

Differential regulation of eotaxin expression by IFN- γ in airway epithelial cells

Satoshi Matsukura, MD, PhD,^a Fumio Kokubu, MD, PhD,^a Hideki Kuga, MD, PhD,^a
Mio Kawaguchi, MD, PhD,^a Koushi Ieki, MD,^a Miho Odaka, MD,^a
Shintarou Suzuki, MD,^a Shin Watanabe, MD,^a Hiroko Takeuchi, BS,^a
Mitsuru Adachi, MD, PhD,^a Cristiana Stellato, MD, PhD,^b and
Robert P. Schleimer, PhD^b Tokyo, Japan, and Baltimore, Md

Background: Eotaxin is a chemokine that binds with high affinity and specificity to the chemokine receptor CCR3 and plays an important role in the pathogenesis of allergic disease. **Objective:** We studied the regulation of eotaxin expression by the T_H1 cytokine IFN- γ and analyzed its molecular mechanisms.

Methods: Levels of eotaxin mRNA and protein expression in the airway epithelial cell line BEAS-2B were determined with RT-PCR and ELISA. Mechanisms of transcriptional regulation were assessed by means of electrophoretic mobility shift assays and luciferase assay with eotaxin promoter-luciferase reporter plasmids.

Results: Although IFN- γ did not directly induce the expression of eotaxin protein, it increased the induction by TNF- α when these cytokines were added simultaneously. In contrast, preincubation of cells with IFN- γ for 24 hours profoundly inhibited the production induced by TNF- α . IFN- γ did not influence the TNF- α -induced binding of nuclear factor κ B to a DNA probe derived from the eotaxin promoter. IFN- γ did not increase the ability of TNF- α to activate the eotaxin promoter. Studies of eotaxin mRNA levels indicate that IFN- γ combined with TNF- α increased the expression of eotaxin mRNA. When cells were preincubated with IFN- γ , there was no inhibition of the appearance of eotaxin mRNA.

Conclusion: These studies demonstrate that IFN- γ enhances eotaxin expression when added in combination with TNF- α and profoundly inhibits eotaxin expression after preincubation. In both cases the available data indicate that the effect is mediated by a posttranscriptional mechanism. (J Allergy Clin Immunol 2003;111:1337-44.)

Key words: Eotaxin, IFN- γ , epithelial cells, asthma

Abbreviations used

EMSA: Electrophoretic mobility shift assay
 γ IRE: IFN- γ -responsive element
IRF: IFN regulatory factor
NF- κ B: Nuclear factor κ B
STAT: Signal transducer and activator of transcription

Asthma is a disease characterized by the infiltration of eosinophils and lymphocytes into airway epithelium and subsequent epithelial damage and tissue remodeling. T_H2 cells and their cytokine products play a crucial role in this process.^{1,2} Eotaxin is a C-C chemokine that was identified by means of the purification of an eosinophil chemoattractant from bronchoalveolar lavage fluid of ovalbumin-sensitized and ovalbumin-challenged guinea pigs.³ CCR3 is a specific receptor for eotaxin and is expressed on important cells in allergic disease, such as eosinophils, basophils, a subset of T_H2 cells, and mast cells.⁴⁻⁸ Eotaxin has been reported to be highly expressed in the epithelium of asthmatic patients and might play an important role in the infiltration of these cells into the airways. Although many different cell types can express eotaxin, airway epithelial cells are a major cell source of eotaxin in the airway.^{9,10}

Previous reports have demonstrated that TNF- α and IL-4 synergistically stimulate eotaxin expression in epithelial cells and fibroblasts.¹¹⁻¹⁴ A similar synergistic effect on the induction of eotaxin expression in airway epithelial cells has been observed with TNF- α and IL-13.¹⁴ Upregulation of eotaxin expression by TNF- α and IL-4/IL-13 might be expected to occur in asthma because these cytokines are known to be produced in asthma and have been implicated in the pathogenesis of asthma. We have demonstrated that the eotaxin promoter has adjacent binding sites for nuclear factor κ B (NF- κ B) and signal transducer and activator of transcription 6 (STAT-6), and these 2 transcription factors activate eotaxin transcription and subsequent mRNA-protein induction in response to TNF- α and IL-4/IL-13 stimulation, respectively.^{13,14}

It has been reported that IFN- γ can potentiate eotaxin expression induced by TNF- α in airway epithelial cells.^{12,15,16} On the other hand, recent reports showed that IFN- γ inhibits eotaxin expression in fibroblasts.^{17,18}

From ^athe First Department of Internal Medicine, Showa University School of Medicine, Tokyo, and ^bJohns Hopkins Asthma and Allergy Center, Baltimore.

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Reprint requests: Satoshi Matsukura, MD, PhD, First Department of Internal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.

