

Association of Glucocorticoid Insensitivity with Increased Expression of Glucocorticoid Receptor β

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Summary

In many chronic inflammatory disorders, glucocorticoid (GC) insensitivity is a challenging clinical problem associated with life-threatening disease progression. The molecular basis of GC insensitivity, however, is unknown. Alternative splicing of the GC receptor (R) pre-messenger RNA generates a second GCR, termed GCR- β , which does not bind GCs but antagonizes the transactivating activity of the classic GCR, termed GCR- α . In the current study, we demonstrate that GC-insensitive asthma is associated with a significantly higher number of GCR- β -immunoreactive cells in peripheral blood than GC-sensitive asthmatics or normal controls. Furthermore, we show that patients with GC-insensitive asthma have cytokine-induced abnormalities in the DNA binding capability of the GCR. These abnormalities can be reproduced by transfection of cell lines with the GCR- β gene resulting in significant reduction of their GCR- α DNA binding capacity. We conclude that increased expression of GCR- β is cytokine inducible and may account for GC insensitivity in this common inflammatory condition.

Glucocorticoid (GC)¹ insensitivity is increasingly being recognized in the management of chronic inflammatory diseases such as asthma, rheumatoid arthritis, systemic lupus erythematosus, and transplantation rejection. It poses a major clinical challenge as GC therapy is the cornerstone of antiinflammatory therapy. Delineation of the molecular basis for GC insensitivity is critical for the development of new treatment approaches for this group of refractory patients, and may provide new insights into the pathogenesis of chronic inflammation. GC insensitivity has been most extensively studied in chronic asthma, as the failure here to respond to GCs is more readily demonstrated than in other inflammatory diseases (1). Previous studies on PBMCs from patients with GC-insensitive asthma have revealed that GCs fail to inhibit their mitogen-induced T cell proliferation and cytokine secretion in vitro (2, 3). In addition, T cells from peripheral blood of GC-insensitive asthmatics, but not GC-sensitive asthmatics, are persistently activated despite high doses of GC therapy (4).

GCs act by binding to a cytoplasmic glucocorticoid receptor (GCR) which then translocates to the nucleus as a transcription factor (5). Recently, we found that the majority of patients with GC-insensitive asthma have a reversible defect in PBMC GCR ligand binding affinity which can be sustained in vitro by the addition of IL-2 and IL-4 but not the individual cytokines (6). Furthermore, in vitro incubation of normal peripheral blood T cells with the combination of IL-2 and IL-4 reduces their GCR ligand binding affinity to the level seen in GC-insensitive asthma (7). Bronchoscopy studies indicate that airway T cells of GC-insensitive, as compared to GC-sensitive, asthma have significantly higher levels of IL-2 and IL-4 gene expression (8). Overall these data support the concept that GC-insensitive asthma results from high level expression of IL-2 and IL-4 which leads to reduced GCR binding affinity and decreased T cell responsiveness to GCs.

Independent studies by other investigators have recently reported that PBMCs from patients with GC-insensitive asthma have a decreased ability of their GCRs to bind its specific DNA recognition sequence (glucocorticoid response elements, i.e., GRE; reference 9). In the current study, we extended our analysis of PBMCs from GC-insensitive asthmatics to demonstrate that our patients previously found to

¹Abbreviations used in this paper: EMSA, electromobility shift assay; FEV₁, forced expiratory volume in one second; GC, glucocorticoid; GRE, glucocorticoid response element; mRNA, messenger RNA.

have abnormally low GCR ligand binding affinity also have a GCR DNA binding defect. More importantly, we report for the first time that the combined GCR ligand and DNA binding defect in GC-insensitive asthma is due to cytokine-induced expression of GCR- β , a newly identified inhibitor of GC action.

Materials and Methods

Patient Selection. Informed consent was obtained from all patients before their entry into this study. Patients with a diagnosis of asthma, based on The American Thoracic Society criteria (10), were selected for evaluation. They were included if they had a morning prebronchodilator FEV₁ <70% of predicted values and a \geq 15% increase in forced expiratory volume in one second (FEV₁) after two inhalations of albuterol (90 μ g/actuation). Patients were excluded if they had evidence for other types of lung disease. Patients were classified as GC-sensitive or GC-insensitive based on their prebronchodilator morning FEV₁ and response to a course of oral prednisone (6). Asthmatic patients were defined as GC-insensitive if they failed to improve their morning prebronchodilator FEV₁ by \geq 15% after a 1-wk course of prednisone at a minimum oral dose of 40 mg/day. Patients were classified as GC-sensitive if they had an increase in baseline FEV₁ of 30% or greater.

