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IL-4 has been shown to be involved in the accumulation of leukocytes, especially eosinophils, at sites of inflammation by acting on vascular endothelial cells. To identify novel molecules involved in the IL-4-dependent eosinophil extravasation, cDNA prepared from HUVEC stimulated with IL-4 was subjected to differential display analysis, which revealed a novel CC chemokine designated as eotaxin-3. The human eotaxin-3 gene has been localized to chromosome 7q11.2, unlike most other CC chemokine genes. The predicted mature protein of 71 aa showed 27–42% identity to other human CC chemokines. The recombinant protein induced a transient increase in the cytosolic Ca2+ concentration and in vitro chemotaxis on eosinophils. Furthermore, in cynomolgus monkeys, the accumulation of eosinophils was observed at the sites where the protein was injected. Eotaxin-3 inhibited the binding of [125I]-eotaxin, but not [125I]-macrophage inflammatory protein-1α, to eosinophils and acted on cell lines transfected with CCR-3, suggesting that eotaxin-3 recognized CCR-3. IL-13 as well as IL-4 up-regulated eotaxin-3 mRNA in HUVEC, whereas neither TNF-α, IL-1β, IFN-γ, nor TNF-α plus IFN-γ did. The expression profile of eotaxin-3 is different from those of eotaxin, RANTES, and monocyte chemoattractant protein-4, which are potent eosinophil-selective chemoattractants and are induced by either TNF-α or TNF-α plus IFN-γ. These results suggest that eotaxin-3 may contribute to the eosinophil accumulation in atopic diseases. The Journal of Immunology, 1999, 163: 1602–1610.

 interleukin 4 (IL-4) is a pleiotropic cytokine secreted by Th2 lymphocytes, basophils, and mast cells, and it affects a variety of target cells in multiple ways like many other cytokines (1). One of the important roles of IL-4 is the regulation of immune responses, including the regulation of IgE synthesis and the development of Th2 lymphocytes. In addition, IL-4 has been shown to be involved in the infiltration and the accumulation of leukocytes, particularly eosinophils, at the sites of inflammation observed in some human allergic diseases. For example, transgenic mice expressing IL-4 exhibited inflammatory responses with the accumulation of eosinophils (2, 3), and selective eosinophil accumulation was also observed in mice to which the IL-4 protein had been administered (4, 5). Furthermore, tissue eosinophilia observed upon allergen-induced airway inflammation in wild-type mice was strongly suppressed in IL-4-deficient mice (6, 7).

In general, leukocyte extravasation from the bloodstream into tissues is a regulated process that involves a series of coordinated interactions between leukocytes and vascular endothelial cells (VEC)3. This process is divided into the following four steps (8, 9): in step 1, the initial interaction between leukocytes and VEC, namely rolling, comprises transient and reversible adhesion mediated mainly by cell adhesion molecules, selectins, and carbohydrate ligands; in step 2, the leukocytes rolling on VEC come into contact with chemoattractants such as chemokines and platelet-activating factor present on the surface of VEC, which stimulate leukocytes to induce integrin adhesiveness; in step 3, the activated leukocytes adhere firmly to VEC through the interaction of integrins with their endothelial counterreceptors, such as ICAM-1 and VCAM-1; and in step 4, the leukocytes adhered to VEC transmigrate through vascular walls and move to the center of inflamed tissues.

The selective recruitment of specific classes of leukocytes to inflamed tissues has been explained by inducible expression of specific molecules on VEC in steps 1, 2, and 3. As to IL-4-induced accumulation of eosinophils, IL-4-induced expression of P-selectin (10) and L-selectin ligands (11, 12) in step 1 and that of VCAM-1 (13, 14) in step 3 have been detected.

Eosinophil-active chemokines identified to date comprise eotaxin (15, 16), eotaxin-2 (17), RANTES (18, 19), monocyte chemoattractant protein (MCP)-2 (20, 21), MCP-3 (20–22), MCP-4 (23–25), and macrophage inflammatory protein (MIP)-1α (19), which belong to the CC subfamily. Among them, eotaxin has been most characterized and is thought to be a potent eosinophil-selective chemoattractant because it acts on a single receptor, CCR3, highly expressed on eosinophils (26, 27). However, eotaxin expression was not up-regulated in IL-4-stimulated HUVEC (28).

3 Abbreviations used in this paper: VEC, vascular endothelial cells; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; [Ca2+]i, free cytosolic Ca2+ concentration.
Eotaxin has not been found in the culture medium of IL-4-stimulated HUVEC but has been in that of IL-4-stimulated dermal fibroblasts (29). Sanz et al. (30) reported that eotaxin-dependent accumulation of eosinophils was observed in rats in response to IL-4, but no evidence has been presented for IL-4-induced expression of eotaxin on VEC. Furthermore, eotaxin repletion to the sites of inflammation was not completely blocked in eotaxin-deficient mice (31). Thus, chemokines that are induced on VEC by IL-4 and involved in the leukocyte-activation step (step 2) in the selective recruitment of eosinophils to inflammatory sites have not been definitely identified so far.

