

Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens

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Background: Microbial superantigens have been described to contribute to the pathogenesis of chronic inflammatory diseases often complicated by insensitivity to glucocorticoid therapy. In bronchial asthma glucocorticoid insensitivity has been associated with increased expression of glucocorticoid receptor β , an endogenous inhibitor of the classic glucocorticoid receptor α . **Objective:** To study a potential mechanism by which superantigens could contribute to poor disease control, we examined their capacity to alter steroid sensitivity and expression of glucocorticoid receptor β .

Methods: The capacity of dexamethasone to inhibit stimulation of PBMCs from 7 healthy subjects with the prototypic superantigens, staphylococcal enterotoxin (SE) B, toxic shock syndrome toxin (TSST)-1 and SEE, versus PHA, was tested. The expression of glucocorticoid receptor β in normal PBMCs after stimulation with SEB, versus PHA, was assessed by immunocytochemistry.

Results: Dexamethasone 10^{-6} mol/L caused a 99% inhibition of PHA-induced PBMC proliferation but only a 19% inhibition of the SEB-induced, 26% inhibition of the TSST-1, and 29% inhibition of the SEE-induced PBMC proliferation ($P < .01$ for all superantigens versus PHA) demonstrating that superantigens can induce steroid insensitivity. Stimulation of normal PBMCs with SEB induced a significant increase of glucocorticoid receptor β compared with PHA and unstimulated cells ($P < .01$).

Conclusion: We have demonstrated the capacity of microbial superantigens to induce glucocorticoid insensitivity, which should be considered in the diagnosis and treatment of patients with superantigen-triggered diseases. These data suggest that superantigens may contribute to glucocorticoid insensitivity through induction of glucocorticoid receptor β . (J Allergy Clin Immunol 2000;105:782-7.)

Key words: Superantigens, dexamethasone, glucocorticoid resistance, glucocorticoid receptor β , inflammation, human PBMCs

Infection and colonization with microbes trigger the onset and aggravate the course of various inflammatory diseases. An important mechanism by which microbes contribute to disease pathogenesis is by the secretion of toxins known as superantigens (SAGs).¹ SAGs bind directly without antigen processing to MHC class II molecules on antigen-presenting cells and trigger a potent activation of T cells expressing selected T-cell receptor β -variable region elements in both CD4⁺ and CD8⁺ T cells. T-cell receptor repertoire analysis of PBMCs or cells from sites of local inflammation demonstrated involvement of SAGs in the pathogenesis of toxic shock syndrome,² Kawasaki disease,³ rheumatoid arthritis,⁴ and recently in severe bronchial asthma.⁵ With use of this approach, SAG involvement has also been implicated in atopic dermatitis⁶⁻⁹ and psoriasis.⁹⁻¹¹

In many SAG-triggered chronic inflammatory diseases immunosuppression with glucocorticoids (GC) is the mainstay of therapy. However, atopic dermatitis is most effectively treated by the combination of GCs with antibiotics.¹² This treatment approach reduces GC doses required for reduction of skin inflammation, suggesting that microbes or microbial products may be involved in the induction of insensitivity to GCs. In bronchial asthma GCs are used to treat airway inflammation and to prevent disease exacerbation. However, during respiratory infections increased doses of inhaled and oral GCs are required to control disease activity. We recently described that SAGs might be involved in maintaining chronic inflammation in a group of asthmatic patients with poorly controlled lung function, although we were not able to identify the specific SAG.⁵ These asthmatics are usually in a progressed disease state frequently associated with decreased sensitivity to GCs. To date, it has been shown that PBMCs of patients with poorly controlled asthma and GC insensitivity have significantly lower responses to the antiproliferative effect of GCs than do healthy subjects^{13,14} but respond well to the suppressive effects of cyclosporin A,¹⁵ suggesting the involvement of cytokines in the pathogenetic process. The mechanism(s) by which cytokines can induce T-cell GC insensitivity is an area of active investigation. In a recent study we found that GC insensitivity in asthma was associated with increased expression of glucocorticoid receptor (GR) β ,¹⁶ a protein isoform of the classical

