Inhibition of Cytokine Gene Transcription by the Human Recombinant Histamine-Releasing Factor in Human T Lymphocytes

Becky M. Vonakis, Rebecca Sora, Jacqueline M. Langdon, Vincenzo Casolaro and Susan M. MacDonald

*J Immunol* 2003; 171:3742-3750; doi: 10.4049/jimmunol.171.7.3742

http://www.jimmunol.org/content/171/7/3742
Inhibition of Cytokine Gene Transcription by the Human Recombinant Histamine-Releasing Factor in Human T Lymphocytes

Becky M. Vonakis, Rebecca Sora, Jacqueline M. Langdon, Vincenzo Casolaro, and Susan M. MacDonald

Human recombinant histamine-releasing factor (HrHRF) preincubation enhances the secretion of histamine, IL-4, and IL-13 from FcεRI-stimulated human basophils. In GM-CSF-primed human eosinophils, HrHRF increases IL-8 production. Our recent experiments were designed to evaluate the effects of HrHRF on human T cell cytokine production. Purified T cells were preincubated with GST-tagged HrHRF, followed by stimulation with PMA and A23187 overnight. A partial inhibition of IL-2 and IL-13 production (30 and 75%, respectively) was detected compared with that in cells treated with PMA/A23187 alone. However, the production of IFN-γ was similar in PMA/A23187-stimulated cells with or without HrHRF. The inhibition of cytokine protein production was dose dependent and specific to the HrHRF portion of GST-HrHRF. The inhibition was not due to endotoxin, since preincubation with polymyxin B and HrHRF gave similar results to that with HrHRF alone. The same pattern and specificity of cytokine regulation were replicated in the Jurkat T cell line as for primary T cells. The PMA/A23187-stimulated activity of a proximal promoter IL-13, IL-4, or IL-2 luciferase construct transfected into Jurkat cells was partially inhibited (60, 32, or 70%, respectively) upon GST-HrHRF preincubation, suggesting that HrHRF functions to inhibit cytokine production in Jurkat cells by preventing gene transcription. The inhibition of IL-2 promoter activation was specific to the HrHRF portion of GST-HrHRF. We conclude that HrHRF, in addition to functioning as a histamine-releasing factor, can differentially modulate the secretion of cytokines from human basophils, eosinophils, T cells, and murine B cells, suggesting that it may induce a complex array of responses at sites of allergic inflammation. The Journal of Immunology, 2003, 171: 3742–3750.

The Johns Hopkins Asthma and Allergy Center, Baltimore, MD 21224
Received for publication February 3, 2003. Accepted for publication July 22, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Abbreviations used in this paper: HrHRF, human recombinant histamine-releasing factor; LPR, late phase response; m, murine; PBT, peripheral blood T cell; PKC, protein kinase C; TCTP, translationally controlled tumor protein.
Materials and Methods

GST-HrHRF and GST preparation

The cDNA encoding GST-HrHRF fusion protein was subcloned by restriction digestion from the vector pGEX-2T into the baculovirus vector, pVL1393. Plasmid DNA was cotransfected into S9 cells for viral isolation and was amplified according to the company’s specifications (Invitrogen, Carlsbad, CA). Subsequently, S9 insect cells were infected with recombinant baculovirus and grown commercially on a large scale in serum-free medium (Paragon Biotech, Baltimore, MD). The cell pellets from 8 liters of S9 cells were resuspended in 800 ml of NBB buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8) to which 800 µl of BD Biosciences (San Diego, CA) protease inhibitor cocktail was added. The suspension was freeze-thawed three times and centrifuged at 9000 rpm for 20 min. Purification of the supernatant, which contained the GST-HrHRF fusion protein, was accomplished by affinity chromatography on immobilized glutathione and was confirmed by SDS-PAGE and Western blotting using a polyclonal anti-HrHRF Ab generated against the recombinant material produced in Escherichia coli. The protein concentration of the purified fusion protein preparation was determined using a Bio-Rad (Hercules, CA) protein assay. The protein concentration of the HrHRF portion of the fusion protein (160 µg/ml) was calculated from the molecular mass of HrHRF (23 kDa) relative to the molecular mass of the fusion protein (50 kDa). HrHRF was dialyzed against physiologic 1x PIPES buffer (25 mM PIPES, 110 mM NaCl, and 5 mM KCl, pH 7.4) for use in all assays. Purified HrHRF was judged to be >95% pure by Coomassie Blue staining of an SDS-PAGE gel. Although the GST-HrHRF fusion protein contains a thrombin cleavage site, our preparations are poorly cleaved upon incubation with thrombin, as has been observed for several other GST-tagged proteins (10). One preparation of GST-HrHRF-infected Sf9 lysate was judged to contain only GST by a positive anti-GST Western blot, but a negative anti-HRF Western blot. The baculovirus-expressed GST was recovered from this GST-HrHRF preparation by sequential chromatography on a G-75 size exclusion column and glutathione beads. The purified 26-kDa protein reacted with an anti-GST Ab, but not with an anti-HRF Ab on Western blot. The purified GST protein was found to be >95% pure by Coomassie Blue staining of an SDS-PAGE gel. In some experiments GST protein purified from porcine liver (Sigma-Aldrich, St. Louis, MO) was compared with baculovirus-expressed GST for its effects on T cell cytokine production.

