

Allergens, IgE, mediators, inflammatory mechanisms

Glucocorticoids suppressed production and gene expression of interleukin-5 by peripheral blood mononuclear cells in atopic patients and normal subjects

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Background: Interleukin-5 (IL-5) is known to play a major role in regulating eosinophil function in atopic diseases, including bronchial asthma. Glucocorticoids are most effective agents for treating these diseases. However, their mechanism remains unclear. We examined the effect of glucocorticoids on the production and gene expression of IL-5 in atopic patients and normal subjects.

Methods: Human peripheral blood mononuclear cells (PBMCs) in five atopic and four normal subjects were cultured with phytohemagglutinin and phorbol 12-myristate 13-acetate (PMA) in the presence of dexamethasone. IL-5 secreted by PBMCs was assayed by ELISA. Gene expression of IL-5 by PBMCs was assessed semiquantitatively by sequential reverse transcription-polymerase chain reaction, and Southern blot analysis.

Results: Phytohemagglutinin/PMA-stimulated PBMCs from all atopic patients and three normal subjects secreted detectable amounts of IL-5, which were suppressed by dexamethasone in a dose-dependent manner, with 85.8% suppression at 10^{-6} mol/L. Gene expression of IL-5 was detected by reverse-transcription polymerase chain reaction in PBMCs from all subjects, even when not stimulated; was increased by stimulation; and was suppressed by dexamethasone. The concentration of dexamethasone resulting in 50% inhibition in IL-5 gene expression did not differ between atopic patients and normal subjects.

Conclusion: These findings indicate that dexamethasone suppressed IL-5 production in atopic human PBMCs through an inhibitory action on the gene expression. These results suggest that the suppression of IL-5 production through the suppression of IL-5 gene expression is one of the most important mechanisms by which glucocorticoids inhibit eosinophil functions in the treatment of atopic diseases, including bronchial asthma. (*J ALLERGY CLIN IMMUNOL* 1994;93:1006-12.)

Key words: Interleukin-5, glucocorticoids, atopy, mononuclear cells, polymerase chain reaction

Eosinophils are thought to play an important role in atopic diseases, including bronchial asthma. Histologically, bronchial asthma is characterized by the infiltration of a large number of activated eosinophils in the airways, which are

assumed to play an important role in the pathogenesis or pathophysiology of bronchial asthma.¹ A number of cytokines with selective actions to eosinophils have been sequenced. One of the most important is interleukin-5 (IL-5), produced by T lymphocytes, which promotes terminal differentiation of precursor cells,² activates mature eosinophils,³ and enhances the in vitro adhesion of human eosinophils to vascular endothelium.⁴ In animal models antibodies to IL-5 selectively block eosinophil accumulation in the lung.⁵ It has been reported that atopic patients show a higher level of IL-5 gene expression by stimulated peripheral blood mononuclear cells (PBMCs) than normal controls.⁶ In patients with asthma correlations

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Received for publication July 12, 1993; revised Oct. 22, 1993; accepted for publication Nov. 15, 1993.

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0091-6749/94 \$3.00 + 0 1/1/54741

Abbreviations used

bp:	Base pairs
EDTA:	Ethylenediaminetetraacetic acid
IC ₅₀ :	Concentration that results in 50% inhibition
IL:	Interleukin
PBMCs:	Peripheral blood mononuclear cells
PHA:	Phorbol 12-myristate 13-acetate
RT-PCR:	Reverse transcription-polymerase chain reaction
SDS:	Sodium dodecylsulfate
SSPE:	Sodium sulfate phosphate EDTA buffer

between IL-5 mRNA expression in the bronchial mucosa and the number of activated T lymphocytes (CD25⁺), secreting eosinophils (EG2⁺), and total eosinophil count were reported.⁷ These findings suggest that IL-5 plays a major role in regulating eosinophil function in bronchial asthma.

In the treatment of atopic diseases, including bronchial asthma, glucocorticoids are widely used as most effective agents. Despite their dramatic effect, their mechanism remains unclear. Glucocorticoids are known to inhibit the synthesis of several lymphokines, such as IL-1, IL-2, IL-3, IL-4, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, interferon- γ , tumor necrosis factor- α and tumor necrosis factor- β .⁸⁻¹⁴ However, its effect on IL-5 production has not yet been determined. To evaluate the role of glucocorticoids in the treatment of atopic diseases, we examined their effect on IL-5 production and its gene expression in atopic patients.