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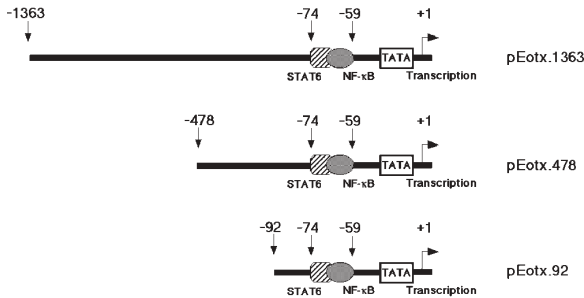


FIG 1. Structures of the eotaxin promoter-luciferase reporter plasmids containing 1363, 478, and 92 bp of the eotaxin promoter region (referred to as pEotx.1363, pEotx.478, and pEotx.92, respectively) are depicted.

Regulation of epithelial cell eotaxin expression by IFN- γ is likely to be relevant to various types of inflammation of the airways, and it is important to better understand this process.

In the present study we show that the effect of IFN- γ on eotaxin expression in airway epithelial cells is dependent on the timing of exposure to the cytokine: preincubation with IFN- γ profoundly inhibited eotaxin expression, whereas cocubation enhanced eotaxin expression when TNF- α was used as a stimulus. We also investigated the possible mechanisms of these effects and present evidence that the regulation of eotaxin transcription by IFN- γ occurs at the posttranscriptional level.

METHODS

Cell culture

BEAS-2B is a human airway epithelial cell line transformed with adenovirus 12-SV40 hybrid virus (a kind gift from Dr Curtis Harris).¹⁹ BEAS-2B cells were cultured in Dulbecco modified Eagle medium/F12 with 10% FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin (Invitrogen Corp, Tokyo, Japan) at 37°C with 5% CO₂ in humidified air.

Assay of eotaxin and RANTES protein release into the culture medium

Concentrations of eotaxin and RANTES in the collected culture medium were determined with a commercially available system for ELISA (R&D systems, Tokyo, Japan), as described previously.¹² The limit of detection in the assay was 5 pg/mL for eotaxin and 8 pg/mL for RANTES.

Nuclear extraction

BEAS-2B cells were treated for 24 hours with or without IFN- γ (50 ng/mL) and then incubated with control medium, IFN- γ (50 ng/mL), or TNF- α (100 ng/mL) for 30 minutes. Then nuclear extracts were prepared on the basis of methods described previously.¹³

Electrophoretic mobility shift assays

DNA-protein binding assays were based on methods described previously.¹³ Aliquots of 5 μ g of nuclear extracts were incubated in 10 μ L of total reaction volume containing 10 mmol/L Tris-HCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 10% glycerol, 0.1% Triton X-100, 50 μ g/mL poly(dI-dC), 0.1 mg/mL BSA, and 50 mmol/L KCl (Invitrogen Corp) with phosphorous 32-labeled oligonucleotide probe at room temperature for 20 minutes with or without

unlabeled oligonucleotide probe. In some experiments, antibodies against p50 (NF- κ B1), p65 (Rel A), STAT-1, and IFN regulatory factor 1 (IRF-1; Santa Cruz Biotech, Tokyo, Japan) were incubated with the mixture for 30 minutes after incubation with labeled probe. The reaction products were analyzed by means of electrophoresis in a 5% polyacrylamide gel with 0.5 $\times$ TBE buffer. The gels were dried and analyzed by means of autoradiography. The sequence of the oligonucleotide probe used in electrophoretic mobility shift assays (EMSAs) is 5'-GGCTTCCTGGAATCTCCACA-3', including the binding sites for STAT-6 and NF- κ B in the eotaxin promoter, as reported previously.¹³

