Induction of Interleukin-4 and Interleukin-5 Expression in Mast Cells Is Inhibited by Glucocorticoids

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Inflammation in asthma and other allergic diseases is characterized by excessive production of immunoglobulin E (IgE) and the influx of leukocytes, especially eosinophils. Interleukin 4 (IL-4) and IL-5 are essential for IgE production and eosinophilia, respectively, and are produced by mast cells in allergic conditions, for which glucocorticoids are widely used therapeutically. We assessed the effect of glucocorticoids on IL-4 and IL-5 mRNA production by the RBL-2H3 cell line, an analog of mucosal mast cells. IL-4 and IL-5 mRNAs were induced by an antigen that is used to cross-link receptor-bound IgE, by calcium ionophore, or by ionophore with phorbol ester and were markedly inhibited by dexamethasone. In cells activated with ionophore and phorbol ester, $10^{-6}$ M dexamethasone reduced the IL-4 and IL-5 mRNA levels to only 12.8 and 5.7%, respectively, of those in cells without dexamethasone, and $10^{-9}$ M dexamethasone caused reductions to 27 and 56%, respectively. Hydrocortisone at $10^{-6}$ and $10^{-7}$ M almost completely inhibited IL-4 and IL-5 mRNA production. Dexamethasone was markedly inhibitory even if it was added after the cells were activated, provided that it was present in the cultures for at least 1.5 h. These studies indicate that the expression of IL-4 and IL-5 mRNAs by mast cells is highly sensitive to glucocorticoids. The data suggest that these inhibitory effects may contribute to the clinical efficacy of glucocorticoids in the therapy of allergic diseases.

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total β-hexosaminidase content. Dexamethasone and hydrocortisone were obtained from Sigma (St. Louis, Mo.); the water-soluble form of dexamethasone was used. For experiments involving antigen activation, a 2,4-dinitrophenol (DNP)-specific monoclonal IgE antibody (75 ng/ml; Sigma) was added at the time that the cells were seeded.

Cells were washed twice with MEM (Gibco BRL) containing 200 mg of CaCl₂ per liter but without fetal calf serum. All of the following activators were diluted in MEM at the indicated concentrations unless stated otherwise: 100 ng of DNP-bovine serum albumin (BSA) per ml (24 molecules of DNP conjugated with 1 molecule of BSA), kindly donated by H. Metzger, National Institutes of Health, Bethesda, Md.; 1,000 nM A23187 (Sigma), and 50 nM phosphoryl myristate acetate PMA (Sigma). In preliminary experiments, IL-4 mRNA was elicited to a similar extent by DNP-BSA concentrations of 10 to 1,000 ng/ml (data not shown). The cells were incubated in the solutions for 30 min, and the supernatants were collected for analysis of β-hexosaminidase secretion. The activated cells were then incubated for the remaining time in MEM without CaCl₂ and were then lysed for RNA extraction. For samples treated with glucocorticoids overnight, prior to activation, cells were maintained in glucocorticoids throughout the time between activation and RNA extraction.

