Identification of a Novel Eosinophil Chemotactic Cytokine (ECF-L) as a Chitinase Family Protein*

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A novel eosinophil chemotactic cytokine (ECF-L) was purified from the culture supernatant of splenocytes of mice by a combination of anion-exchange chromatography, Procion red-agarose affinity chromatography, size exclusion high performance liquid chromatography (HPLC), and reverse phase HPLC. The NH2-terminal amino acid sequence was determined by direct protein sequencing. An ECF-L cDNA clone of 1,506 nucleotides was isolated from a cDNA library, and the nucleotide sequence predicted a mature protein of 397 amino acids. A recombinant ECF-L showed a level of eosinophil chemotactic activity comparable with that of natural ECF-L, and the activity was inhibited by a monoclonal antibody to ECF-L. ECF-L also attracted T lymphocytes and bone marrow polymorphonuclear leukocytes in vitro, whereas it caused selective extravasation of eosinophils in vivo. ECF-L mRNA was highly expressed in spleen, bone marrow, lung, and heart. A comprehensive GenBank data base search revealed that ECF-L is a chitinase family protein. ECF-L retains those amino acids highly conserved among chitinase family proteins, but Asp and Glu residues essential for the proton donation in hydrolysis were replaced by Asn and Gln, respectively. Although ECF-L contains a consensus CXC sequence near the NH2 terminus akin to chemokine family proteins, the rest of ECF-L shows poor homology with chemokines.

Many parasites, especially tissue-invasive helminths, cause local eosinophilia as well as systemic eosinophilia in mammalian hosts. For example, Schistosoma mansoni (1) Schistosoma japonicum (2), Toxocara canis (3), and Mesocestoides corti (4) form eosinophilic inflammatory lesions in host tissue at the site where they invade or lay their eggs. The mechanisms by which eosinophils are recruited to local inflammatory sites via the tissue eosinophilia in parasitic infections (6, 7). Indeed, most multipotent hemopoietic stem cells are located in bone marrow (8). On the other hand, kinetic studies suggest that the spleen is the site where eosinophils mature (9), indicating eosinophil influx from the bone marrow to spleen at the maturation stage.

Previous studies have shown that a splenocyte-derived ECF (ECF-L) is produced in an antigen-specific manner during the course of parasitic infections accompanying systemic and local eosinophilia (7), and CD4+ CD8+ T cells play an important role in ECF-L production (10). Although ECF-L can be discriminated from other ECFs in terms of its physicochemical and immunochemical properties (11), the molecular structure of ECF-L still remains unclear. To obtain further insight into the immunological functions of ECF-L at the molecular level, cloning of cDNA encoding ECF-L is needed.

In this paper, we present the isolation of cDNA encoding ECF-L, the molecular expression of ECF-L, and a structure comparison of ECF-L with related proteins. The results clearly show that ECF-L is a novel eosinophil chemotactic cytokine that belongs to the chitinase protein family.

EXPERIMENTAL PROCEDURES

Mice and Infection—Female C57BL/6 or ddY mice were obtained from Japan SLC (Hamamastu, Japan) and used at 8–10 weeks of age. They were infected intraperitoneally with 30 cercariae of S. japonicum (Japanese strain) or 100 tetrathyridiae of M. corti and were killed by cervical dislocation under ether anesthesia.

Antigens—Soluble egg antigen of S. japonicum was prepared as described previously (12). In short, the eggs were harvested from the intestines of S. japonicum-infected mice by enzymatic digestion using Promase (Kaken, Tokyo, Japan) and collagenase (type I, Sigma Chemical Co., St. Louis, MO). Eggs were suspended in 5.8 mM phosphate-buffered saline (PBS, pH 7.4) and homogenized with a Teflon homogenizer in an ice-chilled water bath. The mixture was centrifuged at 100,000 × g for 1 h. The supernatant was used as S. japonicum egg antigen. Purification of a major 260-kDa antigen (J1) was performed as described previously (13). For preparation of soluble M. corti antigen, tetrathyridiae of M. corti were homogenized with a Teflon homogenizer in an ice-chilled water bath and centrifuged at 100,000 × g for 30 min. The supernatant was used as M. corti antigen.

