



Differential control of eosinophil survival by glucocorticoids*

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Glucocorticoids are effective drugs for eosinophil-related disorders, such as asthma and allergy. Previous studies have demonstrated that glucocorticoids increase eosinophil apoptosis and block the survival effect of submaximal concentrations of interleukin-5 (IL-5). We investigated the effect of glucocorticoids on eosinophil survival in the presence of a higher concentration of IL-5 (1 ng/ml), comparable to IL-5 levels in bronchoalveolar lavage and sputum specimens from patients with asthma. In contrast to incubation in the presence of submaximal concentrations of IL-5, the addition of dexamethasone (DEX) to media containing 1 ng/ml IL-5 led to a significant increase in eosinophil cell viability from $58 \pm 6.9\%$ to $87 \pm 2.4\%$ ($p < 0.005$) after 72 hours in culture. We found that RU486 blocked the DEX effect on cell viability confirming that glucocorticoid receptor functions are required. We investigated the possibility that the glucocorticoid enhancement of eosinophil survival may be due to an effect on IL-5 receptor expression. Our results show that the IL-5 associated decrease in IL-5 receptor α -subunit expression was blocked significantly after 24 hrs in culture with media containing IL-5 plus DEX compared to IL-5 alone. It is tempting to speculate that the observed glucocorticoid enhancement of eosinophil survival in the presence of elevated concentrations of IL-5 could be a mechanism that contributes to glucocorticoid resistance in asthma.

Keywords: eosinophil; glucocorticoids; interleukin-5.

Introduction

Asthma is characterized by airway inflammation and is associated with tissue eosinophilia in the lung.^{1–3} Localized eosinophil accumulation likely results from eosinophil infiltration into the airways through chemotactic and cell adhesion mechanisms^{4,5} and by cytokine-mediated in-

creases in eosinophil cell survival at the target site.^{6–9} The increase in eosinophil cell viability is thought to be mediated by cytokine-derived anti-apoptotic signals due to increased levels of IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the airways.^{7,10} Based on the results of eosinophil cell culture studies using recombinant IL-5 and GM-CSF to promote cell survival, and on biochemical measurements showing increased production of IL-5 and GM-CSF at *in vivo* sites of inflammation, it has been proposed that cytokine stimulation of eosinophil survival in the airways of asthmatics is a contributing factor to the pathology of the disease.^{1,11–13} A better understanding of the signaling components involved in eosinophil survival may provide new targets for the treatment of asthma.

The most widely used anti-inflammatory agents for asthma are glucocorticoids which function by binding to the glucocorticoid receptor, a 90 kDa intracellular transcription factor that modulates specific gene activation and repression.^{14,15} Glucocorticoids inhibit the expression of cytokine genes in bronchial epithelial cells^{16–18} and infiltrating T cells.¹⁹ Glucocorticoid-mediated inhibition of cytokine production in the airways indirectly stimulates eosinophil apoptosis by reducing the level of survival signals that contribute to eosinophilia.^{10,20} High dose glucocorticoid treatment has been shown to induce eosinophil apoptosis directly in cell culture.^{12,21} Also, glucocorticoids directly decrease eosinophil survival in the presence of submaximal concentrations of IL-5 (0.01 ng/ml).²⁰ Taken together, it is possible that inhaled glucocorticoids function in the airways to inhibit eosinophil survival by either direct or indirect mechanisms, or both. Some patients are relatively resistant to the anti-inflammatory effects of glucocorticoids, and the mechanisms of glucocorticoids resistance are unclear.

In order to characterize better the direct effect of glucocorticoids in the control of eosinophil survival under clinically relevant conditions, we measured human eosinophil cell viability in the presence of higher levels of IL-5. Surprisingly, in contrast to the glucocorticoid-induced

*This work was supported by grants from the NIH to RLM (HL-60201) and MH (HL-14136).

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decrease in survival of eosinophils in the presence of sub-maximal levels of IL-5, we found that glucocorticoids enhanced eosinophil survival in primary cultures containing levels of IL-5 in the range of those found in clinical specimens.

Methods

Subjects

Healthy, adult subjects were tested by skin prick with 20 common aero-antigens to determine atopic status. Atopic subjects were those responding to any allergen with a mean wheal of ≥ 3 mm. Allergic subjects had mild to moderate allergic rhinitis, but were not asthmatic. Nonatopics did not respond to any of the allergens tested and did not report any allergic symptoms. The protocol for the studies involving human subjects was approved by the Institutional Review Board of the University of Arizona.

