Rapid publication

Dexamethasone inhibits the binding of nuclear factors to the IL-5 promoter in human CD4+ T cells

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Background: IL-5 is produced by the T_H2 subset of CD4+ T lymphocytes and is necessary for the eosinophilia typical of allergic conditions. Glucocorticoids such as dexamethasone are highly effective inhibitors of eosinophilic inflammation, and one of their effects is inhibition of IL-5 gene expression.
Objective: We wished to examine the effect of dexamethasone on the binding of nuclear factors from primary human CD4+ T lymphocytes to the RE-I and RE-II positively acting regulatory elements of the IL-5 promoter.
Methods: CD4+ T cells, purified from PBMCs by magnetic bead separation, were activated with anti-CD3 antibody and phorbol myristate acetate. Nuclear extracts were tested in electrophoretic mobility shift assays with probes based on RE-I and RE-II.
Results: In extracts from activated cells, the RE-II region of the promoter formed a complex that was shown by supershift assay to contain NFATc. This complex was abolished by treatment of the cells with dexamethasone before activation and was weak or absent in unactivated cells. By contrast, binding to the RE-I region and to the GATA-3 site within RE-I was observed in resting cells and was not affected by activation or treatment with dexamethasone.
Conclusion: Dexamethasone inhibits the inducible binding of factors to the RE-II region but does not affect the constitutive binding to the RE-I region. Characterization of such molecular effects of glucocorticoids could enable the development of specific inhibitors of IL-5 expression that lack the side effects of glucocorticoids. (J Allergy Clin Immunol 2001;108:340-8.)

Key words: *Gene expression, glucocorticoids, electrophoretic mobility shift assay, interleukin, T_H2 cells*

Eosinophils are prominent in the inflammation associated with allergic diseases and parasitic infestation. The cytokine IL-5 plays an essential role in promoting growth, differentiation, survival, and activation of eosinophils. There is extensive evidence that IL-5 correlates with eosinophilia in asthma,[1] and in a recent report, treatment of asthmatic patients with a neutralizing antibody to IL-5 was followed by a marked reduction in sputum and blood eosinophil counts.[2] By contrast, challenge of asthmatic patients with IL-5 induced significant eosinophilia in bronchial mucosa and bronchoalveolar lavage fluid.[3] In animal studies, transgenic mice constitutively expressing IL-5 showed profound eosinophilia in the blood and tissues, suggesting that IL-5 expression was important for the production of eosinophils.[4] Studies on IL-5 gene knockout mice demonstrated that IL-5 was required for pulmonary eosinophilia and airway hyperreactivity in a model of allergic pulmonary inflammation.[5]

Abbreviations used
AP-1: Activator protein 1
CLE0: Conserved lymphocyte element 0
EMSA: Electrophoretic mobility shift assay
NFAT: Nuclear factor of activated T cells
PMA: Phorbol myristate acetate
RE: Regulatory element

The T_H2 subset of CD4+ T lymphocytes produces cytokines such as IL-4 and IL-5, which are prominent in allergic reactions.[6] In allergic asthma, T_H2-type cells are the most numerous cells expressing mRNA for IL-4 and IL-5.[7] The expression of IL-5 is tightly regulated and occurs only in activated cells. DNAse I footprinting studies demonstrated nuclear factor interactions with elements of the IL-5 gene promoter in human T cells.[8] Two of these regulatory elements (REs), RE-I and RE-II, were found to have functional activity in studies on IL-5 promoter–luciferase reporter gene constructs.[8]

RE-I, located –79 to –45 bp upstream of the transcription start site, contains a double-consensus sequence for the GATA family of zinc finger transcription factors. This sequence, located between –70 and –60 bp, has been shown to bind GATA-3 in T cells.[9] This region is critical for IL-5 expression, because its deletion abolished activation of the IL-5 promoter.[9] Increased expression of GATA-3 was observed in bronchoalveolar lavage fluid and bronchial biopsy specimens from atopic asthmatic subjects and was correlated with increased IL-5 expression.[10] The RE-I region also encompasses binding sequences for activator protein 1 (AP-1) members (widely expressed transcription factors composed of fos and jun family proteins),[8] ets proteins,[11] and oct factors.[12]

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TABLE I. Sequences for the EMSA probes[8,35]

Oligonucleotide for EMSA probes		Sequences of the complementary strands
RE-I (-79 to -45)		
	Sense	5′-CATTCTCTATCTGATTGTTAGAAATTATTCATTTC-3′
	Antisense	5′-GAAATGAATAATTTCTAACAATCAGATAGAGAATG-3′
RE-II (-123 to -92)		
	Sense	5′-TAAGGCATTGGAAACATTTAGTTTCACGATAT-3′
	Antisense	5′-ATATCGTGAAACTAAATGTTTCCAATGCCTTA-3′
GATA-3 (-76 to -57)		
	Sense	5′-TCTCTATCTGATTGTTAGAA-3′
	Antisense	5′-TTCTAACAATCAGATAGAGA-3′