Cell Cultures. Peripheral blood was collected in heparinized syringes and PBMCs isolated by Ficoll-Paque[®] (Pharmacia, Piscataway, NJ) gradient centrifugation. PBMCs from normal donors and GC-insensitive asthma patients were isolated and resuspended at a concentration of 10⁶ cells/ml in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal calf serum (HyClone Labs, Logan, UT). Cells were incubated in the absence and presence of IL-2 (50 U/ml; Chiron Corp., Emeryville, CA) and/or IL-4 (50 U/ml; Schering-Plough Research Institute, Bloomfield, NJ) for 48 h at 37°C in 5% CO₂ before analysis.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from PBMCs as previously described in reference 11. GCR DNA binding was analyzed by electrophoretic mobility shift assay (EMSA) using a 30-bp double-stranded oligonucleotide composed of two complementary strands: 5'-TCGACTGATTA-CAAAGTCTTCTTGCTACT-3' and 5'-AGTAGCAAGAA-CAGTTTGTAAATCAGTCGA-3'. The oligonucleotide probe was end labeled with ³²P-ATP and polynucleotide kinase. The labeled probe (50,000 cpm/reaction) was incubated with PBMC nuclear extract protein (~5 μ g) in 20- μ l reactions containing 20 mM Hepes 7.9, 10% glycerol, 3 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, and 1 μ g poly dI:dC for 30 min on ice. Some reactions also contained other oligonucleotides and antibodies to c-Jun or NF- κ B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as indicated for specificity controls. Affinity-purified rabbit anti-human GCR (Affinity BioReagents, Neshanic Station, NJ) was also used for supershift experiments. Reactions were then separated on 6% nondenaturing polyacrylamide gels containing 0.25 \times tris-boric acid EDTA buffer at 250 V until the free oligonucleotide probe had migrated to the bottom of the gel. Gels were then exposed to Kodak XAR5 film (Rochester, NY) and GCR DNA binding was quantitated by densitometry of the developed films.

Saturation binding assays for GCR DNA binding were performed as described above. The reactions contained ~5 μ g of PBMC nuclear extract protein and from 0.1 to 100 ng of labeled glucocorticoid response element (GRE) probe. After the reactions were resolved on polyacrylamide gels, levels of bound and free

probe in each reaction were determined by densitometry of autoradiograms. Dissociation constants (K_d) for GCR DNA binding were derived from the slope of curves of saturation binding data plotted by the method of Scatchard (12).

Western Blot Analysis of GCR. PBMC nuclear extracts were prepared as described above. After correcting for protein concentrations, ~50 μ g of nuclear extract protein was resolved on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. The nitrocellulose blots were blocked with phosphate-buffered saline containing 5% dry milk and 0.1% Tween 20, and then treated with affinity-purified rabbit anti-human GCR (Affinity Bio Reagents, Neshanic Station, NJ). The blots were washed and subsequently treated with goat anti-rabbit IgG conjugated to alkaline phosphatase. After the blots were washed, specific immune complexes were visualized with bromo-chloro-indoyle-phosphate and nitroblue tetrazolium.

Immunohistochemistry Staining. GCR- β immunoreactivity was detected in acetone/methanol-fixed cytopins of PBMCs by the use of a GCR- β -specific polyclonal rabbit antibody raised against human GCR- β . This antibody has previously been shown to be specific for GCR- β (13), and has no cross-reactivity against GCR- α . The specificity of the antibody-antigen reaction for immunocytochemistry was tested by preabsorption of the antibody with excess purified GCR- β antigen which resulted in a loss of reactivity (data not shown). The reaction was visualized using the avidin-biotin-peroxidase complex method as previously described (14). For negative control preparations, the primary antibody was replaced by either nonspecific rabbit immunoglobulin or Tris-buffered saline. The percentage of cells positive for GCR- β in each preparation were enumerated by a blinded assessor counting a minimum of 1,000 total cells.

Statistical Analysis. Group means were expressed as the arithmetic mean \pm standard error of the mean. Statistical comparisons between groups used the Student's *t* test (nonpaired) with the *P* < 0.05 representing a significant result.