Recently, Rolfe et al.¹⁵ reported the inhibition of IL-5 gene expression by dexamethasone from PBMCs in healthy subjects by using the reverse transcription-polymerase chain reaction (RT-PCR) method. In this report we demonstrate that glucocorticoids suppressed the production and gene expression of IL-5 in atopic patients and that the suppression of IL-5 gene expression in atopic patients does not differ from that in normal control subjects.

METHODS

Preparation of PBMCs

PBMCs were isolated from venous blood obtained from five atopic and four normal subjects by gradient centrifugation on Ficoll Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), then washed twice in phosphate-buffered saline. All atopic patients had bronchial asthma or allergic rhinitis or both. These atopic patients were not treated with glucocorticoids

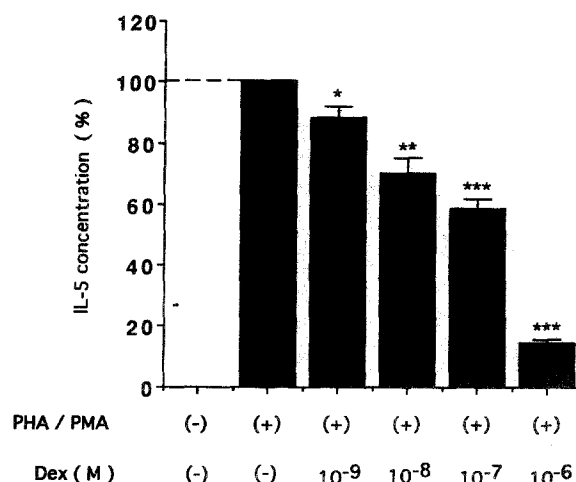


FIG. 1. The effect of dexamethasone on IL-5 production in five atopic patients. PBMC supernatants were assayed for secreted IL-5 by ELISA. IL-5 was not detected (<3.9 pg/ml) without stimulation in all atopic patients. The values are shown as percentages of each PHA/PMA-stimulated value (69.3 $\pm$ 17.7 pg/ml). Each point represents the mean $\pm$ SEM of five experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with PHA/PMA-stimulated value.

and were free from any medication for more than 12 hours.

Cell culture and IL-5 assay

PBMCs were suspended at a final concentration of 5×10^6 cells/ml in RPMI 1640 medium (430-1800 powder; Gibco, Grand Island, N.Y.) supplemented with 10% (vol/vol) fetal bovine serum (Gibco). Cells (10^7) were preincubated for 1 hour in each well of 12-well plates (Costar Corp., Cambridge, Mass.) with or without glucocorticoids (dexamethasone [Banyu, Tokyo] or methylprednisolone, [Upjohn Company, Kalamazoo, Mich.]) and then incubated for 12 hours with 5 μ g/ml phytohemagglutinin (PHA) (Sigma Diagnostics, St. Louis, Mo.) and 10 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma) at 37°C in a humidified atmosphere of 5% CO₂ in air. After culture the cell suspensions were centrifuged, and PBMC supernatants were assayed for accumulated IL-5 by means of ELISA (Suntory, Osaka, Japan) to assess IL-5 production. Sensitivity of the assays was 3.5 pg/ml for IL-5.

RT-PCR

Total RNA was isolated by lysing the cells in RNAzol B (Biotecx Laboratories, Inc., Houston, Texas), following the manufacturer's protocol, precipitated twice in ethanol, and quantified by measurement of absorbance at 260 nm. Total RNA (1 μ g) was converted to first strand cDNA with oligo-dT primer and Moloney murine leukemia virus reverse transcriptase (Gibco), according to the suppliers' instructions. The primer sequences used in PCR for the IL-5 cDNA are 5'-AGAGCCATGAGGATGCTTCT-3' and 5'-GAGT-