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Abbreviations used

DEX:	Dexamethasone
GC:	Glucocorticoid
GR:	Glucocorticoid receptor
MFI:	Mean fluorescence intensity
NaN ₃ :	Sodium azide
SAg:	Superantigen
SE:	Staphylococcal enterotoxin
TSST-1:	Toxic shock syndrome toxin-1

GR α , which has been found to act as a potent inhibitor of GC action.¹⁷⁻¹⁹ GR β is generated by alternative splicing of the GR pre-RNA and can be induced in T cells by the combination of IL-2 and IL-4.¹⁶

Because colonization with SAg-producing microbes in atopic dermatitis and respiratory infections in asthma are associated with both worsening of clinical symptoms and increased requirements of GCs, it was of interest to determine whether there was a pathogenic connection between microbial superantigens and the development of GC insensitivity. We therefore assessed in the current study whether microbial SAgS are involved in the development of insensitivity to GC and their effects on the expression of GR β .

MATERIAL AND METHODS

Lymphocyte proliferation assays

PBMCs from 7 healthy donors were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) gradient centrifugation and suspended in RPMI 1640 media (BioWhittaker, Walkersville, Md) containing 10% heat-inactivated (30 minutes at 56°C) FCS, 40 μ mol/L L-glutamine, 100 U/mL penicillin G sodium, 100 U/mL streptomycin, and 20 mmol/L HEPES buffer solution (GIBCO BRL Life Technologies, Grand Island, NY). PBMCs were plated at 2×10^5 cells per well in 96-well round-bottom culture plates (Nunc, Nunc Brand Products, Denmark) and stimulated with 5 μ g/mL PHA (Boehringer Mannheim, Indianapolis, Ind) or 100 ng/mL staphylococcal enterotoxin (SE) E, SEB, or toxic shock syndrome toxin (TSST)-1 (Toxin Technology, Sarasota, Fla) in the absence or presence of 10^{-10} to 10^{-6} mol/L dexamethasone (DEX) (Sigma Chemical, St Louis, Mo). For dose-response experiments, 3 different concentrations of PHA (10 μ g/mL, 5 μ g/mL, 2.5 μ g/mL) and of SEB (100 ng/mL, 10 ng/mL, 1 ng/mL) were used. Cultures were incubated for 72 hours at 37°C in a humidified, 7% carbon dioxide-containing incubator. Six hours before harvesting, tritiated thymidine (ICN Biomedicals, Costa Mesa, Calif) was added at a final concentration of 1 μ Ci per well. Cells were then harvested (Tomtec Harvester 96, Orange, Conn) onto glass fiber filters (Wallac Oy, Turku, Finland), placed into a liquid scintillation cocktail (Wallac, Milton Keynes, UK), and incorporated tritium was counted in a liquid scintillation counter (1450 Microbeta Plus counter, Wallac Oy). For all proliferation assays the experimental values reported represent the mean of triplicate determinations.

Immunocytochemistry staining for GR β

PBMCs from 5 healthy volunteers were suspended in RPMI 1640 media supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, and HEPES buffer solution. PBMCs were plated at

1×10^6 /mL in 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) and cultured for 48 hours at 37°C in a humidified, 7% carbon dioxide-containing incubator in media alone and in the presence of 5 μ g/mL PHA or 100 ng/mL SEB. In 3 individuals PBMCs were stimulated with PHA or SEB in the presence of 5 μ g/mL anti-IL4 mAb (Pepro Tech, Rocky Hill, NJ). After culture, cells were washed once and resuspended in cold PBS at a concentration of 1×10^6 /mL. Cultured and freshly isolated PBMCs were cytospun at 800 revolutions/min onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde.^{16,20} A GR β -specific polyclonal rabbit antibody raised against human GR β was used to detect GR β immunoreactivity. This antibody has previously been shown to be specific for GR β and has no cross-reactivity against GR α .^{16,19} The reaction was visualized with use of an avidin-alkaline phosphatase complex and fast red technique.²¹ In negative controls the GR β antibody was replaced by nonspecific rabbit Ig. A blinded assessor enumerated the percentage of cells positive for GR β in each preparation counting at least 1000 total cells.