In this study, we have explored novel molecules expressed selectively on HUVEC stimulated by IL-4 using a differential display technique and have identified a novel CC chemokine that attracts human eosinophils. We have named this chemokine eotaxin-3.

Materials and Methods

Materials

HUVEC were purchased from Kurabo (Osaka, Japan). Recombinant human TNF-α and IL-4 were obtained from Genzyme (Cambridge, MA). Recombinant human IL-1β, IL-13, and IFN-γ were obtained from R&D Systems (Minneapolis, MN). Recombinant human eosinophil, MIP-1α, MIP-3, RANTES, and IL-8 were purchased from PeproTech (London, U.K.). [32P]-labeled DNA fragment covering the 3'-terminal portion of eotaxin-3 cDNA (Fig. 1A) was isolated and sequenced using a dye primer cycle sequencing kit and 377 DNA sequencer (Perkin-Elmer, Norwalk, CT).

Fluorescent differential display

HUVEC were propagated in F-12K medium supplemented with 100 μg/ml heparin, 30 μg/ml endothelial cell growth supplement (Becton Dickenson, Franklin Lakes, NJ), and 10% FCS in tissue culture plastic flasks precoated with 2 mg/ml gelatin. The confluent HUVEC monolayers of passage 6 were treated for 17 h with either 10 ng/ml TNF-α or 100 U/ml IL-4. HUVEC cultivated without the addition of these cytokines were used as controls. Total cellular RNA was extracted from the cells by the method of HUVEC treated with IL-4 for 17 h. First-strand synthesis was performed using a 3'-terminal oligo(dT) primer that contained a NotI site. After synthesis of the first strand, the DNAs were blunt-ended with T4 DNA polymerase, digested with NotI and EcoRI adaptors, and then digested with NotI. The PCR reactions for differential display were performed as described by Ito et al. (33) using 80 different arbitrary primers (Operon Technologies, Alameda, CA). The PCR products were electrophoresed on a high-resolution denaturing gel and then analyzed with a FluorImager (Molecular Dynamics, Sunnyvale, CA). The differentially regulated bands were excised from the gel, reamplified, cloned into plasmid pT7Blue(R) (Novagen, Madison, WI), and then sequenced using a dye primer cycle sequencing kit and 377 DNA sequencer (Perkin-Elmer, Norwalk, CT).

Isolation of full-length cDNA of eotaxin-3

A cDNA library was constructed from poly(A)+ RNA prepared from HUVEC treated with IL-4 for 17 h. First-strand synthesis was performed using a 3'-oligo(dT) primer that contained a NotI site. After synthesis of the second strand, the DNAs were blunt-ended with T4 DNA polymerase, ligated with EcoRI adaptors, and then digested with NotI. The cDNAs of 0.4–2.0 kb fragments were inserted into λ vector ZAP Express (Stratagene, La Jolla, CA). Then they were packaged in phage particles using packaging insect cell XL-1 Blue MRF (New England Biolabs, Beverly, MA) (34). The PCR products were electrophoresed on a high-resolution denaturing gel and then analyzed with a FluorImager (Molecular Dynamics, Sunnyvale, CA). The differentially regulated bands were excised from the gel, reamplified, cloned into plasmid pT7Blue(R) (Novagen, Madison, WI), and then sequenced using a dye primer cycle sequencing kit and 377 DNA sequencer (Perkin-Elmer, Norwalk, CT).

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Figure 1. Nucleotide and deduced amino acid sequences of eotaxin-3 cDNA. A, The deduced amino acid sequence is indicated in a single letter code below the cDNA sequence. A putative signal sequence is underlined, and a typical polyadenylation signal is indicated as poly(A). The 3' downstream sequence of the 152nd G residue was obtained through a differential display experiment. The primers used for chromosomal mapping are indicated by arrows. The sequence is available from EMBL/GenBank/DDJB under accession number AB016542. B, Alignment of the amino acid sequence of eotaxin-3 with those of other human CC chemokines. The amino acid sequence of the mature form of eotaxin-3 is aligned with those of 16 other human CC chemokines using the PileUp program of the GCG package (Genetic Computer Group, Madison, WI). The percent identity to eotaxin-3 is indicated on the right.

Expression of recombinant eotaxin-3 by baculovirus-infected insect cells

The BomHI-Pvl 370-bp fragment encoding eotaxin-3 was excised from pHVCO02 and then cloned into the baculovirus transfer vector, pVL1393 (PharMingen, San Diego, CA) to obtain pVL-HVC. The plasmid was co-transfected with BaculoGold viral DNA (PharMingen) into Sf9 cells to construct a recombinant baculovirus, and then the recombinant virus was purified by plaque purification as described (35). Confluent monolayers of Sf21 cells grown in serum-free medium, Ex-Cell 400 (SRB Biosciences, Lenexa, KS) were infected with the recombinant virus (multiplicity of infection = 10) and then incubated at 27°C.

