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Inhibition of interleukin-5 gene expression by dexamethasone

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SUMMARY

The effect of glucocorticoids on interleukin-5 (IL-5) gene expression was assessed in human peripheral blood mononuclear cells. IL-5 expression was stimulated by phytohaemagglutinin (PHA), IL-2, phorbol myristate acetate (PMA) or Ionomycin. A semi-quantitative assay for IL-5 gene expression was developed, based on RNA extraction and the polymerase chain reaction. IL-5 expression in response to PHA was profoundly inhibited by 10^{-6} M dexamethasone, and significant inhibition was detected at doses of dexamethasone as low as 10^{-9} M. When dexamethasone was added to the cells at the same time as PHA, the inhibitory effect could be detected as early as 3 hr. Dexamethasone at 10^{-6} M also profoundly inhibited the IL-5 response to PMA and to IL-2, but the IL-5 response to Ionomycin was not significantly affected. These results suggest that dexamethasone may be capable of interfering with a pathway involving protein kinase C. There is increasing evidence that IL-5 may play a pathogenic role in asthma and other manifestations of acute hypersensitivity. The present findings indicate that inhibition of IL-5 expression may be one of the mechanisms whereby glucocorticoids exert their beneficial effects in diseases such as asthma.

INTRODUCTION

Interleukin-5 (IL-5) is a cytokine, produced by activated T cells, which has prominent effects on eosinophils. IL-5 promotes the terminal differentiation of the committed eosinophil precursor,[1] as well as enhancing the effector capacity of mature eosinophils.[2] In mice infected with the nematode *Nippostrongylus brasiliensis*, injection of a monoclonal antibody to IL-5 completely suppressed the blood eosinophilia and the pulmonary eosinophil infiltrate[3] suggesting the importance of IL-5 for production of eosinophils *in vivo*.

Evidence is accumulating to suggest that IL-5 has a pathogenic role in acute hypersensitivity. Infiltration of T lymphocytes and eosinophils is a feature of the late-phase response to allergen in atopic individuals.[4,5] In a primate model of asthma, inhibition of the eosinophilic response to allergen substantially blocked the development of airway hyper-responsiveness.[6] Products generated by eosinophils induce hyper-responsiveness of human airways *in vitro*.[7] In bronchial biopsies, T lymphocytes from asthmatics with eosinophilic infiltration contained IL-5 mRNA, whereas none could be detected in asthmatics with no eosinophilic infiltration or in normal controls.[8] Release of IL-5 at the site of allergic inflammation could explain the accumulation and activation of eosinophils in these conditions.

Glucocorticoids are a very effective treatment for asthma. They appear to inhibit the release of inflammatory mediators and the influx of eosinophils and basophils associated with the late-phase response to allergen.[9] The major actions of glucocorticoids on inflammatory cells are the modulation of the composition of the cellular infiltrate which occurs in the allergic reactions, as well as the spectrum of mediators, including cytokines, chemical modulators and enzymes, which are released into the tissue.[10] The ability of glucocorticoids to inhibit the release or action of cytokines is likely to be of great importance in the treatment of asthma. Dexamethasone, a synthetic glucocorticoid, is a potent inhibitor of T-cell proliferation induced by antigen or mitogens[11] and is highly inhibitory to the production by human T cells of the cytokines IL-2, interferon-γ (IFN-γ)[12] and IL-4.[13] In fibroblasts, glucocorticoids inhibited expression of granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-8, but not of M-CSF.[14]

In these studies several different stimuli were used to elicit IL-5 expression in human peripheral blood mononuclear cells. The effect of dexamethasone on IL-5 gene expression was assessed by analysis of RNA using the polymerase chain reaction in a semi-quantitative fashion.

MATERIALS AND METHODS

Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy volunteers by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, washed twice in phosphate-buffered saline (PBS) and resuspended at 1×10^6 cells/ml in RPMI-1640, 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 25 mM HEPES buffer (complete medium).

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Cells were cultured at 37° for 20 hr, unless otherwise specified, in medium alone, or with 1 μg/ml phytohaemagglutinin (PHA) (Burroughs-Wellcome, Dartford, U.K.), 4 ng/ml phorbol myristate acetate (PMA) (Sigma Chemical Co., St Louis, MO), 1 μg/ml Ionomycin (Calbiochem, La Jolla, CA), or 10 U/ml recombinant (r)IL-2 (Boehringer Mannheim, Mannheim, Germany). Dexamethasone (Organon, Lane Cove, Australia) was used at 10^{-6} M unless otherwise specified and added at the same time as the above stimuli (unless otherwise specified).

