Fibroblast Chemotaxis Induction by Human Recombinant Interleukin-4

Identification by Synthetic Peptide Analysis of Two Chemotactic Domains Residing in Amino Acid Sequences 70-88 and 89-122

Arnold E. Postlethwaite[*‡||¶] **and Jerome M. Seyer**[§¶]

Division of Connective Tissue Diseases, ‡Departments of Medicine and §Biochemistry, University of Tennessee, Memphis; and the ||Laboratory of Cellular Immunology, ¶Veterans Administration Medical Center, Memphis, Tennessee 38163

Abstract

Interleukin-4 is a T lymphocyte- and mast cell-derived cytokine with pleiotropic properties with biological effects on a variety of target cells including B and T lymphocytes, macrophages, hematopoietic cells, mast cells, and fibroblasts. In addition to the proliferation effect of IL-4 on fibroblasts, which has been previously described, in this report the chemotactic properties of IL-4 for fibroblasts is described. Human recombinant IL-4 induced the chemotactic migration of dermal fibroblasts in vitro in modified Boyden-type chambers at concentrations between 10^{-12} and 10^{-11} M. The chemotactic activity of IL-4 was neutralized by anti-human recombinant IL-4 IgG antibodies. Oligopeptides representing the complete deduced amino acid sequence of human IL-4 were synthesized by the Merrifield technique and tested for their ability to induce fibroblast chemotaxis. Two peptides representing residues 70-88 and 89-122 induced fibroblast migration. Peptide 70-88 was the more potent of the two causing chemotaxis of fibroblasts at 10^{-8}–10^{-6} M while peptide 89-129 induced migration at 10^{-7}–10^{-5} M. Although the mechanism by which IL-4 and these two peptides induce fibroblast chemotaxis is unknown, each of these three compounds were able to chemotactically desensitize fibroblasts to the chemotactic effects of the other two but not to a structurally unrelated chemotactic cytokine, transforming growth factor β − 1. These studies suggest that IL-4 might function in vivo to induce the accumulation of fibroblasts at sites of tissue injury, inflammatory and immune reactions in which T lymphocytes and mast cells participate. (*J. Clin. Invest.* 1990. 87:2147–2152.) Key words: cytokines • fibrogenesis • wound healing • oligopeptides • lymphokines

Introduction

IL-4, formerly called B cell stimulatory factor-1, was originally characterized as a costimulant with anti-immunoglobulin antibodies for resting murine B cells, causing them to enter the S-phase of growth (1). Although other effects of IL-4 on B cells have also been described including stimulation of expression of class II antigens and production of IgGl and IgE, it is now apparent that this T cell and mast cell product affects a variety of different target cells; It is a growth factor for normal T cells and T cell lines (1–3). IL-4 promotes maturation of myeloid precursors to monocyte-macrophages, synergizes with colony-stimulating factors such as granulocyte colony stimulatory factor to promote growth of granulocytic colonies, and with erythropoietin to stimulate colony formation by immature and mature erythroid precursors and in establishment of megakaryocytic colonies (4–7). IL-4 is synthesized by mast cell lines and acts as a costimulator with IL-3 in promoting growth of mast cells (8, 9). IL-4 is capable of activating macrophages, augmenting expression of class II antigens, and nonspecific cytotoxicity (7, 10, 11). It also modulates cytokine gene expression, down regulating both IL-1 α and β, and TNF α mRNA (12).

Receptors have been identified not only on hemotopoietic cells such as normal B cells, resting and activated T cells, macrophages, and mast cell tumor lines, but also on fibroblasts (13, 14). Murine dermal and lung fibroblasts have been reported to proliferate in the presence of murine IL-4 (15). These observations in fibroblasts prompted us to determine whether IL-4 could act as a chemoattractant for these cells. In this study, we have observed that human recombinant (hr)IL-4 is indeed a potent chemotactic agent for fibroblasts. Structure-function studies employing synthetic peptides have identified two peptides, 70-88 and 89-122, that are able to induce fibroblast chemotaxis. Furthermore, these peptides can specifically deactivate fibroblasts so that they do not chemotax to hrIL-4 or to each other, suggesting that the fibroblast chemotactic property resides within these amino acid residues of IL-4.