Analysis of IL-4 and IL-5 mRNAs. The cells were harvested 4 h after activation unless otherwise stated. Total cellular RNA was extracted as described previously (8), the RNA concentration was estimated by measuring the optical density at 260 nm, and cDNA was prepared as described previously (23) from 1 µg of RNA in 50-µl volumes containing oligo(dT) (4 ng/µl) and 4 µl of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). PCR was performed with cDNA derived from 0.1 µg of total RNA with 1 U of Taq polymerase (Boehringer Mannheim, Mannheim, Germany) and 250 ng of each amplification primer as described previously (23). PCR was performed in a Gene Machine (Innovenics, Melbourne, Victoria, Australia) for 26 cycles for IL-4, 35 cycles for IL-5, and 22 cycles for β-actin. Denaturation, annealing, and extension conditions were 95°C for 60 s, 58°C for 30 s, and 75°C for 30 s, respectively. The primers, based on published sequences (22, 25, 36), were 5′-ACCTGCTGTACCGTCCTGTC-3′ and 5′-TTGGAGGACGGTGAACCTC-3′ for IL-4, 5′-CTCTTGGAGGAACTGAGGAC-3′ and 5′-CTCTTGGCAAGTAAATCCAGGA-3′ for IL-5, and 5′-TAACCACTTGGGAGATATG-3′ for β-actin. The expected product sizes were 351 bp for IL-4, 239 bp for IL-5, and 202 bp for β-actin. The primers were designed to anneal to different exons so that any contaminating genomic DNA in the cDNA samples would contain one or more introns and would yield a product larger than that derived from cDNA. PCR products were electrophoresed in 2% agarose gels and were stained with ethidium bromide. The sizes of the PCR products were determined with reference to molecular size markers (dnX174 cleaved with HaeIII; Boehringer Mannheim). In some experiments, the gels were photographed with reversed-image film (Polaroid), and band intensities were measured by laser densitometry (Molecular Dynamics, Sunnyvale, Calif.). In other experiments, the specificities of the products were confirmed by Southern blotting. The gels were transferred to a nylon membrane (Hybond N+; Amersham, Amersham, Buckinghamshire, England) under vacuum, and the membranes were hybridized to oligonucleotide probes designed to anneal to the PCR product but not to the amplification primers. The hybridization probes were 5′-TACCTCGGTGTCGTTAAGAACG-3′ for IL-4, 5′-TCGATGTTCGCCTCCT-3′ for IL-5, and 5′-CAGCCATGTTCGATTC-3′ for β-actin. The expected product sizes were 351 bp for IL-4, 239 bp for IL-5, and 202 bp for β-actin. The membranes were washed and exposed to X-ray film (DuPont, Wilmington, Del.). After development of the films, band intensities were determined by laser densitometry as described above.

Reverse transcription-PCR (RT-PCR) for β-actin was performed with all samples, but the samples had been reduced or heated to inactivate the reverse transcriptase for 5 min after the cells had been activated. Supernatants (20 µl) were incubated with 20 µl of 5 M p-nitrophenyl-N-acetyl-D-glucosamine (Sigma) in 0.05 M sodium carbonate buffer (pH 4.5) in triplicate at a 96-well plate at 37°C for 2 h. At the end of the incubation, 200 µl of 0.1 M sodium carbonate-sodium bicarbonate buffer (pH 9.8) was added, and the absorbance at 405 nm was read in an enzyme-linked immunosorbent assay plate reader (Diagnostica Pasteur). The release of β-hexosaminidase was expressed as a percentage of the total β-hexosaminidase present in unactivated cells. Any effect of phenol red in the medium was accounted for by the inclusion of monosodium as a control. The cell lysate was obtained by incubating cells with 1 ml of 0.1% Triton X-100 for 10 min. Spontaneous release in the absence of stimuli was in the range of 2 to 6% of the total β-hexosaminidase and was subtracted from the values given above. Release from cells stimulated with antigen or with the combination of PMA and A23187 (PMA-A23187) was typically in the range of 40 to 50% of total β-hexosaminidase. The means for triplicate samples were determined, and these were used to calculate the means and standard errors of the means (SEM) for replicate experiments. Data were then expressed as a percentage of the release from stimulated cells not treated with glucocorticoids.

RESULTS

Cytokine expression by RBL-2H3 cells. The capacity of RBL-2H3 cells to express cytokines was assessed in response to antigen cross-linking of receptor-bound IgE, to PMA, and to the calcium ionophore A23187. At 4 h after activation, cells were lysed and RT-PCR was performed for IL-4, IL-5, and β-actin mRNA. The sizes of the principal products determined by gel electrophoresis were within 10 bp of their predicted sizes, and their identities were confirmed by Southern transfer and hybridization to radiolabelled oligonucleotide probes (Fig. 1). In the Southern blots, in addition to the principal bands, some faint bands were observed that were hybridized to the β-actin probe but not to the cytokine probes. These bands were likely derived from the noncross-linked controls. In some experiments, the specificities of the probes were confirmed by Southern blotting. The gels were transferred to a nylon membrane (Hybond N+; Amersham, Amersham, Buckinghamshire, England) under vacuum, and the membranes were hybridized to oligonucleotide probes designed to anneal to the PCR product but not to the amplification primers. The hybridization probes were 5′-TACCTCGGTGTCGTTAAGAACG-3′ for IL-4, 5′-TCGATGTTCGCCTCCT-3′ for IL-5, and 5′-CAGCCATGTTCGATTC-3′ for β-actin. The expected product sizes were 351 bp for IL-4, 239 bp for IL-5, and 202 bp for β-actin. The membranes were washed and exposed to X-ray film (DuPont, Wilmington, Del.). After development of the films, band intensities were determined by laser densitometry as described above.