RNA extraction and cDNA synthesis

Pelleted PBMC were lysed with 100 μl of a denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0·5% sarcosyl, 0·1 M 2-mercaptoethanol. Twenty micrograms of tRNA was added and RNA was extracted by the acid-guanidium-thiocyanate method as previously described[15] and resuspended in 50 μl of water treated with diethylpyrocarbonate (DEPC) (Fluka Chemie AG, Buchs, Switzerland).[16] cDNA was made from 5×10^5 cell equivalents of total cell RNA which was heated to 65° for 5 min and made up in 50 μl with dNTP at 250 μM each, 200 ng of oligo dT_{12-18} (Pharmacia), 4 U AMV Reverse Transcriptase (Pharmacia), 2 U of RNasin (Promega, Madison, WI), 1 × reverse transcriptase (RT) buffer, and DEPC water. 1 × RT buffer contained 50 mM Tris pH 8·3, 8 mM $MgCl_2$, 30 mM KCl and 10 mM DTT. The samples were incubated at 42° for 60 min then heated to 65° for 5 min, and stored at −70°.

Polymerase chain reaction (PCR)

For the β-actin PCR, 4×10^4 cell equivalents of cDNA were amplified, whereas for the IL-5 PCR 5×10^4 cell equivalents of cDNA were used, unless otherwise specified. Reaction mixtures contained dNTP (200 μM final concentration of each), 250 ng of each primer, 1 × PCR buffer, 0·8 U of Hot Tub DNA polymerase (Amersham International, Amersham, U.K.) and DEPC water to a final volume of 50 μl. 1 × PCR buffer was 50 mM Tris-HCl pH 8·2, 1·5 or 2·0 mM $MgCl_2$, 50 mM KCl and 0·001% (wt/vol) gelatin.[17]

Samples were overlaid with mineral oil and amplified by repeated cycles of denaturation at 95° for 1 min, annealing at 58–60° (depending on the primers used) for 30 seconds, and extension at 72° for 30 seconds, using a Gene Machine (Innovonics, Victoria, Australia) or a Thermal Cycler (Hybaid, U.K.). The annealing temperatures for IL-5 and β-actin were 58° and 60° respectively, corresponding to the Tm of each primer. Immediately upon completion of the PCR the samples were slowly cooled from 72 to 37° over 2 hr to reduce the formation of single-stranded product.[18] The primers were designed to span an intron, so that any genomic DNA co-purified with the RNA would yield a larger fragment, thus not contaminating the cDNA of interest. The hybridization primer was designed to bind to a region of cDNA between the two amplification primers. The primers are shown in Table 1.

Analysis of PCR products

The aqueous phase was precipitated with ethanol and the DNA pellet was resuspended and electrophoresed in 1·2% agarose gels, transferred to Hybond N+ nylon membrane (Amersham) in 0·4 M NaOH, neutralized in 2 × SSC (300 mM NaCl, 40 mM Na Citrate, pH 7), and prehybridized in 7% SDS, 0·25 M NaPi pH 7·2, 1 mM EDTA for a minimum of 2 hr at 50°. One hundred and twenty nanograms of hybridization primer, end labelled with ^{32}P using T4 polynucleotide kinase (Pharmacia), was added to the prehybridization buffer and the membranes were hybridized overnight at 50°.

The blots were washed twice with 2 × SSC 0·1% SDS at room temperature for 5 min, and once with 1 × SSC 0·1% SDS at 50° for 15 min. Autoradiography was performed at −70° using Cronex X-ray film (DuPont, Sydney, Australia) or Hyperfilm MP (Amersham). The intensities of the bands were quantitated by a scanning densitometer (LKB). Each band on the autoradiograph was converted to a curve by the densitometer, the area of which was calculated using Gaussian integration. Films with different exposures were measured to ensure that the intensities of the bands were within the linear range of the densitometer. The mean and standard deviation of triplicate area values were calculated for IL-5 and for β-actin and the quotients of the IL-5: β-actin were calculated and expressed as a per cent of the maximum response in each experiment.

RESULTS

Semi-quantitation of the PCR

In initial experiments, the expression of IL-5 mRNA elicited by PHA was strongly inhibited by dexamethasone at a dose of 10^{-6} M. In order to develop a semi-quantitative PCR, studies were performed where identical samples were amplified for different numbers of cycles to establish an appropriate termination point for the PCR.