Eosinophil isolation

Peripheral blood was collected from subjects by venipuncture and eosinophils were isolated by density-gradient centrifugation and immunomagnetic separation using techniques adapted from Miltenyi and Hansel^{22,23}. Twenty milliliters of citrated blood were mixed with 4 ml of 4.5% dextran in 0.9% saline and allowed to sediment for 45 min at room temperature. The leukocyte-rich upper layer was collected into a 50 ml polypropylene tube and underlayered with 8 ml of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). After centrifugation, the plasma, mononuclear cell layer, and Ficoll layer above the granulocyte-containing pellet were removed. The pellet was washed twice with Hank's balanced salt solution (HBSS) without Ca^{+2} or Mg^{+2} , containing 0.1% gelatin. Contaminating red blood cells were lysed with 0.8 ml sterile distilled water for 5–10 sec, then 7.2 ml 10X HBSS added to stop the reaction, and the volume taken to 50 ml with HBSS containing 2% heat inactivated fetal calf serum (HBSS + FCS) and centrifuged for 12 min at $300 \times g$. The cells were washed once more with HBSS + FCS. After determining the total cell count, anti-CD16 coated magnetic beads (Miltenyi Biotec, Auburn, CA) were mixed with the granulocytes in a ratio of 100 μl beads per 10^8 cells in a volume of 300 μl and incubated at 4°C for 45 min with periodic mixing. After incubation, the cells were resuspended in 2 ml of HBSS + FCS, added to the top of the MACS-CS separation column (Miltenyi Biotec) which had been equilibrated with HBSS + FCS and then placed in the magnetic separator. The column was eluted with 3 to 5 bed volumes of HBSS + FCS. The

eosinophil-containing effluent was collected, centrifuged for 5 min and the pellet resuspended in 2 ml of RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% heat inactivated FCS (RPMI complete media). Aliquots were taken for total cell count determination and cytospin slide preparation. The percentage of eosinophils was determined by microscopy from the stained cytospin slide.

Eosinophil cultures

Eosinophils were resuspended to a concentration of 1×10^5 cells/ml in RPMI complete media and duplicate 1 ml cultures were aliquoted into 24 well tissue culture plates. IL-5 (0.01, 0.1, 0.3, or 1 ng/ml), DEX (10^{-6} , 10^{-7} , 10^{-8} M final; Sigma), RU-486 (10^{-6} final, a gift from Roussel-UCLAF) or diluent (control) were added to the appropriate wells and cultures were incubated at 37°C , 5% CO_2 for 24, 48 or 72 hours. Cells were harvested from the culture plates, washed twice with phosphate buffered saline (PBS), then resuspended in 500 μl PBS. Propidium iodide (5 $\mu\text{g}/\text{ml}$ final concentration) was added 5 min prior to collection on a FACScan flow cytometer using PC-Lysys II software (Becton Dickinson, San Jose, CA).²⁴ The percentage of viable cells for each control and treated sample were calculated.

Caspase activation assay

Caspase activation was assessed with the fluorogenic substrate for caspase-3-like proteases, PhiPhiLuxG₆D₂ (OncoImmunin, Inc., College Park, MD), according to the manufacturer's recommendations. PhiPhiLuxG₆D₂ is a fluorogenic substrate that is cleaved in a DEVD-dependent manner to produce rhodamine molecules, which fluoresce.^{25,26} Briefly, freshly isolated eosinophils or eosinophils cultured for 72 hrs were treated with 10 μM PhiPhiLuxG₆D₂ substrate for 60 min at 37°C . Following treatment, cells were washed, resuspended in 500 μl of ice cold flow cytometry dilution buffer, and kept on ice until flow cytometric analysis. Fluorescence emission was determined using the FL-2 channel of a FACScan flow cytometer (Becton Dickinson).

Analysis of eosinophil IL-5 receptor density

Eosinophils were stained with R-phycoerythrin (PE) labeled mouse anti-human IL-5 receptor α -chain antibody (CDw125, Pharmingen, San Diego, CA) or isotype control. Data were collected from 10^4 cells on a FACScan flow cytometer. The percentage of positive cells was established

by subtracting the background fluorescence determined by analysis of cells stained with the PE-labeled isotype control.