Construction of eotaxin promoter-luciferase reporter plasmids

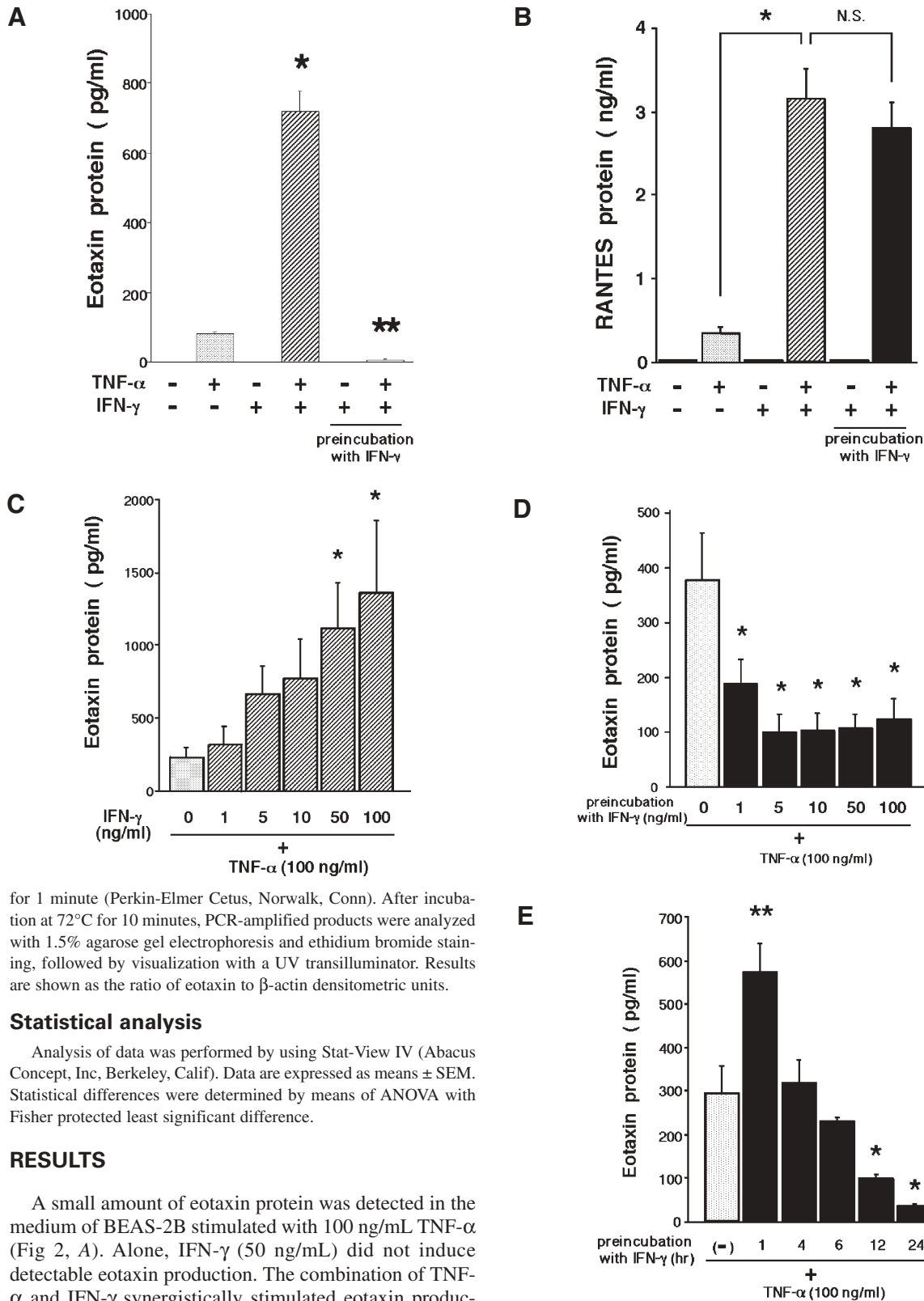
Methods of construction of eotaxin promoter-luciferase reporter plasmids have been described previously.¹³ A 1363-bp fragment of the promoter region of the gene encoding eotaxin (site -1363 to -1) was amplified by means of PCR and ligated into *Mlu*I and *Bgl*II sites of a firefly-luciferase reporter pGL3-basic vector (Promega, Madison, Wis), and the construct is referred to as pEotx.1363. pEotx.478 and pEotx.92 were constructed by deleting portions of the 5' end of the eotaxin promoter sequence of pEotx.1363 (Fig 1).

Transient transfections and luciferase assay

BEAS-2B cells were seeded into 6-well plates and allowed to grow to 50% to 70% confluence. Cells were transfected with 1 μ g of reporter plasmids and 10 ng of a control renilla-luciferase vector pRL-TK (Promega, Tokyo, Japan) by using 3 μ L of Fugene 6 transfection reagent (Roche Diagnostics, Tokyo, Japan) and incubated for 24 hours in 2 mL of medium. After incubation with or without IFN- γ (50 ng/mL) for 24 hours, additional stimuli were then added, and 6 hours later, cells were washed twice with Ca²⁺- and Mg²⁺-free PBS, solubilized by means of incubation in 500 μ L of lysis buffer for 20 minutes, transferred to microtubes, and centrifuged to pellet cellular debris. The supernatants were stored at -80°C until luciferase activity was measured with the Dual-Luciferase Assay System (Promega) and a Gene-Light 55 luminometer (Microtech Nichion, Chiba, Japan). The firefly-luciferase activity of the eotaxin promoter-luciferase reporter plasmid was normalized by using renilla luciferase activity and calculated as fold induction compared with the control value.