The autoradiograph of a cycle number experiment on IL-5 is shown in Fig. 1 and densitometric data is plotted against cycle number in Fig. 2. Each sample contained 5×10^4 cell equivalents of cDNA and tubes were removed from the PCR machine after cycle numbers 24, 26, 28 and 30. There was rapid accumulation of IL-5 PCR products between cycles 24 and 30 in the samples treated with PHA, and accumulation of lower levels of PCR products in the samples treated with PHA and 10^{-6} M dexamethasone. For subsequent experiments with IL-5, the cycle number of 28 was chosen. Similar experiments with 4×10^4 cell equivalents led to the choice of 18 cycles for β-actin (Fig. 3).

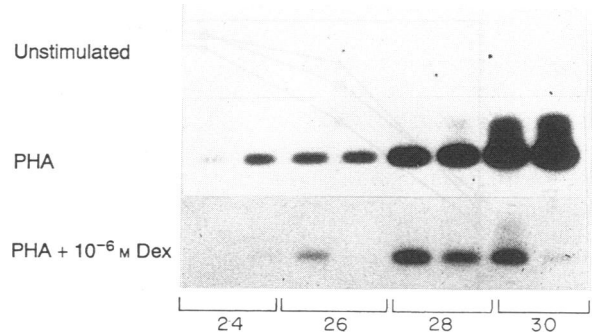

Figure 1. The effect of the number of PCR cycles on IL-5 expression. Identical samples were amplified by PCR for 24, 26, 28 or 30 cycles as shown. Autoradiographs of duplicate samples are shown for each cycle number. PBMC were either unstimulated, or treated with PHA alone, or PHA together with 10^{-6} M dexamethasone (Dex). PHA and dexamethasone were given at the same time and all samples were harvested after 20 hr incubation.

Table 1. Primer sequences

Gene	Primers	Expected fragment size (bp)	Ref.
IL-5			
Amp.	5'CGGATCCCACAGAAATTCCCACAA3'	328	19
Amp.	5'TGATATCCACTCGGTGTTCATTAC3'		
Hyb.	5'CTTCAGTGCACAGTTGGTGAT3'		
β-actin			
Amp.	5'TCACCAACTGGGACGACATG3'	204	20
Amp.	5'GTACAGGGATAGCACAGCCT3'		
Hyb.	5'CAGCCATGTACGTTGCTATC3'		

The sequences of the amplification (Amp.) and hybridization (Hyb.) primers used for each gene and the expected size of the amplified cDNA fragments are shown.

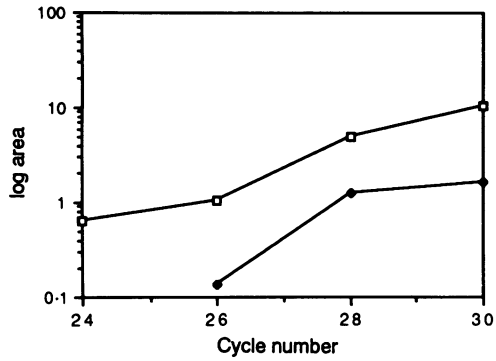

Figure 2. Densitometric scanning data derived from the autoradiograph shown in Fig. 1. The 'area' in arbitrary units (derived from densitometric analysis) is plotted against the cycle number. No IL-5 expression was detected in the unstimulated cells. The means of duplicate samples are shown for each cycle number. PHA (□); PHA + 10^{-6} M Dex (◆).

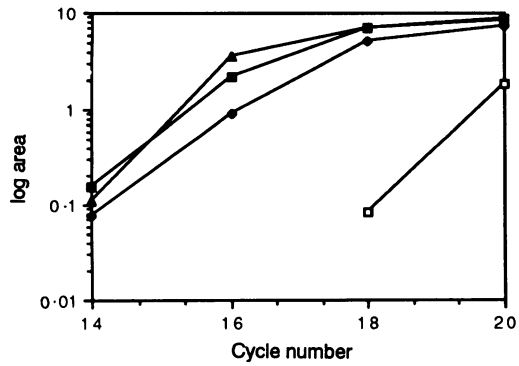

Figure 3. Densitometric scanning data from an experiment on β-actin amplification, from a similar experiment to that described in Figs 1 and 2. The means of duplicate samples are shown for each cycle number. Unstimulated (□); PHA (◆); PHA + 10^{-6} M Dex (■); PHA + 10^{-11} M Dex (▲).