Statistical methods

Statistical analyses were performed using the Statistical Package for the Social Sciences for the PC, version 4.0.1 (SPSS Inc., Chicago, IL). The mean percentage of duplicate wells for each sample was calculated, then the group mean \pm SEM for each treatment determined. Student's unpaired *t*-test was used to determine the statistical significance of differences in the means of responses between atopic and nonatopics. One-way ANOVA was used to determine differences in treatment effects. To determine the significance of DEX enhancement on cell viability of IL-5 cultures at 48 hours for atopic and nonatopic samples (Table 1), the percent viability values were logit transformed. For analysis of the effects of the glucocorticoid receptor antagonist RU-486, and IL-5 receptor density, the Wilcoxon Signed-Rank Test was used.

Results

Effects of DEX in the presence of IL-5

In preliminary experiments, we investigated the effect IL-5 (0.01–1.0 ng/ml) on eosinophil survival after 72 hours in culture. As previously reported,²⁰ the response was dose dependent with a significant increase in eosinophil survival at 0.01 ng/ml and maximal effect at 0.1 ng/ml (data not shown). The addition of DEX to media containing submaximal amounts of IL-5 (0.01 ng/ml), resulted in de-

Table 1. Effect of IL-5 and DEX treatment on caspase activation

Culture condition	Time of incubation (hrs)	Mean fluorescence intensity	Percent cell viability (%)
Control media	0	170 \pm 19	96 \pm 2
Control media	72	541 \pm 31	10 \pm 10
DEX (10^{-8} M)	72	562 \pm 35	4 \pm 1
IL-5 (1 ng/ml)	72	230 \pm 28*	69 \pm 8
IL-5 (1 ng/ml) + DEX (10^{-8} M)	72	173 \pm 22*	90 \pm 2

Freshly isolated and cultured eosinophils in the absence (control media) or presence of IL-5, DEX, or in combination with IL-5 and DEX were treated with PhiPhiLuxG6D2 substrate (10 μ M) and MFI (relative units) determined by flow cytometry analysis as described in Methods. Percent cell viability was determined by flow cytometric analysis of propidium iodide uptake. The MFI for eosinophils treated with IL-5 + DEX is significantly lower (**p* = 0.03) than eosinophils treated with IL-5 alone.

creased cell survival at concentrations of DEX $\geq 10^{-7}$ M (data not shown), consistent with previous reports.^{20,27,28} To investigate the effect of DEX on eosinophil survival in the presence of elevated concentrations of IL-5, comparable to those found in sputum and bronchoalveolar lavage fluid of asthmatic patients, we cultured eosinophils in media containing 1 ng/ml IL-5. Figure 1 shows the results of 12 independent experiments after 48 and 72 hrs in culture. These data demonstrate a statistically significant increase in cell viability in cultures containing IL-5 + DEX, as compared to IL-5 alone. At both time points, it was observed that the DEX-enhancing effects on cell viability were maximal at 10^{-8} to 10^{-7} M DEX. Specifically, cell viability at 72 hrs was increased from 58 \pm 6.9% to 87 \pm 2.4% (*p* < 0.005) when 10^{-8} M DEX was included in the IL-5 (1 ng/ml) containing media.

Figure 1. Glucocorticoid treatment enhances IL-5-mediated eosinophil survival. Eosinophils from twelve individuals were cultured in media containing IL-5 (1 ng/ml) or in media containing both IL-5 (1 ng/ml) and DEX (10^{-10} – 10^{-6} M) for 48 or 72 hours. The mean percent cell viability under each condition is shown. Dexamethasone concentrations showing a significant increase in cell viability, as compared to control cultures, are shown (**p* < 0.005).