The RE-II region, from –123 to –92 bp, contains a binding site for the nuclear factor of activated T cells (NFAT) transcription factor.[8,13] NFAT proteins translocate to the nucleus from the cytosol after cellular activation.[14] NFAT family members often cooperate with AP-1 in DNA binding and transactivation.[15] Two NFAT members, NFATp and NFATc, play an important role in the regulation of T_H1 and T_H2 cytokine gene expression. NFATp-deficient mice developed pleural eosinophilia and pulmonary inflammation and increased expression of IL-4 and IL-5.[16] Therefore, it has been proposed that NFATp might inhibit the T_H2 allergic response. By contrast, IL-4 expression was reduced in the absence of NFATc,[17,18] suggesting that NFATc might increase T_H2 gene expression. In double NFATc and NFATp knockouts, IL-5 production was significantly reduced.[19]

Glucocorticoids are highly effective in the treatment of asthma and other allergic conditions. Their effects include reduction of the influx of inflammatory cells, such as eosinophils.[20] Glucocorticoids bind to and activate cytoplasmic receptors, enabling them to translocate to the nucleus, where they influence gene expression.[20]

We first reported that the expression of IL-5 is inhibited by dexamethasone, a synthetic glucocorticoid.[21] The production of a wide variety of other inflammatory cytokines, including IL-2, is inhibited by glucocorticoids.[20] Glucocorticoids inhibit IL-2 gene transcription by the direct interaction of the activated glucocorticoid receptor with AP-1 to prevent it from binding to the IL-2 promoter.[22,23]

The transcription binding sites on the IL-5 gene promoter that are specifically affected by glucocorticoids have not yet been identified. Hence, this report investigates the effect of dexamethasone on the binding of nuclear factors to RE-I and RE-II of the human IL-5 gene promoter. We have found that binding to the RE-II region is induced by activation and inhibited by dexamethasone whereas binding to RE-I occurs in resting cells and is not affected by activation or by dexamethasone.

MATERIALS AND METHODS

Cell culture

Blood samples were collected from healthy volunteer donors. The procedures were approved by the Committee on Experimental Procedures Involving Human Subjects of the University of New South Wales. PBMCs were isolated through use of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Adherent cells were removed by incubation in complete medium overnight at 37°C in 5% CO_2. The complete medium consisted of Dulbecco modified Eagle medium, 10% (v/v) FCS, 2 mmol/L L-glutamine, and 20 mmol/L HEPES. Suspension cells were placed in 80 μL of sterile cold column buffer (PBS, 5% [v/v] FCS, 2 mmol/L EDTA) and 10 μL of paramagnetic beads conjugated with anti-CD4 monoclonal antibodies (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) per 10^7 cells for 30 minutes at 6° to 12°C; swirling was done every 10 minutes. The cells were then resuspended in 500 μL of column buffer per 10^8 cells and passed through a magnetic cell separation (MACS) column (VS^+) attached to a magnetic separator (Miltenyi Biotec GmbH). Three aliquots of column buffer were passed through the column and discarded. The column was removed from the separator and allowed to demagnetize for 5 minutes. Cells were then collected through the application of 2 rounds of 5 mL column buffer and determined by flow cytometry to be greater than 96% $CD4^+CD3^+$ (hereinafter referred to as *$CD4^+$ T cells*).

The $CD4^+$ T cells were resuspended at 1×10^6 cells/mL in complete medium and activated with 1 μg/mL OKT3 (anti-CD3) antibody (kindly provided by Janssen-Cilag Pharmaceuticals, Sydney, Australia), which had been adhered to the multiwell flat-bottom tissue culture plates in PBS overnight at 4°C, and 6 ng/mL phorbol myristate acetate (PMA; Sigma Chemical Co, St Louis, Mo). The cells were cultured at 37°C in 5% CO_2. Water-soluble dexamethasone (10^{-6} mol/L; Sigma) was added to cells immediately before activation.

RT-PCR

Total cellular RNA was extracted through use of Trizol reagent (GibcoBRL, Gaithersburg, Md) according to the manufacturer's instructions and resuspended in RNAse-free H_2O. cDNA, synthesized from 5×10^5 cell equivalents of total cellular RNA, was amplified through use of PCR with 25 cycles for β-actin and 35 cycles for IL-5, as previously described.[21]

IL-5 secretion

IL-5 secretion was measured by means of a sandwich ELISA through use of TRFK5 (Pharmingen, San Diego, Calif) as capture antibody and biotinylated JES1-5A10 (Pharmingen) as detection antibody, according to the manufacturer's instructions. Culture supernatants were harvested 24 hours after activation. The minimum detection limit was 5 pg/mL. Duplicates of each sample and standard (recombinant human IL-5, Pharmingen) were performed in each assay.