The amount of β-actin expression in the unstimulated cells was significantly less than for the cells activated by PHA, consistent with the lower recovery of total RNA from resting cells. Dexamethasone at doses of 10^{-11} M and 10^{-6} M did not affect the level of β-actin expression in the activated cells (Fig. 3). These results indicate that dexamethasone did not down-regulate the expression of all genes, and that it was feasible to use β-actin as a control for the IL-5 results.

Dexamethasone inhibits IL-5 expression in response to PHA stimulation

The effect of dexamethasone on IL-5 gene expression in response to PHA was assessed in PBMC from 12 different healthy volunteers. PBMC were cultured in the presence of PHA or PHA together with dexamethasone. In each individual, IL-5 expression, measured at 20 hr after stimulation, was strongly inhibited by 10^{-6} M dexamethasone. The mean was 93·0% and the SEM 2·3% ($n = 12$).

Human PBMC were then cultured in the presence of medium alone, PHA, and PHA with 10-fold dilutions of dexamethasone ranging from 10^{-11} M to 10^{-6} M. Dexamethasone inhibited IL-5 gene expression induced by PHA, in a dose-related fashion (Fig. 4). In these experiments, dexamethasone, at 10^{-6} M and 10^{-7} M, suppressed IL-5 gene expression by 95–100%. 10^{-8} M and 10^{-9} M caused marked inhibition, but no effect was seen at 10^{-10} M and 10^{-11} M.

At the concentration of 10^{-6} M, dexamethasone is known to induce apoptosis in T-cell hybridomas, thymocytes and murine T-helper cell lines.[21] To ensure that the inhibition of IL-5 gene expression by dexamethasone was not due to reduced cell viability, cell counts were performed at 20 and 48 hr after stimulation of the cells with PHA and exposure to dexamethasone at the two concentrations of 10^{-11} M and 10^{-6} M, by trypan blue exclusion. The viability in all samples was greater than 95% (data not shown). This finding is consistent with the lack of effect of these doses of dexamethasone on β-actin expression (Fig. 3).

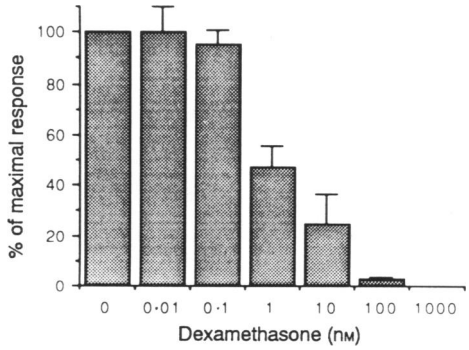

Figure 4. Concentration responses to dexamethasone. Cells were cultured in the presence of PHA and various concentrations of dexamethasone for 20 hr. The IL-5 data shown have been derived from scanning densitometry normalized for β-actin expression. The corrected IL-5 values are presented as a percentage of the response to PHA alone. Results are shown as mean $\pm$ SD for triplicate determinations from one experiment. Similar results were obtained in PBMC from two other donors studied in this manner.

Time–course of the inhibitory action of dexamethasone

IL-5 expression was assessed in PBMC before stimulation, and from 3 to 48 hr after PHA stimulation in the presence and absence of 10^{-6} M dexamethasone. IL-5 expression was not detected in the unstimulated cells or in the cells treated with dexamethasone alone at any time-point (data not shown). In the PBMC stimulated with PHA alone, IL-5 expression was strongly detectable at all time-points (Fig. 5), even as early as 3 hr after stimulation (Fig. 5). In cells treated with PHA together with 10^{-6} M dexamethasone, IL-5 expression was inhibited at all time-points.

Experiments were performed to assess the effect of dexamethasone when added after activation. Cells were activated with PHA at time 0 and dexamethasone was added from time 0 to 4 hr afterwards. All cells were harvested 20 hr after PHA stimulation. The addition of dexamethasone at any of the time-points inhibited IL-5 gene expression by more than 75% (Table 2).