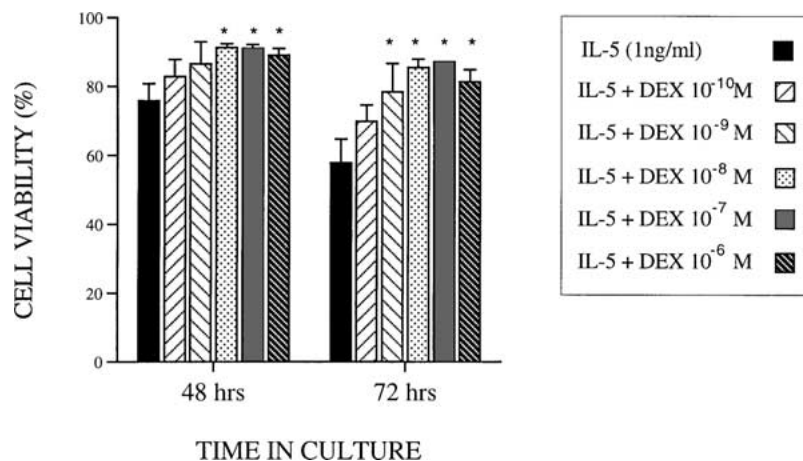


Table 2. DEX treatment enhances IL-5 mediated survival of eosinophils isolated from atopic and nonatopic individuals

Sample	Allergic status	Peripheral blood EOS/ μ l	Percent cell viability at 48 hrs		
			Control	IL-5	IL-5 + DEX
1	Atopic	425	15.9	87.4	89.8
2	Atopic	516	9.2	45.1	90.7
3	Atopic	353	8.3	60.0	93.9
4	Atopic	1567	10.2	46.9	84.6
5	Atopic	791	12.8	89.8	97.0
6	Atopic	710	11.3	88.0	95.3
Mean	–	727 \pm 165*	11.3 \pm 1.1**	69.5 \pm 8.7***	91.9 \pm 1.8***
7	Nonatopic	400	16.9	76.7	91.7
8	Nonatopic	303	26.7	88.8	85.3
9	Nonatopic	243	17.1	95.4	96.3
10	Nonatopic	233	19.0	71.3	90.7
11	Nonatopic	140	45.7	87.9	91.8
12	Nonatopic	230	17.5	72.5	89.3
Mean	–	258 \pm 32*	23.8 \pm 4.2**	82.1 \pm 3.7	90.9 \pm 1.3

The number of eosinophils/ μ l whole blood and mean percent cell viability for duplicate samples after 48 hrs in culture are shown for each eosinophil sample from atopic and nonatopic individuals. The number of eosinophils in the blood of atopic subjects was significantly elevated compared to nonatopic subjects ($p < 0.05$). There was a significant increase in cell viability at 48 hrs of control culture conditions for eosinophils isolated from nonatopic versus atopic subjects ($p = 0.02$). DEX enhanced significantly cell viability in IL-5 treated cultures of eosinophils from atopic subjects ($p = 0.007$, by logit transformation analysis). A similar trend was observed for the nonatopic subjects ($p = 0.07$).

DEX-enhanced cell viability is due to a decrease in caspase activation

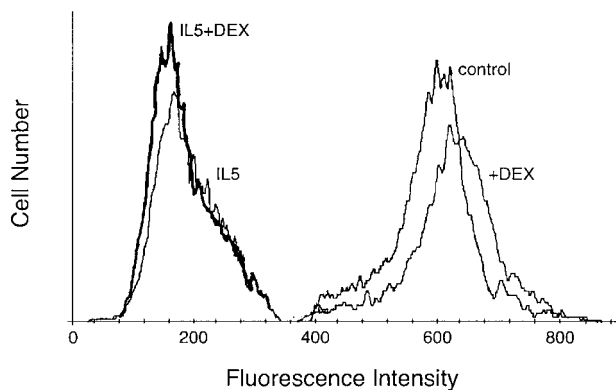
To determine if the unexpected DEX-enhancement of cell viability in the presence of 1 ng/ml IL-5 was due to inhibition of apoptosis, caspase-3 activation was measured directly in live cells using a bifluorophore-derivitized peptide substrate, PhiPhiLuxG₆D₂. PhiPhiLuxG₆D₂ is a fluorogenic substrate that is cleaved in a DEVD-dependent manner.^{25,26} Primary eosinophils were purified and either analyzed immediately or maintained in culture under various conditions for 72 hrs prior to incubation with the cell caspase substrate. Figure 2 shows the result of a representative experiment, and Table 1 summarizes data obtained from three independent primary eosinophil preparations. It can be seen that cells incubated for 72 hrs in the absence of IL-5 (control) display a high-level of caspase activity, based on mean fluorescent intensity (MFI) values. Figure 2 and Table 1 reveal that inclusion of 10⁻⁸ M DEX to cultures containing 1 ng/ml IL-5 causes a decrease in the MFI value. The DEX enhanced survival corresponds to a decrease in the number of cells undergoing apoptosis as measured by a decrease in caspase-activation.