Reverse transcriptase-polymerase chain reaction

Gene expression of eotaxin and β -actin was analyzed by means of RT-PCR, as previously described.²⁰ BEAS-2B cells were treated for 24 hours with or without IFN- γ (50 ng/mL) and then incubated with control medium, IFN- γ (50 ng/mL), or TNF- α (100 ng/mL) for 24 hours, and total RNA was extracted with ISOGEN reagent (Nippon-Gene, Tokyo, Japan). First-strand cDNAs were synthesized by using a cDNA extraction kit (Amersham Pharmacia Biotech, Tokyo, Japan). After cDNAs were denatured at 94°C for 5 minutes, the PCR was performed with 5 pmol each of the forward and reverse primers, 5 μ L of cDNA, 0.6 U of Taq polymerase (Roche Diagnostics), and 2.5 μ L of PCR reaction buffer (100 mmol/L Tris-HCl, 15 mmol/L MgCl₂, and 500 mmol/L KCl; Roche Diagnostics); distilled water was added to bring the reaction volume to 25 μ L. The sequences of the primers for eotaxin were 5'-CCAACCACCTGCTGCTTTAACCTG-3' (forward) and 5'-GCTTTGGAG TTGGA-GATTT TTGG-3' (reverse), and those for β -actin were 5'-GTGGGGCGCCCCAGGCACCA-3' (forward) and 5'-CTCCT-TAATGTCACGCACGATTTC-3' (reverse). The amplification reaction was performed for 30 cycles, with denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C



for 1 minute (Perkin-Elmer Cetus, Norwalk, Conn). After incubation at 72°C for 10 minutes, PCR-amplified products were analyzed with 1.5% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with a UV transilluminator. Results are shown as the ratio of eotaxin to β -actin densitometric units.

Statistical analysis

Analysis of data was performed by using Stat-View IV (Abacus Concept, Inc, Berkeley, Calif). Data are expressed as means $\pm$ SEM. Statistical differences were determined by means of ANOVA with Fisher protected least significant difference.

RESULTS

A small amount of eotaxin protein was detected in the medium of BEAS-2B stimulated with 100 ng/mL TNF- α (Fig 2, A). Alone, IFN- γ (50 ng/mL) did not induce detectable eotaxin production. The combination of TNF- α and IFN- γ synergistically stimulated eotaxin production ($P < .05$ vs either alone). Pretreatment with 50 ng/mL IFN- γ for 24 hours before TNF- α stimulation markedly inhibited expression of eotaxin production induced by TNF- α ($P < .05$ vs TNF- α alone). To confirm

FIG 2. Release of eotaxin (A, C, D, and E) or RANTES (B) protein into the medium by BEAS-2B cells. Concentrations of eotaxin or RANTES protein in the media are presented as the means $\pm$ SEM of a total of 6 independent experiments.

