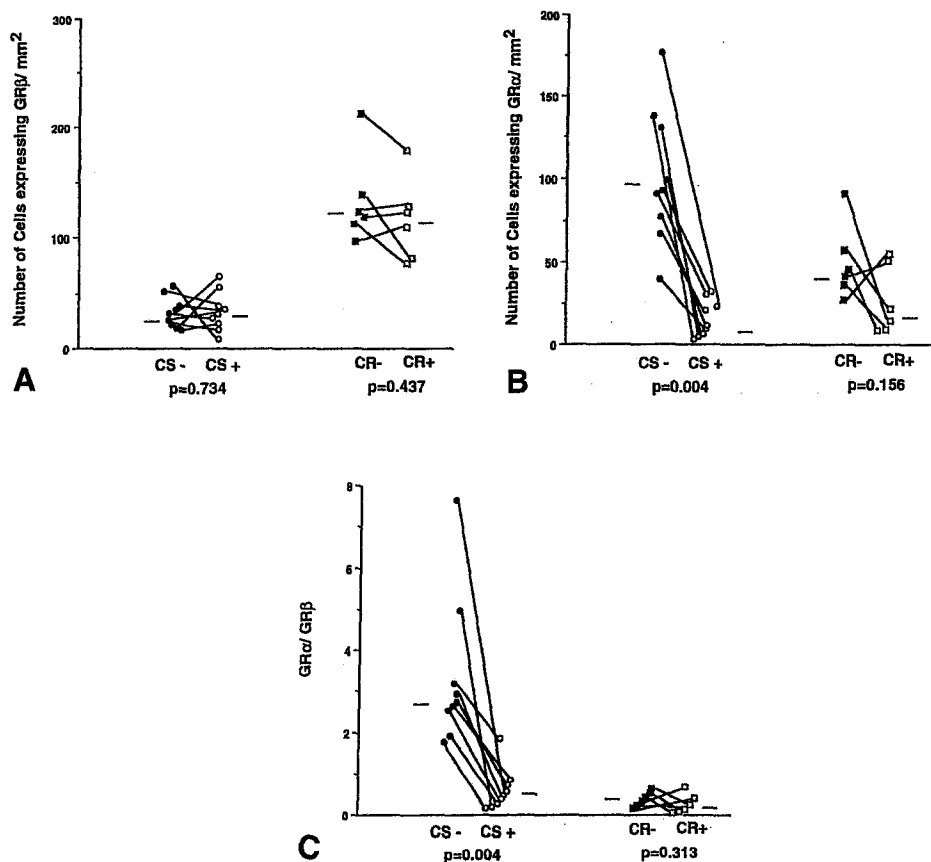


FIG 2. Number of cells expressing GR β (A); GR α (B); and α -GR/ β GR (C) in skin biopsies of the tuberculin responses from patients with asthma who are glucocorticoid sensitive (CS) and glucocorticoid resistant (CR) after either placebo (-) or prednisolone (+) therapy. Each point represents data from an individual patient; bars, medians.

RESULTS

Clinical findings and total numbers of inflammatory cells

The sizes of the cell-mediated reaction in glucocorticoid-sensitive and -resistant groups have previously been reported.¹³ In subjects with glucocorticoid-sensitive asthma, prednisolone suppressed the cutaneous induration from 25 ± 2 mm to 10 ± 1 mm (mean $\pm$ SEM) ($n = 9$; $P < .003$). In the subjects with glucocorticoid-resistant asthma the size of the cutaneous reaction did not change significantly: 13.5 ± 2 mm during placebo administration and 12 ± 1 mm (mean $\pm$ SEM) ($n = 6$; $P = .23$) after prednisolone treatment. There was no significant difference in the size of the tuberculin response between the glucocorticoid-sensitive and -resistant groups during the placebo part of the study ($P = .3$). There was no significant difference observed between subjects who were glucocorticoid sensitive or subjects who were resistant for total inflammatory cells per square millimeter ($1985/\text{mm}^2$ [range, 464-3704/ mm^2] and $1306/\text{mm}^2$ [range, 528-2098/ mm^2], respectively; $P = .181$).