Mean fluorescence intensity of GR α and GR β

PBMCs from 8 healthy volunteers were cultured for 48 hours in media alone and in the presence of 5 μ g/mL PHA or 100 ng/mL SEB, as described above. After culture, cells were washed once and resuspended in PBS. Staining was performed at room temperature in 96-well round-bottom culture plates. After fixing in PBS containing 3% paraformaldehyde and 3% sucrose, cells were washed and permeabilized in PBS containing 0.2% Triton-X and 0.02% sodium azide (NaN₃). After washing, nonspecific binding was blocked by incubation in IMDM media (GIBCO BRL Life Technologies) containing 5% FCS and 0.02% NaN₃. Cells were then stained for 1 hour in blocking solution with either the GR β -specific polyclonal rabbit antibody used above or a GR α -specific polyclonal rabbit antibody.¹⁹ In negative controls the specific antibodies were replaced by nonspecific rabbit Ig. After washing, cells were incubated for 1 hour in blocking solution containing cyanine 3-conjugated, affinity-purified donkey-antirabbit-F(ab')₂ fragments (Jackson Immuno Research Laboratories, West Grove, Pa). Stained cells were suspended in PBS containing 5% FCS and 0.02% NaN₃ and were analyzed with a Becton Dickinson FACScalibur, with use of Cellquest software. Intracellular staining was verified by fluorescence microscopy. In 3 individuals PBMCs were stimulated with 100 ng/mL SEB or 1 ng/mL SEB in the absence or presence of 5 μ g/mL anti-IL4 mAb or both, anti-IL4 mAb, and 5 μ g/mL anti-IL2 mAb (Pepro Tech). These cells were stained and analyzed for GR β only.

Statistical analysis

Lymphocyte proliferation to 4 different stimuli (PHA, SEB, TSST-1, and SEE) in the absence or presence of increasing doses of DEX were compared by a 2-way repeated-measures ANOVA test. The expression of GR β + cells in PBMCs by immunocytochemistry staining after different culture conditions was compared with use of Fisher's protected multicomparisons procedure. The overall α level for the set of comparisons was fixed at .05 for statistical significance. *P* values were adjusted for the multicomparisons procedure. The expression of GR α and GR β in PBMCs by intracellular staining was compared with use of the ANOVA *t* test.

Study subjects

Informed consent was obtained from all healthy donors before enrollment in this study, which was approved by the National Jewish Medical and Research Center Institutional Review Board and the Scientific Advisory Committee of the University of Colorado Health Science Center General Clinical Research Center in Denver.