Electrophoretic mobility shift assays (EMSAs)

Aliquots of 5×10^6 cells were washed twice in ice-cold PBS and

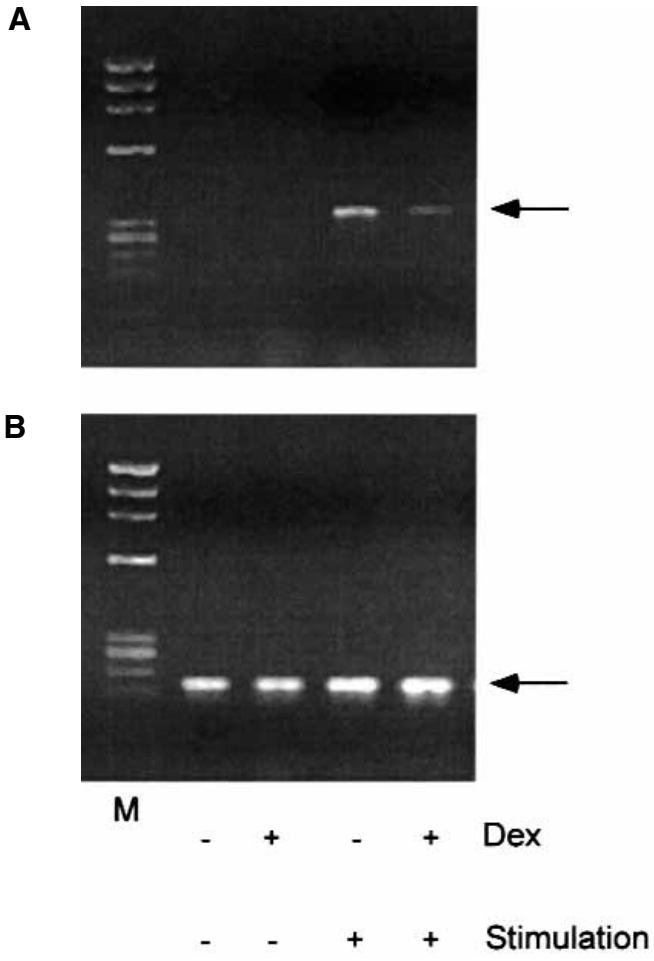

FIG 1. RT-PCR. cDNA was prepared from $CD4^+$ T cells, unstimulated or else stimulated for 6 hours with OKT3 and PMA , and then treated with or without dexamethasone *(Dex)*. cDNA was amplified for IL-5 (**A**) and β-actin (**B**). *M* indicates DNA size markers. Apparent mobilities of IL-5 and β-actin bands *(arrows)* are consistent with predicted sizes of 328 bp and 204 bp, respectively.[21]

resuspended in 250 μL ice-cold low-salt buffer for 15 minutes. The low-salt buffer consisted of 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 100 μmol/L EDTA, 500 μmol/L Pefabloc (Pentapharm LTD, Basel, Switzerland), and 1 mmol/L dithiothreitol (DTT). NP-40 was added to a final concentration of 0.6% (v/v), and the tubes were vortexed and centrifuged for 30 seconds. The supernatants were removed, and the pellets were washed once with low-salt buffer and resuspended in 50 μL high-salt buffer (50 mmol/L HEPES [pH 7.9], 50 mmol/L KCl, 100 μmol/L EDTA, 10% glycerol, 1 mmol/L Pefabloc, 1 mmol/L DTT). Samples were rotated for 30 minutes at 4°C and then centrifuged at 12,000*g* for 10 minutes at 4°C. The supernatants (nuclear extracts) were transferred to new microcentrifuge tubes on dry ice and stored at –70°C until required. The protein content of the nuclear extracts was determined through use of bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill), according to the manufacturer's instructions.

Oligonucleotides corresponding to the sequences of RE-I and RE-II (Table I) were synthesized with 5′-GGG overhangs (Geneworks, Adelaide, Australia). Complemetary oligonucleotides (2 μg each) were annealed in 200 μL H_20 by being heated to 70°C for 20 minutes and then cooled slowly. The RE-I and RE-II probes were radiolabeled by filling in the overhanging ends through use of Klenow fragment and [α-^{32}P]dCTP (ICN Biomedicals, Aurora, Ohio). The GATA-3 probes lacked overhangs and were end-labeled through use of T4 polynucleotide kinase and [α-^{32}P]dATP (ICN Biomedicals).