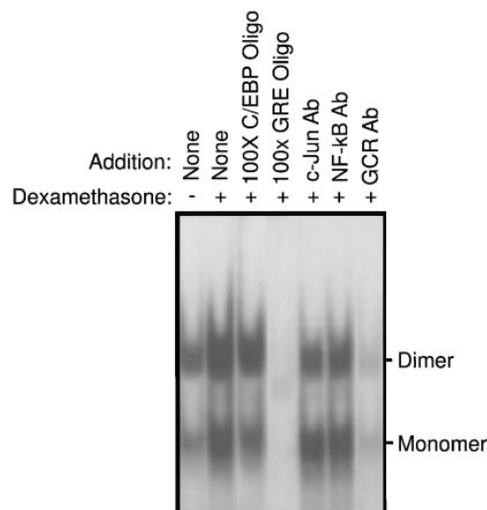
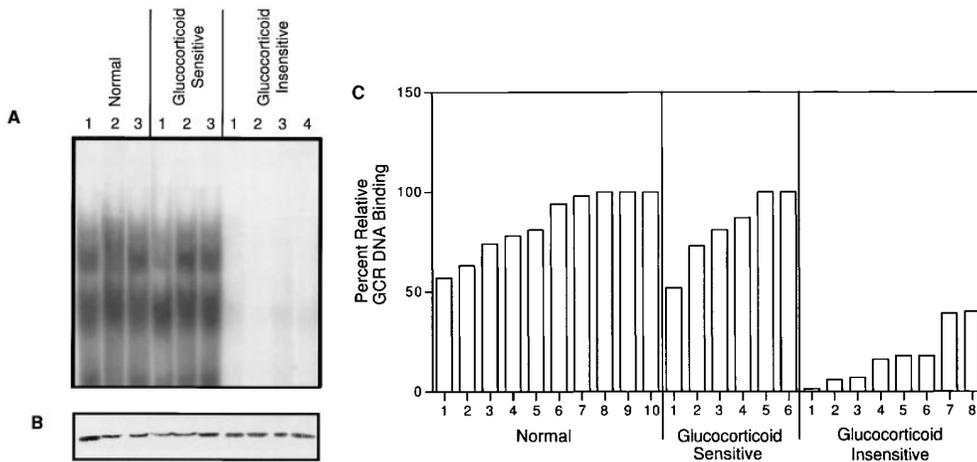


Figure 1. Specificity of electrophoretic mobility shift blot for GCR-GRE interactions using nuclear extracts from a normal subject. The specific monomeric and dimeric GCR-GRE retarded bands are indicated by arrows. The effect of dexamethasone (1 μ M) on the density of the GCR-GRE band is shown at 60 min. Specificity is shown by the inhibitory effect of 100-fold excess of unlabeled oligonucleotide and anti-GCR antibody on GCR-GRE binding.



clear extract protein were assayed in each lane of *A* and *B*. (*C*) The average, relative GCR DNA binding in nuclear extracts from PBMCs of normal, GC-sensitive, and GC-insensitive subjects determined by densitometric quantitation of multiple experiments. Levels are shown relative to the maximum binding observed in normal subjects.

Results

An EMSA was used to measure the interaction between GCR and its specific DNA recognition sequence or GRE. The first step in these studies was to confirm that the DNA binding activity observed in our EMSAs was due to GCR DNA binding. As shown in Fig. 1, two retarded bands, corresponding to monomeric and dimeric GCRs, were observed in EMSAs performed with a 30-bp oligonucleotide containing the consensus GRE. Although these two bands were observed in reactions performed with nuclear extracts from untreated cells, their intensity was much higher in reactions containing extracts from dexamethasone-treated cells. A 100-fold excess of cold nonspecific oligonucleotide containing the consensus CCAAT/enhancer binding protein binding sequence had no effect on the DNA binding activity, but excess cold GRE oligonucleotide completely inhibited formation of the two retarded bands. Likewise, antibodies to the GCR completely blocked complex formation, whereas antibodies to other transcription factors had no effect on DNA binding. The ability of dexamethasone to enhance complex formation and the ability of GCR specific antibodies and oligonucleotides to inhibit their formation clearly indicates that the DNA binding activity observed in these reactions is due to the GCR.

We next compared GCR DNA binding in nuclear extracts from 10 normal subjects, 6 GC-sensitive, and 8 GC-insensitive individuals (Fig. 2). In these experiments, PBMCs were treated with dexamethasone to translocate the GCRs to the nucleus. We found significant levels of GCR DNA binding in nuclear extracts from normal and GC-sensitive individuals (Fig. 2, *A*). When quantitated by densitometry of the autoradiographs, no significant difference in GCR DNA binding levels was noted between these two populations (Fig. 2, *C*). In contrast, nuclear extracts from GC-insensitive patients demonstrated significantly lower ($P < 0.001$) levels of GCR DNA binding than normals or GC-sensitive individuals (Fig. 2, *A* and *C*). The decreased binding in these sam-

ples was not due to decreased GCR protein levels, as Western blot analysis of the nuclear extracts from normal, GC-sensitive, and GC-insensitive subjects revealed comparable levels of GCR protein (Fig. 2, *B*).