Eosinophils, Neutrophils, or Macrophages—For collection of eosinophil-rich peritoneal exudate cells, ddY mice were infected intraperitoneally with 100 larvae of M. corti. 3 weeks later, peritoneal lavage was harvested. The cells were washed twice with PBS and suspended in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) containing 2% FBS (Life Technologies, Inc.). To remove adherent cells, the cell suspension was incubated in a tissue culture flask (Nunc) at 37 °C for 30 min. The nonadherent cells were used for eosinophil chemotaxis experiments. By this method, more than 1 × 10⁷ eosinophils were collected from one mouse, and the purity of the eosinophils was 50–70%. Major contaminants were macrophages and neutrophils. Neutrophil-rich or macrophage-rich peritoneal exudate cells were obtained from normal ddY mice that received an intraperitoneal injection of 2 ml of 0.1% oyster glycogen (Nacalai Tesque, Kyoto, Japan) 2 h or 48 h before use, for neutrophils or macrophages, respectively. Neutrophil-rich (>90%) or macrophage-rich (40–50%) cell suspensions from three

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The abbreviations used are: ECF(s), eosinophil chemotactic factor(s); ECF-L, splenocyte-derived ECF; PBS, phosphate-buffered saline; FBS, fetal bovine serum; RANTES, regulated on activation normal T cell expressed; GRO, growth-related oncogene; MCP, monocyte chemotactic protein; HPLC, high performance liquid chromatography; mAbs, monoclonal antibody; bp, base pair; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinopropanesulfuric acid; MIP, macrophage inflammatory protein; PMN, polymorphonuclear leukocyte.

Increased eosinopoiesis in bone marrow is observed prior to the tissue eosinophilia in parasitic infections (6, 7). Indeed, most multipotent hemopoietic stem cells are located in bone marrow (8). On the other hand, kinetic studies suggest that the spleen is the site where eosinophils mature (9), indicating eosinophil influx from the bone marrow to spleen at the maturation stage.

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animals were pooled and used for the respective chemotaxis experiments.

**T Lymphocytes**—T lymphocytes for chemotaxis indicator cells were prepared as a T cell line. Female C57BL/6 mice were immunized with 20 μg of ovalbumin (Sigma) in complete Freund's adjuvant. 12 days later, 105 spleen cells were cultured in ECF-L (10 μg/ml) as adjuvant (Difco Laboratories, Detroit, MI). 2 weeks after the second immunization, they were bled, and the serum was separated. Establishment of a monoclonal antibody (mAb) to ECF-L was carried out according to a method described previously (14) with modifications. In short, female Lewis rats were immunized twice with the purified ECF-L (10 μg) emulsified in Freund's complete adjuvant. The mAbs were removed 2 weeks after the last immunization and were hybridized to Sp2/0. A clone (E3.1) was selected, and the mAb was purified from the culture supernatant of hybridomas by gel chromatography on Sephacryl S300 (Amersham Pharmacia Biotech) for experimental use. The isotype of E3.1 was determined as IgM by a kit (Binding Site, Birmingham, U. K.).

**Construction of the Baculovirus Transfer Vector and Generation of the Recombinant Virus**—A BamHI site was created 6 bp upstream of the translation initiation codon and an EcoRI site 6 bp downstream of the stop codon to obtain a full-length cDNA by polymerase chain reaction, and the cDNA was digested with the BamHI and EcoRI. The BamHI-EcoRI fragment was then ligated to pVL1393 (Invitrogen). The recombinant pVL construct was co-transfected into Spodoptera frugiperda SF21 insect cells with a modified Autographa californica nuclear polyhedrosis virus DNA (BaculoPlus, Pharmingen), and the resultant viral pool was collected 4 days later.