PBT purification

PBTs were purified by countercurrent elutriation from the residual cells of anonymous donors undergoing leukapheresis and were routinely found to be 80–85% CD3+ by flow cytometry and immunofluorescence (11). By flow cytometry, the PBTs used in this study were found to be 60% CD3+ CD4+, 25% CD3+ CD8+, 5% CD19+, and <3% CD14+. The relative ratio of CD4+ to CD8+ cells in our preparation is similar to the 2:1 ratio seen in human peripheral blood before fractionation. Contamination of the PBT preparation with basophils was <1%, as assessed by Alcian Blue staining.

Stimulation of cytokine production

PBTs or Jurkat T cells (obtained from Dr. J. Strominger, Harvard University, Boston, MA) were resuspended in T cell medium consisting of RPMI 1640 containing 8.7% heat-inactivated FBS, 8.7 mM HEPES buffer, 1.75 mM Glutamax-I, 0.8 mM sodium pyruvate, 12.6 ng/ml gentamicin, 88 U/ml penicillin, and 88 µg/ml streptomycin. All T cell medium components were purchased from Invitrogen, except for FBS and sodium pyruvate, which were obtained from Sigma-Aldrich. PBTs were incubated at 2 x 10⁶ cells/ml for 4.5 h with GST-HrHRF, GST, or PIPES buffer in 24-well plates in a humidified 37°C incubator (5% CO₂). Jurkat cells were preincubated with GST-HrHRF in T cell growth medium or medium alone for the times indicated, usually 4 h. PMA (Sigma-Aldrich) and A23187 (Calbiochem, La Jolla, CA) stock solutions were prepared in DMSO and then diluted in medium before addition to the cells. The two stimuli were added for 24 h, and the culture supernatants were subsequently collected for quantification of cytokines by ELISA. Alternatively, cells were co-stimulated via their TCRs and CD28 by anti-CD3 and anti-CD28 Abs (both from BD Biosciences). ELISAs for IL-2 (BioSource International, Camarillo, CA), IL-13 (Immunotech, Westbrook, ME), and IFN-γ (BioSource) were conducted according to the manufacturer’s instructions. To calculate the percentage of maximum cytokine concentration (Fig. 1), the concentration of IL-2 or IFN-γ detected in buffer-preincubated cells stimulated with 10 ng/ml PMA and 150 µg/ml A23187 was set at 100%. The cytokine concentrations from the remaining conditions in the experiment were then expressed as a percentage of that 100% value. The percentages of maximum cytokine concentration values for the three individual experiments were then averaged, and the SEM was calculated. Similar calculations were performed for IL-13 concentrations, except the value for buffer-preincubated cells stimulated with 10 ng/ml PMA and 25 µg/ml A23187 was set at 100%.

Endotoxin determination in recombinant protein preparations

The endotoxin levels of the purified recombinant GST-HrHRF and GST protein preparations were determined using the Limulus amebocyte lysate test (BioWhittaker, Walkersville, MD).
Assessment of potential effects of endotoxin on cytokine production
Polyoxymyxin B sulfate has been shown to bind and neutralize endotoxin (12). In some experiments preincubating PBTs with GST-HrHRF or GST was conducted in the presence of 50 μg/ml polyoxymyxin B sulfate (Sigma-Aldrich), followed by PMA/A23187 stimulation to assess the contribution of endotoxin, if any, to cytokine production.

Measurement of apoptosis
Jurkat T cells were preincubated with buffer, 8 μg/ml GST-HrHRF, or GST for 4.5 h, followed by overnight stimulation with 150 ng/ml A23187 and 10 ng/ml PMA (or buffer as a control). The percentage of cells undergoing apoptosis was determined using flow cytometry after labeling the cells using the TACS-annexin V-biotin kit from R&D Systems (Minneapolis, MN). Cells in the early stage of apoptosis after each type of treatment were labeled using annexin V, while cells in late stage apoptosis/necrosis were stained by propidium iodide.