Enhanced cell survival in IL-5 + DEX media as a function of allergic status

Table 2 summarizes the cell viability data from the 48 hr timepoints for the control, IL-5 and IL-5 + DEX cell

cultures using eosinophil preparations from atopic and nonatopic blood donors. It was found that the mean cell viability for control cultures using eosinophils obtained from atopic individuals was significantly lower than the cell viability of control cultures using eosinophils from nonatopic individuals ($p = 0.02$). Viability of eosinophils from atopic donors in the IL-5 containing

Figure 2. The effects of IL-5 and DEX on caspase activation in isolated human eosinophils. Caspase activation was assessed with the fluorogenic substrate for caspase-3-like proteases, PhiPhiLuxG₆D₂, as described in Methods. Fluorescence intensity was determined by flow cytometric analysis. Eosinophils were cultured for 72 hrs with either no treatment ("control"), IL-5 (1 ng/ml; "IL5"), DEX (10⁻⁸ M; "+DEX"), or with IL-5 and DEX ("IL5 + DEX").



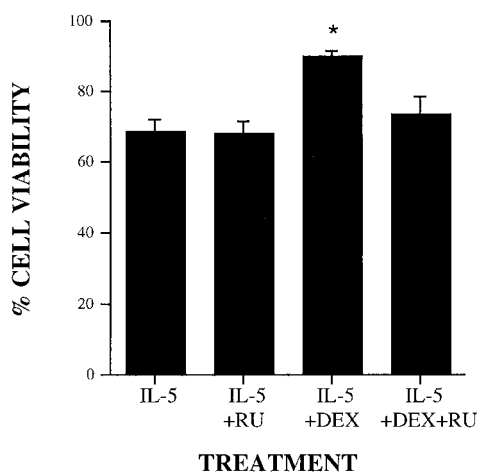
cell cultures, also tended to be less than eosinophils from nonatopic donors, although this difference did not reach significance.

To determine if the observed DEX enhancement of cell viability in IL-5 media (Figure 1) was a function of the allergic status of the subjects, we compared the cell viability of eosinophils from atopic and nonatopic individuals cultured in IL-5 + DEX (10^{-8} M) media at 48 hours. The viability of eosinophils in IL-5 + DEX cell cultures was found to be nearly identical for the atopic and nonatopic individuals used in this study (Table 2). Taken together, these results indicate that the absolute level of DEX-mediated enhancement of eosinophil survival in IL-5 containing cultures was not strictly dependent on the allergic status of the blood donor, however, the magnitude of the observed enhancement was shown to be determined by the level of cell viability achieved in IL-5-supplemented media.

Effect of RU486 on DEX enhancement of eosinophil survival

To investigate the mechanism of DEX enhancement of eosinophil viability in the presence of IL-5, we examined the effect of the glucocorticoid receptor antagonist RU486 in the cell survival assay. As shown in Figure 3,

Figure 3. Inhibition of glucocorticoid enhancement of IL-5 mediated survival by the glucocorticoid receptor antagonist, RU486. Percent eosinophil viability is shown following 72 hrs cell culture in the presence of IL-5 (1 ng/ml) and the presence or absence of RU486 (RU, 10^{-6} M) and DEX (10^{-8} M). Percent cell viability ($n = 4$) was significantly greater following treatment with the combination IL-5 and DEX compared to other treatments (* $p = 0.0004$, Wilcoxon Signed-Rank test, p value corrected for multiple comparisons). There was no significant effect of RU486 on eosinophil survival in control experiments in the absence of IL-5 and DEX (data not shown).



RU486 (10^{-6} M) had no demonstrable effect on IL-5 mediated cell survival, but blocked the DEX enhancement of IL-5 mediated survival. Thus, glucocorticoid receptor functions are required for the DEX enhancement of the IL-5 effect on eosinophil survival.