Number of cells expressing GR β and GR α

Significantly lower number of cells positive for GR β were observed in the glucocorticoid-sensitive compared

with the resistant group ($26/\text{mm}^2$ [range, 22-53/ mm^2] and $122/\text{mm}^2$ [range, 98-213/ mm^2], respectively; $P = .0004$) (Fig 1, A). In contrast, no significant difference was observed in the number of cells expressing GR α between the glucocorticoid-sensitive and -resistant group ($93/\text{mm}^2$ [range, 39-176/ mm^2] and $44/\text{mm}^2$ [range, 28-160/ mm^2], respectively; $P = .066$) (Fig 1, B). An 8-fold higher GR α /GR β ratio was observed in the glucocorticoid-sensitive group when compared with the resistant group (2.75 [range, 1.82-7.64/ mm^2] and 0.38 [range, 0.22-0.75/ mm^2], respectively; $P = .0004$) (Fig 1, C).

Effect of prednisolone on the number of cells expressing GR α and GR β

No significant decrease was noted in the total number of inflammatory cells after prednisolone treatment in either of the groups (from $1985.00/\text{mm}^2$ [range, 464-3704/ mm^2] to $1343/\text{mm}^2$ [range, 684-1836/ mm^2] in patients who were glucocorticoid sensitive, $P = .074$; and from $1306/\text{mm}^2$ [range, 528-2098/ mm^2] to $1184.00/\text{mm}^2$ [range, 806-1851/ mm^2] in patients who were glucocorticoid resistant, $P = .844$).

Corticosteroids had no effect on the number of cells expressing GR β in either the glucocorticoid-sensitive or