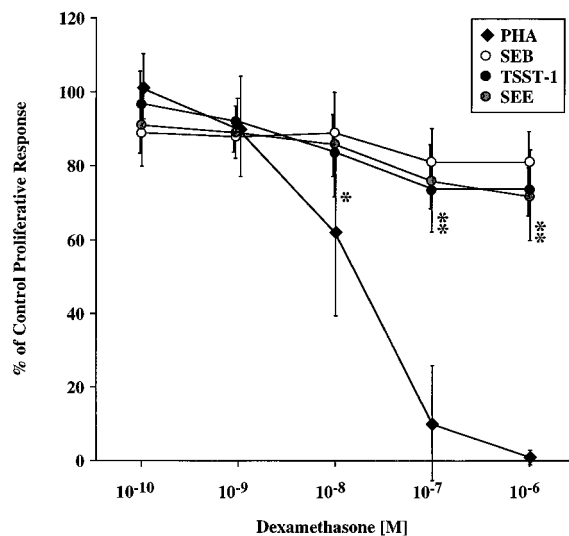


FIG 1. Effect of DEX on PBMC response to SAGs in comparison to PHA. PBMCs of 7 healthy donors were incubated for 72 hours with PHA (5 μ g/mL) or the SAGs SEB, TSST-1, and SEE (100 ng/mL) in the presence or absence of DEX (10^{-10} to 10^{-6} mol/L). SAG-induced PBMC proliferation was significantly less sensitive to DEX than stimulation with PHA. Proliferation response under various conditions was expressed as the percent of mitogen-induced tritiated thymidine incorporation in the absence of DEX compared with the addition of DEX. Data are expressed as mean $\pm$ SEM with a 2-way repeated-measures ANOVA to determine significance (asterisk, $P < .05$; two asterisks, $P < .01$). The mean (range) of the induced lymphocyte proliferation was 171,229 counts/min (104,378–256,596 counts/min) for 5 μ g/mL PHA, 190,952 counts/min (138,718–228,948 counts/min) for 100 ng/mL SEE, 236,839 counts/min (159,696–314,511 counts/min) for 100 ng/mL TSST-1, and 289,719 counts/min (213,326–393,281 counts/min) for 100 ng/mL SEB.

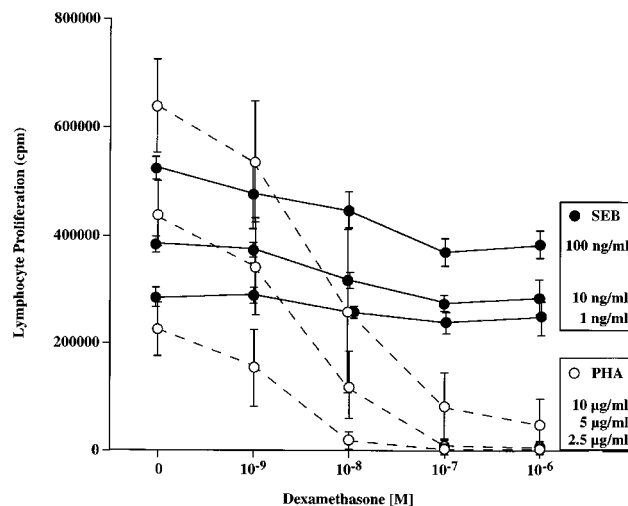


FIG 2. Effect of DEX on PBMC response to different concentrations of SEB in comparison to PHA. PBMCs from 3 healthy donors were stimulated for 72 hours with PHA (2.5, 5, and 10 μ g/mL) or SEB (1, 10, and 100 ng/mL) in the presence or absence of DEX (10^{-9} to 10^{-6} mol/L). Lymphocyte proliferation induced by different concentrations of SEB is insensitive to DEX, whereas proliferation induced by different concentrations of PHA is highly sensitive to DEX. Results are expressed as means $\pm$ SEM.

RESULTS

Effect of DEX on in vitro incubation of PBMCs with SAGs

Because SAGs have been described to contribute to the pathogenesis of various chronic inflammatory diseases frequently associated with a reduced response to GC therapy, we assessed the GC sensitivity of normal PBMCs stimulated with microbial SAGs. PBMCs from 7 healthy volunteers were stimulated with the prototypic SAGs SEB, TSST-1, and SEE compared with PHA in the pres-

ence of increasing concentrations of DEX. In Fig 1 the responses are expressed as percent of the corresponding culture with the stimulus alone (= 100%). After stimulation with PHA, DEX induced a highly significant decrease of T-cell proliferation, particularly at 10^{-6} mol/L DEX ($P < .01$). In contrast, DEX inhibited significantly less the proliferation of T cells stimulated with SAGs. As shown in Fig 1, DEX at concentrations of 10^{-6} mol/L and 10^{-7} mol/L inhibited the lymphocyte proliferation induced by SEB, TSST-1, and SEE significantly less than PHA