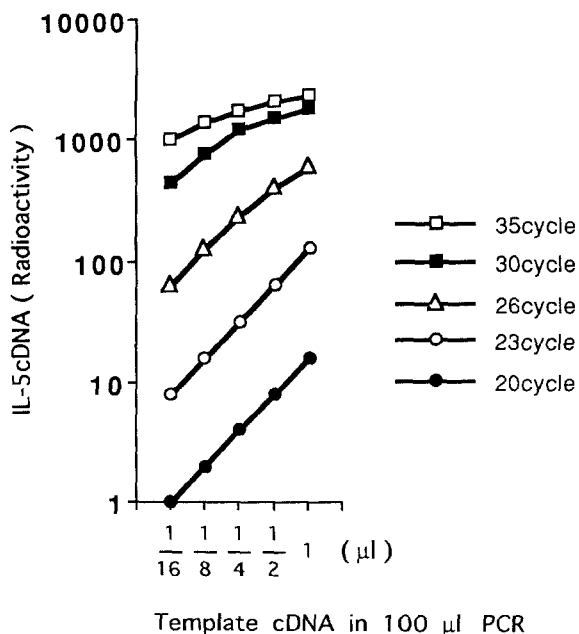


FIG. 2. The effect of the number of PCR cycles on the amplification for IL-5 cDNA. The IL-5 cDNA fragment was amplified proportionally to the cDNA template at 20 and 23 cycles, but not at more than 26 cycles.

TTCTTGGTGTAAATGAACAC-3', an amplified cDNA size of 389 base pairs. The primer sequences for the actin cDNA are 5'-ATGGGTCAGAAGGATTCCTAT-3' and 5'-TGACTTA-GTTGCGTTACAC-3', an amplified cDNA size of 1017 bp. One twentieth of the cDNA synthesis reaction volume was combined at a final volume of 100 µl for PCR amplification by using each primer and 2.4 units of Tth DNA polymerase (Toyobo, Osaka, Japan). For semiquantitative analysis of IL-5 mRNA, PCR was performed for 20 cycles, each cycle consisting of denaturation at 94° C (45 seconds), annealing at 54° C (1 minute), and elongation at 72° C (1 minute). And for analysis of β -actin mRNA also, PCR was performed for 20 cycles, each cycle consisting of denaturation at 94° C (1 minute), annealing at 54° C (1 minute), and elongation at 72° C (3 minutes).

Southern blot analysis

The PCR-amplified products underwent electrophoresis on 1.5% agarose gels in Tris-acetic acid-EDTA buffer and were transferred to nylon membranes (Hybond N⁺, Amersham Corp., Arlington Heights, Ill.). Hybridization was carried out in a solution with a final concentration of 5× sodium sulfate phosphate EDTA buffer (SSPE), 5× Denhardt's solution, 0.5% sodium dodecylsulfate (SDS), 20 µg/ml denatured sperm DNA, and 5 × 10⁵ cpm/ml of phosphorous 32 labeled IL-5 and β -actin oligonucleotide probes for 14 hours. After hybridization, membranes were washed three times in 1 × SSPE/0.1% SDS at room temperature for 30 minutes,

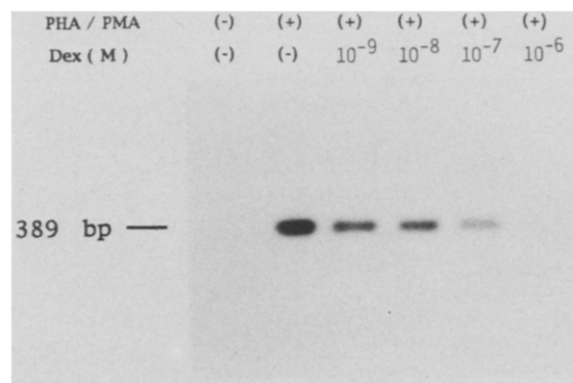


FIG. 3. Southern blot analysis showing the effect of dexamethasone on the induced expression of the IL-5 gene. One representative autoradiograph in atopic patients. The length of the amplified product was 389 bp for IL-5 cDNA.

then once in 1 × SSPE/0.1% SDS at 40° C for 10 minutes. Autoradiography was performed by exposing x-ray film (Fuji Photo Film Co., Tokyo) to washed membranes. The cDNA levels were calculated on the basis of the hybridization signals measured by a Fujix Bio-Image Analyzer BAS 2000 (Fuji Photo Film Co.). ³²P-labeled IL-5 and β -actin oligonucleotide probes, 5'-TCTACTC-ATCGAACTCTGCT-3', and 5'-GATGACCCAGATCATGTTTG-3', respectively, were labeled with T4 polynucleotide kinase (Toyobo), according to the manufacturer's recommendation.

Statistical analysis

Data are expressed as means ± SEM. For mean comparisons, one-way analysis of variance or unpaired two-tailed Student's *t* test was used. Differences were considered statistically significant at *p* < 0.05.