The 20-μL EMSA binding reactions contained 5 μg of total nuclear protein and 0.1 μg poly dI-dC in binding buffer (10 mmol/L HEPES [pH 7.9], 100 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 4 mmol/L Pefabloc). Reactions were incubated with 4 ng of labeled probe for 20 minutes at room temperature. In competition assays, nuclear extracts were preincubated for 10 minutes at room temperature with a 20-fold molar excess of unlabeled, double-stranded oligonucleotides before the addition of the labeled probe. In supershift assays, nuclear extracts were incubated with monoclonal IgG mouse anti-NFATc antibody (2μg; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) for 30 minutes at room temperature before addition of the probe. The complexes were then resolved by electrophoresis in 5% nondenaturing polyacrylamide gels at 150 V for 3 hours at room temperature in 90 mmol/L TRIS, 90 mmol/L boric acid, and 2 mmol/L EDTA. Gels were dried before autoradiography at –70°C with Biomax MS/MR film (Eastman Kodak Co, Rochester, NY).

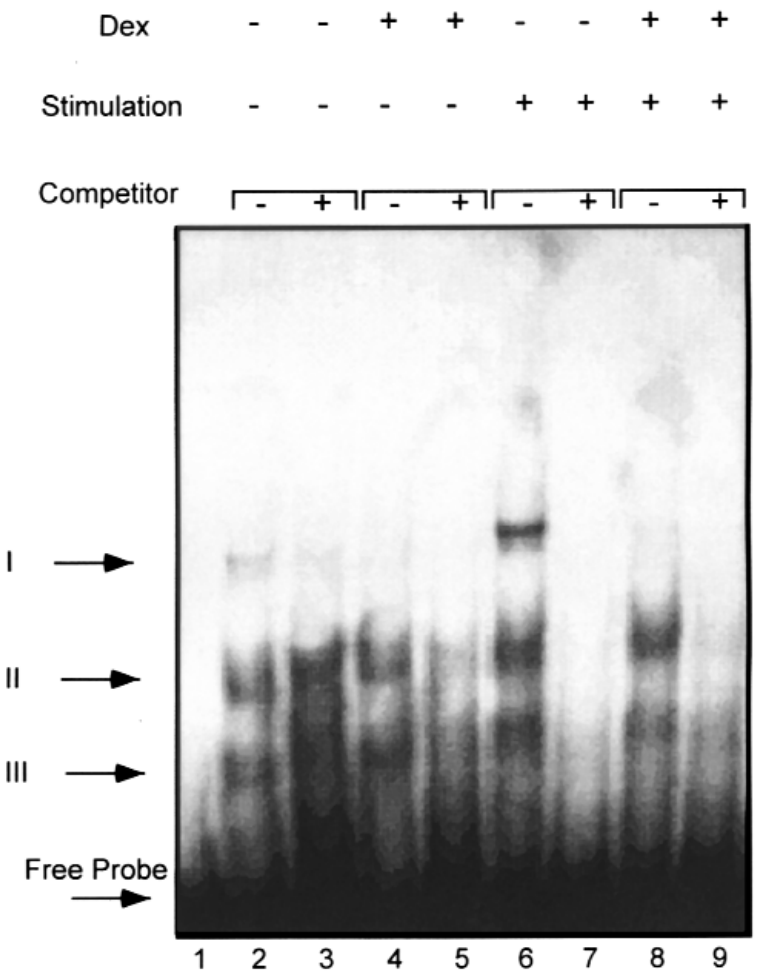

FIG 2. Effect of dexamethasone *(Dex)* on binding to RE-II. Nuclear extracts from CD4+ T lymphocytes, unstimulated or else stimulated with OKT3 and PMA and treated with or without dexamethasone, were tested in EMSA. Two reactions for each set of conditions—one with and the other without 20-fold molar excess of unlabeled RE-II oligonucleotide as competitor—were loaded in adjacent wells. Complexes I, II, and III are marked. *Lane 1* shows labeled probe alone. Data are representative of separate experiments on 3 different donors.

RESULTS

Response element II

To confirm that IL-5 mRNA was induced by activation of CD4+ T cells with OKT3 and PMA, RT-PCR was performed on total RNA from resting and activated cells. IL-5 mRNA was absent in resting cells and readily detected 6 hours after activation (Fig 1), as has been reported previously.[24] Dexamethasone (10^{-6} mol/L) almost completely inhibited IL-5 mRNA expression (Fig 1), as previously observed,[21] and reduced IL-5 secretion to undetectable levels (data not shown).