**Preparation of Recombinant Proteins**—SF21 insect cells were cultured in serum-free medium containing 10% fetal bovine serum (Life Technologies, Inc.) at 27 °C. To produce recombinant proteins, SF21 cells seeded at a density of 106 cells/ml were infected with the recombinant virus. The cells were harvested 96 h after infection, washed with a solution of 0.15 M NaCl in 0.02 M phosphate buffer, pH 7.4, homogenized in a Tiss-HCl, pH 7.6, and centrifuged at 10,000 × g for 20 min. Recombinant ECF-L was purified from the supernatant using a Procion red-agarose, G3000SW, and C18 columns following the same steps as for the purification of natural ECF-L from the culture supernatant of splenocytes.

**SDS-PAGE and Immunoblot**—SDS-PAGE was performed on 10% polyacrylamide gel by using the discontinuous buffer system of Laemmli (16) with precast Rainbow protein molecular weight markers (Amersham Pharmacia Biotech). Western blotting onto a nitrocellulose filter (Amersham Pharmacia Biotech) was performed as described (17). Blots were immunostained with 1,000 dilutions of anti-ECF-L antiserum and 2,000 dilutions of alkaline phosphatase-labeled goat anti-rat IgG (EY Laboratories, San Mateo, CA). Antibodies were detected with substrate containing 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.1 mg/ml nitro blue tetrazolium, and 4 μg/ml MgCl2 in 100 mM Tris-HCl, pH 9.6.

**Northern Blot Analysis**—Total RNA from each organ was isolated using Isogen (Nippon Gene, Tokyo, Japan) from female C57BL/6 mice infected with 106 tetrathyridiae of S. japonicum 8 weeks after infection and gently mixed at 65 °C, blots were hybridized to 32P-labeled polymerase chain reaction fragments of ECF-L and mouse sperm DNA at 65 °C, blots were hybridized with random primed 32P-labeled DNA probes for 16 h under identical conditions. Filters were washed three times with 1 × SSC containing 0.1% SDS at 65 °C and then exposed to x-ray film (BioMax, Eastman Kodak, Rochester, New York)."
Analysis of Interaction with Chitins—Chitinase activity was measured by a chromogenic assay using 200 mM p-nitrophenyl β-D-N,N,N-triacetylchitotriose (Sigma) in 0.05 M phosphate buffer, pH 6.8, as the substrate. Chitinase from Serratia marcescens (Sigma) was used as a positive control. Chitin bead (Life Technologies, Inc.) chromatography (1.0 × 3.3-cm column) was performed with PBS at a flow rate of 3.0 ml/h, and 0.6-ml fractions were collected.

RESULTS

Purification of ECF-L—For purification of ECF-L protein, the medium conditioned by spleen cells obtained from S. japonicum-infected mice was concentrated, dialyzed against Tris-HCl buffer, and applied to a DE52 anion-exchange column. ECF activity was eluted at around the 0.15 M NaCl region of the gradient (Fig. 1A). Active fractions were pooled, concentrated by ultrafiltration, dialyzed against Tris-HCl buffer, and then applied to the Procion red-agarose affinity column. Elution profiles are shown in Fig. 1B. The major ECF activity was eluted at around 0.5 M NaCl. ECF-positive fractions were pooled, concentrated, and chromatographed by G3000SW HPLC (Fig. 1C). ECF-positive fractions were pooled and purified further on a reverse phase HPLC column (Fig. 1D). A single absorbance peak at 230 nm coincided with the peak of ECF activity. The purified ECF-L showed homogeneity on SDS-PAGE analysis (Fig. 1E) with a molecular mass of 43-kDa, and the specific eosinophil chemotactic activity was approximately 100 times that of the crude supernatant (Fig. 1F).