Transfection and promoter studies
Reporter plasmids consisting of the proximal promoters for IL-2 (312 bp), IL-4 (270 bp), or IL-13 (312 bp) were prepared in the vector pGL3 Basic (Promega Corp., Madison, WI). Deletion constructs of the IL-13 promoter (151 and 123 bp) were prepared in the same vector. Jurkat T cells were transiently transfected using either polyethyleneimine (1.2 μg polyethyleneimine and 1 μg of reporter plasmid, 5 × 10^6 cells) or electroporation (300 V, 1050 microfarads, 0.4-cm gap width cuvettes; Bio-Rad GenePulsar II instrument) with 10 μg of reporter plasmid; 10 × 10^6 cells. After 24 h, the cells were preincubated with 8 μg/ml HrHRF (which contains 9 μg/ml GST), 9 μg/ml GST, or growth medium alone for 3–4.5 h and stimulated with 10 ng/ml PMA and 500 ng/ml A23187 overnight. Production of luciferase in cell lysates was determined using a kit from BD Biosciences and was monitored on a Monolight 3010C luminometer (San Diego, CA). Relative light units for each sample were normalized to the total protein content of the lysate using the bicinchoninic acid protein assay (Pierce, Rockford, IL). To control for transfection efficiency, Jurkat cells were only used between 2 and 6 wk in culture and were transfected under identical conditions with different reporter plasmid preparations. Furthermore, the transfection efficiency of Jurkat cells by electroporation consistently averaged 40%, as determined by analyzing the fluorescence of cells cotransfected with a green fluorescence protein expression vector (data not shown).

Results
Preincubation with HrHRF inhibits primary T cell cytokine production induced by stimulation with PMA and calcium ionophore
Human T lymphocytes were isolated by countercurrent elutriation from leukopheresis packs as described in Materials and Methods. HrHRF has been shown to prime human basophils for enhanced histamine release after anti-IgE stimulation as well as to directly induce degranulation in a subset of donors (6, 7). Therefore, we first tested the ability of HrHRF (8 μg/ml GST-tagged HrHRF) to directly induce the secretion of the cytokines IL-2, IL-13, and IFN-γ after a 24-h incubation. No cytokine production, as determined by ELISA, was detected in the collected culture supernatants (data not shown). In subsequent experiments purified human T cells were preincubated with 8 μg/ml HrHRF or buffer for 4 h, followed by a 20-h stimulation with 10 ng/ml PMA and 10–250 μg/ml A23187. Quantification of secreted IL-2 indicated a dose-dependent production of IL-2 in buffer-preincubated cells between 100 and 250 μg/ml A23187, with a 150 μg/ml calcium ionophore stimulus being optimal (Fig. 1A). The maximum IL-2 produced varied between donors and ranged from 1750–5040 U/ml (n = 3). Increasing the PMA concentration to 50 ng/ml did not enhance production of the cytokines tested (results not shown). However, rather than priming, preincubation with HrHRF partially inhibited IL-2 secretion (by 30%) at doses of stimuli between 150 and 250 μg/ml A23187.

Testing of the same culture supernatants indicated that production of IL-13 was maximal at 25–50 μg/ml calcium ionophore in buffer-preincubated cells, with a maximum of 870–4040 pg/ml IL-13 produced (n = 3 donors). HrHRF preincubation partially inhibited IL-13 production (up to 75%) at 10–50 μg/ml A23187 (Fig. 1B). Equivalent concentrations of IL-13 were released into the supernatants of stimulated T cells at 100–250 μg/ml A23187 regardless of whether they were preincubated with HrHRF or buffer.

Quantification of PMA/A2318- stimulated T cell supernatants from buffer-preincubated lymphocytes resulted in IFN-γ production at A23187 doses between 100 and 250 μg/ml, with a maximum of 65–130 ng/ml IFN-γ produced (n = 3 donors). In contrast to IL-2 and IL-13, IFN-γ in the supernatants of HrHRF- or buffer-preincubated cells was not statistically different at five of the seven doses of A23187 tested and varied between donors, leading us to conclude that HrHRF does not affect IFN-γ production induced by this stimulus (Fig. 1C).