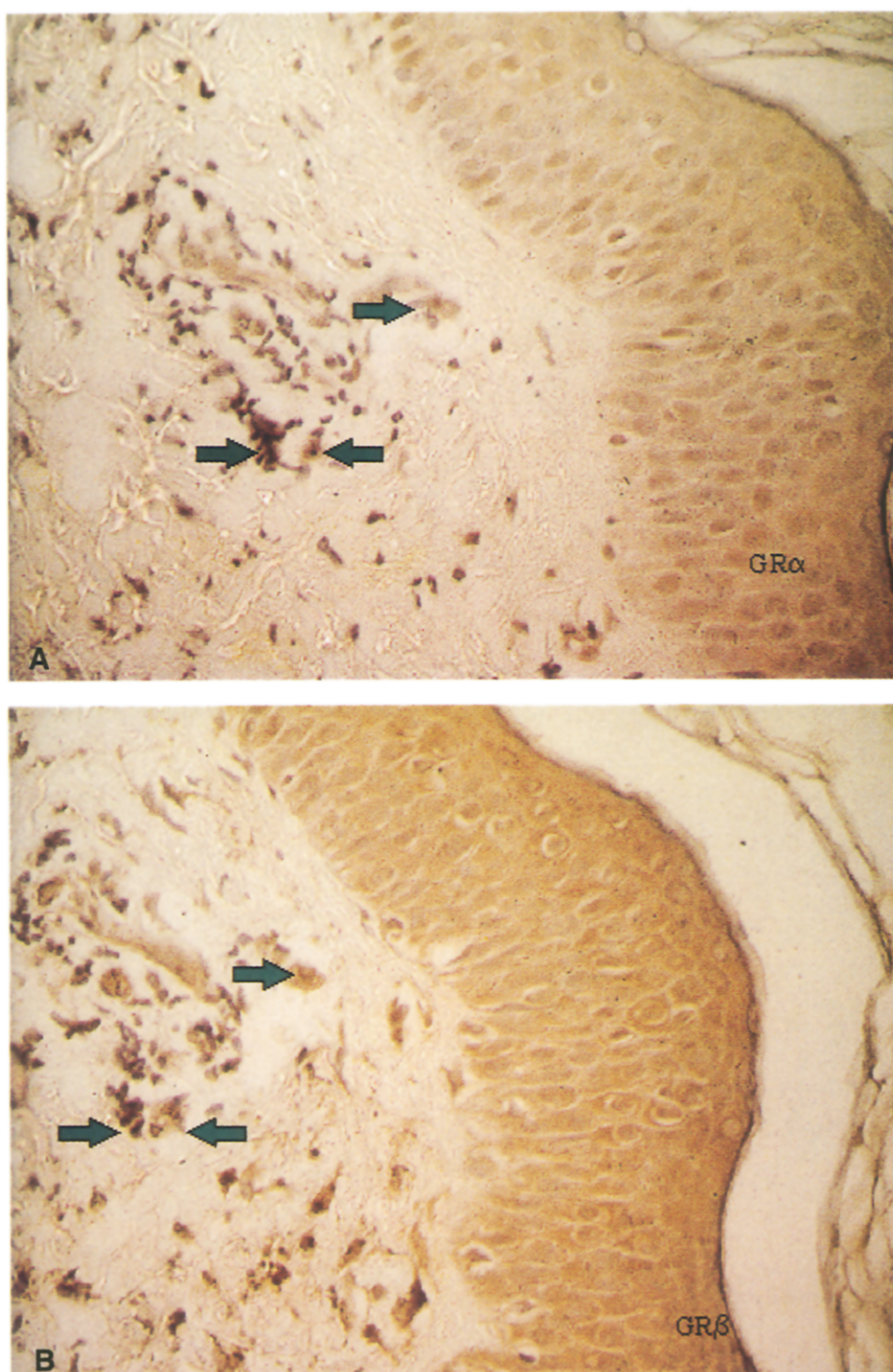


FIG 3. Photographs of consecutive sections stained with rabbit antihuman GR α (A) and GR β (B) polyclonal antibodies, respectively. Arrows, Positive cells for both antibodies showing cellular colocalization. Positivity is shown by the brown staining. Nuclei are counterstained in blue.

-resistant group (from 26/mm² [range, 22-53/mm²] to 30/mm² [range, 9-65/mm²], $P = .734$; and from 122/mm² [range, 98-213/mm²] to 113/mm² [range, 77-177/mm²], $P = .437$, respectively) (Fig 2, A).

Although corticosteroids produced a highly significant downregulation in the number of cells expressing GR α in the glucocorticoid-sensitive group (from 93/mm² [range, 39-176/mm²] to 9/mm² [range, 3-31/mm²]; $P = .004$),