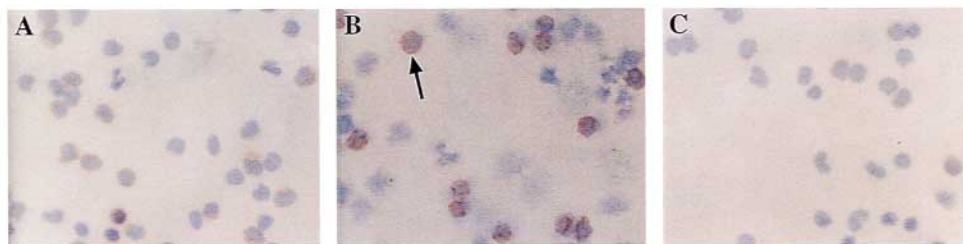


FIG 3. Effect of SEB on number of GR β + PBMCs. Immunocytochemical staining of 4% paraformaldehyde-fixed PBMCs with use of an affinity-purified rabbit polyclonal antibody to GR β was performed. The antigen-antibody complex was visualized with fast red. The number of GR β + PBMCs is increased by culture in the presence of SEB. **A**, An example of a PBMC preparation incubated in media for 48 hours. **B**, An example of PBMCs that were stimulated with SEB (100 ng/mL) for 48 hours. Note the large number of GR β +. **C**, An example of a negative control where the GR β antibody was replaced with nonspecific rabbit Ig.

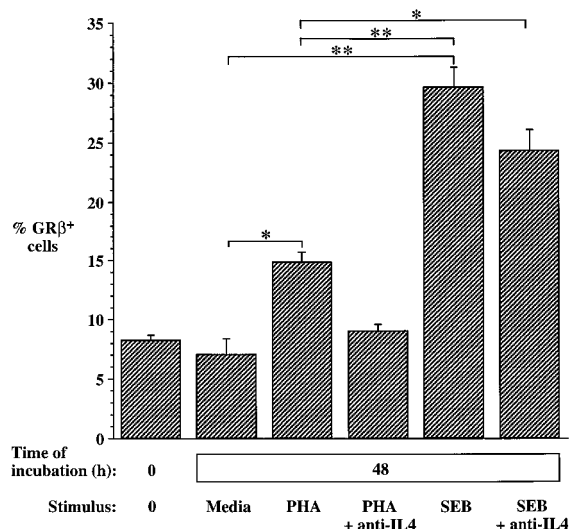


FIG 4. Effect of SEB on number of GR β + PBMCs in comparison with PHA. Freshly isolated PBMCs from healthy donors and their cells after 48 hours of incubation in culture alone or in the presence of 5 μ g/mL PHA or 100 ng/mL SEB were processed and stained for GR β immunoreactivity as described in Material and Methods. In 3 subjects PBMCs were also cultured with PHA or SEB in the presence of anti-IL-4 mAbs (5 μ g/mL). GR β + cells were scored in each preparation by a blinded assessor by counting 1000 cells and expressing them as a percentage of the total cell population. The percentage of GR β + cells in normal PBMCs was significantly higher after stimulation with SEB than with PHA or culture medium alone. PHA induced a significant increase in GR β + cells compared with PBMCs cultured in medium alone. The PHA-induced increase of GR β + cells was completely abolished by anti-IL-4 mAbs. However, the increased number of GR β + cells induced by SEB remained elevated despite coculture with anti-IL-4 mAbs.

did ($P < .01$ in each case). This result was also present at DEX concentrations of 10^{-8} mol/L ($P < .01$ for SEB and TSST-1 versus PHA, $P < .05$ for SEB versus PHA). To rule out a dosing effect, we carried out dose-response experiments for PHA versus SEB (Fig 2) with PBMCs from 3 healthy donors stimulated in the presence of DEX. Lymphocyte proliferation induced by 10 μ g/mL PHA, which was higher than after stimulation with 100 ng/mL SEB used in our previous experiments (Fig 1), was almost

completely inhibited by increasing doses of DEX. This sensitivity to DEX inhibition was present for all concentrations of PHA tested, whereas stimulation with decreasing concentrations of SEB remained relatively insensitive to the inhibitory effect of DEX.