Determination of the endotoxin levels in our baculovirus-expressed HrHRF preparation using the Limulus amebocyte lysate test indicated that only trace amounts were added to our T cell cultures (75 pg/ml endotoxin in 8 μg/ml HrHRF). Furthermore, addition of 50 pg/ml polyoxymyxin B sulfate to bind and neutralize endotoxin did not reverse the HrHRF-induced inhibition of IL-2 or IL-13, indicating that the observed inhibitory effects are not due to endotoxin (results not shown).

Preincubation with HrHRF inhibits IL-13 production induced by costimulation through the TCR and CD28
We next tested the ability of HrHRF to modulate IL-13 production when human T cells were stimulated via their TCR and CD28. As shown in Fig. 2, incubation of buffer-preincubated T cells with 3 μg/ml anti-CD3 and 0.6–5.0 μg/ml anti-CD28 for 24 h resulted in the secretion of 290–380 pg/ml IL-13, while HrHRF-preincubated cells produced only 200–215 pg/ml IL-13, a 31–43% partial inhibition (n = 1). The combination of PMA and A23187 acts to activate protein kinase C (PKC) and increase cytosolic calcium in T cells, thereby bypassing the TCR (13). The ability of HrHRF to block cytokine production induced by both stimuli indicates that HrHRF must be acting at the level of PKC activation/calcium signaling or between PKC/calcium and cytokine transcription in the nucleus.

Dose dependence and specificity of the HrHRF-induced inhibition of IL-2 and IL-13 secretion in PMA/A23187-treated T cells
To test the dose dependence and specificity of the HrHRF-mediated modulation of cytokine production, we preincubated purified HrHRF INHIBITS T CELL CYTOKINE GENE TRANSCRIPTION
were preincubated with 1–8 μg/ml HrHRF (which contains 1.1–9 μg/ml GST) or an equivalent amount of GST alone (1.1–9 μg/ml). The cells were then incubated with a dose of stimulus chosen to maximize the inhibition of cytokine production (10 ng/ml PMA plus 25 μg/ml A23187 for IL-13 or 10 ng/ml PMA plus 150 μg/ml A23187 for IL-2 and IFN-γ). As shown in Fig. 3A, a reduction in the concentration of GST-HrHRF fusion protein preincubated with the cells from 8–1 μg/ml resulted in an increase in IL-13 production from 37.6 ± 9.4% of buffer-treated control cells to 89.2 ± 14.2% of control. The partial inhibition of IL-13 was specific to the HrHRF portion of the fusion protein, since preincubation of the cells with 9 μg/ml GST produced 98.3 ± 14.5% of the IL-13 from buffer control cells.

The secretion of IL-2 was also partially inhibited in a dose-dependent fashion by the concentration of HrHRF preincubated with the T cells, although less markedly. As shown in Fig. 3B, a reduction in the concentration of GST-HrHRF fusion protein preincubated with the cells from 8–1 μg/ml resulted in an increase in IL-2 production from 58.7 ± 7.1% to 112.4 ± 6.7% of that in buffer-treated control cells. As seen with IL-13, the partial inhibition of secreted IL-2 was specific to HrHRF rather than GST, as preincubation of the cells with 9 μg/ml GST resulted in 99.6 ± 0.7% of the IL-2 in buffer-treated cells. In contrast, cells treated with 8 μg/ml GST-HrHRF or 9 μg/ml GST produced similar amounts of IFN-γ (60.5 ± 17.1 or 81.5 ± 26% of buffer control-treated cells, respectively; Fig. 3C).

Our two recombinant protein preparations contained similar trace amounts of endotoxin (75 pg/ml in 8 μg/ml GST-HrHRF; 27 pg/ml in 9 μg/ml GST), but exhibited a differential ability to inhibit IL-13 and IL-2 secretion, providing further evidence that the observed HrHRF-induced inhibition of cytokine production is not due to endotoxin.

The pattern and specificity of HrHRF-induced inhibition of cytokine protein production in Jurkat T cells are identical with those in primary T cells

To explore the mechanism by which HrHRF regulates cytokine production in a transfectable T cell, we conducted a series of experiments with the Jurkat T cell line. We first tested the ability of HrHRF to modulate the production of IL-2, IL-13, and IFN-γ from Jurkat T cells stimulated with 10 ng/ml PMA and 12.5–250 μg/ml A23187 for 24 h. For all three cytokines examined, 10 ng/ml PMA and 150 μg/ml A23187 were the optimal stimuli from growth medium-preincubated Jurkat cells (Fig. 4). The secretion of IL-2 and IL-13 was partially inhibited (up to 70%) after preincubation of the cells with 8 μg/ml GST-HrHRF for 4 h compared with that in medium-preincubated cells (Fig. 4, A and B). In contrast, no statistically significant differences were detected in IFN-γ production from cells preincubated with medium or HrHRF at the stimulus doses tested (Fig. 4C).