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mRNAs for IL-4 and IL-5 were expressed at low levels in unstimulated cells. After stimulation with antigen, mRNA levels for IL-4 and IL-5 were markedly increased. Stimulation with the combination of PMA and A23187 had similar effects (Fig. 1a). IL-4 mRNA was very readily detected, with bands in ethidium bromide-stained gels being visible after only 26 cycles of PCR amplification of cDNA derived from 0.1 µg of total RNA. Stimulation of cells with A23187 alone was almost as effective as PMA-A23187 in inducing IL-4 and IL-5 mRNAs. However, PMA alone had little or no effect (Fig. 1b). The expression of IL-4 and IL-5 mRNAs in response to the different activators correlated with granular secretion, measured by the release of β-hexosaminidase (see the legend to Fig. 1).

To define the peak time of mRNA production, cells were harvested from 15 min to 8 h after activation. There was differential induction of IL-4 and IL-5 (Fig. 2). IL-4 mRNA was
induced earlier, with markedly increased production detectable within 1 h. IL-4 mRNA levels peaked at 2 h and returned to baseline levels at 8 h. IL-5 had a slower induction rate, with little change within the first hour, peak expression 4 h after stimulation, and a slower decay of mRNA levels than those for IL-4 mRNA. In these experiments, IL-5 mRNA was detectable in unstimulated cells and IL-5 mRNA levels were not significantly increased by PMA alone. On the basis of these findings, subsequent mRNA harvest was performed 4 h after activation.

Effects of glucocorticoids. The effect of the synthetic glucocorticoid dexamethasone on the production of IL-4 and IL-5 mRNAs in RBL-2H3 cells was determined. Concentrations of dexamethasone ranging from $10^{-2}$ to $10^{-11}$ M were tested in the presence of PMA-A23187 (Fig. 3). Dexamethasone at $10^{-10}$ M reduced the level of IL-4 mRNA production significantly and at $10^{-6}$ to $10^{-8}$ M almost abolished it (Fig. 3a), with a 50% inhibitory concentration (IC$_{50}$) of $10^{-10.3}$ M. Dexamethasone at $10^{-7}$ M reduced IL-5 mRNA levels significantly and at $10^{-7}$ and $10^{-6}$ M reduced the mRNA to very low levels (Fig. 3b), with an IC$_{50}$ of $10^{-8.8}$ M. At the higher doses tested, dexamethasone was moderately inhibitory to granule secretion, as measured by β-hexosaminidase release. However, even at doses as high as $10^{-6}$ M, only 40 to 50% inhibition of granule release was observed (Fig. 3c).

The naturally occurring glucocorticoid hormone hydrocortisone had effects similar to those of dexamethasone. There was a dramatic concentration-dependent decrease in the levels of expression of both IL-4 and IL-5, with the mRNAs of both cytokines essentially being abolished at hydrocortisone concentrations of $10^{-6}$ M (Fig. 4a and b). As with dexamethasone, IL-4 mRNA production was more sensitive than IL-5 mRNA production to hydrocortisone. The IC$_{50}$s were $10^{-8.9}$ M for IL-4 and $10^{-8.0}$ M for IL-5. Granule secretion was only moderately inhibited by the highest concentrations of hydrocortisone (Fig. 4c).

Studies were then performed to determine the effect of dexamethasone on cells activated by antigen-induced cross-linking of IgE receptors. These experiments were performed because this is a more physiological method of mast cell activation than PMA-A23187 stimulation. Cells were incubated with IgE antibody overnight and were exposed to specific antigen. Production of IL-4 and IL-5 mRNAs was markedly inhibited by dexamethasone concentrations from $10^{-10}$ to $10^{-7}$ M (Fig. 5). The IC$_{50}$s were $10^{-8.6}$ M for IL-4 and $10^{-8.2}$ M for IL-5. These effects were similar to those described in Fig. 3 for cells stimulated with PMA-A23187. However, the inhibitory effect of dexamethasone on the secretion of β-hexosaminidase was much greater for cells stimulated with antigen (Fig. 5c) than for cells stimulated with PMA-A23187 (Fig. 3c).