Isolation of ECF-L cDNA—The cDNA library of bone marrow cells from mice infected with S. japonicum was screened with an anti-ECF-L polyclonal antibody or mAb E3.1b. Of approximately 1,000,000 plaques screened, one plaque was found to be positive. The positive clone was picked and purified through three successive rounds of replating antibody screening. The insert was purified and subcloned into Bluescript SK2. As shown in Fig. 2, sequence analysis revealed that the cDNA was 1,506 bp long with a 1,197-bp open reading frame that could encode a protein 397 amino acids long. The putative amino acid sequence included the NH2-terminal 21-amino acid endoplasmic reticulum signal peptide followed immediately by the NH2-terminal sequence of natural ECF-L as determined by a protein microsequencer (YQLMXYYTISWAKDRPIEG). The molecular mass of mature ECF-L, calculated from the deduced amino acid sequence, was 42,370 Da. This value is close to the one determined experimentally by SDS-PAGE (Fig. 1E). The calculated isoelectric point (pI 5.3) was somewhat higher than that determined experimentally (pI 3.6) by isoelectric focusing (11). The sequence does not contain any consensus N-linked glycosylation sites (NXS, NXT) (18). In addition, the mRNA contains an approximately 300-bp-long untranslated region with a polyadenylation signal (AAUAAA) toward the 3′-end.

Recombinant ECF-L was prepared by a baculovirus expression system and purified by a combination of anion-exchange...
chromatography on DE52, affinity chromatography on Procion red-agarose, gel chromatography on G3000SW, and reverse phase HPLC. The purified recombinant protein showed a single band on SDS-PAGE analysis with a molecular mass of 43 kDa (data not shown). The ECF activity of the recombinant ECF-L was comparable with that of the natural ECF-L (Fig. 3A) and was completely inhibited by mAb to ECF-L (E3.1b) as well as natural ECF-L (Fig. 3B).

We next examined the cellular specificity of ECF-L as a chemoattractant. Because the optimum chemotactic activity of RANTES, GRO, and MCP-1 was achieved at 10^{-8} M in our assay system, the same molar concentration of ECF-L was employed for the comparative chemotaxis assays. As shown in Fig. 4, both natural and recombinant ECF-L exhibited chemotactic activity for T lymphocytes and bone marrow cells as well as for eosinophils. Most of the migrated bone marrow cells in the membrane showed PMN-like features with segmented nuclei. In contrast, ECF-L exhibited a limited chemotactic activity for mature neutrophils and no detectable chemotactic activity for macrophages. This cellular specificity of ECF-L was similar to that of RANTES. On the other hand, GRO showed chemotactic activity for mature neutrophils but not for bone marrow cells.

To examine whether ECF-L could induce eosinophils to extravasate into the inflammatory site, recombinant ECF-L was injected intradermally on the back of mice infected with M. corti. As shown in Fig. 5, large numbers of permeated eosinophils were observed at the site where ECF-L was injected. 90 ± 6% of the migrated leukocytes outside the blood vessel were eosinophilic when 29 ± 4% of all leukocytes in the blood were eosinophils in the treated mice. The number of migrated eosinophils increased dose-dependently with ECF-L. The increase in eosinophil migration was observed at as low as 5 × 10^{-9} M (1 pmol).

Production of ECF-L in a Parasitic Infection—Kinetics of the ECF-L production by splenocytes were examined in M. corti-infected mice. As shown in Fig. 6A, production of eosinophil chemotactic activity by splenocytes was detectable as early as 2 weeks postinfection by Western blot analysis in an antigen-specific manner and increased with time. The amount of ECF-L detected was correlated with the eosinophil chemotactic activity of the culture supernatant of splenocytes (Fig. 6B). When the ECF-L mRNA expression of each organ was examined at 2 weeks after M. corti infection, high level mRNA expression was detected in spleen, bone marrow, lung, and heart (Fig. 6C). Lower levels of mRNA were also detected in liver, thymus, and small intestine. A message for ECF-L was generally undetectable in normal mice by Northern blot analysis, whereas a low level of mRNA was detected by reverse transcription-polymerase chain reaction in bone marrow, spleen, and thymus in normal mice (data not shown). Nucleotide sequences of the reverse transcription-polymerase chain reaction products (1–1,195) from the liver, spleen, and bone marrow mRNA of ddY mouse were identical to the sequence of the isolated cDNA clone from the library (Fig. 2).