RESULTS

PBMC supernatants were assayed for secreted IL-5 by ELISA in atopic patients. Unstimulated PBMCs did not produce detectable amounts of IL-5 in any of the five patients. PHA/PMA-stimulated PBMCs produced 69.3 ± 17.7 pg/ml (mean ± SEM) of IL-5, and dexamethasone suppressed IL-5 production in a dose-dependent manner, with 85.8% ± 1.5% suppression at 10⁻⁶ mol/L (Fig. 1). The mean concentration of dexamethasone resulting in 50% inhibition (IC₅₀) was 1.6 × 10⁻⁷ mol/L. Similarly, methylprednisolone (10⁻⁶ mol/L) significantly suppressed IL-5 production by stimulated PBMCs of atopic patients by 91.0 ± 5.8% (*n* = 3; *p* < 0.05). In normal subjects 3 of 4 stimulated PBMCs produced detectable amounts of IL-5 (mean, 13.4 ± 3.0 pg/ml). Dexamethasone (10⁻⁶ mol/L) significantly suppressed IL-5 production. The dose-dependent re-

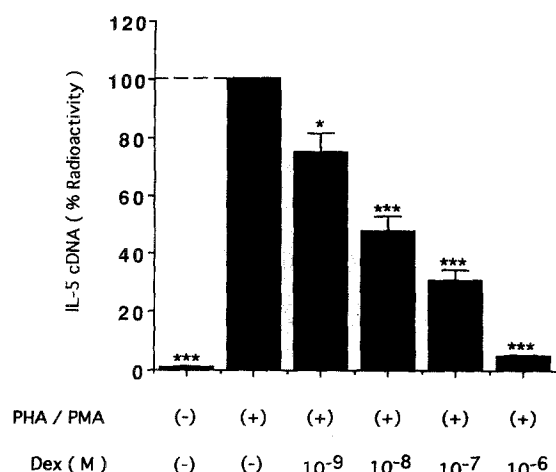


FIG. 4. The effect of dexamethasone on IL-5 gene expression in five atopic patients, assessed by Southern blot analysis. The cDNA levels of IL-5 were determined by the image analyzer. The values are shown as percentages of each PHA/PMA-stimulated value. Each point represents the mean $\pm$ SEM of five experiments. * $p < 0.05$, *** $p < 0.001$ compared with PHA/PMA-stimulated value.

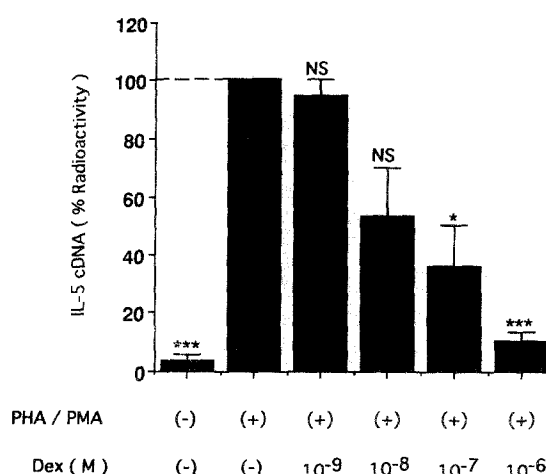


FIG. 5. The effect of dexamethasone on IL-5 gene expression in four normal subjects. The values are shown as percentages of each PHA/PMA-stimulated value. Each point represents the mean $\pm$ SEM of four experiments. NS, Not significant. * $p < 0.05$, *** $p < 0.001$ compared with PHA/PMA-stimulated value.

sponse to dexamethasone could not be assessed, because the concentration of IL-5 was too low for statistical analysis.

To assess the level of IL-5 cDNA semiquantitatively, we first determined the number of PCR cycles at which the saturation of amplification started. The cDNA template that showed the thickest band after 30 PCR cycles was used. Different amounts of the same cDNA template were amplified for 20 to 35 cycles by using IL-5 specific primers. Each PCR product was subjected to a dot blot hybridization with a ³²P-labeled IL-5 oligonucleotide probe. There was a linear relationship between the level of amplified IL-5 cDNA fragment and the amount of the template at 20 and 23 cycles, but not between those at more than 26 cycles (Fig. 2). Therefore we amplified the IL-5 cDNA fragment for 20 cycles for semiquantitative analysis, and the PCR products were transferred to nylon membranes after electrophoresis to prevent possible contamination of genomic DNA. This was followed by Southern blot analysis.