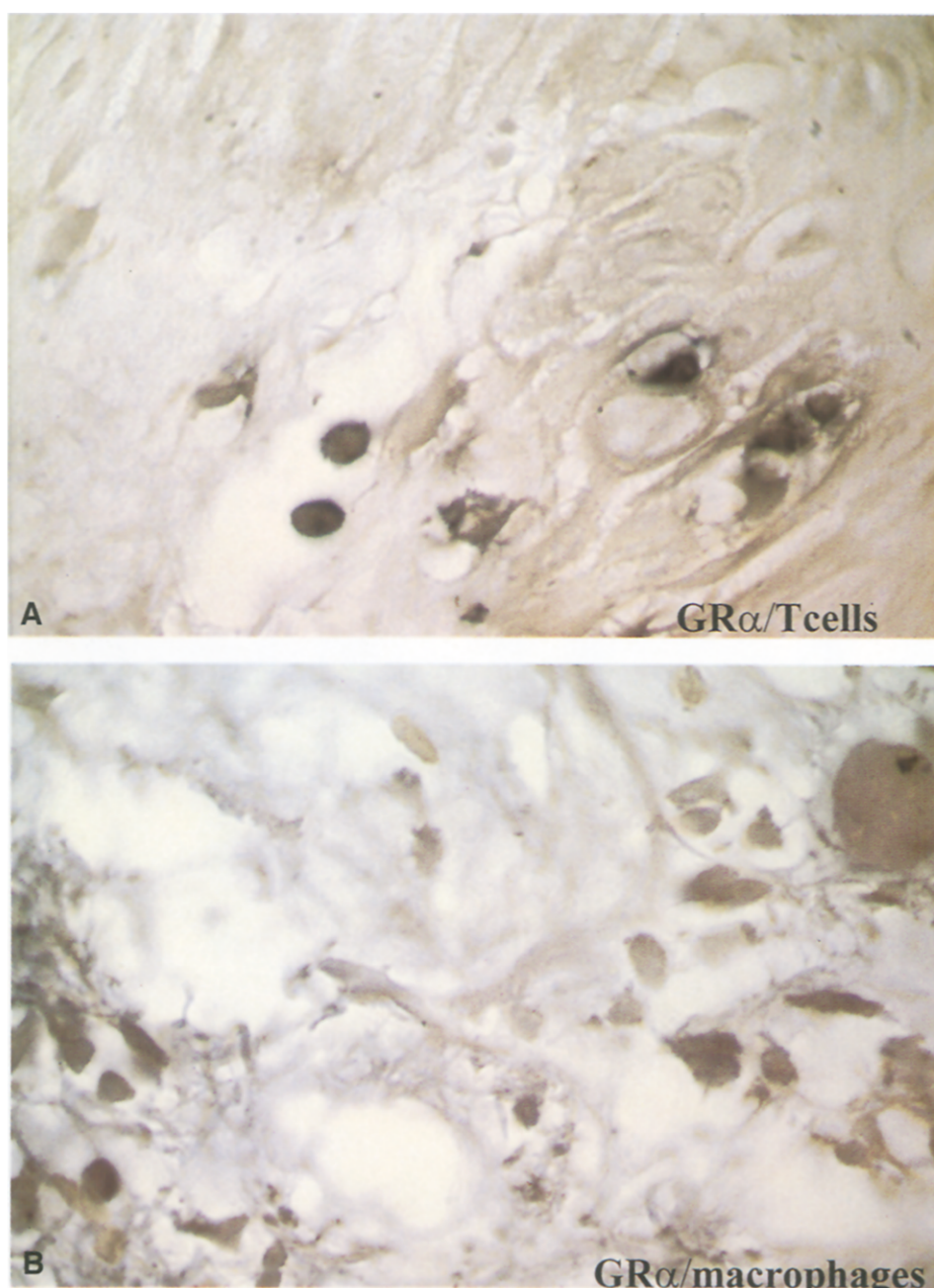


FIG 4. Photographs of skin sections double stained for GR α and T cells (**A**); GR α and macrophages (**B**); GR β and T cells (**C**); GR β and macrophages (**D**). Blue staining indicates positivity for either T cells or macrophages, and brown staining indicates positivity for either GR α or GR β .

there was no significant effect on the number of cells expressing GR α in the glucocorticoid-resistant group (from 44/mm² [range, 28-160/mm²] to 18/mm² [range, 9-54/mm²]; $P = .156$) (Fig 2, *B*).

Although corticosteroids produced a highly significant downregulation of the GR α /GR β ratio in the glucocorticoid-sensitive group (from 2.75 [range, 1.82-7.64] to 0.46 [range, 0.15-1.87]; $P = .004$), there was no significant effect on the GR α /GR β ratio in the resistant group

(from 0.38 [range, 0.22-0.75] to 0.16 [range, 0.069-0.68]; $P = .313$, (Fig 2, *C*).

There was a negative correlation between the reduction in cutaneous induration after glucocorticoids and the number of positive cells for GR β in the placebo arm ($r = -0.732$; $P = .0019$) and a positive correlation between the reduction in cutaneous induration and the GR α /GR β ratio ($r = 0.8004$; $P = .0003$), suggesting that the greater the relative expression of GR β , the lower response to steroids.