Methods

Fibroblast chemotaxis. Fibroblasts maintained in monolayer cultures and grown from infant foreskin explants were used as indicator cells in chemotaxis studies. Maintenance medium for fibroblast cultures consisted of Eagle's minimum essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 9% fetal bovine serum, nonessential amino acids, 50 μg/ml ascorbic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B. All samples were tested in quadruplicate and fibroblast migration was measured as previously described using blind-well Boyden-type chambers equipped with gelatin-treated polycarbonate filters (8 micron pore size; Nucleopore Corp., Pleasanton, CA) (16). Nuclei of fibroblasts migrating through the 8 micron pores and present on the lower surface of each filter were quantitated in 20 randomly selected oil immersion fields (OIF)[1] (magnification, 1,000). Results for each sample are expressed where indicated as the mean±SEM of the 4 replicates.

Chemoattractants. hrIL-4 was purchased from Genzyme Corp., Boston, MA and had a specific activity of 10^8 proliferation U/mg of protein. It was judged to be 95% pure by silver staining of SDS-PAGE gels. Porcine platelet-derived TGF-β1 was purchased from R & D Sys-

Address correspondence and reprint requests to Dr. Arnold E. Postlethwaite, Director, Division of Connective Tissue Diseases, Department of Medicine, University of Tennessee, 956 Court Avenue, G326, Memphis, TN 38163.

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1. *Abbreviations used in this paper:* IL-4R, IL-4 receptor; OIF, oil immersion fields.

tems, Minneapolis, MN, and was 97% pure as assessed by NH_2-terminal sequence analysis and silver staining of SDS-PAGE gels.

Anti-hrIL-4 antibodies. Specific neutralizing rabbit anti-hrIL-4 Ig was purchased from Genzyme Corp. and was > 90% IgG. The antibody preparation was diluted in PBS and incubated at 15 μg/15 μl with continuous shaking on a rocker platform for 14 h at 4°C with hrIL-4 (1.25 ng in 1 ml PBS) or TGF-β1 (10 pg in 1 ml PBS).

Synthesis of human IL-4 peptides. The published amino acid sequence of precursor human IL-4 by Yokota et al. (17) was used to construct peptides of various lengths. Peptides were synthesized on a Beckman Instruments, Inc. (San Diego, CA) automated peptide synthesizer (model 990) by the solid-phase method of Merrifield (18). The peptides were purified by gel filtration and reverse phase HPLC, and the amino acid composition of each peptide was confirmed by use of a Beckman 121 MB automatic amino acid analyzer (19, 20).

Results

Chemotaxis of fibroblasts to hrIL-4. On numerous occasions using different batches of hrIL-4 and fibroblast lines from different donors, hrIL-4 consistently induced maximal migration of fibroblasts at concentrations ranging between 1 and 50 pg/ml (7.1×10^{-14}–3.6×10^{-12} M). A representative experiment is shown in Fig. 1. As has been found with other fibroblast chemoattractants, the response curve was bell shaped (21, 22). To determine whether hrIL-4 was chemotactic, and/or chemokinetic for fibroblasts, a checkerboard analysis of the Zigmond-Hirsch type was performed wherein different concentrations of hrIL-4 were added to the upper (cell) and lower (test) compartments of the modified blind-well Boyden-type chambers, and migration of fibroblasts was measured (23). hrIL-4-induced increased migration of fibroblasts when it was present in the lower compartment of the chambers relative to the upper compartment (Table I). This experiment was repeated twice with different cell lines and similar results were obtained (data not shown). This indicates that IL-4 induces chemotaxis rather than chemokinesis of fibroblasts.

Inhibition of hrIL-4 induced chemotaxis by anti-hrIL-4 antibodies. Polyclonal rabbit anti-hrIL-4 IgG effectively neutralized the chemotactic property of hrIL-4 but had no effect on the chemotactic activity of TGF-β1 (Table II). This experiment was repeated and similar results were obtained (data not shown).

Chemotaxis of fibroblasts to synthetic IL-4 peptides 70-88 and 89-122. In an effort to identify the amino acid sequences in human IL-4 responsible for its fibroblast chemotactic property, peptides 1-24, 25-47, 48-69, 70-88, 89-122, and 123-153 were synthesized from the published deduced sequences of precursor IL-4 as described in Methods (Table III). These peptides were tested at different concentrations ranging from 10^{-10} to 10^{-4} M on three different occasions with different fibroblast lines for their ability to induce fibroblast migration. Two peptides, 70-88 and 89-122, consistently caused fibroblast migration in a dose-dependent manner (a representative experiment is shown in Fig. 2). Peptide 70-88 was the more potent of the two inducing migration at 10^{-8} to 10^{-7} M (Fig. 2). To assess whether these two peptides stimulate chemotaxis or chemokinesis of fibroblasts, Zigmond-Hirsch checkerboard analyses were performed. Peptide 70-88 and 89-122 each induced only chemotactic migration of fibroblasts, causing increased migration when present in the lower versus the upper compartment of the modified Boyden chambers (Tables IV and V).