We next investigated the specificity and dose dependence of the HrHRF-mediated inhibition of IL-2 production in Jurkat cells. Jurkat cells were preincubated with 9 μg/ml GST, 8 μg/ml GST-HrHRF (which contains 9 μg/ml GST), or growth medium for 4 h.

of GST (1.1–9 μg/ml), followed by stimulation with PMA (10 ng/ml) and A23187 (25 μg/ml for IL-13, 150 μg/ml for IL-2 and IFN-γ). Cytokines were quantified by ELISA on the cell supernatants. A, IL-13; B, IL-2; C, IFN-γ. The data are expressed as the percentage of cytokine secreted by buffer-preincubated control cells and represent the mean ± SEM from three separate donors. The range of cytokines detected in supernatants from buffer-preincubated (100% control) samples was 1965–3853 U/ml IL-2, 0.5–2.8 ng/ml IL-13, and 36.6–151 ng/ml IFN-γ.
The cells were then stimulated overnight with 10 ng/ml PMA and 150 μg/ml A23187. The results, as depicted in Fig. 5, indicate that the GST-HrHRF-mediated inhibition is relatively specific for HrHRF, since 47.2 ± 8.5% inhibition of IL-2 secretion was observed compared with 9.6 ± 7.8% inhibition with baculovirus GST (average ± SEM; n = 3). In a single experiment, preincubation with GST (9 μg/ml) purified from porcine liver gave only 1.3% inhibition of IL-2 production, further confirming the specificity of the IL-2 inhibition. Furthermore, the HrHRF-mediated inhibition of IL-2 production was dose dependent, since reducing the concentration of HrHRF incubated with the cells from 8 to 1 μg/ml reduced the inhibition from 47.2 ± 8.5 to 15.0 ± 1.3%. In comparison, preincubation with 1 μg/ml GST did not affect PMA/A23187-induced IL-2 production from Jurkat cells (1.6 ± 1.3% inhibition).

Simultaneous addition of HrHRF and PMA/A23187 stimuli is equivalent to a 4-h HrHRF preincubation in inhibiting production of IL-13 and IL-2 protein in Jurkat T cells.

To begin to understand the mechanism by which HrHRF limits the production of specific cytokines, we varied the time the cells were preincubated with HrHRF (or medium as a control) before addition of stimulus (10 ng/ml PMA and 150 μg/ml A23187). As expected, the length of the preincubation period with medium (0–4 h) did not significantly affect the amount of either IL-2 or IL-13 produced after stimulation with PMA and A23187 (Fig. 5, A or B, respectively). However, to our surprise, preincubation with HrHRF for 0.5 h was equivalent to 4 h in partially inhibiting either IL-2 or IL-13 production (by 50–60%) compared with cells preincubated with medium for the same time (Fig. 6, A or B, respectively). Furthermore, even simultaneous addition of HrHRF and stimulus (0-h preincubation) was effective at partially preventing the secretion of IL-2 or IL-13. These data suggest that the production of IL-2 or IL-13 was partially inhibited by HrHRF.

**FIGURE 4.** The pattern of HrHRF-induced inhibition of cytokine protein production in Jurkat T cells is identical with that seen in primary T cells. Jurkat T cells were preincubated and stimulated, and secreted cytokines were quantified as described in Fig. 1. A, IL-2; B, IL-13; C, IFN-γ. The data represent the mean ± SEM from three separate experiments.

**FIGURE 5.** Dose dependence and specificity of the HrHRF-induced inhibition of IL-2 secretion in PMA/A23187-treated Jurkat T cells. Jurkat cells were preincubated with 1.1 or 9 μg/ml GST, 1 or 8 μg/ml GST-HrHRF (which contains 1.1 or 9 μg/ml GST, respectively), or growth medium for 4 h. The cells were then stimulated overnight (10 ng/ml PMA and 150 μg/ml A23187), and secreted IL-2 was quantified by ELISA. The percent inhibition of IL-2 induced by GST-HrHRF preincubation was calculated by subtracting the concentration of IL-2 for GST-HrHRF-treated cells from that of medium-treated cells and then dividing by the value for medium-treated cells. The data represent the mean ± SEM from three separate experiments. The concentrations of IL-2 in the supernatants of medium-preincubated cells were 46.6, 126.4, and 147.4 U/ml in the three experiments conducted.
post-translational modification of a putative HrHRF-induced inhibitory factor does not require time to accumulate before stimulus addition to be effective. Furthermore, these data suggest that HrHRF may generate an inhibitory factor that competes for binding to intracellular effector sites with a stimulatory factor inducible by both TCR stimulation and PMA/A23187 treatment.