The low levels of GCR DNA binding in the GC-insensitive samples appears to be due to decreased GCR DNA binding affinity as compared to binding in normal and GC-sensitive individuals determined by saturation binding experiments. Fig. 3 shows a Scatchard plot of a representative saturation binding experiment comparing GCR DNA binding in a nuclear extract from a GC-sensitive subject to binding in a nuclear extract from a GC-insensitive patient. The slope of the curve derived from the GC-sensitive patient indicates a K_d of $\sim 10^{-9}$ M, whereas the curve obtained from the GC-insensitive patient indicated a lower affinity interaction with a K_d of 10^{-8} M.

We next tested the effect of combination IL-2 and IL-4 on GCR DNA binding in nuclear extracts from GC-insensitive individuals. For these experiments, nuclear extracts

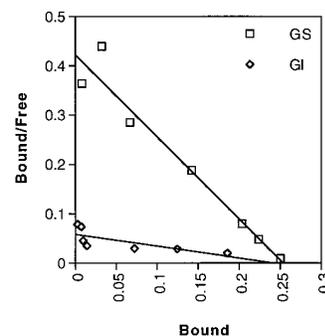


Figure 3. Saturation binding analysis of GCR DNA binding affinity in nuclear extracts from GC-sensitive and GC-insensitive patients. Nuclear extracts were prepared from PBMCs of a GC-sensitive and a GC-insensitive asthmatic. Approximately 5 μ g of nuclear extract protein from each subject was incubated in reactions with concentrations of 32 P-labeled GRE probe from 0.1 to 100 ng. The reactions were separated on polyacrylamide gels and exposed to film. The resulting autoradiograms were subjected to densitometry to quantitate levels of bound and free probe. Both shifted bands were included in the bound probe levels. The figure shows the binding data plotted by the method of Scatchard.

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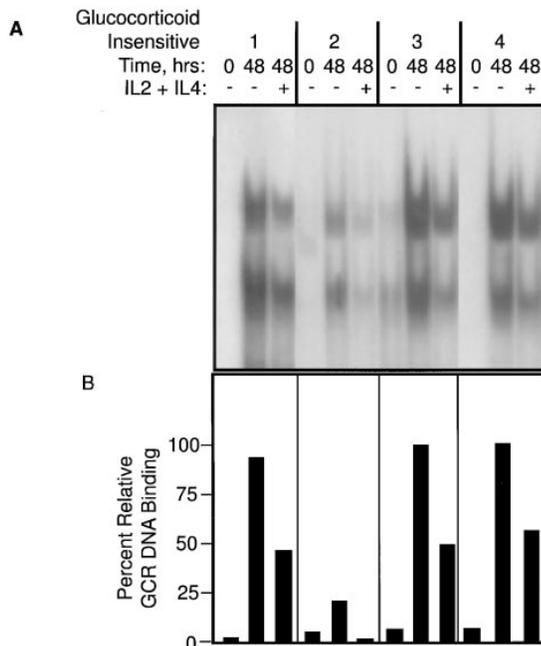


Figure 4. Reversibility and cytokine induction of GCR DNA binding abnormalities in GC-insensitive asthmatics. In these experiments, nuclear extracts were prepared from freshly isolated PBMCs or after incubation with culture medium or the combination of IL-2 (50 U/ml) and IL-4 (50 U/ml) for 48 h at 37°C in 5% CO₂. GCR DNA binding was then analyzed by EMSA as described in the Materials and Methods section.

were prepared from freshly isolated PBMCs or from cells incubated in culture for 48 h in the presence or absence of IL-2 and IL-4. Fig. 4 shows the results of four experiments using PBMCs from four GC-insensitive patients. As expected, very little GCR DNA binding was present in freshly isolated PBMCs from any of the patients. However, after 48 h in the absence of IL-2 and IL-4, there was a significant ($P < 0.01$) increase in GCR DNA binding in all four samples. Interestingly, this increase in GCR DNA binding was blocked or inhibited in the cells cultured in IL-2 and IL-4. These data suggest that the combination of these two cytokines plays a significant role in triggering and maintaining the GCR DNA binding defect.