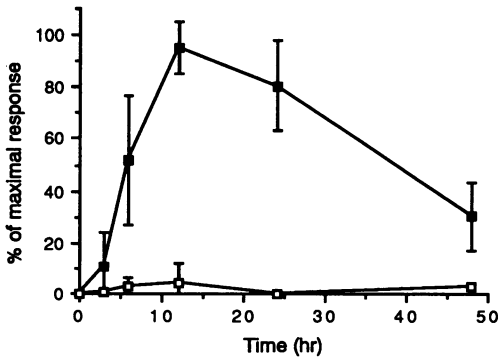

Figure 5. Time–course of the inhibitory effect of dexamethasone. Cells were stimulated with PHA, in the presence or absence of 10^{-6} M dexamethasone at the same time. Cells were harvested at 3, 6, 12, 24 and 48 hr thereafter. Data are the means $\pm$ SD of four determinations of IL-5 expression, each in a separate PCR. Each sample was normalized for β-actin expression. PHA (■); PHA + 10^{-6} M Dex (□).

Table 2. Effect of adding dexamethasone at various times

Time (hr)*	% of maximal response†
0	19·22 $\pm$ 4·97
1	10·27 $\pm$ 8·13
4	23·66 $\pm$ 18·93

* Cells were stimulated with PHA and 10^{-6} M dexamethasone was added from 0 to 4 hr after the addition of PHA, and cells were harvested at 20 hr.

† IL-5 values were quantitated and normalized for β-actin expression. Percentages were calculated with respect to the maximal response (PHA in the absence of dexamethasone). Results shown are means $\pm$ SD of triplicate samples.

Effect of different stimuli on the inhibitory action of dexamethasone

Experiments were performed with several different agents known to stimulate IL-5 expression. PBMC were cultured in the presence of PHA, PMA, Ionomycin, or rIL-2 with and without dexamethasone for 20 hr before the RNA was extracted and amplified by PCR. As can be seen in Table 3, in the absence of dexamethasone, all these stimuli evoked readily detectable IL-5 expression. Dexamethasone completely inhibited the IL-5 response to PMA, and to rIL-2, but had little effect on IL-5 expression in response to Ionomycin. These data are normalized in relation to the β-actin results and the densitometric findings are presented in Table 3.

DISCUSSION

In this study, dexamethasone at doses of 10^{-9} M to 10^{-6} M has been shown to inhibit IL-5 gene expression in unfractionated PBMC from normal human volunteers. It is notable that the half-maximal effect was seen at a dexamethasone concentration of about 10^{-9} M (Fig. 4). This concentration of dexamethasone is the same order of magnitude as that reported for the inhibition of collagenase gene expression.[22]

PBMC are comprised of a mixture of cells including T cells, B cells, monocytes, and natural killer (NK) cells. There is a possibility that the effect of dexamethasone on these other subsets may contribute to the inhibition of IL-5 gene expression. In the case of another T-cell cytokine, IL-2, inhibition of gene expression by dexamethasone has been demonstrated in purified T cells and T-cell clones,[23,24] suggesting that in this instance dexamethasone acts directly on the T cells. Therefore it might be expected that dexamethasone would inhibit IL-5 gene expression in purified T cells. IL-5 expression has been reported in mast cells,[25] but it is unlikely that the PBMC used in the present experiments are contaminated with significant numbers of mast cells or basophils.

IL-5 expression was assessed by the analysis of RNA using the PCR. This approach is specific and very sensitive, and the assay itself is not affected by dexamethasone or other agents that might be carried over from the experiment. Several steps were taken to develop a semi-quantitative PCR. Studies were performed where identical samples were amplified for different

Table 3. Effect of dexamethasone (Dex) on different stimuli

Treatment	Donor	IL-5* (densitometric units)	β-actin* (densitometric units)	IL-5/β-actin	% inhibition
IL-2	1	0·470	5·340	0·088	
IL-2+Dex	1	0·071	7·187	0·010	88
IL-2	2	0·927	6·513	0·142	
IL-2+Dex	2	0·184	5·120	0·036	75
PHA	3	9·772	4·872	2·006	
PHA+Dex	3	1·105	11·098	0·100	95
PMA	3	10·804	8·974	1·204	
PMA+Dex	3	0	7·543	0	100
Ion	3	8·910	17·284	0·516	
Ion+Dex	3	6·908	15·172	0·455	12

Cells were stimulated with the above agents and 10^{-6} M dexamethasone at 0 hr.
Cells were harvested 20 hr after stimulation.
* These values represent areas under the curve as calculated by the densitometer. The ratio of IL-5 to β-actin was then calculated and from this the percentage inhibition of the maximal response was calculated.

numbers of cycles (Figs 1–3) so that the PCR could be terminated while products were accumulating at an exponential rate. Since very small samples of RNA and cDNA are difficult to measure, the quality of the samples was checked by amplifying another gene, β-actin, the expression of which was not affected by dexamethasone (Fig. 3). Samples were always measured in duplicate or triplicate, because small differences in amplification efficiencies from tube to tube can cause large differences in overall PCR yield. Finally, to obtain quantitative data, densitometry was performed on autoradiographs. The effect of glucocorticoids on IL-5 protein production remains to be determined. Similar effects on IL-5 mRNA and protein might be expected because stimulated T cells do not store cytokines in appreciable quantities.