Chemotaxis desensitization studies. Chemoattractant or receptor specific desensitization has been demonstrated for chemotaxins for neutrophils and fibroblasts (24–26). To determine whether IL-4-specific desensitization could be achieved by peptide 70-88 and/or 89-122 and vice versa, experiments were performed in which the fibroblasts in the upper compartments were exposed to chemotactic levels of either hrIL-4, peptide 70-88, peptide 89-122, or to media, and their subsequent migration to these chemoattractants and an unrelated cytokine, TGF-β1, was assessed. The migration of fibroblasts to

*Table I. Zigmond-Hirsch Checkerboard Analysis of hrIL-4-induced Fibroblast Migration**

hrIL-4 concentration in upper compartment (M)	hrIL-4 concentration in lower compartment (M)				
	0	2.9×10^{-12}	1.1×15^{-11}	4.5×10^{-11}	8.9×10^{-11}
	Fibroblasts per 20 OIF (mean±SEM)				
0	7±1	26±3	35±4	43±3	62±6
2.9×10^{-12}	8±1	8±1	28±4	31±5	43±4
1.1×19^{-11}	9±1	9±1	8±1	19±2	29±2
4.5×10^{-11}	7±1	8±1	9±1	8±1	18±3
8.9×10^{-11}	8±1	10±2	10±1	9±1	7±1

* Various concentrations of hrIL-4 were added as indicated to the upper and/or lower compartments of modified blind-well Boyden chambers in quadruplicate, and migration of fibroblasts was quantitated.

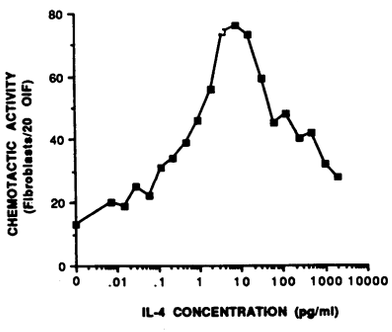

Figure 1. hrIL-4 was tested at the concentrations indicated for its ability to induce fibroblast migration.

Table II. Inhibition of hrIL-4-induced Chemotaxis by Anti-hrIL-4 Antibodies

Condition*	Chemotactic activity
	Fibroblasts per 20 OIF
hr-IL4 (1.25 ng in 1 ml) + PBS (15 μl)	123±7
hrIL-4 (1.25 ng/ml in 1 ml PBS) + anti-hrIL-4 Ig (15 μg in 15 μl)	24±2
TGFβ-1 (10 pg in 1 ml PBS) + PBS (15 μl)	100±3
TGFβ-1 (10 pg in 1 ml PBS) + anti-hrIL-4 Ig (15 μg in 15 μl)	107±18
PBS	22±2

* hrIL-4 or TGFβ-1 samples were incubated for 14 h at 4°C with or without rabbit anti-hrIL-4 Ig. The samples were then tested in an assay for their ability to induce fibroblast migration.

*Table III. Synthetic IL-4 Peptides**

Residues	Amino acid sequences
‡1–24	Met Gly Leu Thr Ser Gln Leu Leu Pro Pro Leu Phe Phe Leu Leu Ala Cys Ala Gly Asn Phe Val His Gly
25–47	His Lys Cys Asp Ile Thr Leu Gln Glu Ile Ile Lys Thr Leu Asn Ser Leu Thr Glu Gln Lys Thr Leu
48–69	Cys Thr Glu Leu Thr Val Thr Asp Ile Phe Ala Ala Ser Lys Asn Thr Thr Glu Lys Glu Thr Phe
70–88	Cys Arg Ala Ala Thr Val Leu Arg Gln Phe Tyr Ser His His Glu Lys Asp Thr Arg
89–122	Cys Leu Gly Ala Thr Ala Gln Gln Phe His Arg His Lys Gln Leu Ile Arg Phe Leu Lys Arg Leu Asp Arg Asn Leu Tyr Gly Leu Ala Gly Leu Asn Ser
123–153	Cys Pro Val Lys Glu Ala Asn Gln Ser Thr Leu Glu Asn Phe Leu Glu Arg Leu Lys Thr Ile Met Arg Glu Lys Tyr Ser Lys Cys Ser Ser

* Peptides were constructed from the deduced sequence of human Il-4 (17). Peptides were synthesized by the solid-phase method of Merrifield on a Beckman Instruments, Inc. automated peptide synthesizer (model 990) (2, 23). ‡ These 234 amino terminal residues are cleaved off during intracellular processing of precursor IL-4 to mature IL-4 (17).

hrIL-4, peptide 70-88 and 89-122 but not to TGF-β1, was inhibited by either hrIL-4, peptide 70-88, or peptide 89-122, indicating specific desensitization was achieved by hrIL-4, and peptides 70-88 and 89-122 (Table VI). Similar results were obtained when this experiment was repeated (data not shown). These results suggest that these synthetic peptides mediate fibroblast chemotaxis by a mechanism (or perhaps receptor) similar to that used by hrIL-4.