Purification of the recombinant eotaxin-3 protein

The culture medium of the recombinant baculovirus-infected Sf21 cells was applied to a heparin-Sepharose (Amersham Pharmacia Biotech) column preequilibrated with 50 mM sodium phosphate, pH 6.5. The column

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was washed with 10-bed volumes of the same buffer, and then bound proteins were eluted with a linear gradient of 0–1 M NaCl in 50 mM sodium phosphate, pH 6.5. Each fraction was analyzed with SDS-PAGE followed by silver staining, and the fractions containing the recombinant eotaxin-3 protein were pooled and diluted with 1.6 volumes of 50 mM sodium phosphate, pH 6.5. Then, Sepharose (Amersham Pharmacia Biotech) pre-equilibrated with 50 mM sodium phosphate, pH 6.5, containing 0.4 M NaCl was added, followed by gentle mixing at 4°C for 12 h. The resin was packed into a column and washed with 50 mM sodium phosphate, pH 6.5, containing 0.4 M NaCl, and then the bound proteins were eluted with 1 M NaCl. The eluted proteins were applied to a PD-10 column (Amersham Pharmacia Biotech) pre-equilibrated with 50 mM sodium phosphate, pH 7.3, containing 0.4 M NaCl. The recombinant eotaxin-3 protein thus purified was concentrated to an appropriate concentration using a Centricon-3 (Millipore, Bedford, MA). Protein concentrations were determined by the method of Bradford (36) using BSA as a standard. N-terminal sequence analysis was performed with a protein sequencer (PPSQ-10; Shimadzu, Tokyo, Japan).

Preparation of human leukocytes

Human granulocytes and PBMC were separated from the venous blood of healthy donors by Percoll (1.085 g/ml) centrifugation at room temperature as described (37). To isolate granulocytes, RBC were removed by hypotonic lysis after centrifugation. To enrich eosinophils, CD16-positive cells, i.e., neutrophils, were removed, and to enrich eosinophils, very late Ag-4-positive cells, i.e., eosinophils, were removed from the granulocyte fraction, respectively, using an immunomagnetic bead technique, as described (37). The contents of the leukocytes determined by analysis of Diff-Quik (International Reagents, Kobe, Japan)-stained cyt centrifugation preparations were as follows: the PBMC fraction contained 70% lymphocytes and 30% monocytes, the neutrophil-rich preparation 95% neutrophils, and the eosinophil-rich preparation 95% eosinophils.

Construction of cell lines stably expressing CCR3

A plasmid pcDNA-CCR3 carrying human CCR3 gene was generously provided by Dr. Kouji Matsushima (University of Tokyo, Tokyo, Japan). A CMV promoter on the plasmid was replaced with a CAG promoter on plasmid pAdexCAtw (38) as follows. The Sall-ClaI 1.8-kb fragment was excised from pAdexCAtw and cloned into pcBluescript KS+(−). The resulting plasmid was digested with Sall, treated with T4 DNA polymerase, and digested with HinIII. The 1.8-kb fragment encoding CAG promoter was excised and ligated with the NraI-HindIII 5.8-kb fragment of pcDNA-CCR3 to construct pcDNA (CAG)-CCR3. K562 cells were transfected with pcDNA(CAG)-CCR3 by electroporation and grown in the presence of 0.8 mg/ml G418 sulfate to obtain G418-resistant clones. From those clones, a CCR3-transfectant was selected, which showed specific binding to 125I-eotaxin.

Ca2+ mobilization assay

Peripheral blood cells were loaded with 1 nmol fura-2/AM (Wako Pure Chemicals, Osaka, Japan) per 1 × 10⁶ cells for 30 min at 37°C in 1 ml of loading buffer (1 mM CaCl₂, 1 mM MgCl₂, 125 mM NaCl, 5 mM KCl, 0.5 mM glucose, 20 mM HEPES, pH 7.5) containing 0.025% BSA. The cells were washed with the loading buffer containing 0.5% BSA, resuspended in the same buffer at 1 × 10⁷ cells/ml, and then placed in a continuously stirred cuvette at 37°C. As for the CCR3-transfected K562 cells, HBSS was used for loading fura-2/AM, washing, and resuspension. The cells were excited at 340 and 380 nm in a fluorescence spectrophotometer (CAF-110; Jasco Corp., Tokyo, Japan) and then the relative ratio of the fluorescence (340 nm/380 nm) emitted at 500 nm was recorded.