Degree of HrHRF-induced inhibition of IL-2 and IL-13 increases between 3 and 24 h in Jurkat T cells

In the next set of experiments we preincubated Jurkat T cells with HrHRF or medium alone for 4 h, added PMA and A23187, and harvested the culture supernatants 3, 8, or 24 h later. Testing the supernatants from medium-preincubated cells for secreted IL-2 indicated that production of the cytokine was 7.5 ± 1.9% at 3 h and 54 ± 11.5% at 8 h of the value reached 24 h poststimulation (Fig. 7A). Compared with medium-treated cells, HrHRF-treated cells produced proportionally less IL-2 with time (37.3% inhibition at 3 h, 45.5% at 8 h, 55.5% at 24 h). As shown in Fig. 7B, the production of IL-13 from stimulated Jurkat cells increased with time, as expected. HrHRF-treated cells also produced proportionally less IL-13 with time, compared with medium-treated cells (40% inhibition at 3 h, 49.2% at 8 h, 65.4% at 24 h).

Effect of HrHRF-induced reduction in IL-2 secretion on T cell proliferation or apoptosis

We have begun to investigate the consequences of HrHRF-induced reductions in IL-2 production on PBT proliferation and Jurkat T cell apoptosis. In two separate experiments PBTs were counted by hemocytometer after buffer or HrHRF preincubation and PMA/A23187 stimulation. There was no decrease in the number of cells recovered after HrHRF treatment, indicating that the reduction in IL-2 is not associated with decreased proliferation of T cells. After preincubation with buffer, GST, or GST-HrHRF, Jurkat cells were stimulated with PMA/A23187 (or buffer as a control) and labeled

FIGURE 6. Simultaneous addition of HrHRF and PMA/A23187 stimuli is equivalent to a 4-h HrHRF preincubation in inhibiting the production of IL-13 and IL-2 protein in Jurkat T cells. Jurkat T cells were preincubated for 0, 0.5, or 4 h with growth medium with or without 8 μg/ml HrHRF before addition of stimulus (10 ng/ml PMA and 150 μg/ml A23187) for 24 h. A, IL-2; B, IL-13. The data are expressed as the percentage of cytokine recovered from cells preincubated with medium alone for 0.5 h and represent the mean ± SEM from three separate experiments.

FIGURE 7. The degree of HrHRF-induced inhibition of IL-2 and IL-13 increases between 3 and 24 h in Jurkat T cells. Jurkat T cells were preincubated with 8 μg/ml HrHRF or medium alone for 4 h, then stimulated for 24 h (10 ng/ml PMA and 150 μg/ml A23187), and the culture supernatants were harvested 3, 8, or 24 h later. A, IL-2; B, IL-13. The data are expressed as the percentage of cytokine recovered from cells preincubated with medium alone after stimulation for 24 h and represent the mean ± SEM from three separate experiments.
with annexin V and propidium iodide. The percentage of cells undergoing apoptosis was determined using flow cytometry. Stimulation increased the percentage of cells undergoing apoptosis and necrosis slightly, but preincubation with GST-HrHRF or GST did not affect this process (data not shown).

Activation of the proximal IL-13, IL-2, or IL-4 promoter by PMA/A23187 is prevented by preincubation of Jurkat T cells with HrHRF

Since production of the cytokines IL-2 and IL-13 is regulated at the transcriptional level, we tested the effect of HrHRF preincubation on activation of the proximal promoter for IL-2 or IL-13. Jurkat T cells were transiently transfected with reporter plasmids, consisting of the 312-bp proximal promoters for either IL-2 or IL-13 coupled to luciferase using polyethyleneimine (see Materials and Methods). After 24 h the cells were incubated with HrHRF or buffer for 4.5 h and then stimulated with PMA and A23187 overnight. Promoter activation was measured by the relative light units from the luciferase expressed in cell lysates. Stimulated Jurkat cells showed a 35-fold activation of the IL-2 promoter construct relative to unstimulated cells, which was partially inhibited, in a dose-dependent fashion, by preincubation with 2–8 μg/ml HrHRF (Fig. 8A).