Sequence Homology with Other Proteins—A comprehensive search of GenBank or EMBL nucleic acid data bases revealed that ECF-L possesses significant homology with prokaryotic chitinases, class III plant chitinases, fungus chitinase, insect and nematode chitinase, and chitinase family proteins distributed in vertebrate animals. An optimal alignment of 20 repre-
sentative chitinases or chitinase family proteins was performed using the multiple sequence alignment program, Clustal X. 49 residues are highly conserved in more than 80% of the 20 representative chitinase family proteins, and 13 residues are completely conserved in all chitinase family proteins including ECF-L (Fig. 2). ECF-L retains 44 residues (90%) out of the 49 highly conserved. Fig. 7 shows an alignment of a conservative region where the catalytic center of the chitinases is located (19). Aspartic acid, essential for catalytic activity (19), is conserved in all of the chitinases and chitinase family proteins other than ECF-L. Although the other essential acid for catalytic activity (19), glutamic acid, is conserved in all of the chitinases, it is generally replaced by leucine or isoleucine in chitinase family proteins that have no chitinolytic activity (20–25). In ECF-L, both the glutamic acid and the aspartic acid are replaced by glutamine and asparagine, respectively.

ECF-L possesses the CXC consensus sequence near the NH₂ terminus which is typical of CXC chemokines. However, for ECF-L with CC chemokines or CXC chemokines (Fig. 8) poor alignments including for second and third cysteine residues were obtained.

Analysis of Interaction with Chitins—We could not detect any chitinase activity in purified ECF-L at any concentration up to 0.5 mg/ml. On the other hand, natural ECF-L eluted far behind (162%) the bed volume of the chitin column, whereas bovine serum albumin or other unrelated proteins eluted at 47% of the bed volume.

In this paper, we described the purification, cDNA cloning, and molecular characterization of a novel eosinophil chemotactic cytokine, ECF-L, which is produced in parasitic infections upon stimulation of specific antigens. Comparisons of the inferred protein sequence against all of the sequences in the GenBank or EMBL data base revealed a high degree of similarity to chitinase belonging to family 18 of glycosyl hydrolases (26) and vertebrate chitinase family proteins without chitinase activity (20–25). Sequence alignments revealed that ECF-L is an independent molecule different from other known ECF cytokines such as interleukin-5 (27), RANTES (28), eotaxin (29), or ecalectin (30).

Chitinases catalyze the hydrolysis of β-1,4-N-acetylgalactosamine linkages in chitin and chitodextrins and are widely distributed among a variety of species such as bacteria, fungi, nematodes, plants, insects, and vertebrates. In plants, chitinases themselves likely form part of innate immune system important for host defense against invading pathogenic bacteria and fungi (31). Insect or nematode chitinases are likely essential to certain life cycle events such as molting of the larval exoskeleton or casting of the egg shell (32). Similarly, protozoans increase chitin synthesis (33) and chitinase activity (34) during encystation. On the other hand, proteins of the so-called chitinase family are distributed in mammals and have no detectable chitinolytic activity (20–25). The actual physiological roles of the mammalian chitinase family proteins remain to be clarified. The present study has revealed a signif-

**FIG. 3.** Characterization of recombinant ECF-L. A, comparison of recombinant ECF-L with natural ECF-L. Eosinophil chemotactic activity of crude (closed circles) or purified (open circles) ECF-L is shown. Each point represents the mean ± S.E. of four samples. B, specific inhibition of eosinophil chemotaxis of ECF-L by mAb. Purified natural (open circles) or recombinant (closed circles) ECF-L was preincubated with anti-ECF-L mAb at 20 °C for 20 min and examined for eosinophil chemotaxis. An inhibition study of eosinophil chemotaxis of natural (open squares) or recombinant (closed squares) ECF-L for control mAb was also performed under the same experimental conditions.