Competition binding assay of 125I-eotaxin

Cells were mixed with either 0.1 nM 125I-eotaxin or 125I-MIP-1α for 1 h at 37°C in binding buffer (50 mM HEPES, pH 7.5, 1 mM CaCl₂, 5 mM MgCl₂, 0.02% sodium azide) containing various concentrations of the respective unlabeled chemokine. After incubation, the cells were collected by centrifugation at 8,000 × g for 3 min, suspended in 150 µl of washing buffer (binding buffer plus 0.5 M NaCl), and then centrifuged at 16,000 × g for 3 min. The resulting pellets were transferred in 300 µl of the same buffer to polystyrene tubes for γ counting.

In vitro chemotaxis assay

Chemotaxis for eosinophils was assessed in 96-well microplate chambers (Neuro Probe, Cabin John, MD) using polycarbonate filters with 5-µm pores (Neuro Probe). Cell suspensions and chemokine dilutions were made with RPMI 1640 medium supplemented with 1% (v/v) FCS. Aliquots (350 µl) of chemokines were placed in the wells of the lower compartment, and 200-µl aliquots of the eosinophil suspension (1 × 10⁶ cells/ml) were placed in the upper wells of the chamber. In some experiments, various concentrations of chemokines were mixed with the cells in the upper compartment (chequerboard analysis). The chamber was incubated for 60 min at 37°C in humidified air containing 5% CO₂. After incubation, nonmigrating cells were scraped from the upper surface of the filter and then the lower compartment to which the filter was attached was centrifuged at 200 × g for 10 min. The migrated eosinophils were counted essentially as described (39). After removing the medium carefully, the cells were lysed with 50 µl of 0.3% (w/v) cetethyltrimethylammonium bromide, followed by the addition of 100 µl of eosinophil peroxidase substrate (2.2 mM o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO), 8.8 mM hydrogen peroxide in 50 mM sodium citrate, pH 5.0) to each well. After 5 min at room temperature, the color reaction was terminated by the addition of 50 µl of 4 N H₂SO₄. The OD₄₅₅ was monitored and converted to the number of migrating cells using purified eosinophils as a standard. Assays for PBMC and neutrophils were performed on chemo TX-96 chambers (Neuro Probe) using 5-µm pore polycarbonate filters. Cell suspensions and chemokine dilutions were prepared as described above. Aliquots (28 µl) of chemokines were placed in the wells of lower compartment, whereas 60 µl aliquots of the PBMC suspension (3 × 10⁶ cells/ml) or neutrophil suspension (3 × 10⁵ cells/ml) were put on the upper wells of the chamber. The chamber was incubated for 90 min for PBMC or for 60 min for neutrophils at 37°C, and migrated cells were collected as described above. After removing the medium carefully, 20 µl of RPMI 640 containing 10% FCS and 10 µl of MTT (sodium salt-1-(phenylmethylcarboxyl)-3,4-tetrazolium)-bist(4-methoxy-6-nitro)benzene sulfonic acid hydrate solution (Boehringer Mannheim, Mannheim, Germany) were added to each well and incubated at 37°C for 3 h. The value of the OD₄₉₀ minus OD₃₇₅ was determined and converted to the number of migrating cells using purified PBMC or neutrophils as a standard. These cell-counting procedures were found to be highly reproducible and comparable to a direct cell-counting method by microscopic observation.

In vivo assessment of leukocyte recruitment

Thirty micromolar eotaxin-3, purified as described above, and 10 µM eotaxin in PBS were diluted to 8.5 µM and 1 µM, respectively, with pyrogen-free isotonic saline. Two wells (100 µl) of 100 µl isotonic saline as a control were then each administered intradermally at two sites on the back of a male cynomolgus monkey that had been anesthetized by injection of 10 mg/kg ketamine. Then, full skin thickness punch biopsy samples of 8-mm diameter were taken from the injection sites 4 h after. The biopsies were fixed in 10% formalin and then processed by standard histologic techniques. After embedding in paraffin blocks, the pieces were cut into 3-mm sections, which were stained with Giemsa solution plus hematoyxlin and eosin, and the leukocyte infiltrates were evaluated by two independent observers. In each section, eosinophils and neutrophils that had migrated out of the vessels were counted at 400-fold magnification in five randomly selected fields including postcapillary venules using a grid of 0.22 × 0.22 mm, and the number of eosinophils and neutrophils per mm² were calculated.

Northern blot analysis

Total RNA from HUVEC with or without cytokine treatment was electrophoresed on a formaldehyde-1.0% agarose gel and then transferred to a Biodyne A membrane (Pall BioSupport, East Hills, NY). The digoxigenin-labeled cDNA of eotaxin-3 was synthesized by amplification of a region corresponding to the sequence from 190 to 383 in Fig. 1A, using a PCR digoxigenin labeling mix (Hoffmann-LaRoche, Basel, Switzerland), and hybridization was performed according to the method described in the manufacturer’s manual.