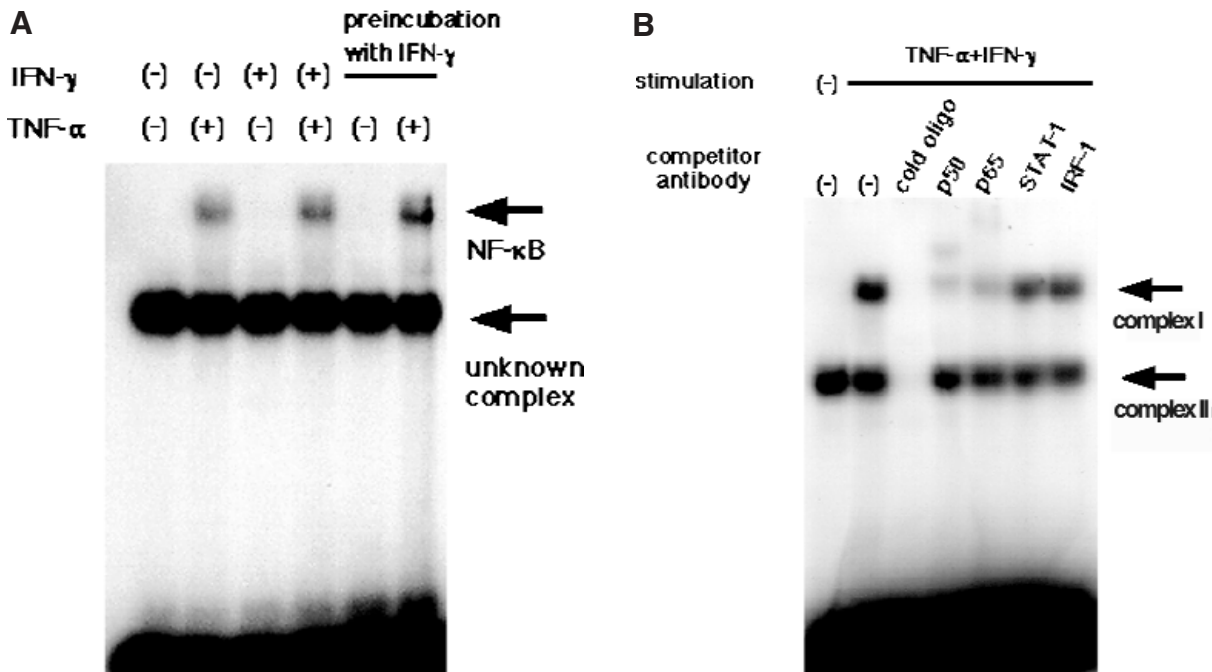


FIG 3. IFN- γ does not alter formation of a binding complex of NF- κ B (p50/p65) with a DNA probe derived from the proximal region of the eotaxin promoter. **A**, TNF- α induced formation of a binding complex, which was not changed by costimulation or preincubation with IFN- γ . **B**, The binding complex was confirmed to consist of p50/p65 NF- κ B family by using antibodies as indicated. Results are representative of 3 independent experiments.

that the inhibitory effect of preincubation with IFN- γ was not due to a generalized inhibition of cellular responses, we analyzed the effect of IFN- γ on RANTES production in BEAS-2B cells. RANTES protein production was increased by TNF- α , and this response was dramatically enhanced by IFN- γ (Fig 2, *B*). Preincubation with IFN- γ did not further increase RANTES expression nor did it inhibit the induction of RANTES by TNF- α . These data indicate that the effect of IFN- γ is distinct for the expression of each chemokine. We next confirmed the concentration and time-dependent effects of both activating and inhibitory effects of IFN- γ . The synergistic effect of IFN- γ on the expression of eotaxin protein induced by TNF- α (100 ng/mL) depended on the concentration of IFN- γ used (Fig 2, *C*). Likewise, the inhibition of eotaxin protein expression with preincubation was dependent on the concentration of IFN- γ (Fig 2, *D*). Interestingly, the inhibitory effects of IFN- γ required lower concentrations than the activating effects (Fig 2, *C* and *D*). The inhibitory effect of preincubation with IFN- γ (50 ng/mL) also depended on the time of preincubation, and a significant inhibitory effect was observed only with preincubation with IFN- γ for more than 12 hours (Fig 2, *E*).

Unfortunately, several laboratories, including our laboratories in Tokyo and Baltimore, as well as the laboratory of Luster et al in Boston (personal communication), have found that primary epithelial cell isolates make very little eotaxin. Because epithelial cells are well documented to express large amounts of eotaxin *in vivo*, we believe that this is a technical problem. It precludes repeating these studies in primary cells for the time being, however.

We next focused experiments on the regulation of eotaxin transcription by IFN- γ . First, we hypothesized that IFN- γ might influence the binding of NF- κ B to the NF- κ B binding site in the eotaxin promoter.²¹ EMSAs with nuclear extracts from BEAS-2B cells stimulated with 100 ng/mL TNF- α resulted in formation of a strong binding complex (Fig 3, *A*). This binding complex was confirmed to be a heterodimer of p50 and p65 NF- κ B Rel family members, as reported earlier.¹³ Binding of NF- κ B was not affected by means of either simultaneous stimulation or preincubation with IFN- γ . Formation of the complex in extracts from cells stimulated with the combination of IFN- γ and TNF- α was inhibited by an excess of unlabeled oligonucleotide and was supershifted by anti-p50 and anti-p65 NF- κ B Rel family antibodies but was not inhibited by anti-STAT-1 or anti-IRF-1 antibodies (Fig 3, *B*). These experiments indicate that IFN- γ neither inhibits nor enhances the ability of TNF- α to activate NF- κ B.

We next analyzed the effect of IFN- γ on the transcriptional activity of the eotaxin promoter. TNF- α (100 ng/mL) activated the eotaxin promoter-luciferase reporter plasmid pEotx.1363, as reported previously (2.4 ± 0.1 -fold induction; Fig 4, *A*).¹³ IFN- γ (50 ng/mL) alone did not affect the eotaxin promoter activity. Simultaneous stimulation with IFN- γ and TNF- α was no different than stimulation with TNF- α alone (2.1 ± 0.1 -fold induction). Pretreatment with IFN- γ only modestly inhibited the activity induced by TNF- α (1.6 ± 0.2 -fold induction). This pattern of regulation of eotaxin promoter activity was also observed with pEotx.478 (Fig 4, *B*). Interestingly, the inhibitory effect of preincubation with