GR β expression after stimulation with SEB

Because high-level GR β expression has been associated with GC insensitivity,¹⁶ it was of interest to determine whether SAg induced GR β . In these experiments, PBMCs from healthy control subjects were evaluated for GR β expression by immunocytochemistry after stimulation with SEB compared with PHA. Positive staining for GR β was observed on all samples stained with anti-GR β (Fig 3, A and B) but not the Ig isotype control (Fig 3, C). This staining was blocked in the presence of purified GR β immunizing peptide, indicating that the staining was specific for GR β .

Compared with PBMCs after culture in media alone, SEB induced a significantly higher number of GR β + cells ($P < .01$). Furthermore, incubation with SEB for 48 hours caused a significantly higher increase in GR β + cells than incubation with PHA ($P < .01$), as shown in Fig 4. The highly increased expression of GR β induced by SEB remained elevated despite culture in the presence of anti-IL-4 mAbs and was significantly higher compared with PHA ($P < .01$). Culture in media alone resulted in a decrease of GR β + cells compared with freshly isolated PBMCs, which could be prevented by culture with PHA (Fig 4). In contrast to stimulation with SEB, the increase of GR β + cells caused by PHA, which was significant compared with culture in media alone ($P < .01$), was decreased below baseline values (time 0) when cultured in presence of anti-IL-4 mAbs.

Because anti-IL-4 mAbs alone did not significantly reduce the number of GR β + cells in PBMCs stimulated with 100 ng/mL SEB, PBMCs from 3 healthy volunteers were stimulated in the absence and presence of anti-IL-4 and also anti-IL-4 and anti-IL-2 mAbs to further evaluate the relative expression of GR β by mean fluorescence intensity (MFI) with intracellular staining. The MFI of GR β significantly increased ($P < .05$) in the blasting cell population (MFI 342.4 ± 32.2 SEM) after SEB stimulation. However, the MFI of GR β in PBMCs stimulated with 100 ng/mL SEB did not change significantly in the presence of anti-IL-4 mAbs (MFI 351.1 ± 53.0 SEM) or both, anti-IL-4 and

anti-IL-2 mAbs (MFI 301.2 + 33.9 SEM). To rule out that we masked an effect of the blocking antibodies on GR β expression by stimulating PBMCs with too high a dose of SEB, we repeated the experiment with a lower dose of SEB. After culture with 1 ng/mL SEB, the MFI of GR β in the the resting cell population of 61.6 (+ 4.8 SEM) was similar to the MFI of cells cultured in media. The evaluation of the blasting cell population revealed again a significant increase ($P < .05$) in the GR β expression (MFI 329.8 + 36.2 SEM), which was not significantly altered by anti-IL-4 mAbs (MFI 320.8 + 24.7 SEM) or by anti-IL-4 and anti-IL-2 mAbs (MFI 342.47 + 41.4 SEM).

Glucocorticoid receptor α and β expression after stimulation with SEB

Because GR β was described to act as an inhibitor of the classical GR α ,¹⁷⁻¹⁹ it was of interest to further evaluate the effects of SEB stimulation on the relative expression of both GR isoforms. To quantify the increase in GR α - and GR β -MFI in different stimulation conditions, we expressed the respective MFI measured in blasting cells as percentage of the MFI from resting cells. In PHA-stimulated PBMCs, we found an increase of 356.7% (+21.4% MFI) in the GR α -MFI ($P < .05$) and of 403.7% (+22.8%) in the GR β -MFI ($P < .05$). In SEB-stimulated PBMCs the GR α -MFI was increased 497.7% (+34.5% SEM, $P < .05$), and the GR β -MFI was increased 626.3% (+35.4% SEM, $P < .05$). In PBMCs stimulated with SEB, the increase of both the GR α - and the GR β -MFI was significantly higher than in the PHA-culture condition ($P < .01$). Most important, however, the increase in GR β -MFI was significantly higher than in GR α -MFI in SEB-stimulated PBMCs ($P < .05$), demonstrating a shift toward overexpression of GR β in the SEB-culture condition.