Fig. 3 represents one of the autoradiographs in atopic patients. No band was observed without stimulation, and a thick band, at a calculated size of 389 bp, was observed when stimulated with PHA and PMA, which decreased with increasing concentrations of dexamethasone.

With the use of the Image Analyzer, the IL-5

cDNA band was detected without PHA/PMA stimulation in all atopic patients. Activation of PBMCs with PHA/PMA increased the level of IL-5 cDNA by 212 ± 103 orders of magnitude (Fig. 4). Dexamethasone suppressed the level of IL-5 cDNA in a dose-dependent manner, and the mean IC_{50} was calculated to be 1.0×10^{-8} mol/L.

In normal subjects the IL-5 cDNA band was also detected without PHA/PMA stimulation and increased with PHA/PMA stimulation (Fig. 5). Dexamethasone suppressed the level of IL-5 cDNA in stimulated PBMCs from normal subjects in a dose-dependent manner, as well as in atopic patients. The mean IC_{50} was 2.6×10^{-8} mol/L in normal subjects, which was not significantly different from that of atopic patients. To confirm the quality of extracted RNA and the reverse transcribed cDNA, we also amplified the cDNA of β -actin and quantified it by Southern blot analysis as an internal control. The amplified β -actin cDNA fragment from each first strand cDNA sample obtained from the same individual revealed a similar level. Then, for evaluation of IL-5 gene expression, we used the actual radioactivity of IL-5 cDNA determined by Southern blot analysis without normalization by the amount of amplified β -actin cDNA.

To assess the effect of dexamethasone on IL-5 production induced by an autocrine or paracrine mechanism via IL-2 or IL-4, respectively, PBMCs

from four atopic patients were preincubated with or without 10^{-6} mol/L dexamethasone for 1 hour and then incubated for 12 hours with 10 ng/ml PMA in the presence of 50 U/ml IL-2 or 3000 U/ml IL-4. PBMC supernatants were assayed for secreted IL-5 by ELISA. The concentration of IL-5 was not detected in any patients without stimulation or with PMA alone. The concentration of IL-5 was detected to be 20.6 ± 4.9 pg/ml with PHA/PMA stimulation and 12.2 ± 2.8 pg/ml with IL-2/PMA stimulation. IL-5 production was not detected by IL-4/PMA stimulation in any patients. Dexamethasone (10^{-6} mol/L) suppressed IL-5 production induced by PMA/IL-2 to below the limit of detection.

DISCUSSION

This is the first report to show direct evidence of the inhibition of IL-5 production in atopic patients by glucocorticoids. We evaluated the effect of dexamethasone on the production and gene expression of IL-5 by PBMCs obtained from atopic patients and normal subjects. Staynov and Lee⁶ reported that atopic patients show a higher level of IL-5 gene expression by stimulated PBMCs (69.3 ± 17.3 pg/ml) than normal control subjects (13.4 ± 3.0 pg/ml), which suggests a higher production of IL-5 in atopic patients. Our results confirmed a higher production of IL-5 by atopic PBMCs than by normal ones. In atopic patients dexamethasone suppressed the IL-5 production by PHA/PMA-stimulated PBMCs in a dose-dependent manner, reaching 86% suppression at 10^{-6} mol/L. It is particularly important that IL-5 production was significantly suppressed in atopic patients by dexamethasone in a dose range of 10^{-9} to 10^{-6} mol/L, which was similar to that which might be achieved after therapeutic administration of corticosteroids.¹⁶⁻¹⁸ Similarly, methylprednisolone suppressed the IL-5 production by 91% at 10^{-6} mol/L, suggesting that the suppression of IL-5 production is a common effect of all glucocorticoids.

Dexamethasone suppressed IL-5 gene expression, as well as IL-5 production. In atopic patients dexamethasone suppressed IL-5 production and gene expression, with 11.6% and 25.2% suppression at 10^{-9} mol/L, with 30.1% and 52.6% suppression at 10^{-8} mol/L, with 41.8% and 69.5% suppression at 10^{-7} mol/L, and with 85.8% and 95.3% suppression at 10^{-6} mol/L, respectively (Figs. 1 and 4). There was a significant difference between IL-5 production and gene expres-

sion at a concentration of greater than 10^{-8} mol/L dexamethasone. Since dexamethasone suppressed IL-5 gene expression rather than IL-5 production, it is suggested that this suppression is the most efficient at the gene expression level. However, additional effects of dexamethasone on other processes, including translation and secretion, to control IL-5 production cannot be ruled out by the present experiment. The discrepancy of the sensitivity to dexamethasone between IL-5 production and its gene expression might be explained by a mechanism that depends on the time course: the IL-5 cDNA level indicates the transient level of IL-5 mRNA at 12 hours, whereas the IL-5 concentration in the culture medium indicates not the transient level of IL-5 translation, production, or secretion rate, but the accumulation of protein secreted for 12 hours. Further studies will be required for precise evaluation.