Nuclear proteins were extracted and tested in EMSA binding reactions with radiolabeled RE-II probe. Three complexes of different mobilities were present (Fig 2). The complex with the slowest mobility, complex I, was detected in resting cells and was markedly increased after activation (Fig 2, *lane 2* vs *lane 6*), suggesting the involvement of one or more factors induced by activation. The intensities of the other 2 complexes remained unchanged after activation, suggesting that the nuclear factors in these 2 complexes are constitutively expressed.

A control of preincubation of the nuclear extracts with a 20-fold molar excess unlabeled competitor was performed. The control data revealed that complex I was abolished both in resting and activated cells, indicating the specificity of binding. The effects of competition on complexes II and III were less consistent, so further investigation focused on complex I (Fig 2).

We next examined the effect of dexamethasone on binding to RE-II. In activated cells, dexamethasone abolished complex I (Fig 2, *lane 8 vs lane 6*). In unactivated cells, the weak binding to complex I was totally inhibited by treatment with dexamethasone (Fig 2, *lane 4 vs*

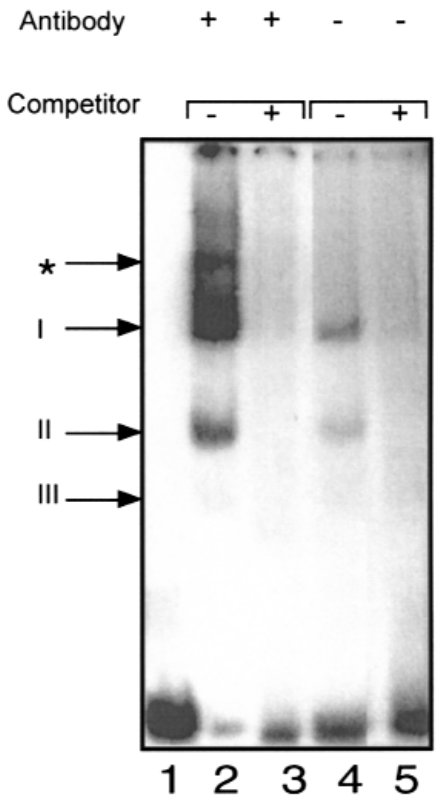

FIG 3. NFATc contributes to inducible complex formation with RE-II. Nuclear extracts from CD4+ T lymphocytes, stimulated with OKT3 and PMA, were tested in EMSA with or without prior addition of anti-NFATc antibody. Complexes I, II, and III are marked, and the supershifted complex is indicated by an *asterisk*. Two reactions for each condition—one with and the other without 20-fold molar excess of unlabeled RE-II competitor—were loaded in adjacent wells. Data are representative of experiments in 3 different donors.

lane 2). This inhibitory effect of dexamethasone on complex I (Fig 2) was similar to its effect on IL-5 mRNA (Fig 1); however, in untreated cells there was no detectable IL-5 mRNA, whereas complex I was detected, albeit weakly, in the EMSA.

To investigate the composition of the inducible complex I that binds RE-II, we performed supershift assays. Because RE-II has been shown to bind NFATc,[8] we examined whether NFATc was a component of the dexamethasone-sensitive complex I (Fig 3). Addition of anti-NFATc antibody resulted in a substantial (though incomplete) supershift of complex I (Fig 3, *lane 2 vs lane 4*). Incubation of nuclear extract with a 20-fold molar excess unlabeled competitor before the addition of anti-NFATc antibody abolished all complex formation (including the supershifted), demonstrating that all complexes bound specifically to the RE-II probe (Fig 3, *lane 3*).

Response element I

The binding of nuclear extracts from CD4+ T cells to RE-I was then examined. In unactivated cells, 3 complexes, designated I, II, and III, were observed in 3 separate donors. Activation for 4 to 24 hours with OKT3 and PMA had no consistent effect on any of the complexes (Fig 4, *A*). When extracts were preincubated with a 20-fold molar excess of unlabeled probe, all 3 complexes disappeared or were markedly reduced at all time points.

The effect of dexamethasone treatment of the cells was investigated. Formation of the 3 complexes was not reduced by dexamethasone treatment of both resting cells and cells harvested at all times after activation (Fig 4, *B*). Again, preincubation with unlabeled probe markedly diminished or abolished all 3 complexes.