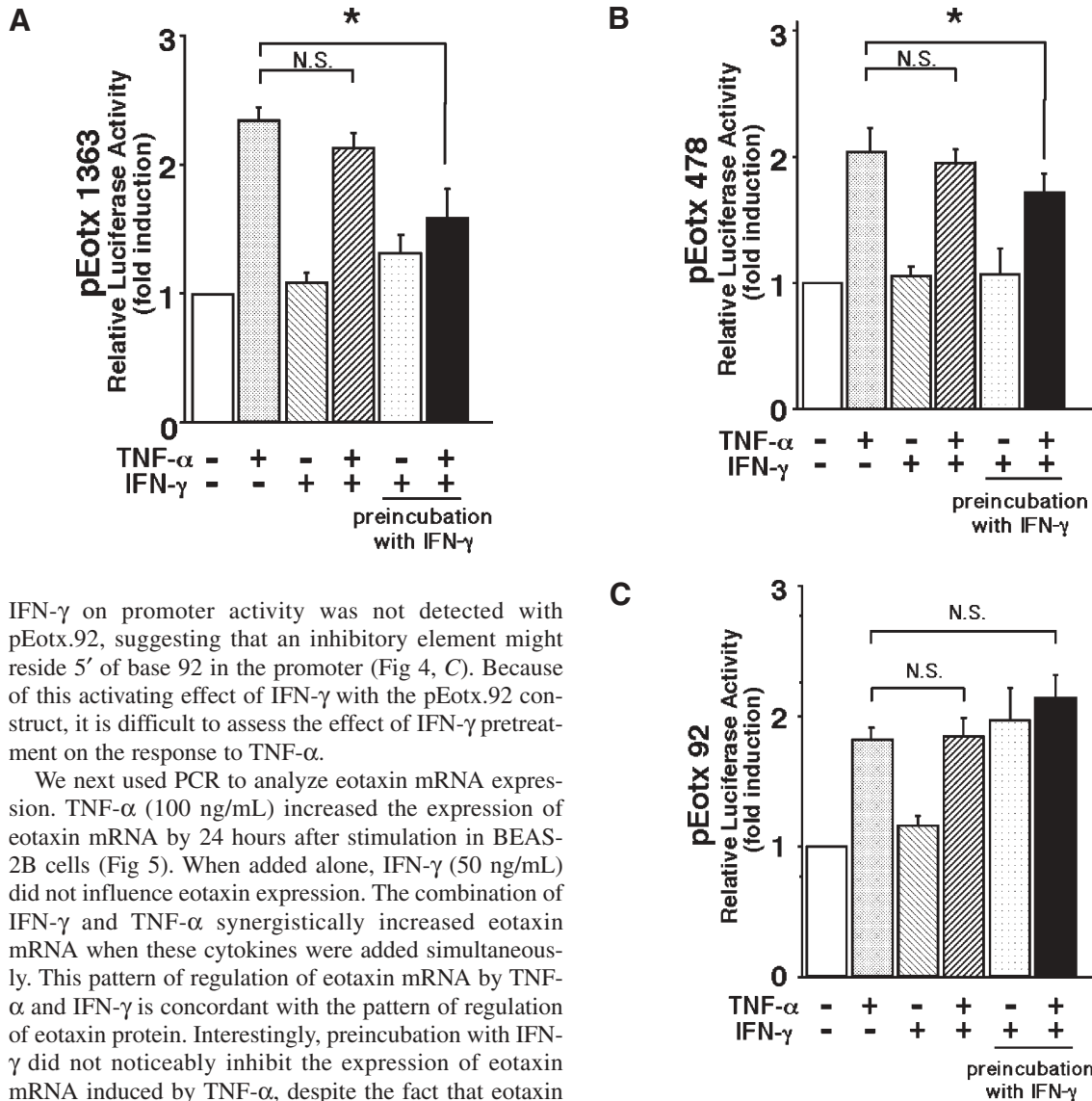


FIG 4. Effect of IFN- γ on eotaxin promoter activity. BEAS-2B cells were transfected with the eotaxin promoter-luciferase reporter plasmids shown in Fig 1 (pEotx.1363, pEotx.478, and pEotx.92) and the control vector pRL-TK. The relative luciferase activity was calculated as fold induction compared with the control value. The data are presented as the means $\pm$ SEM of a total of 3 independent experiments. * $P < .05$. **A**, pEotx.1363; **B**, pEotx.478; and **C**, pEotx.92.

IFN- γ on promoter activity was not detected with pEotx.92, suggesting that an inhibitory element might reside 5' of base 92 in the promoter (Fig 4, C). Because of this activating effect of IFN- γ with the pEotx.92 construct, it is difficult to assess the effect of IFN- γ pretreatment on the response to TNF- α .

We next used PCR to analyze eotaxin mRNA expression. TNF- α (100 ng/mL) increased the expression of eotaxin mRNA by 24 hours after stimulation in BEAS-2B cells (Fig 5). When added alone, IFN- γ (50 ng/mL) did not influence eotaxin expression. The combination of IFN- γ and TNF- α synergistically increased eotaxin mRNA when these cytokines were added simultaneously. This pattern of regulation of eotaxin mRNA by TNF- α and IFN- γ is concordant with the pattern of regulation of eotaxin protein. Interestingly, preincubation with IFN- γ did not noticeably inhibit the expression of eotaxin mRNA induced by TNF- α , despite the fact that eotaxin protein production was remarkably inhibited by means of preincubation with IFN- γ (Fig 2, A). We confirmed that the level of amplified gene encoding eotaxin was dependent on the number of cycles, and the linearity of amplification was observed with 30 cycles (data not shown).