Results

Differential display and isolation of cDNA of eotaxin-3

Three mRNA samples were prepared from IL-4-stimulated, TNF-α-stimulated, and unstimulated HUVEC, respectively, and then subjected to differential display analysis. HUVEC cultivated without the addition of these cytokines were used as a control. On the analysis of 80 different PCR reactions, two bands were identified that were abundant only for the sample from IL-4-stimulated HUVEC. These materials were reamplified, cloned, sequenced, and then subjected to database analysis (data not shown). One was a part of dUTP pyrophosphatase cDNA. This may be a technical
artifact because up-regulation of this mRNA was not observed in IL-4-stimulated HUVEC by RT-PCR (data not shown). The other had a novel sequence exhibiting significant homology to the carboxyl-terminal halves of some known CC chemokines. To isolate the full-length cDNA of the latter, we constructed an IL-4-treated HUVEC cDNA library and obtained several independent positive clones. The nucleotide sequence of the most 5'−extended one among them is shown in Fig. 1. The full-length cDNA contains an open reading frame consisting of 94 aa with a predicted molecular mass of 10,647. The predicted polypeptide sequence has a highly hydrophobic amino-terminal region (Fig. 1), characteristic of a signal peptide with a putative cleavage site between Ala 23 and Thr 24. The predicted mature protein of 71 aa shows significant homology to human CC chemokines (Fig. 1).

FIGURE 2. Expression of eotaxin-3 mRNA in HUVEC stimulated with IL-4. A. Five micrograms of total RNA was isolated from HUVEC 6 h after stimulation with 0, 3, 10, 100, 300, or 3000 U/ml IL-4 and was subjected to Northern blot analysis. B. Total RNA (5 μg) was isolated from HUVEC 0, 1, 2, 4, 8, or 24 h after stimulation with 100 U/ml IL-4. The blots both in A and B were hybridized sequentially with eotaxin-3 and G3PDH cDNA probes.

FIGURE 3. SDS-PAGE analysis of the recombinant eotaxin-3. The recombinant eotaxin-3 was purified from the culture medium of recombinant baculovirus-infected Sf21 cells using a heparin-Sepharose column and SP Sepharose. The pooled fractions from the heparin-Sepharose column (lane 1) and SP Sepharose chromatography (lane 2) were analyzed by SDS-PAGE on a 15% (w/w) polyacrylamide gel, followed by silver staining. Lane M, Molecular mass marker proteins.

FIGURE 4. Changes in the [Ca 2+], in human leukocytes. A. Fura-2-loaded human blood eosinophils were stimulated with serial concentrations of eotaxin-3 (●) or eotaxin (○), and changes of the relative fluorescence ratio were measured. Duplicate experiments were performed, and the values whose error ranges were within 5% were averaged. B. Fura-2-loaded human blood PBMC and neutrophils were stimulated with 0.8 μM eotaxin-3 or 0.1 μM of another CC chemokine, as indicated by arrows. The results are representative of two separate experiments. C. Cross-desensitization of human blood eosinophils. Fura-2-loaded eosinophils were stimulated sequentially at 90-s intervals with 0.8 μM eotaxin-3 and 0.1 μM of another CC chemokine. The chemokines were added at the times indicated by arrows. The results are representative of two separate experiments.
We designated this novel CC chemokine as eotaxin-3 because it was functionally similar to eotaxin and eotaxin-2 (Refs. 15–17 and see below). The expression level of eotaxin-3 mRNA was dependent on the concentration of IL-4 (Fig. 2A). Similar expression level was observed when HUVEC at passage 3, 4, 6, 12, and 15 were stimulated with IL-4 (data not shown). Furthermore, the expression of eotaxin-3 mRNA was found to continue at least for 24 h by stimulation with 100 U/ml IL-4 (Fig. 2B). No known CC chemokine from other species showing extensive homology with eotaxin-3 was found, suggesting an ortholog of eotaxin-3 has not been identified in other species.

### Chromosomal mapping of the human eotaxin-3 gene

The chromosomal localization of the human eotaxin-3 gene was investigated by PCR using human-hamster somatic cell hybrids. The expected 152-bp fragment, corresponding to the carboxyl-terminal and 3' untranslated regions of eotaxin-3 (Fig. 1A), was amplified with two kinds of somatic cell DNA fragments containing human chromosome 7 (data not shown). To map the eotaxin-3 gene more precisely, radiation hybrid mapping was conducted. The result was 0100000110 0000000110 0101010010 0011010000 0110111001 1010000100 0001100010 1100001000 1101001010 000 (Whitehead Institute/MIT Center for Genomic Research order), where 0 and 1 represent negative and positive results in the PCR assay, respectively. These results indicate that the gene is located at 7q11.2 between chromosomal markers D7S489 and D7S669.