**FIG. 4.** Cellular specificity of ECF-L as a chemoattractant in vitro. The chemotactic activity of recombinant ECF-L (rECF-L) or natural ECF-L (ECF-L) was examined in the absence (closed column) or presence (open column) of 20 μg/ml anti-ECF-L mAb (ECF3). ECF-L was preincubated with anti-ECF-L mAb at 20 °C for 20 min and examined for chemotactic activity. The chemotactic activity of ECF-L for eosinophils (A), neutrophils (B), macrophages (C), T lymphocytes (D), and bone marrow cells (E) was examined. RANTES was used for the positive control of chemotactic activity for eosinophils, T lymphocytes, or bone marrow cells, GRO for neutrophils, and MCP-1 for macrophages at 10⁻⁸ M, respectively. PBS was used for the negative control.

**DISCUSSION**
icant physiological role for a novel chitinase family protein in mammals.

Eosinophils contain many cytotoxic mediators including eosinophil peroxidase, major basic protein, and eosinophil cationic protein. Eosinophils have been observed to be degranulated accompanying eosinophil infiltration around the tissue-invasive stages of helminth infection. In vitro experiments have shown that eosinophils possess helminthotoxic activity in a manner that involves the release of eosinophil granule proteins.

**FIG. 5.** Extravasation of eosinophils induced by ECF-L. Typical pictures of extravasated eosinophils in the skin of M. corti-infected mice (3 weeks postinfection) injected with 25 μl of PBS (A) or 4 × 10⁻⁸ M (B), 2 × 10⁻⁷ M (C), or 10⁻⁴ M (D) of recombinant ECF-L. 90 ± 7% of the infiltrated leukocytes are eosinophils with red colored large granules.

**FIG. 6.** Production of ECF-L in M. corti infection. A, splenocytes at 0 (0 W), 2 (2 W) or 4 (4 W) weeks after infection with M. corti were incubated without (0) or with 10 or 100 μg/ml M. corti larval antigen. Production of ECF-L was examined by Western blot analysis. B, splenocytes at 0, 2, or 4 weeks after infection with M. corti were incubated without (closed column) or with 10 (gray column) or 100 (open column) μg/ml M. corti larval antigen. Supernatants were examined for eosinophil chemotactic activity. C, Northern blot analysis of ECF-L mRNA expression. Liver (lane 1), spleen (lane 2), thymus (lane 3), brain (lane 4), bone marrow (lane 5), lung (lane 6), heart (lane 7), kidney (lane 8), or small intestine (lane 9) from M. corti-infected mice at 2 weeks after infection was examined for ECF-L or GAPDH mRNA expression. The position of rRNA is indicated on the left.

**FIG. 7.** Aligned ECF-L amino acid sequence of a highly conserved region with 19 chitinase family proteins. The deduced amino acid sequence of ECF-L is aligned with the sequences of human chondrocyte protein YKL-39 (GenBank™ accession no. U49835), human cartilage gp-39, human oviductal glycoprotein, Mesocricetus auratus oviductin, Mus musculus BRP39 protein, Sus scrofa heparin-binding glycoprotein, Human Chitinase, Chelonus sp. venom chitinase, Anopheles gambiae chitinase, Manduca sexta chitinase, Brugia malayi chitinase, Onocercra volvis chitinase, Nicotiana tabacum Chitinase class V, Entamoeba histritica chitinase, Aphanocladium album chitinase, Acanthocheilonema viteae chitinase, Stenotrophomonas maltophilia chitinase, Serratia marcescens chitinase A, Bacillus circulans chitinase A1, Bacillus licheniformis chitinase A1, and Bacillus circulans chitinase A1. Boxed letters show chitinases that possess chitinolytic activity. The reverse type residues indicate amino acids that are identical in at least 50% of the members of this group of proteins. The asterisk and double asterisk represent the positions of aspartic acid and glutamic acid essential for proton donation in hydrolysis (19), respectively.