To determine whether the GATA-3 site in RE-I is responsible for the binding observed in Fig 4, we investigated the effects of a 20-bp fragment of RE-I containing the GATA-3 site (Table I). First, we assessed the effect of the GATA-3 probe on binding of nuclear extracts to the RE-I probe, which contains 35 bp of the IL-5 promoter. In extracts from unactivated cells, preincubation with unlabeled RE-I substantially reduced complex I formation, even at a 10-fold molar concentration excess (Fig 5). When the GATA-3 competitor was tested, the intensity of complex I was reduced in a concentration-dependent manner (Fig 5, *lanes 4-6*), but the complex could still be detected at 40-fold molar excess. This suggests that the GATA-3 competitor is competing for the protein(s) that form complex I. Second, we performed EMSA with

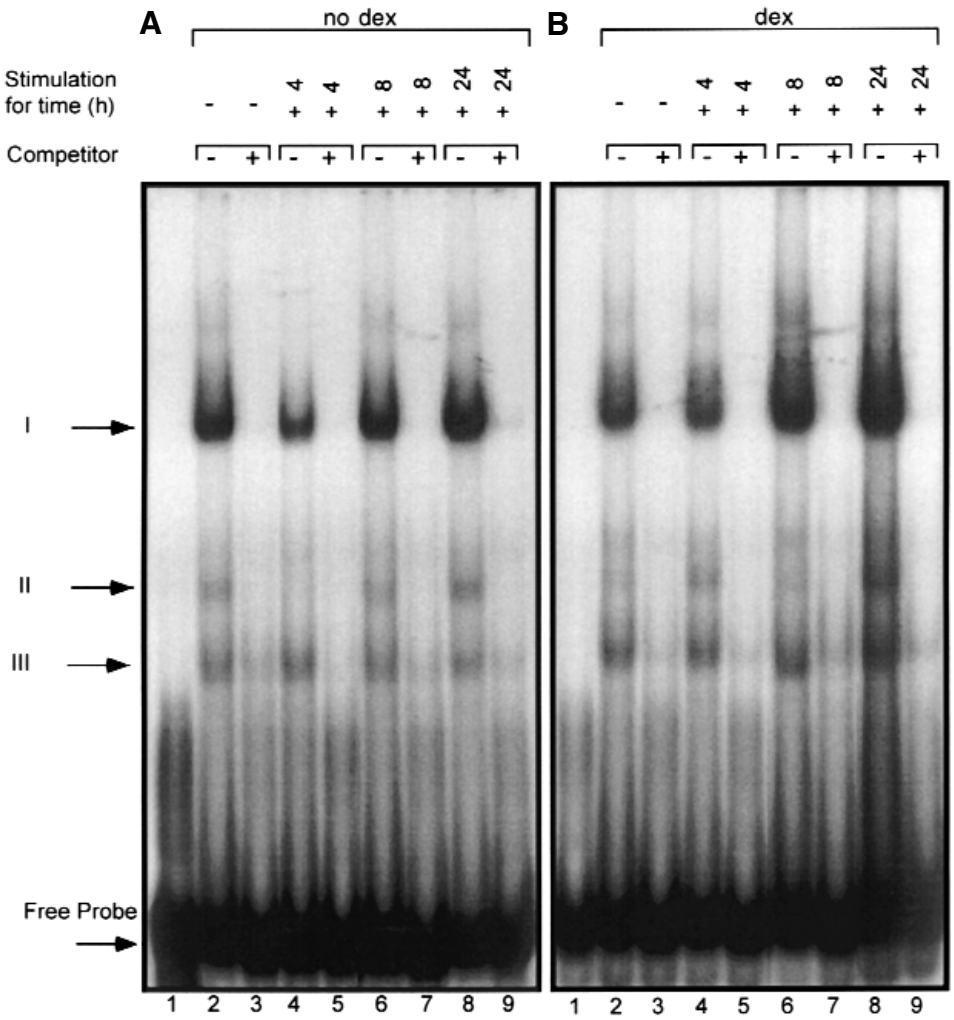

FIG 4. Binding to RE-I. Nuclear extracts from CD4+ T cells stimulated with OKT3 and PMA for various amounts of time were used in the EMSA binding reactions. Cells were treated without *(panel A)* and with *(panel B)* dexamethasone *(dex)*. Competitor reactions with 20-fold molar concentration excess of unlabeled RE-I oligonucleotide were conducted for each extract and run in adjacent lanes. Complexes I, II, and III are marked. The data are representative of experiments in 3 different donors.

labeled GATA-3 probe. Only 1 complex was detected with the GATA-3 probe, and it was present at similar intensities in extracts from unactivated, activated, and activated and dexamethasone-treated cells (Fig 6). Unlabeled GATA-3 probe, at a 40-fold molar excess, competed for most of the binding to the GATA-3 probe, and a 20-fold molar excess of unlabeled RE-I almost abolished complex formation (Fig 6). The observation that binding to the GATA-3 and RE-I probes was constitutive and not affected by activation or dexamethasone suggests that the 2 probes bind common nuclear factors.