IL-5 gene expression was induced by the stimuli PHA, PMA, Ionomycin and rIL-2, and dexamethasone inhibited the response to all of these agents except Ionomycin (Table 3). PHA activates T lymphocytes, at least in part, by the generation of inositol triphosphate (IP_3), which raises intracellular calcium ion concentration, and diacylglycerol (DAG), which activates protein kinase C (PKC). The effects of IP_3 and DAG can be mimicked by calcium ionophores (e.g. Ionomycin) and phorbol esters (e.g. PMA) respectively.[26] Inhibition of the IL-5 response to PMA but not to Ionomycin (Table 3) suggests that dexamethasone interferes with a pathway requiring protein kinase C, but not that involving increased intracellular calcium. It is difficult to reconcile the findings with Ionomycin and those with PHA, given that PHA elicits raised intracellular calcium ion concentration. Given that Ionomycin is a strong stimulus, the possibility was considered that higher doses of PHA than used in these experiments (1 μg/ml) might not be susceptible to dexamethasone. However, dexamethasone inhibited IL-5 expression induced by doses of PHA up to 30 μg/ml (data not shown). Therefore the difference between PHA and Ionomycin is not explained by the low dose of PHA used in the experiment shown in Table 3.

Many genes, such as those for extracellular proteases and cytokines, are negatively regulated by glucocorticoids, but the mechanism whereby glucocorticoids inhibit the rate of gene transcription has been a matter of controversy.[27] In the case of inhibition of collagenase and stromolysin gene expression by dexamethasone, the glucocorticoid–glucocorticoid receptor complex binds to the AP-1 complex, thereby inhibiting the interaction of AP-1 with DNA.[22,28] AP-1, a heterodimer of the proto-oncogenes *fos* and *jun*, binds to specific DNA sequences in genes whose expression is stimulated by phorbol esters. The phorbol ester PMA stimulated IL-5 gene expression (Table 3), an effect which may be mediated by the AP-1 complex. The published sequence of the 5′ region of the human IL-5 gene[19] does contain a possible AP-1 response element sequence. The negative effects of glucocorticoids on IL-5 expression might therefore be mediated, at least in part, by inhibition of binding of the AP-1 complex to its response element.

This is unlikely to be the sole mechanism of glucocorticoid effects on IL-5 gene expression, because dexamethasone also inhibited IL-5 expression induced by IL-2 (Table 3). IL-2 uses an intracellular signal transduction pathway which is independent of PKC and does not require extracellular calcium.[26] Furthermore, total mRNA levels are a reflection of both the rate of gene transcription and the stability of the mRNA. In the case of IL-2 expression in T cells, glucocorticoids inhibit the rate of gene transcription and accelerate mRNA degradation.[23] It would be interesting to test whether glucocorticoids affect both these parameters in the case of IL-5.

It would be appropriate to extend the present studies to cells in mucosal or cutaneous sites, and to assess the effects of glucocorticoids on the response to allergens. There are differences in the T-lymphocyte populations in the tissues and in the peripheral blood. T lymphocytes in the lung are predominantly $CD45RO^+$ cells, while in the blood there are roughly equal proportions of $CD45RO^+$ and $CD45RA^+$ cells.[29] Within PBMC, the major cell population involved in IL-5 expression is

the $CD4^+$ $CD45RO^+$ subset.[18] IL-5 expression has been demonstrated in cells in the respiratory mucosa of asthmatic individuals[8] but whether these cells are $CD4^+$ $CD45RO^+$ T cells has not yet been evaluated.

In conclusion, dexamethasone inhibits IL-5 gene expression in unfractionated PBMC. This effect may be mediated, at least in part, by interference with the protein kinase C activation pathway. Further study of the interaction of glucocorticoids with the IL-5 gene enhancer will provide useful information for elucidating the molecular pathway(s) resulting in IL-5 gene activation. Inhibition of IL-5 gene expression may represent one of the mechanisms through which glucocorticoid hormones exert their anti-inflammatory actions, particularly in diseases such as asthma.

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