The actual mechanisms by which cytokines or immune activation might induce a decrease in GCR-GRE binding are unknown. Cloning of the human GCR cDNA and gene revealed that alternative splicing of the GCR pre-messenger RNA (mRNA) gives rise to an additional homologous mRNA and protein isoform, termed GCR- β , that is distinct from the ligand-activated classical GCR, GCR- α . Both mRNAs contain the first eight exons of the GCR gene (15). The remainder is derived by alternative splicing of the nu-

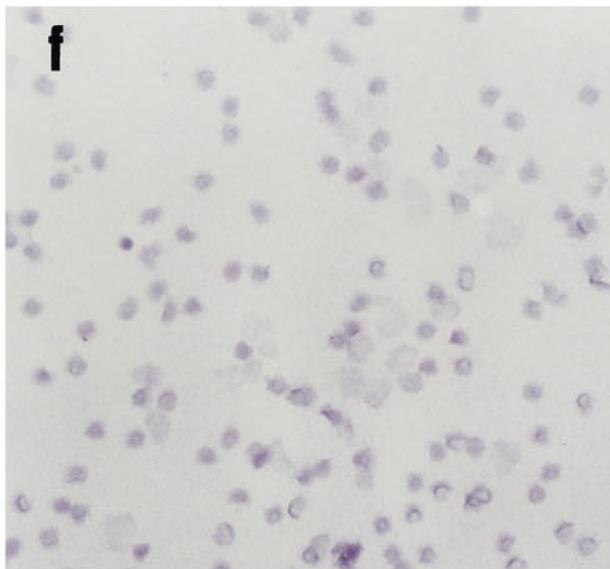
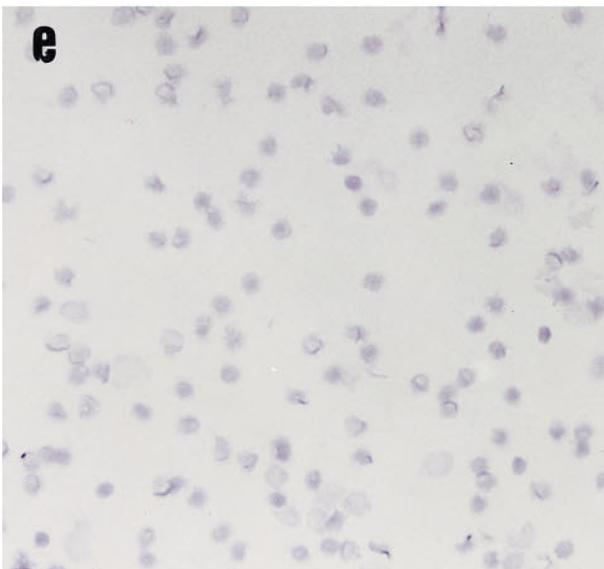
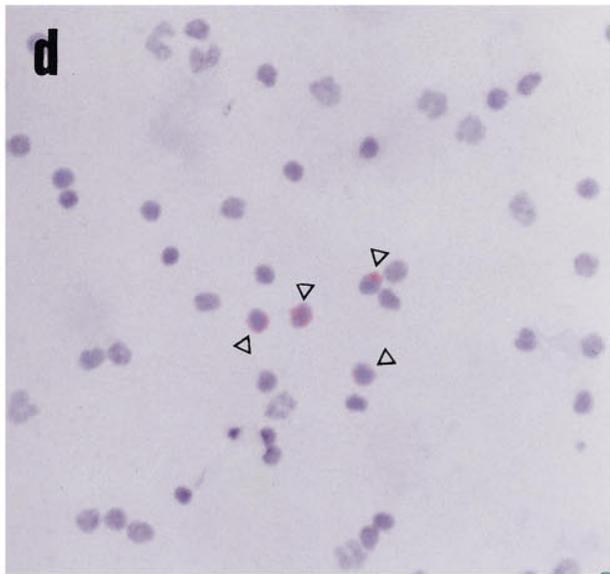
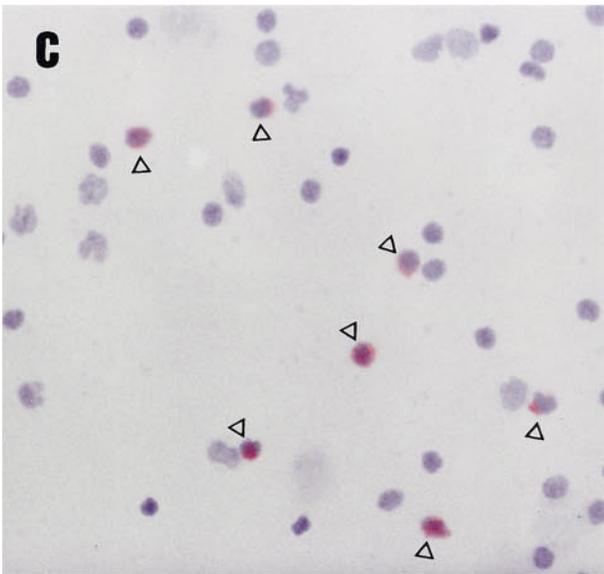
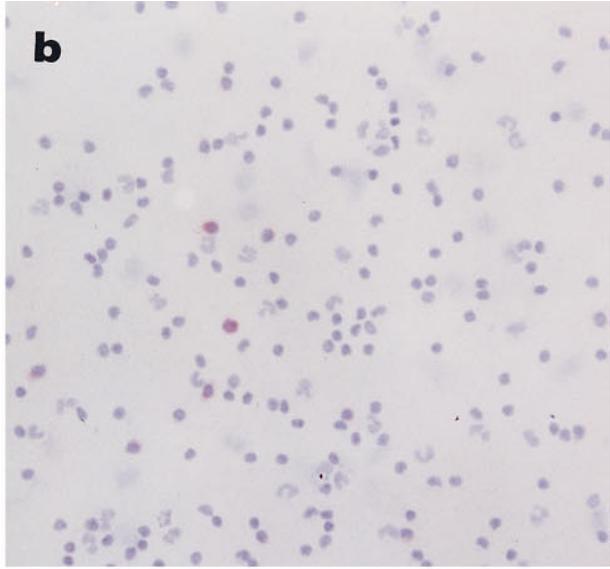
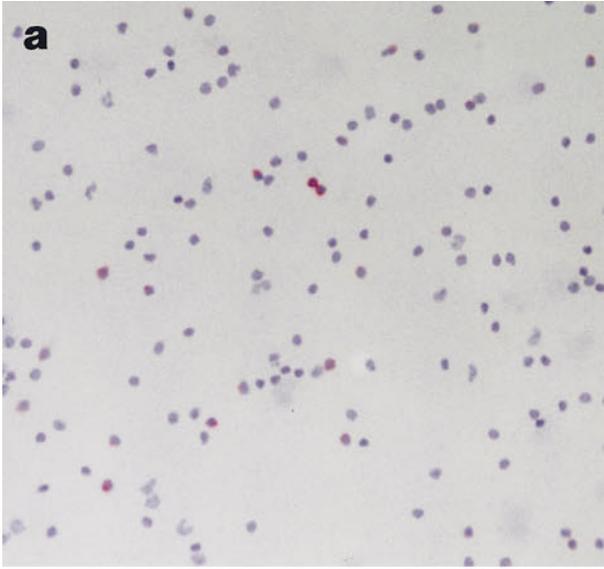
μ g pUC-19	50	49	45	40	0
μ g pHook-1	5	5	5	5	5
μ g pRSV-GCR β	0	1	5	10	50