DISCUSSION

Dexamethasone markedly reduces IL-5 mRNA abundance in activated T cells (Fig 1).[21] This effect involves inhibition of gene transcription, inasmuch as dexamethasone significantly inhibited reporter gene activity driven by the proximal 511-bp region upstream of the human IL-5 gene.[25] We have demonstrated for the first time (to our knowledge) that dexamethasone inhibits inducible binding of nuclear factors to the RE-II region on the human IL-5 gene promoter (Fig 2). Thus we propose that the effect of dexamethasone on gene transcription, and hence on RNA abundance, involves inhibition of binding to RE-II. EMSA with the RE-II probe revealed that complex I was weak in resting cells and markedly induced by cellular activation (Fig 2). Supershift assays showed that complex I contained NFATc (Fig 3), which is consistent with the findings of Stranick et al,[8] who reported the binding of NFATc to the RE-II sequence.[8] These researchers found that this complex was abolished by prior treatment of cells with cyclosporin A. Together, these data suggest the interesting possibility that dexamethasone and CsA affect the same region of the IL-5 promoter.

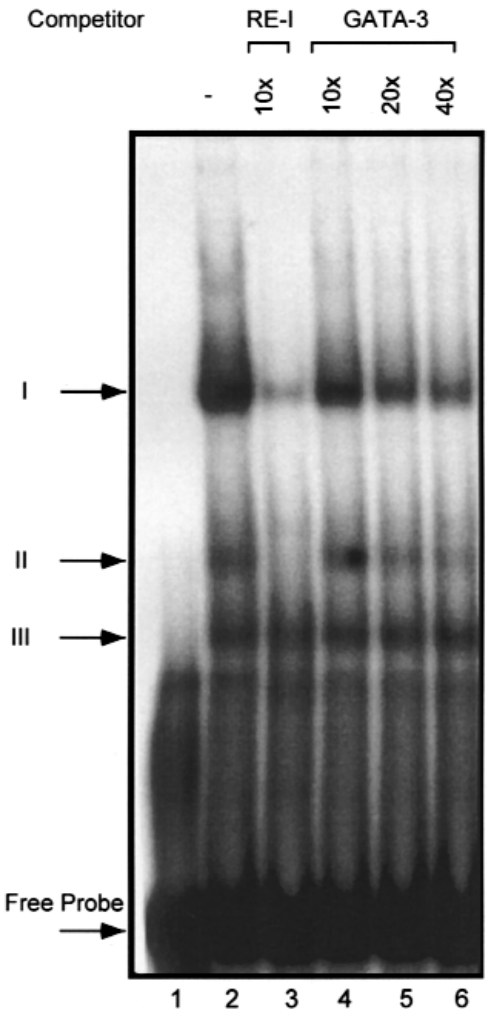

FIG 5. GATA-3 competes with RE-I. Nuclear extracts from unstimulated CD4+ T cells were tested in EMSA with labeled RE-I probe. Where indicated, samples were preincubated with unlabeled probe at 10-, 20-, or 40-fold molar excess for GATA-3 and at 10-fold molar excess for RE-I. Complexes I, II, and III are marked.

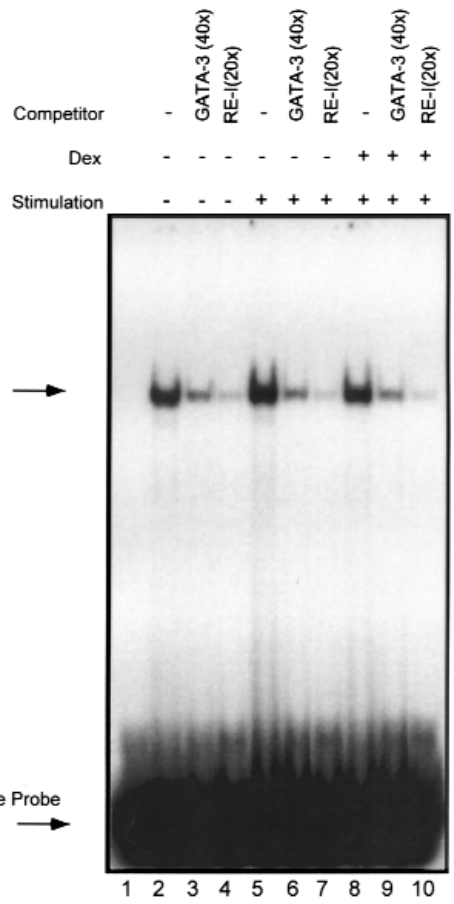

FIG 6. Binding to GATA-3 probe. Nuclear extracts were prepared from CD4+ T cells that had been unstimulated, stimulated with OKT3 and PMA, or stimulated and treated with dexamethasone *(Dex)*. Where indicated, extracts were preincubated with unlabeled GATA-3 probe at 40-fold molar excess or unlabeled RE-I probe at 20-fold molar excess. EMSA was then performed with labeled GATA-3 probe. The shifted complex is marked by an arrow.