Effect of DEX on IL-5 receptor expression

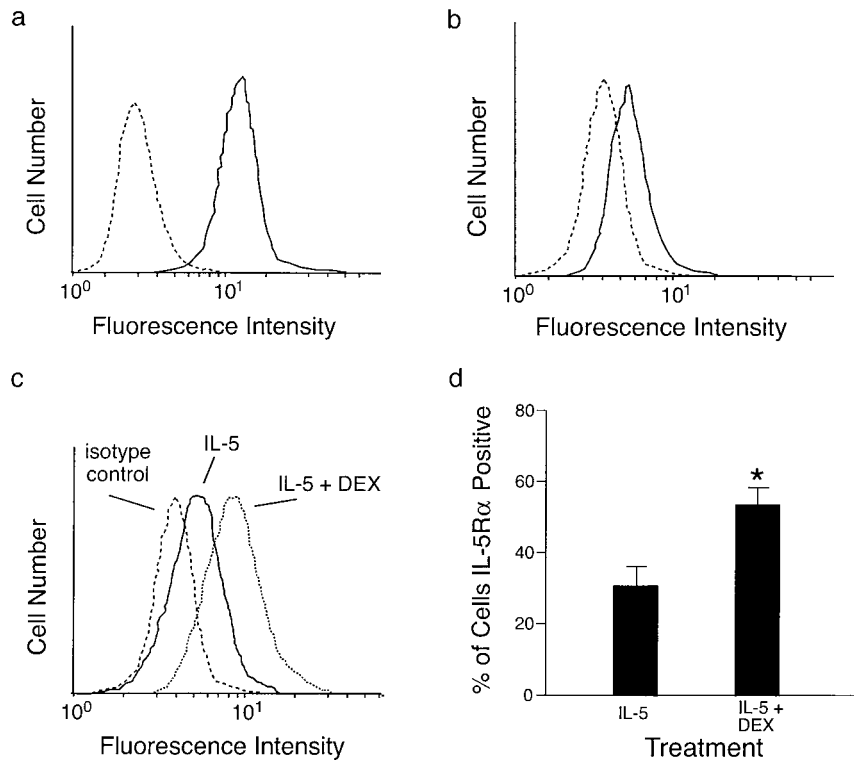
To investigate the possibility that the improved eosinophil survival was associated with elevated surface expression of the IL-5 receptor, we determined the effect of DEX on eosinophil IL-5 receptor α -subunit (IL-5R α) expression by flow cytometry. Immediately following eosinophil isolation, IL-5R α was present on $92.8 \pm 0.9\%$ ($n = 3$) of the cells (Figure 4a). After 24 hrs in culture with IL-5 (1 ng/ml), there was a marked decrease ($31.4 \pm 6.2\%$ positive cells) in detectable IL-5R α levels (Figure 4b and d). Addition of DEX (10^{-8} M) significantly blocked the decrease in IL-5R α expression after 24 hrs in culture in the presence of IL-5 (Figure 4c and d).

Discussion

In this study, we have examined the direct effects of glucocorticoids on eosinophil survival in the presence of clinically relevant concentrations of IL-5. The novel finding in our investigation is that in the presence of higher concentrations of IL-5 (1 ng/ml), comparable to those reported in bronchoalveolar lavage and sputum specimens from patients with asthma, the effect of glucocorticoids is to prolong rather than decrease eosinophil survival. Our results do not contradict earlier studies demonstrating that glucocorticoids decrease eosinophil survival in presence of submaximal concentrations of IL-5 (0.01 ng/ml).^{20,27,28} In clinical studies examining IL-5 levels in patients with asthma, bronchoalveolar lavage and sputum levels have ranged from 0.05 ng/ml to over 1 ng/ml in asthmatic patients.²⁹⁻³¹ In preliminary studies in our primary culture system, we confirmed previous work demonstrating that in the presence of submaximal concentrations of IL-5, glucocorticoids decreased survival of eosinophils (see Results).