In the experiments described above, dexamethasone was added overnight, prior to activation of the cells. The effects of adding it at a range of times prior to and after activation were assessed (Fig. 6). Cells were activated at time zero and 4 h later were harvested for RNA extraction. Dexamethasone was added at different times, from 16 h before activation to 4 h after activation, i.e., immediately prior to cell lysis for RNA
Dexamethasone markedly inhibited IL-4 and IL-5 mRNA production when it was added 16 and 2 h prior to activation. It was also markedly inhibitory when it was added at the time of activation and at later times, as late as 2.5 h after activation, which was only 1.5 h before the cells were harvested for RNA extraction (Fig. 6). The effects were variable when dexamethasone was added only 1 h prior to harvest (3 h after activation), and there was no effect when it was added 30 min prior to harvest (3.5 h after activation).

**DISCUSSION**

In this paper we demonstrate that expression of IL-4 and IL-5 can be induced in the RBL-2H3 cell line, an analog of mucosal mast cells, by cross-linking of receptor-bound IgE or by a calcium ionophore. Induction of IL-4 and IL-5 mRNAs was strongly inhibited by the glucocorticoids dexamethasone and hydrocortisone. These findings are consistent with reports that glucocorticoids inhibit the expression or production of a number of cytokines by mast cells, including tumor necrosis factor alpha, IL-1β, IL-3, IL-6, and IL-8 (18, 19, 38, 39, 41). Inhibition of IL-4 mRNA by dexamethasone was recently reported in the human mast cell line HMC-1 (38). In T cells, by contrast, there are conflicting reports on the effect of glucocorticoids on IL-4 production. High concentrations of glucocorticoids were reported to inhibit IL-4 production by human blood T lymphocytes or mononuclear cells (7, 42) and by CD4+ T cells from rat lymph nodes (27). In a study with mice, however, low concentrations of dexamethasone from $10^{-11}$ to $10^{-8}$ M were reported to enhance IL-4 production by lymph node or spleen cells (10). In the present studies, dexamethasone and hydrocortisone doses from $10^{-11}$ to $10^{-6}$ M were tested, and there was no evidence of the enhancement of IL-4 or IL-5 mRNA production at any concentration, but there was marked inhibition at higher concentrations (Fig. 3 and 4).

The present findings confirm and extend other recent observations on the effects of glucocorticoids on the production of IL-5 mRNA by mast cells. Dexamethasone inhibits the expression of IL-5 in rat peritoneal mast cells (41) and in human lung explants stimulated with IgE (14). In these studies with cells prepared from tissue, the possibility that glucocorticoids might be acting on another cell population to induce a factor that could inhibit IL-5 mRNA production by mast cells cannot be completely excluded. The use of the RBL-2H3 mast cell line in the present study demonstrates unequivocally that these inhibitory effects of glucocorticoids on IL-5 expression are directly on the mast cells. In cells activated with PMA-A23187, lower concentrations of either dexamethasone or hydrocortisone were required to inhibit IL-4 mRNA compared with the concentrations required to inhibit IL-5 mRNA (Fig. 3 and 4). This effect suggests that the cells may use different signaling pathways for IL-4 and IL-5 mRNA expression. However, differences in glucocorticoid concentrations were not observed in cells activated by antigen (Fig. 5).

These findings raise the possibility that the beneficial therapeutic effects of glucocorticoids in allergic diseases may be mediated, at least in part, by inhibition of production of IL-4.
and IL-5 by mast cells. An intriguing and novel observation was that dexamethasone may be added after activation but still markedly reduce the abundance of cytokine mRNA. When dexamethasone was added 2.5 h after activation and the cells were harvested 4 h after activation, the inhibition of IL-4 and IL-5 expression was as marked as when dexamethasone was added prior to activation (Fig. 6). These findings also suggest that in a therapeutic setting, IL-4 and IL-5 production can be inhibited by glucocorticoids after mast cells have been activated by an allergen. This effect may contribute to the clinical efficacy of therapy with glucocorticoids when they are introduced after the onset of an asthma attack.