### Preparation of the recombinant eotaxin-3 protein

The recombinant eotaxin-3 protein was purified from the culture medium of baculovirus-infected insect cells. On SDS-PAGE, a 10-kDa major material was detected, showing about 95% purity (Fig. 3). Because this material was not detected in the culture medium of the insect cells infected with a control virus which had no recombinant eotaxin-3 cDNA (data not shown), this should be a product of the recombinant eotaxin-3 gene. N-terminal amino acid sequence analysis of the purified eotaxin-3 protein showed that the mature form of human eotaxin-3 started at Thr24 (data not shown).

### Leukocyte responses and receptor usage

We monitored changes in the free cytosolic Ca$^{2+}$ level ([Ca$^{2+}$]$_i$) in leukocytes after stimulation with eotaxin-3 (Fig. 4). Like eotaxin, a rapid and drastic increase in [Ca$^{2+}$]$_i$ was observed when eotaxin-3 acted on eosinophils. The dose of eotaxin-3 conferring a maximal response on eosinophils was $\approx 10$ nM, which was $\approx 10$-fold higher than that of eotaxin (Fig. 4A). The increase in [Ca$^{2+}$]$_i$ was not observed toward PBMC and neutrophils stimulated with an excess concentration of eotaxin-3, unlike MCP-3 or IL-8 (Fig. 4B). Desensitization experiments were then performed with eosinophils to determine the receptor selectivity (Fig. 4C). Preexposure of eosinophils to eotaxin-3 completely blocked the responses to eotaxin and MIP-1α. Furthermore, activation of eosinophils by eotaxin-3 was completely prevented by prior stimulation with eotaxin, but was hardly affected by preexposure to MIP-1α. These relationships between eotaxin-3 and MIP-1α were almost the same as those between eotaxin and MIP-1α.

![FIGURE 5. Competitive inhibition of 125I-eotaxin (A) and 125I-MIP-1α (B) binding to human blood eosinophils. Human blood eosinophils were incubated with 0.1 nM of a 125I-labeled chemokine and serial concentrations of unlabeled eotaxin-3 (●), eotaxin (○), or MIP-1α (□) for 1 h at 37°C. The bound 125I-labeled chemokines were measured. Three independent experiments were performed, and the results were expressed as the mean ± SD. C represents 0 nM chemokine.](http://www.jimmunol.org/content/1606/10/1606.full)

![FIGURE 6. A. Changes in the [Ca$^{2+}$]$_i$ in K562 cells stably expressing CCR3. The fura-2-loaded cells were stimulated with 0.1 μM eotaxin-3, eotaxin, or MIP-1α, as indicated by arrows. The results are representative of two separate experiments. B. Competitive inhibition of 125I-eotaxin binding to K562 cells stably expressing CCR3. The cells were incubated with 0.1 nM of a 125I-eotaxin and serial concentrations of unlabeled eotaxin-3 (●), eotaxin (○), or MIP-1α (□) for 1 h at 37°C. The bound 125I-labeled chemokines were measured. Three independent experiments were performed, and the results were expressed as the mean ± SD. C represents 0 nM chemokine.](http://www.jimmunol.org/content/1606/10/1606.full)
The receptor specificity of the action of eotaxin-3 on eosinophils was further analyzed by means of competitive binding studies (Fig. 5). Eotaxin-3 was a potent inhibitor of 125I-eotaxin binding to eosinophils, with an ID\textsubscript{50} value of ~10 nM, which was ~10-fold higher than that of eotaxin. On the other hand, 125I-MIP-1a binding was hardly affected by the addition of eotaxin-3 up to 300 nM. Eotaxin and MIP-1a have been shown to recognize CCR3 and CCR1, respectively (26, 27). Taken together, these results suggest that eotaxin-3 preferentially recognizes a receptor for eotaxin, CCR3, on eosinophils. The receptor usage of eotaxin-3 was examined using K562 cells stably expressing CCR3 (Fig. 6, A and B). The cells also exhibited calcium flux responses when stimulated with eotaxin-3 as well as eotaxin. Furthermore, eotaxin-3 competitively inhibited 125I-eotaxin binding to the K562 cells as eotaxin did. Eotaxin-3 had no effect on K562 cells not expressing CCR3 (data not shown). These results also suggest that eotaxin-3 recognizes CCR3. We cannot yet explain why [Ca\textsuperscript{2+}]\textsubscript{i} in eosinophils after stimulation with MIP-1a was completely prevented by prior stimulation with either eotaxin or eotaxin-3, although they were not potent inhibitors of 125I-MIP-1a binding (Fig. 4C, Fig. 5B).

**In vitro and in vivo chemotactic activity of eotaxin-3**

Eotaxin-3 induced strong chemotaxis on eosinophils with a typically bimodal manner, but not for PBMC and neutrophils (Fig. 7). We observed that MCP-3 and RANTES preferentially acted on monocytes and lymphocytes, respectively, by Diff-Quik staining of the migrated cells (data not shown). Checkerboard analysis revealed that the migration of eosinophils toward eotaxin-3 was “chemotactic” rather than “chemokinetic” (data not shown).