DISCUSSION

Understanding the mechanism by which SAgS might contribute to poor disease control in SAg-triggered chronic inflammatory diseases would be of great interest. In this study we therefore investigated whether SAgS could induce GC insensitivity because GCs play a crucial role in the control of chronic inflammation. Our data demonstrate that prototypic SAgS can render PBMCs of healthy individuals highly resistant to the T-cell inhibitory effect of DEX. PBMCs of the same individuals, incubated with PHA, responded to equal concentrations of DEX with an almost complete inhibition of their proliferation response. This observation could not be accounted for by a simple dosing effect because concentrations of PHA and SAgS that caused a similar level of T-lymphocyte proliferation revealed GC sensitivity for PHA and GC resistance for SAg-stimulated cells. Furthermore, we have found that SAgS, but not PHA, induce GC insensitivity over a wide range of mitogen concentrations.

To investigate a potential mechanism by which SAgS induce steroid resistance, we assessed the expression of GR β in SAg-stimulated normal PBMCs. Recent studies suggest that GR β , an endogenous inhibitor of the classic

GR α , might play an important role in determining tissue sensitivity to GCs.¹⁷⁻¹⁹ GR β arises from alternative splicing of the GCR premessenger RNA and lacks the ability to bind GCs. It forms heterodimers with hormone-bound GR α and may thereby inhibit GC action.²² Overexpression of GR β in different cell lines by transfection with GR β plasmids inhibits GR binding affinity¹⁶ and transcription of GC-responsive genes¹⁷ in a dose-dependent manner. Disease relevance for GR β has recently been demonstrated in GC-insensitive asthma, which is associated with an increased GR β expression in PBMCs.¹⁶

Our results indicate that incubation of normal PBMCs with SEB significantly increased the percentage of GR β + cells compared with PHA and unstimulated cells. The GR β expression induced in vitro by SEB was even higher than previously observed in the PBMCs of GC-insensitive asthmatic subjects¹⁶ and may therefore contribute significantly to the high level of GC insensitivity during superantigenic stimulation because PBMCs of patients with GC-insensitive asthma also lack a response to GCs. Furthermore, we detected a significantly higher increase in the expression of GR β than of the classic GR α in SEB-stimulated PBMCs, demonstrating a shift toward overexpression of GR β and induction of a GC-insensitive state. Interestingly, incubation with PHA induced more GR β expression than the medium control in normal PBMCs. However, the mechanism for PHA-induced GR β appears to be different from SAg-induced GR β expression. Not only is the level of GR β expression significantly higher with SAgS compared with PHA, coinubation with anti-IL-4 completely abolished the PHA-induced increase in GR β .

Our results indicate that the PHA-induced GR β expression is dependent on increased production of cytokines, such as IL-2 and IL-4, which has previously been shown to be a mechanism for allergen-induced GR binding abnormalities.²³ In contrast, the GR β expression in SEB-stimulated PBMCs stayed highly elevated despite blocking antibodies to IL-4 or to the combination of IL-4 and IL-2. Further studies are needed to determine the mechanism for SAg-induced GR β ; however, our current experiments suggest that GC insensitivity can be induced by several mechanisms of cellular activation.

In conclusion, we have demonstrated here an important pathogenetic role for microbial SAgS in the induction of insensitivity to GCs. SAg-producing microbes may cause a potent T-cell activation in their target tissues, which maintains chronic inflammation if not appropriately treated. Because SAgS may be important in the development of GC insensitivity, it is essential to diagnose and treat infections and colonizations with SAg-producing microbes in SAg-triggered inflammatory diseases to avoid increasing insensitivity to GCs, the cornerstone of anti-inflammatory therapy in many inflammatory diseases. Our current results provide a potential rationale for recent observations that certain antibiotics and intravenous Igs, known to have high antibody titers against SAgS, may be effective in the treatment of diseases triggered by SAgS.²⁴⁻²⁶

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