It is interesting to speculate whether the glucocorticoid concentrations used in the present experiments are similar to those achieved therapeutically. In a pharmacokinetic study with humans, a single oral dose of 20 mg of hydrocortisone, a modest glucocorticoid dose, was followed by a peak concentration in plasma of $0.8 \times 10^{-6}$ M (11). In the present experiments, IL-4 and IL-5 expression was almost completely abolished by $10^{-7}$ and $10^{-6}$ M hydrocortisone (Fig. 4). It is difficult to make exact comparisons between concentrations in vivo and in vitro because of differences in protein binding, the more rapid elimination of glucocorticoid in vivo, and different kinetics of receptor occupation during changing extracellular concentrations. Nevertheless, it is reasonable to propose that the concentrations of glucocorticoids achieved in the therapy of allergic conditions are sufficient to inhibit IL-4 and IL-5 mRNA expression. Dexamethasone was approximately 1 order of magnitude more potent than hydrocortisone (Fig. 3 and 4), a difference consistent with previous observations (5).

In T cells, IL-4 and IL-5 mRNA abundance is principally regulated by control of the rate of gene transcription rather than by alterations in mRNA stability (24, 29). From the data presented in Fig. 6, the levels of IL-4 mRNA 4 h after activation were very low in cells given dexamethasone 2.5 h after activation, by which time IL-4 mRNA levels have already peaked (Fig. 2). In activated T cells, the half-life of IL-4 mRNA was 60 min (3). If IL-4 has a similar half-life in mast cells, the findings in Fig. 6 could not be fully accounted for by effects on the rate of gene transcription, and acceleration of mRNA decay might also be involved. In the case of IL-5, peak mRNA expression was not reached until 4 h after activation. In mitogen-stimulated T cells, dexamethasone reduced total mRNA levels by inhibition of IL-5 gene transcription, without affecting mRNA stability (29a). The data therefore suggest that the effects of glucocorticoids on IL-5 in mast cells are based on inhibition of the rate of gene transcription.

The stimuli required for IL-4 and IL-5 gene expression are similar to those required for granule release (Fig. 1b). Thus, cross-linking of IgE receptors or calcium ionophore alone, but not PMA alone, is sufficient for both responses. IgE receptor cross-linking initiates a sequence of intracellular events leading to an increased intracellular calcium ion concentration. These elements of the signaling pathways may be required for both granule release and cytokine mRNA production. In cells stimulated with PMA-A23187, secretion was only partially inhibited by glucocorticoids (Fig. 3 and 4), whereas in cells stimulated with antigen, secretion of granule contents was markedly inhibited (Fig. 5), as has been reported previously (9, 40). The data in Fig. 3 and 4 suggest that the effects of glucocorticoids on cytokine mRNA production may involve components of the signaling pathway not required for granule release. A number of possible mechanisms whereby glucocorticoids might inhibit expression of cytokine genes have been described. Glucocorticoids inhibit the effects of transcription factors AP-1 and NF-kB, which are involved in the expression of cytokine genes. The glucocorticoid receptor-hormone complex interacts directly with AP-1 to prevent it from binding to its DNA motifs in promoter regions (16). Glucocorticoids stimulate production of I-kB, which inhibits the translocation of NF-kB from the cytoplasm to the nucleus (1, 32). Glucocorticoids can also accelerate mRNA degradation, as has been described in the reduction of IL-2 mRNA levels induced with dexamethasone in T cells (4). The effects of glucocorticoids on IL-4 and IL-5 mRNA production in mast cells may be mediated by these and/or other mechanisms.

The relative importance of mast cells as producers of cytokines in allergic conditions is controversial. Mast cells are the most numerous cells that contain IL-4 and IL-5 proteins in immunohistochemical studies with biopsy specimens from asthmatic patients (6). By contrast, in situ hybridization studies, IL-4 and IL-5 mRNAs were found more frequently in T cells than in mast cells (43). Mast cells may be more effective early in allergic reactions because they are able to be activated rapidly after the arrival of antigen, which cross-links surface-bound IgE. By contrast, before T cells can be activated, antigen must be processed and presented by other cells. The rapid induction of cytokine mRNA described in Fig. 2 is consistent with a role for mast cells in the initiation of clinical allergic reactions.

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REFERENCES