**ECF-L**

- Human chondrocyte protein YKL-39
- Human cartilage gp-39
- Human oviductal glycoprotein
- Mesocricetus auratus oviductin
- Mus musculus BRP39 protein
- Sus scrofa heparin-binding glycoprotein
- Human Chitinase
- Chelonus sp. venom chitinase
- Anopheles gambiae chitinase
- Manduca sexta chitinase
- Brugia malayi chitinase
- Onocercra volvis chitinase
- Nicotiana tabacum Chitinase class V
- Entamoeba histritica chitinase
- Aphanocladium album chitinase
- Acanthocheilonema viteae chitinase
- Stenotrophomonas maltophilia chitinase
- Serratia marcescens chitinase A
- Bacillus circulans chitinase A1
- Bacillus licheniformis chitinase A1
against the larvae of parasites of *Schistosoma* (35), *Brugia* (36), *Trichinella* (37), and *Strongyloides* (38). Production of ECF-L at local sites would contribute to the triggering of the influx of eosinophils that act as effectors of parasite killing. Thus, ECF-L may have evolved from a chitinase as an immune molecule from invading parasites by means of accumulating eosinophils followed by secreting toxic substances rather than directly digesting parasites.

Chitinase-related proteins of vertebrates share relatively weak amino acid sequence homology with chitinases as a whole. To elucidate the possible function of the conserved residues of ECF-L, the alignment of ECF-L and 19 sequences of representative chitinase-related proteins was performed (Figs. 2 and 5). 13% of the amino acids (49 residues) were highly conserved in more than 80% of the representative chitinase or chitinase family proteins. The ratio of aromatic amino acids (Phe, Tyr, Trp) in the highly conserved residues (34.7%) is relatively high compared with those among all of the residues (14.8%) of ECF-L. Glycine was also prominent in the conserved residues (20.4%) compared with that in total ECF-L (8.5%).

The tertiary structures of some chitinase proteins have been resolved: these proteins all share the feature of a ββα barrel topology (39, 40). Based on the amino acid sequence homology, the locations of these conserved amino acids of ECF-L are assumed by analogy. Some highly conserved aromatic amino acids of ECF-L (Tyr-6, Trp-10, Phe-37, Tyr-246, Trp-339) likely form hydrophobic clusters in the active cleft, and two (Phe-37, Trp-339) of these residues form a cis-peptide bond that is assumed to play a key role in substrate recognition. In terms of position, the glycine residues (Gly-76 and Gly-77) are highly conserved; they likely locate at the end of the third β-strand where a conserved phenylalanine (Phe-37) is closely located. All but one of the highly conserved glycine residues are also located in the β-strand or βα-loops, indicating that replacement by other residues may not be permitted to avoid a steric hindrance. ECF-L shares the highly conserved aromatic amino acids and glycines that form a substrate recognition site, and this fact suggests that ECF-L retains a similar ability for the recognition or conformational change of chitin-related carbohydrates. In fact, elution of ECF-L from the chitin beads column clearly took longer than estimated probably because of the interaction with chitina. As for the carbohydrate recognition of ECF, human ecalctin that binds to carbohydrates (β-galactosides) possesses ECF activity (30). The ability to recognize carbohydrate may be another aspect of ECF-L in the protective immunity against parasitic infections because chitin-related carbohydrates are an important component of the exoskeleton of larvae and egg shells in nematodes (32).