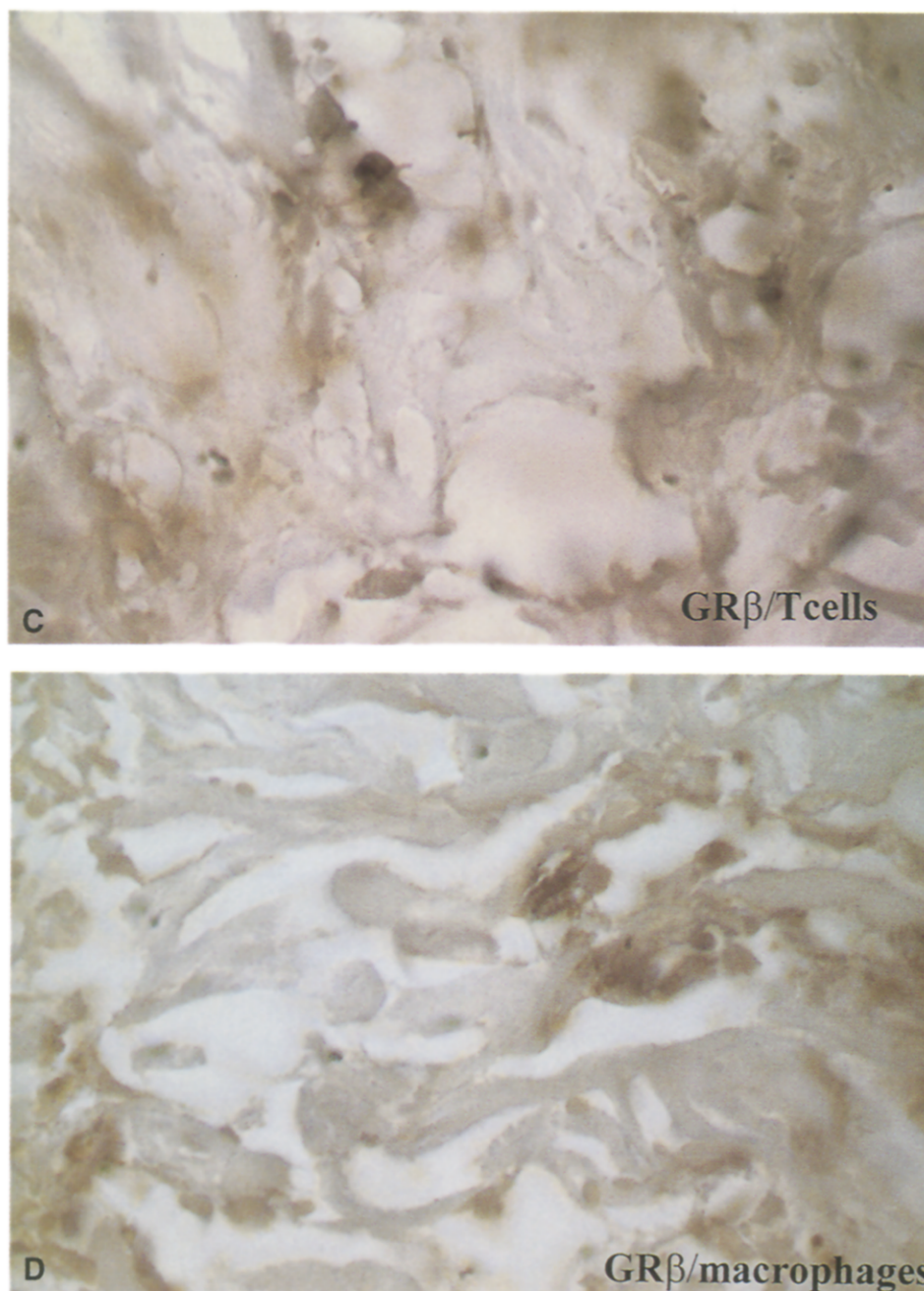


FIG 4. Continued.