To test the specificity of the HrHRF-induced inhibition of IL-2 promoter activation, Jurkat cells were transfected by electroporation with an IL-2-luciferase reporter construct. After 24 h the cells were preincubated with 8 μg/ml GST-HrHRF, an equivalent amount of GST, or growth medium for 3 h. The cells were then stimulated overnight with 10 ng/ml PMA and 500 μg/ml A23187. In two experiments 59% inhibition of IL-2 promoter-driven luciferase activity was observed upon GST-HrHRF preincubation compared with medium preincubation (Fig. 8B). In contrast, GST preincubation produced only 17.2% inhibition of IL-2 promoter activation compared with that in medium-preincubated Jurkat cells. Therefore, the inhibition of IL-2 transcription induced by GST-HrHRF appears to be relatively specific for the HrHRF portion of the molecule.

We then transfected Jurkat cells with three different IL-13 reporter plasmids (312, 151, and 123 bp) to localize the IL-13 promoter region targeted by HrHRF. Stimulated Jurkat cells induced the 312-bp IL-13 promoter nearly 7-fold, which was partially inhibited (60%) by preincubation with 8 μg/ml HrHRF (Fig. 8C). A deletion mutant containing the proximal 151 bp of the IL-13 promoter was more weakly induced upon PMA/A23187 stimulation and was partially inhibited (40%) by HrHRF. A second mutant containing only the proximal 123 bp of the IL-13 promoter and

FIGURE 8. Activation of a proximal IL-13 or IL-2 promoter by PMA/A23187 is inhibited by preincubation of Jurkat T cells with GST-HrHRF, but not GST alone. A, Jurkat T cells were transiently transfected using polyethyleneimine with a reporter construct containing a 312-bp proximal promoter for IL-2. After 24 h, the cells were preincubated with 2–8 μg/ml HrHRF or medium alone for 4.5 h, then stimulated (10 ng/ml PMA and 500 μg/ml A23187) or not (DMSO) overnight before assay for cell-associated luciferase activity as described in Materials and Methods. B, Jurkat cells were transiently transfected by electroporation with a 312-bp IL-2 proximal promoter-luciferase construct and then preincubated with 9 μg/ml GST, 8 μg/ml GST-HrHRF (which contains 9 μg/ml GST), or growth medium for 3 h. The cells were then stimulated overnight (10 ng/ml PMA and 150 μg/ml A23187), and cell-associated luciferase activity was quantified. C, Jurkat T cells were transfected using polyethyleneimine with various IL-13 proximal promoter constructs (312, 151, or 123 bp). After 24 h, the cells were preincubated with 8 μg/ml HrHRF or medium alone for 4.5 h, then stimulated (10 ng/ml PMA and 1 μg/ml A23187) or not (DMSO) overnight before assay for cell-associated luciferase activity. The data in all three panels are expressed as the fold activation (stimulated cellular luciferase/resting cellular luciferase) and represent the mean ± SEM from four separate experiments (A and C) or the mean ± range from two separate experiments (B).
therefore lacking NFAT binding sites was not induced by PMA/A23187, as reported previously (14). In additional experiments, PMA/A23187-induced activation of a luciferase reporter construct containing a 270-bp IL-4 proximal promoter was partially inhibited (32.9 ± 8.5%; n = 3) after preincubation with 8 µg/ml HrHRF. These data suggest that HrHRF induces signals in primary T cells and Jurkat T cells that partially disrupt transcription of the cytokines IL-2, IL-4, and IL-13.

### Discussion

Previous studies from our laboratory have indicated that HrHRF can both directly stimulate cytokine secretion from basophils as well as prime basophils and eosinophils for enhanced secretion of cytokines (6–8). In our current study we demonstrate that preincubation with GST-tagged HrHRF, but not GST alone, can partially inhibit the secretion of IL-2 and IL-13 protein in a dose-dependent manner from both purified peripheral blood T cells and Jurkat T cells. HrHRF was not able to directly stimulate secretion of the cytokines tested (IL-2, IL-13, and IFN-γ) from PBTs after treatment with PMA/A23187. The inhibition was found to be specific to the HrHRF portion of our GST-HrHRF fusion protein, dose dependent on the concentration of HrHRF added during the preincubation period, and independent of endotoxin (Figs. 3 and 5). Additional specificity was seen in the cytokines affected and the sensitivity of each cytokine to HrHRF-induced inhibition (Figs. 1 and 4). Therefore, HrHRF can modulate cytokine production stimulated through several types of plasma membrane receptors, including FcRs (such as FcεRI and TCR) as well as cytokine receptors (such as those for TNF-α and GM-CSF) (2, 8). Signaling intermediates in common to these receptors include the use of nonreceptor protein tyrosine kinases, PKC isoforms, and increases in cytosolic calcium, which may allow HrHRF to modulate cytokine secretion from diverse hemopoietic cell types (13).