DISCUSSION

We demonstrate here that the T_H1 cytokine IFN- γ regulates expression of eotaxin in a time-dependent manner. Although IFN- γ alone did not induce the expression of eotaxin, simultaneous treatment with IFN- γ potentiated the expression of eotaxin protein induced by TNF- α , which is in agreement with previous reports.^{12,15} Interestingly, preincubation with IFN- γ significantly inhibited the induction of eotaxin protein expression by TNF- α .

IFN- γ is a T_H1-type cytokine believed to play an important role in the regulation of allergic disease. Administration of T_H1-inducing stimuli suppresses T_H2 responses and manifestations of allergic disease, such as

asthma.^{2,22} However, it has been reported that IFN- γ might contribute to the generation of airway inflammation and acute deterioration of asthma.²³⁻²⁵ Our results suggest that the chronic presence of IFN- γ could counteract the development of allergic inflammation. Our results also suggest that acute production of IFN- γ , as might occur during a viral infection, could enhance production of eotaxin and exacerbate allergic inflammation.

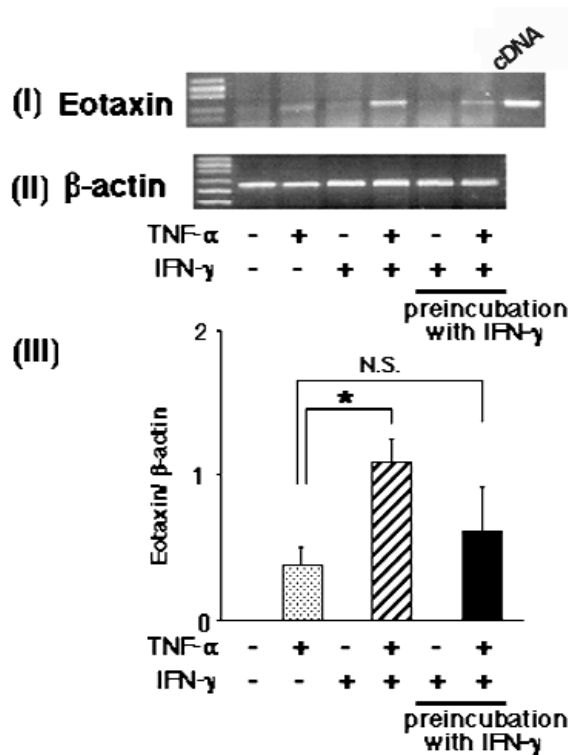


FIG 5. Effect of IFN- γ on eotaxin mRNA expression. RNA was extracted from BEAS-2B cells that had been treated for 24 hours with or without IFN- γ (50 ng/mL), followed by incubation with control medium or indicated cytokines for 24 hours. Extracted RNA was subjected to RT-PCR: *I*, eotaxin mRNA expression; *II*, β -actin mRNA expression; *III*, mean $\pm$ SEM of the densitometric analysis of eotaxin mRNA expression normalized with β -actin expression. The data are presented as a representative experiment (*I* and *II*) or as the mean $\pm$ SEM of a total of 3 independent experiments (*III*). * $P < .05$.

Our studies indicate that IFN- γ can either potentiate or inhibit eotaxin induction, depending on the timing of exposure. IFN- γ is known to induce a wide variety of genes related to inflammation. In many cases STAT-1 and IRF-1 are pivotal transcription factors that mediate the response to IFN- γ . These transcription factors are known to cooperate with NF- κ B, an important mediator of the response to TNF- α .^{21,26} We first hypothesized that IFN- γ might activate STAT-1 or IRF-1 and that these transcription factors might bind to the eotaxin promoter region. However, we could not identify any putative binding sequences (TTCN3GAA for STAT-1 or tandem repeats of GAAA for IRF-1) in the eotaxin promoter spanning 1363 bp.²⁷ Hein et al²⁸ reported 3 kb of the sequence of the 5' upstream region of the eotaxin gene. In our analysis of this sequence, there is one consensus sequence of TTCN3GAA in a site -1998 to -2006 bp of the upstream region of the eotaxin gene. We examined whether IFN- γ induces formation of a binding complex of STAT-1 with this region by using the DNA probe 5'-GGCATTCTTGGAACCA-3' derived from site -1994

to -2010 bp, but we could not detect a binding complex after stimulation with IFN- γ (data not shown).