During the preparation of this article, Rolfe et al.¹⁵ reported a suppressive effect of dexamethasone on IL-5 gene expression by stimulated PBMCs in healthy subjects by using the RT-PCR method. Essentially, our data support theirs with regard to the gene expression of IL-5 in normal subjects. However, 10^{-9} mol/L dexamethasone suppressed IL-5 gene expression more than 50% (5.5% in our study). We cultured PBMCs with PHA and PMA for 12 hours, but they cultured PBMCs for 24 hours. This difference might cause a difference in the inhibitory effect. However, the number of subjects in both studies was too small to make definite conclusions.

Our results did not indicate a significant difference in the sensitivity (IC_{50}) to dexamethasone in IL-5 gene expression between atopic and normal subjects, although atopic patients demonstrated a higher level of IL-5 production by PHA/PMA. Since the sensitivity in atopic PBMCs to dexamethasone in IL-5 gene expression was similar to that in normal ones, the difference in sensitivity of lymphocyte to glucocorticoid does not explain the difference in IL-5 production between atopic patients and normal subjects. Factors other than the hyposensitivity of T lymphocytes to intrinsic glucocorticoids may play a more important role. Among normal healthy persons, both glucocorticoid-sensitive and resistant groups have been distinguished by lymphocyte blastogenesis.¹⁹ Carmichael et al.²⁰ described two groups of patients with chronic asthma who were clinically sensitive and resistant to treatment with prednisolone. And Corrigan et al.^{21, 22} showed an insensitivity of

T lymphocytes to dexamethasone in inhibiting thymidine uptake and IL-2 production by PHA stimulation in patients with asthma that is resistant to prednisolone. They suggested that clinical glucocorticoid resistance might be at least partly related to a relative insensitivity of T lymphocytes to glucocorticoids. Although our results suggest that the sensitivity of PBMCs to glucocorticoids is not a major determinant of the difference in IL-5 production between atopic patients and normal subjects, the sensitivity might result in the different IL-5 production among atopic patients. The role of sensitivity to glucocorticoids in controlling IL-5 production in atopic pathophysiology should be clarified further.

In murine T-helper type 2 lymphocytes, differential regulation of IL-5 and IL-4 gene expression has been reported.²¹ IL-5 was shown to be induced by IL-4 as an autocrine or by IL-2 as a paracrine system, but IL-4 was not. According to our results IL-2 directly induced IL-5 production, but IL-4 did not. However, we could not determine the effect of IL-4 precisely because we did not assess IL-5 gene expression, which might have been a sufficiently sensitive indicator of the effect. Even if IL-5 gene expression were detected, the effect of IL-4 would be much smaller than that of IL-2. Because gene expressions of IL-2 and IL-4 were remarkably induced by PHA and PMA (data not shown), it is suggested that IL-5 production is partly induced by IL-2, an autocrine/paracrine mechanism when stimulated by PHA and PMA. Since dexamethasone obviously suppressed IL-2 gene expression by PHA/PMA-stimulated PBMCs (data not shown), dexamethasone is suggested to suppress IL-5 production through both IL-2-dependent and IL-2-independent pathways. It is important for the next study to elucidate the molecular mechanisms of the suppressive effect of glucocorticoids on IL-5 gene expression and its production and to determine the possible role of suppression of IL-5 production by glucocorticoids in the treatment of atopic diseases.

In conclusion, this report indicates that glucocorticoids suppressed the production and gene expression of IL-5 in atopic patients, that glucocorticoids suppressed IL-5 production at the gene expression level, and that the sensitivity of atopic PBMCs to dexamethasone was not different from that of normal PBMCs. The suppression of IL-5 production through the suppression of IL-5 gene expression is suggested to be one of the most important mechanisms by which glucocorticoids

inhibit eosinophil functions in the treatment of atopic diseases, including bronchial asthma.

We thank Mr. Ken Chiba, Miyagi Red Cross Blood Center, for help in preparing the sample.

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