Both the RE-I and GATA-3 probes displayed constitutive binding, which was unaffected by dexamethasone (Figs 4-6). Constitutive expression of GATA-3 in human naive T cells and T_H2 cells[26] supports the notion that GATA-3 protein contributes to the observed constitutive binding to the RE-I probe and to the shorter GATA-3 probe. The GATA-3 probe, however, did not compete for all of the binding to the RE-I probe (Fig 5), and it formed only 1 specific complex (Fig 6), whereas the RE-I probe formed 3 (Figs 4 and 5). These findings indicate that there are binding sites in the RE-I probe outside the region encompassed by the GATA-3 probe. Thus it is likely that complex I (the band with the slowest mobility; Fig 4) consists of a number of different proteins, including GATA-3.

The RE-I probe (–79 to –45), but not the GATA-3 probe (–76 to –57), substantially overlaps the conserved lymphocyte element 0 (CLE0; –54 to –40). CLE0, found in the promoters of the GM-CSF, IL-4, and IL-5 genes,[27] is essential for IL-5 expression.[9] In the IL-5 promoter, CLE0 has been reported to bind the transcription factors Oct-1,[28] Ets1, and Ets2[11] as well as the inducible factor AP-1.[9] It was observed that Oct-1 was constitutively bound to the CLE0 element of the IL-5 promoter in the human cell line PER-117.[28] Ets1 and Ets2 transcription factors were shown to bind to the CLE0 element and cooperate with GATA-3 to activate the IL-5 promoter in human cells.[11] We propose that Oct-1 and/or Ets 1/2 cooperate with GATA-3 to generate the 3 specific complexes observed with the RE-I probe.

Our findings with regard to the effects of glucocorticoids on IL-5 gene regulation can be considered in the context of a recent model that proposes 2 steps in the regulation of T_H2 cytokine gene expression.[29,30] In the first step, primary activation of naive T cells, in the presence of IL-4, leads to opening of the chromatin around the gene loci of T_H2 cytokines such as IL-4 and IL-5. Over a

period of days, the genes become accessible to transcription factors and bind constitutively expressed factors such as GATA-3. In the second step, subsequent activation leads to T_H2 gene transcription as a result of the binding of inducible transcription factors such as NFATc and AP-1. In the present study, freshly isolated human T cells were activated in vitro; of such cells, approximately 1% have been reported to express IL-5 within several hours.[31] According to the model, these cells are likely to have been previously activated in vivo; their T_H2 cytokine gene loci have become accessible to transcription factors, and GATA-3 has bound to the IL-5 promoter.

We have found that constitutive binding to RE-I and the GATA-3 probe is not affected by dexamethasone (Figs 4-6). We propose that in vitro addition of glucocorticoids does not affect the first step of the model and that existing levels of GATA-3 protein in the nucleus remain unchanged. By contrast, we have shown that dexamethasone inhibits inducible binding to RE-II, which includes the NFATc site (Figs 2 and 3). We therefore propose that glucocorticoids affect the second step of the model—ie, the binding of inducible transcription factors required for IL-5 expression, such as NFATc and AP-1. Maximal binding of NFAT requires DNA binding by AP-1 family members,[15] and activated glucocorticoid receptors have been shown to prevent the binding of AP-1 to DNA.[32] RE-II is similar to a region in the human IL-2 promoter, which has been shown to contain NFAT and AP-1 binding sites. Dexamethasone has been noted to inhibit AP-1 binding to this site in the IL-2 promoter.[23,33] Therefore, we postulate that dexamethasone inhibits the binding of NFATc- and AP-1–like factors to RE-II in the IL-5 promoter.

In conclusion, this study has demonstrated that dexamethasone inhibits the binding of nuclear proteins to the RE-II region of the human IL-5 promoter, and we propose that this effect is a principal mediator of glucocorticoid inhibition of IL-5 transcription. By contrast, factor binding to the RE-I region was dexamethasone-resistant, implying that glucocorticoids do not affect the function of GATA-3. Commonly used topical glucocorticoids such as beclomethasone dipropionate and fluticasone propionate also inhibit IL-5 production,[34] and it is likely that these drugs act on the IL-5 promoter in the way that dexamethasone does. Identification of molecular effects of glucocorticoids could allow the development of specific inhibitors of IL-5 expression that lack the extensive side effects of glucocorticoids.

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