The binding complex induced by the combination of TNF- α and IFN- γ with a DNA probe derived from the proximal region of the eotaxin promoter was confirmed to be a p50/p65 NF- κ B heterodimer and was not altered by anti-STAT-1 (Fig 3, *B*). Members of the STAT family are known to bind to the DNA consensus sequence TTCN3GAA. As mentioned earlier, STAT-6 binds to the proximal region of the eotaxin promoter. However, the consensus sequence for this binding site is TTCN4GAA, which is reported to be a specific motif for only STAT-6 among this family.^{29,30} Our findings that IFN- γ does not induce binding of STAT-1 to the proximal region of the eotaxin promoter is in agreement with the findings of these previous reports.

One possible mechanism of synergy between TNF- α and IFN- γ might be the activation of NF- κ B or the upregulation of TNF receptors by IFN- γ .²¹ In these studies TNF- α activated NF- κ B and induced the binding of NF- κ B to the DNA probe derived from eotaxin promoter, but IFN- γ did not alter formation of this binding complex. Hein et al²⁸ and Garcia-Zepeda et al³¹ reported that multiple putative IFN- γ -responsive elements (γ IREs) might exist in the eotaxin promoter. γ IRE is known to mediate transcriptional activation of MHC class II by IFN- γ .³² Although the eotaxin promoter-luciferase reporter plasmids pEotx.1363 and pEotx.478 contain numerous consensus binding sites for γ IRE, IFN- γ did not induce promoter activity of these reporters or enhance activity induced by TNF- α in our studies, indicating that γ IRE might be not involved in the activation of the eotaxin promoter. The reporter data suggest that IFN- γ does not increase transcription either alone or in combination with TNF- α , whereas the findings with RT-PCR suggest that IFN- γ increased steady state eotaxin mRNA levels in the presence of TNF- α . Together, these results suggest that IFN- γ either increases transcription through interactions with the endogenous promoter but not the reporter or that the effect is due to posttranscriptional influences of IFN- γ .

The pattern of regulation of chemokine expression by cytokines might differ among cell types and experimental systems. Regulation of eotaxin expression by cytokines appears to be similar in several cell types. Various investigators reported that IL-4/IL-13 and TNF- α stimulate eotaxin expression, and the combination of these cytokines acts in a synergistic manner in fibroblasts.^{17,33} A similar synergism of TNF- α and IL-4/IL-13 on eotaxin expression has been observed in airway epithelial cells. We demonstrated that TNF- α activates NF- κ B, whereas IL-4 and IL-13 activate STAT-6, and these 2 transcription factors activate eotaxin transcription.^{13,14} Hoeck and Woisetschlager³³ recently demonstrated that NF- κ B and STAT-6 similarly regulate eotaxin transcription in fibroblasts. These reports indicate that there is common role for these transcription factors in the regulation of eotaxin in both fibroblasts and epithelial cells. Teran et al¹⁷ and Miyamasu et al¹⁸ reported that IFN- γ inhibited eotaxin protein expression induced by

TNF- α in fibroblasts. Our results also indicate that IFN- γ is a powerful inhibitor of eotaxin expression. Preincubation with IFN- γ for 24 hours inhibited eotaxin protein expression in airway epithelial cells by greater than 95%. We hypothesized that IFN- γ might inhibit the formation of the NF- κ B-DNA binding complex. Direct assay for formation of this complex with EMSAs showed that IFN- γ did not change the binding of NF- κ B to the eotaxin promoter-derived DNA. Preincubation with IFN- γ only modestly inhibited eotaxin promoter activity induced by TNF- α . Studies using 5'-deleted eotaxin promoter-luciferase reporter plasmid indicate that the responsible region for this modest inhibitory effect might exist distal to 93 bp in the eotaxin promoter. This inhibitory effect of preincubation with IFN- γ on eotaxin transcription, as detected by means of PCR or with reporters, was modest when compared with the nearly complete inhibition of eotaxin protein production.

Recent studies indicate that IFN- γ can exert a repressive effect on target protein translation. Ben-Asouli et al³⁴ reported that IFN- γ downregulates its own translation through the double-stranded RNA-activated protein kinase PKR. Mazumder and Fox³⁵ demonstrated that incubation of cells with IFN- γ for 24 hours, but not for 8 hours, induced inhibition of translation of ceruloplasmin. They suggested that a factor distinct from PKR was synthesized by means of incubation of cells with IFN- γ for 24 hours, and this factor might suppress binding of the translational machinery to the 3'-untranslated region of ceruloplasmin mRNA. On the basis of the dissociation of effects of IFN- γ on eotaxin transcription (as detected on the basis of mRNA levels and reporters) and production of eotaxin protein (as detected on the basis of ELISA), it is reasonable to hypothesize that IFN- γ might decrease eotaxin protein synthesis through inhibition of eotaxin translation. Further studies focusing on the translation of eotaxin, including the role of PKR and other factors, are clearly needed to further test this hypothesis.

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