As for the catalytic activity of chitinases, a site-directed mutagenesis study of chitinase A1 from *Bacillus circulans* showed that artificial mutation of either glutamic acid at position 204 (Glu → Gln or aspartic acid at position 200 (Asp → Asn) resulted in a remarkable loss of catalytic activity (19), indicating that the carbonic acids of both residues are essential for proton donation in hydrolysis. In ECF-L, both the essential glutamic acid (corresponding location in ECF-L: Gln-119) and aspartic acid (corresponding location in ECF-L: Asn-115) are modulated in the natural protein, explaining the undetectable level of chitinolytic activity. In other vertebrate chitinase family proteins that have no catalytic activity, glutamic acid is replaced by a similar sized hydrophobic side chain such as leucine in synovial protein (21) and oviductal glycoprotein (22), or with isoleucine, such as in major secreted protein of human articular chondrocytes (20) and heparin-binding glycoprotein (25). On the other hand, the essential aspartic acid is generally conserved. Thus ECF-L possesses a unique blocking form of two essential carbonic acids in contrast to other vertebrate chitinase family proteins, and this fact may suggest a unique function of ECF-L among chitinase family proteins without chitinase activity.

It has been proposed that blocking parasite-derived chitinases could block the transmission of malaria (41) by inhibiting the chitinase activity needed for the penetration of the peritrophic membrane in anopheline mosquitoes. Similarly, microfilarial stage-specific chitinase has been shown to be a candidate antigen for a transmission-blocking vaccine against filariasis (42). The sequence similarity between the chitinase family proteins of the host molecules and parasite-derived chitinases suggests some restriction in the use of chitinases as vaccine molecules.

ECF-L possesses a CXC sequence near the NH₂ terminus of the mature molecule. The CXC or CC sequence is a typical motif shared in many chemokine family proteins but not in other chitinase family proteins. Amino acid sequence alignments of ECF-L with CXC or CC chemokines revealed no sequence similarity even around the CXC sequence (Fig. 7) where the critical receptor binding region exists (43, 44). The other two cysteine residues conserved in all of the chemokines are not found in ECF-L. Moreover, the tertiary structure of interleukin-8, a CXC chemokine, exhibits topography similar to that of the αα2 domains of the human class I histocompatibility antigen HLA-A2 (45) and to share no similarity with chitinases (39, 40). This would consistently indicate that ECF-L is an independent molecule, not one of these chemokines.

We found that ECF-L attracts not only eosinophils but also T lymphocytes and bone marrow PMNs. ECF-L possesses a specificity similar to RANTES as a chemotactrant in that it attracts CD4⁺ memory T lymphocytes (46), eosinophils, and bone marrow cells (Fig. 4). This indicates that the receptor(s) for ECF-L are related to that for RANTES (47, 48). The specific inhibitory effect of anti-ECF-L mAb is likely understood in terms of the steric hindrance that resulted in the obstruction of chemotactic epitope(s) on ECF-L.

Recent evidence suggests that eosinophils bind to vascular endothelial cells, and their transendothelial migrations are mediated by interactions of various adhesion molecules and their ligand molecules (49). ECFs produced in the inflammatory site would play a critical role in the exit of eosinophils from vasculature and their attraction to the inflammatory site. In
ECF-L, Novel Eosinophil Chemotactic Cytokine

the present study, we showed that ECF-L could induce the specific extravasation of eosinophils into the injected site at as low as 1 pmol. This result suggests that eosinophil-specific transendothelial migration could be caused by ECF-L when an appropriate amount of ECF-L is produced outside the blood vessel. The discrepancy in the cellular specificity of ECF-L in vivo (Fig. 5) and in vitro (Fig. 4) studies would be the result of complicated mechanisms of transendothelial processes in vivo including the effects of adhesion molecules (49).

With regard to whether enough ECF-L is generated locally to cause tissue eosinophilia around the larvae, little evidence of this is presently available. Expression of ECF-L mRNA was not limited to the liver where the M. corti larvae locate. So far as we could ascertain, spleen, bone marrow, lung, and heart are the dominant organs of production of mRNA encoding ECF-L.

Several eosinophil chemotactic cytokines have been shown to have multiple functions (27, 50). In addition to their role in the recruitment of eosinophils from the site where PMNs are differentiated to the inflammatory site and to their priming effects, ECF-L may participate in the removal of excess eosinophils from the circulation to spleen or bone marrow where they can be treated and/or reused. The possible novel functions of ECF-L remain to be clarified.

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