The in vivo chemotactic activity of eotaxin-3 was investigated in three adult cynomolgus monkeys. The in vivo activity of eotaxin was detected at 100 pmol per site (17). Because the in vitro sp. act. of eotaxin-3 showed ~10-fold lower than that of eotaxin as described above, each monkey was injected intradermally with 850 pmol of eotaxin-3, 100 pmol eotaxin, and isotonic saline alone. Histological assessment and quantitative image analysis of skin biopsies were performed at 4 h after injection as described (17). In the three monkeys, infiltrating eosinophils that exhibited the characteristic nucleus and Giemsa staining of granules were observed in association with the venular walls at the sites of injection of either eotaxin or eotaxin-3, whereas isotonic saline alone had no effect (Fig. 8, Table I). Nonspecific dermal recruitment of neutrophils was apparent in all specimens, including those injected with isotonic saline alone (Table I). Furthermore, recruitment of monocytes and lymphocytes at the sites injected with eotaxin and eotaxin-3 were not observed.

**Expression profile of eotaxin-3**

Because eotaxin-3 mRNA was expressed in IL-4-stimulated HUVEC, the changes in its mRNA level after treatment with various cytokines were studied. Eotaxin-3 mRNA was up-regulated by another Th2 cytokine, IL-13, having biological activity similar to that of IL-4 (40), but not by either TNF-\alpha, IL-1\beta, a Th1 cytokine, IFN-\gamma, or TNF-\alpha plus IFN-\gamma (Fig. 9). TNF-\alpha and IL-1\beta proteins used could induce VCAM-1 mRNA, respectively, and TNF-\alpha plus IFN-\gamma could induce RANTES mRNA in HUVEC, indicating that these cytokines were effective (data not shown).
The eotaxin-3 gene has been localized to 7q11.2, where the chemokine, and secondary lymphoid-tissue chemokine, liver and activation-regulated chemokine, EBII-ligand 17q11.2, and the genes of thymus and activation-regulated basophils (41) and Th2 cells (42), eotaxin-3 should also act on eosinophils. Because CCR3 has been shown to be expressed on both in vitro and in vivo. The activity of in vitro chemotaxis and mobilization toward eosinophils was lost after the addition of an anti-eotaxin-3 mAb, and the culture medium of the control virus-infected insect cells had no effect on eosinophils (data not shown), indicating that eosinophil-chemotactic activity was derived from eotaxin-3. Eotaxin-3 preferentially recognized CCR3, a receptor for eotaxin, but not CCR1, a receptor for MIP-1α, on eosinophils. Because CCR3 has been shown to be expressed on basophils (41) and Th2 cells (42), eotaxin-3 should also act on these cells.

Most CC chemokine genes are clustered on chromosome 17q11.2, and the genes of thymus and activation-regulated chemokine, liver and activation-regulated chemokine, EBI1-ligand chemokine, and secondary lymphoid-tissue chemokine were mapped to 16q13, 2q33–37, 9p13, and 9p13, respectively (43, 44). The eotaxin-3 gene has been localized to 7q11.2, where the eotaxin-2 gene is mapped (45). This suggests that eotaxin-3 may be evolutionarily related to eotaxin-2.

Eosinophils are involved in allergic diseases, such as asthma, rhinitis, and atopic dermatitis (46). Elucidation of the mechanisms underlying the accumulation of eosinophils in inflamed tissues is of critical importance for understanding the onset and progress of these eosinophilic diseases. Eosinophils predominantly reside in tissues (47). However, eosinophils infiltrating from the bloodstream are likely involved in these diseases because IL-4, a central mediator of allergic inflammation, acts on VEC and induces cell adhesion molecules, such as P-selectin, L-selectin ligand, and VCAM-1, which are responsible for eosinophil infiltration (10–14). Chemokines that are involved in the IL-4-dependent recruitment of eosinophils have not been definitely identified yet. Eotaxin, MCP-4, and RANTES, which have been well characterized as potent eosinophil-selective chemoattractants, were not induced on stimulation of HUVEC with IL-4 (24, 28, 48). Because eotaxin-3 mRNA was significantly induced in HUVEC stimulated with IL-4, eotaxin-3 protein may be involved in the IL-4-dependent activation of eosinophils on VEC. We could not detect the expression of eotaxin-3 protein both in IL-4-stimulated HUVEC and their culture medium (data not shown). The eotaxin-3 protein might be unstable or inefficiently expressed in the cultivated cells. Recently, eosinophil accumulation through the action of eotaxin was observed in rat skin stimulated with IL-4 (30). Eotaxin was found in the culture medium of IL-4-stimulated human dermal fibroblasts but not in that of IL-4-stimulated HUVEC (29). Therefore, in atopic diseases, it is possible to speculate that eosinophils are activated by eotaxin-3 expressed on VEC, and then extravasate from the bloodstream to an inflamed tissue, and then migrate to the center of the tissue through the action of eotaxin.