Preincubation with HrHRF was found to partially inhibit IL-2 and IL-13 secretion from PBTs stimulated through both the TCR/CD-28 and PMA/A23187 (Figs. 1 and 2). The site of action for HrHRF in this experimental system appears to lie between calcium and PKC and transcription in the nucleus. Also, simultaneous addition of HrHRF and PMA/A23187 resulted in partial inhibition of IL-2 and IL-13 production equivalent to that of a 4.5-h HrHRF preincubation (Fig. 6). We interpret this result to indicate that the inhibitory signal generated by HrHRF does not require time to accumulate or be synthesized before stimulus (PMA/A23187) addition. Furthermore, these data suggest that HrHRF may generate an inhibitory factor that competes for binding to intracellular effector sites with a stimulatory factor inducible by both TCR stimulation and PMA/A23187 treatment. Even though previously published data indicated that a 15-min preincubation with HrHRF primed basophils for cytokine secretion (7), more recent data demonstrate that this priming effect occurs with simultaneous addition of HrHRF (data not shown). Therefore, the HrHRF-induced inhibitory signal in T cells has the same kinetics as the signal generated during priming of basophils for histamine release.

The ability of HrHRF to prevent transcription of both Th1 (IL-2) and Th2 (IL-4, IL-13) cytokines is indicated by the partial reduction of luciferase activity after HrHRF preincubation of Jurkat cells transfected with reporter constructs (Fig. 8 and Results). A comparison of the proximal promoters for these cytokines indicates that all three contain binding sites for the widely expressed transcription factors NFAT and NF-xB (15, 16). The proximal promoter for IFN-γ also contains sites for binding NFAT and NF-xB, but the lack of statistically significant HrHRF-induced inhibition of IFN-γ secretion may reflect qualitative differences in the potency of the inhibitory signal (17) or compensation by IFN-γ-specific transcription factors (18).

The partial inhibition of cytokine secretion observed in T cells, rather than priming for secretion reported for basophils, B cells, and eosinophils, may reflect cell type-specific differences in isoform expression of the targeted signaling molecule(s). For instance, PKC isoform expression may contribute to the inhibition of cytokine secretion in T cells vs. the enhanced secretion seen in the other cell types discussed. PKC-θ is uniquely expressed in T cells and is activated by both stimuli used in this study, making it a potential site for interaction with an HrHRF-induced inhibitory signal (19, 20). In contrast, human basophils express PKC-βI, βIII, δ, and ε, but these enzymes are not activated by stimulation through the FcεRI, as measured by PKC translocation from the cytoplasm to the nucleus (21). PMA does induce PKC-βI, βIII, and δ, but not PKC-δ, translocation in basophils. However, Schroeder et al. (22) have demonstrated that secretion of IL-4 from basophils induced by the calcium ionophore, ionomycin, is inhibited by the simultaneous addition of PMA at a post-transcriptional step. For PBTs, the same authors found that the combination of PMA and ionomycin was synergistic in inducing the secretion of IL-4. These data thus suggest that a PKC-activating signal, such as PMA, can have opposing effects on cytokine production in basophils vs T cells.

Subsequent to acute allergy exposure, there is a recrudescence of symptoms in many individuals, which has been termed the late phase response (LPR) (23). Furthermore, HRF is present in skin blister fluids after allergen challenge in human donors during the LPR as well as in nasal lavage fluids from unstimulated individuals (1, 24). HrHRF has now been shown to affect the cytokine production of many of the cells participating in the LPR, namely, basophils, eosinophils, and T cells. One may speculate that at localized sites of late phase allergic inflammation, HRF may have opposing effects by stimulating basophils and eosinophils to release proinflammatory mediators while acting on T cells to limit cytokine production. Our ongoing research to understand the molecular details of HrHRF-mediated cytokine modulation should allow the role of this broadly active factor in allergic inflammation to be more thoroughly understood.

### Acknowledgments

We thank John Schmidt for technical assistance in conducting IL-2, IL-4, and IL-13 promoter assays, and Valerie Alexander for T cell purification.

### References