Figure 5. Overexpression of GCR- β reduces GCR DNA binding affinity. HepG2 cells grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum cotransfected with the indicated amounts of the GCR- β expression plasmid, pRSV-GCR- β , and 5 μ g pHook-1 plasmid by CaPO₄/DNA coprecipitation. Carrier DNA (pUC19) was added to the precipitation reactions to bring them to 60 μ g total DNA. After 48 h, transfected cells were separated from untransfected cells using the Capture-Tec System (Invitrogen) according to the manufacturer's directions. Nuclear extracts were prepared from the cells and equal amounts of nuclear extract protein (5 μ g each) were subjected to electrophoretic mobility shift with labeled GRE probe as described above.

cleotide sequence encoded by the last exon of the GCR gene, corresponding to either exon 9 α or 9 β . The two protein isoforms have the same first 727 NH₂-terminal amino acids. GCR- β differs from GCR- α only in its COOH terminus with replacement of the last 50 amino acids of GCR- α with a unique 15 amino acid sequence. These differences render GCR- β unable to bind GC hormones, thereby antagonizing the transactivating activity of the classic GCR- α molecule (16, 17). Recent studies also indicate that, in the presence of GC, GCR- β heterodimerizes with ligand-bound GCR- α and translocates into the nucleus where it acts as a dominant negative inhibitor of the classic GCR (13).