Eotaxin-3 mRNA was not induced in HUVEC stimulated with IFN-γ. On the contrary, eotaxin, MCP-4, and RANTES mRNAs were induced in HUVEC stimulated with either IFN-γ or TNF-α plus IFN-γ (24, 28, 48), suggesting that these three chemokines play roles at the site where Th1 cells predominantly exist. An inhalant allergen patch test on atopic dermatitis patients revealed that the expression of IL-4 mRNA was increased, but that of IFN-γ mRNA was not, within 24 h. However, the level of IFN-γ mRNA increased more than that of IL-4 mRNA within 48 h (49). In this case, eotaxin-3 might contribute to the early phase response, whereas eotaxin, MCP-4, or RANTES might mainly act in the late phase to maintain the inflammatory response.

FIGURE 9. Induction of eotaxin-3 expression in HUVEC by various cytokines. Five micrograms of total RNA was isolated from HUVEC 6 or 24 h after stimulation with each of the indicated cytokines and then was subjected to Northern blot analysis. The concentration of cytokines was 10 ng/ml except for IL-4, for which it was 100 U/ml (1.32 ng/ml). The blot was hybridized sequentially with eotaxin-3 and G3PDH cDNA probes.

Table 1. In vivo infiltration of eosinophils and neutrophils

<table>
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<tr>
<th>Monkey</th>
<th>Eo</th>
<th>Ne</th>
<th>Eo</th>
<th>Ne</th>
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<td>314 ± 229</td>
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* Each cynomolgus monkey was injected intradermally with 850 pmol eotaxin-3, 100 pmol eotaxin, and isotonic saline alone at two sites, respectively. After 4 h, punch biopsies were taken and the eosinophils (Eo) and neutrophils (Ne) that had migrated out of vessels were counted. The relative density (number cells/mm² ± SD) of the cells was determined for two sections each of random fields/section.

Discussion

We identified a novel human CC chemokine, eotaxin-3, mRNA of which is expressed in IL-4-stimulated HUVEC, using a differential display technique. The recombinant eotaxin-3 protein was purified from the culture medium of the baculovirus-infected insect cells with about 95% purity. This purified protein showed a potent chemotactic activity for eosinophils but not for PBMC and neutrophils both in vitro and in vivo. The activity of in vitro chemotaxis and Ca²⁺ mobilization toward eosinophils was lost after the addition of an anti-eotaxin-3 mAb, and the culture medium of the control virus-infected insect cells had no effect on eosinophils (data not shown), indicating that eosinophil-chemotactic activity was derived from eotaxin-3. Eotaxin-3 preferentially recognized CCR3, a receptor for eotaxin, but not CCR1, a receptor for MIP-1α, on eosinophils. Because CCR3 has been shown to be expressed on basophils (41) and Th2 cells (42), eotaxin-3 should also act on these cells.

VCAM-1, which are responsible for eosinophil infiltration (10–14), Chemokines that are involved in the IL-4-dependent recruitment of eosinophils have not been definitely identified yet. Eotaxin, MCP-4, and RANTES, which have been well characterized as potent eosinophil-selective chemoattractants, were not induced on stimulation of HUVEC with IL-4 (24, 28, 48). Because eotaxin-3 mRNA was significantly induced in HUVEC stimulated with IL-4, eotaxin-3 protein may be involved in the IL-4-dependent activation of eosinophils on VEC. We could not detect the expression of eotaxin-3 protein both in IL-4-stimulated HUVEC and their culture medium (data not shown). The eotaxin-3 protein might be unstable or inefficiently expressed in the cultivated cells. Recently, eosinophil accumulation through the action of eotaxin was observed in rat skin stimulated with IL-4 (30). Eotaxin was found in the culture medium of IL-4-stimulated human dermal fibroblasts but not in that of IL-4-stimulated HUVEC (29). Therefore, in atopic diseases, it is possible to speculate that eosinophils are activated by eotaxin-3 expressed on VEC, and then extravasate from the bloodstream to an inflamed tissue, and then migrate to the center of the tissue through the action of eotaxin.

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study into consideration, over-expressed IL-4 may induce eotaxin-3 at the inflamed sites in the heart and lungs of the mice, which may contribute to the accumulation of eosinophils in these inflamed tissues.

In this study, we have shown that a novel chemokine designated as eotaxin-3 mRNA is induced on VEC by IL-4, and the recombina nt protein preferentially activates eosinophils. These results suggest that it may play an important role in inflammatory allergic diseases such as asthma and atopic dermatitis. It will be important to obtain definitive evidence for the involvement of eotaxin-3 in the onset and progress of these allergic diseases either by determining the eotaxin-3 levels in allergic patients or by analyzing experimental animal allergic models.

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References