Cellular colocalization of GR α and GR β

Through the use of consecutive sections stained with anti-GR α and anti-GR β polyclonal antibodies, both iso-types were seen to be expressed by the same cell (Fig 3). Double staining of sections for T cells and macrophages and GR α or GR β showed that both cell types expressed GR α and GR β (Fig 4).

DISCUSSION

We demonstrate that significantly higher numbers of inflammatory cells expressing GR β immunoreactivity

were observed in the glucocorticoid-resistant tuberculin responses as compared with those in the glucocorticoid-sensitive group after placebo therapy. In contrast, no significant difference was observed in the number of cells expressing GR α immunoreactivity between the 2 groups of patients. An 8-fold higher GR α /GR β ratio was observed in the glucocorticoid-sensitive group when compared with the glucocorticoid-resistant group. The mechanism for the enhanced expression of GR β is unknown. We have previously demonstrated that PBMC of patients with asthma who are glucocorticoid resistant have increased AP-1 and decreased DNA binding activi-

ties.^{14,15} This is likely to be caused by an enhanced transcription of inducible *c-Fos* because *c-fos* anti-sense oligonucleotides increased GR-DNA binding in PBMC of glucocorticoid-resistant subjects after stimulation with dexamethasone.¹⁶ This work was recently extended to show an increase in *c-Fos* in the tuberculin-induced model of dermal inflammation used in this present study.¹⁷ Additional work is clearly necessary to establish whether increased *c-Fos* alters the expression of GR β . Nevertheless, the finding that there is a significant increase in the number of inflammatory cells expressing GR β in this model of cutaneous inflammation supports the hypothesis that GR β may be a critical factor regulating target cell responsiveness to glucocorticoids, and that high levels of GR β may contribute to glucocorticoid resistance. These data support the findings of Hamid et al,¹⁸ who showed elevated percentages of both cells in bronchoalveolar lavage fluid and PBMCs expressing immunoreactivity to β -GR in those subjects with asthma who were glucocorticoid resistant as compared with those who were glucocorticoid sensitive.¹⁸

Because previous work has shown that glucocorticoids can modulate expression of GR mRNA in bronchial epithelium,¹⁹ it was especially pertinent to address whether there were any differential effects of prednisolone therapy on GR β and GR α expression between people with asthma who are glucocorticoid sensitive or glucocorticoid resistant. After prednisolone treatment, there was no significant change in the numbers of inflammatory cells expressing GR β immunoreactivity in either the glucocorticoid-resistant or glucocorticoid-sensitive subjects. Prednisolone did not significantly change the numbers of cells expressing GR α immunoreactivity in the glucocorticoid-resistant group, consistent with their cellular refractoriness to the effects of glucocorticoids,^{12,14-16} but reduced GR α immunoreactive cells in glucocorticoid-sensitive subjects, probably via an effect on GR α gene transcription.²⁰ The finding that the greater the GR α expression, the greater the response to the inhibitory effects of glucocorticoids, emphasizes the putative role of GR α in the response to steroids. The ratio of GR α /GR β was significantly reduced by prednisolone in glucocorticoid-sensitive subjects, but there was no significant alteration of GR α /GR β in glucocorticoid-resistant subjects after glucocorticoids. Thus systemic administration of prednisolone reduced the expression of GR α relative to GR β in patients with asthma who were glucocorticoid sensitive to the level seen in patients who were glucocorticoid resistant, suggesting that treatment with high doses of glucocorticoid, even for a short time period, has the potential to render inflammatory cells subsequently insensitive to glucocorticoids. This finding has clinical implications because the mainstay of treatment for severe asthma is glucocorticoids, and some patients are using high doses of the drug for a prolonged

period. Despite an initial response, any additional improvement may be impeded because the cells are now expressing predominant GR β , which would not be anticipated to be steroid responsive.

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