An explanation for the observed enhancement of IL-5 mediated survival by DEX is our finding that glucocorticoid treatment inhibits loss of IL-5 receptor over time (Figure 4), thereby increasing eosinophil sensitivity to IL-5. The IL-5 receptor consists of a cytokine-specific α chain (IL-5R α) and a signaling β chain, which is shared by receptors for interleukin-3 and GM-CSF. Responsiveness of eosinophils to IL-5 appears to be controlled at the level of expression of IL-5R α .³² In our studies as well as those reported by other investigators, exposure of human eosinophils to IL-5 decreases expression of IL-5R α .^{33,34}

Figure 4. Effect of IL-5 and DEX on IL-5R α expression in eosinophils. Eosinophils were stained with either PE-labeled antibody to the IL-5R α subunit or isotype control and analyzed by flow cytometry. (a) Histogram derived from flow cytometric analysis of eosinophils analyzed for IL-5R α at the time of isolation prior to culture with IL-5. The tracing shown by the broken line represents the background fluorescence determined by the PE-labeled isotype control. The histogram shown is representative of three individually performed experiments. (b) Histogram of flow cytometric analysis for eosinophils after 24 hrs in culture with IL-5 (1 ng/ml). As in panel 4a, the tracing represented by the broken line is the background fluorescence. The histogram shown is representative of four individually performed experiments. (c) Representative experiment comparing fluorescence intensity for eosinophils cultured for 24 hrs in IL-5 (1 ng/ml) alone or IL-5 + DEX (10^{-8} M). In all experiments ($n = 4$), the mean fluorescence intensity was IL-5 + DEX > IL-5 alone. (d) Mean values for the percentage of IL-5R α positive cells determined by subtraction of isotype control value from the value obtained with the IL-5R α specific antibody. The percentage of positive cells for treatment with IL-5 + DEX was significantly greater than for treatment with IL-5 alone (* $p = 0.01$, Wilcoxon Signed-Rank test).



A recent investigation has demonstrated that decreased expression of IL-5R α on the surface of human eosinophils occurs clinically and is associated with loss of eosinophil responsiveness to IL-5.³⁵ These findings support a relationship between IL-5R α expression and eosinophil survival in the presence of IL-5.

In contrast to the general concept that glucocorticoids function in an inhibitory manner to regulate inflammatory and immune responses, it has been shown that receptors for several cytokines are induced in various cell types by glucocorticoids.³⁶ Furthermore, a recent report suggests that glucocorticoid-induced upregulation of the IL-7 receptor produces an enhanced biological response to IL-7. Positive effects of glucocorticoids on T cell function have been demonstrated by upregulation of the IL-7 receptor α subunit (IL-7R α).³⁷ Thus, glucocorticoids may have differential effects on the expression of cytokines and their receptors, and cytokine regulated responses.

We observed that eosinophils isolated from atopic individuals had reduced viability in control cultures

(lacking IL-5 and DEX), as compared to eosinophils from nonatopics under the same conditions ($p \leq 0.02$) (Table 2). We found no significant survival differences for eosinophils from atopic and nonatopic subjects in the presence of IL-5 or IL-5 plus DEX. Our finding is in contrast to Wedi and coworkers who found prolonged survival of eosinophils from atopic subjects compared to controls.³⁸ The basis for the difference in results between studies is unclear.

Conclusion

We found that the addition of 10^{-8} M DEX to media containing higher concentrations of IL-5 (1 ng/ml), in the range of those found in bronchoalveolar lavage and sputum specimens from asthmatic patients, produced a significant increase in eosinophil cell viability. The effect of DEX to enhance eosinophil survival in the presence of IL-5 was associated with higher expression of the IL-5

receptor α -subunit. These data suggest that the mechanism of DEX enhancement of eosinophil survival may involve a DEX-induced increase in IL-5 receptor expression, which is supported by recent evidence that IL-5 mediated eosinophil activation is controlled at the level of IL-5R α expression. Our findings are likely of clinical importance because downregulation of IL-5R α has been observed in clinical specimens and is associated with decreased eosinophil responsiveness.³⁵ In our studies, the effect on IL-5R α expression was noted at low concentrations of DEX (10 nM), well within the pharmacological range *in vivo*. Furthermore, the glucocorticoid augmentation of eosinophil survival was observed at IL-5 concentrations in the range reported in sputum and bronchoalveolar lavage fluid from symptomatic patients with asthma.^{29–31} In the presence of high local concentrations of IL-5, it is tempting to speculate that glucocorticoid enhancement of eosinophil survival could be a mechanism that contributes to glucocorticoid resistance in asthmatic patients.

Acknowledgments

The authors thank Marilyn Smith, R.N., and Lydia de la Ossa, R.N., for performing skin prick tests, and Mark Brown, M.D., for sample acquisition.

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