To determine whether overexpression of GCR- β might also result in reduced GCR DNA binding activity, HepG2 cell lines were transfected with increasing amounts of a plasmid, pRSV-GCR- β , from which the full-length GCR- β protein was expressed. The cells were cotransfected with the plasmid, pHook-1, which allowed us to separate the transfected cells from untransfected cells by using the Capture-Tec System (Invitrogen, Carlsbad, CA). Nuclear extracts from the transfected, GCR- β -expressing cells were subjected to EMSA using the labeled GRE probe. As shown in Fig. 5, the introduction of GCR- β into HepG2 cells inhibits GCR DNA binding activity in nuclear extracts. The ability of GCR- β to block GCR DNA binding exhibited concentration dependence as very little inhibition of binding was noted in nuclear extracts from cells transfected with 1 or 5 μ g of pRSV-GCR- β . However, 10 μ g of GCR- β plasmid decreased GCR DNA binding by ~25%, and 50 μ g of plasmid blocked binding by 70%. Thus, the overexpression of GCR- β in HepG2 cells inhibited GCR DNA binding activity.

Figure 6. Immunohistochemistry staining of PBMCs for expression of GCR- β . Low power magnification ($\times 100$) of cytopsin preparations from PBMCs from (a) GC-insensitive and (b) GC-sensitive patients immunostained for GCR- β . Note the increase in the number of GCR- β -immunoreactive cells in the GC-insensitive compared to GC-sensitive asthmatics. High power magnification ($\times 400$) of GCR- β immunoreactivity in PBMCs of (c) GC-insensitive and (d) GC-sensitive patients. Representative examples of PBMCs from GC-insensitive patients showing (e) immunoglobulin isotype control for GCR- β and (f) competitive inhibition of GCR- β immunoreactivity using preabsorption of the antibody with the GCR- β peptide.



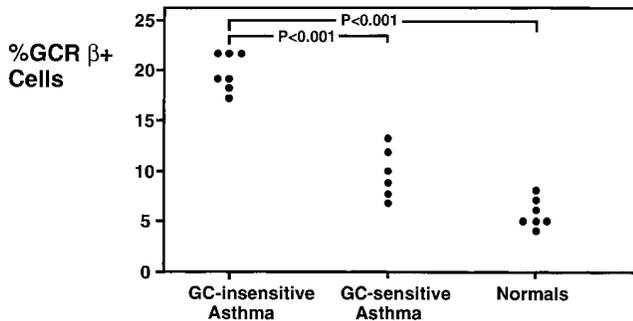


Figure 7. Percent of GCR- β^+ cells in freshly isolated PBMCs from GC-insensitive asthmatics versus GC-sensitive asthmatics and control subjects. PBMCs were processed and stained for GCR- β immunoreactivity as described in Materials and Methods. Cells positive for GCR- β were scored in each preparation, by a blinded assessor, by counting 1,000 cells and expressing them as a percentage of the total cell population.

These data suggest that the increased expression of GCR- β could account for GC-insensitive asthma. To test this hypothesis, PBMCs cytospun from seven GC-insensitive asthmatics, six GC-sensitive asthmatics, and seven normal subjects were stained by immunohistochemistry technique for the expression of GCR- β . Positive staining for GCR- β was observed on all samples stained with anti-GCR- β (see Fig. 6, *a-d*), but not the immunoglobulin isotype control (Fig. 6 *e*). This staining was blocked in the presence of purified GCR- β immunizing peptide indicating that the staining was specific for GCR- β (Fig. 6 *f*). As shown in Fig. 7, GC-insensitive asthma was associated with a significantly higher percentage of GCR- β^+ cells (mean \pm SEM = $20 \pm 0.8\%$; $P < 0.001$) than GC-sensitive asthmatics ($9 \pm 1\%$) or normal controls ($6 \pm 1\%$). This abnormal GCR- β expression in GC-insensitive PBMCs decreased significantly ($P < 0.01$) toward normal after 48 h in culture media (Fig. 8). However, incubation with combination IL-2 and IL-4 sustained this abnormality. Furthermore, combination IL-2 and IL-4 induced significantly greater ($P < 0.001$) GCR- β expression in normal PBMCs as compared to culture medium alone or baseline values (Fig. 8).

Discussion

In the present study, we have demonstrated that patients with GC-insensitive asthma have alterations in PBMC GCR ligand and DNA binding affinity. These GCR binding abnormalities appear to be the result of increased expression of GCR- β , a molecule which we have demonstrated to inhibit the action of GCs in a concentration-dependent manner when its plasmid is transfected into COS-7 cells (16). In this set of studies, we found that expression of GCR- β was cytokine inducible in PBMCs from normal subjects, and reversed to normal levels in GC-insensitive cells incubated in the absence of cytokines.

Since IL-2 and IL-4 are T cell growth factors, the possibility that preincubation with these cytokines could result in a selective expansion of GC-insensitive cells has been previously considered (7). However, this appears to be un-

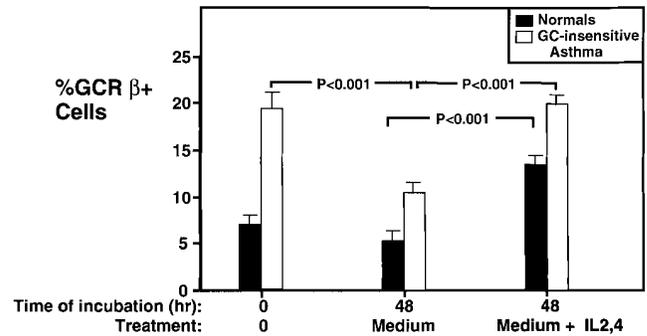


Figure 8. Effect of combination IL-2 and IL-4 on percent GCR- β -immunoreactive cells in PBMCs from normal subjects and GC-insensitive asthmatics. PBMC from four normal donors and three GC-insensitive asthma patients were analyzed for GCR- β immunoreactivity as described in Fig. 5. PBMCs were analyzed for GCR- β immunoreactivity, freshly isolated (time 0) and after incubation in culture medium or the combination of IL-2 and IL-4 for 48 h.

likely since the combination of IL-2 and IL-4 did not induce lymphocyte proliferation, nor was there any change in the proportion of CD4 and CD8 cells over this 48-h time period. Furthermore, incubation with IL-2 alone or IL-4 alone had no effect on GCR binding. Unpublished observations from our laboratory also indicate that GCR alterations induced by the combination of IL-2 and IL-4 begins 1 h after their addition to PBMCs, supporting the concept that these cytokines are not simply acting as T cell growth factors in our cell culture system (Leung, D.Y.M., and S.J. Szefer, unpublished observations).

The mechanism(s) by which combination IL-2 and IL-4 enhances the expression of GCR- β is currently unknown. GCR- α and GCR- β are generated through differential splicing of the same GCR pre-mRNA transcript (13, 16). Changes in GCR- α and GCR- β levels could be due to either differences in the rates of alternative splicing, or in the rates of RNA or protein degradation. Further studies are required to delineate the role of these mechanisms in the pathogenesis of GC insensitivity.

This is the first report demonstrating that cytokine-induced expression of GCR- β may be directly involved in the development of GC-insensitivity in patients with chronic asthma. These observations have important implications as the pathogenesis of other clinical conditions associated with inflammation-induced GC insensitivity may also involve similar mechanisms. During the past three decades there has been a progressive increase in morbidity and mortality due to chronic asthma. This increase in asthma severity has been attributed to changes in our environment, particularly with regard to allergen exposure and air pollution, both of which stimulate airway inflammation in asthma (18, 19). Recent epidemiologic data also indicate that early treatment with inhaled GCs to gain control of immune activation and inflammation, is critical for successful response to GCs (20, 21). Our current observation that immune activation induces the expression of GCR- β and thereby reduces functional responses to GCs is consistent

with the concept that inflammation dampens responses to endogenous and exogenous GCs.

Patients with GC-insensitive asthma have a tissue-specific GC insensitivity and are therefore often subjected to continued high dose treatment with GCs, despite the onset of serious adverse GC effects and poor clinical response to GC therapy. The current study provides a novel marker to

identify patients with GC insensitivity who require alternative forms of antiinflammatory and immunomodulatory therapy. An understanding of the mechanisms by which GCs fail to resolve inflammation in asthma will provide important insights into the pathogenesis of asthma, especially as it relates to progressive deterioration, and should result in the rational design of innovative treatment approaches.

We thank Maureen Plourd-Sandoval for assistance in preparing this manuscript, as well as Lillester A. Colton and Amy Fehringer for their technical assistance.

This work was supported in part by Public Health Services Research grants HL36577, AR41256, and HL37260, and General Clinical Research Center grant 5 MO1 RR00051 from the Division of Research Resources, the University of Colorado Cancer Center, an American Lung Association Asthma Research Center Grant, Medical Research Council Canada, and Inspiraplex.

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Received for publication 28 May 1997 and in revised form 20 August 1997.

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