Discussion

Fibroblast migration was stimulated in vitro by hrIL-4 at concentrations ranging between 10^{-12} and 10^{-11} M. Increased migration of fibroblasts occurred only when a positive concentration gradient of hrIL-4 was present in the chemotaxis chambers (concentration higher in lower sample compartment than in upper cell compartment). The chemotactic property of hrIL-4 was inhibited by neutralizing polyclonal anti-hrIL-4 IgG. IL-4 peptides ranging in length from 19 to 34 residues were synthesized and tested for their ability to induce fibroblast migration. Two oligopeptides consisting of residues 70-88 and 89-129 elicited chemotactic migration of fibroblasts. Peptide 70-88 was more potent (inducing migration at 10^{-8} to 10^{-6} M) than peptide 89-122 (inducing migration at 10^{-7} to 10^{-5} M) in stimulating fibroblast chemotaxis. Both of these peptides desensitized fibroblasts to the chemotactic effect of hrIL-4 but did not desensitize fibroblasts to the chemotactic effect of an unrelated chemotactic cytokine, TGF-β1. hrIL-4 desensitized fibroblasts to the chemotactic effect of peptides 70-88 and 89-129 but not to TGF-β1.

The injury to connective tissue by immunological, mechanical, physical, or chemical insults is followed by an orderly repair process wherein fibroblasts migrate into the area from surrounding locations, expand their numbers by proliferation, and then synthesize and remodel new matrix to constitute scar

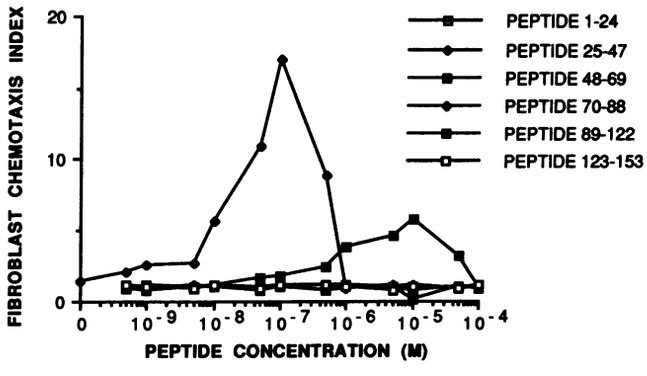

Figure 2. Six synthetic IL-4 oligopeptides were tested in different assays for their ability to induce fibroblast migration at the concentrations indicated. Results are expressed as a "Chemotaxis Index" which was calculated by dividing control migration of fibroblasts (per 20 OIF) obtained with serum-free maintenance medium into the number of cells migrating in 20 OIF with peptide in a given assay. Fibronectin (0.1 μg/ml) served as a positive chemoattractant for each assay and always gave a Chemotaxis Index of > 10.

*Table IV. Zigmond-Hirsch Checkerboard Analysis of IL-4 Peptide 70-88 Induced Fibroblast Migration**

Peptide 70-88 concentration in upper compartment (M)	Peptide 70-88 concentration in lower compartment (M)				
	0	1.6×10^{-8}	3.2×10^{-8}	6.4×10^{-8}	1.3×10^{-7}
	Fibroblasts per 20 OIF (mean±SEM)				
0	16±2	35±4	53±3	78±12	107±11
1.6×10^{-8}	14±2	14±1	45±3	57±8	73±12
3.2×10^{-8}	19±3	12±2	22±4	41±4	62±5
6.4×10^{-8}	15±2	20±4	17±3	19±2	46±4
1.3×10^{-7}	11±1	12±2	14±2	12±1	11±1

* Various concentrations of synthetic IL-4 peptide 70-88 were added as indicated to the upper and/or lower compartments of modified blind-well Boyden chambers in quadruplicate, and migration of fibroblasts was quantitated.

*Table V. Zigmond-Hirsch Checkerboard Analysis of IL-4 Peptide 89-122 Induced Fibroblast Migration**

Peptide 89-122 concentration in upper compartment (M)	Peptide 89-122 concentration in lower compartment (M)				
	0	3.7×10^{-7}	7.3×10^{-7}	1.5×10^{-6}	2.2×10^{-6}
	Fibroblasts per 20 OIF (mean±SEM)				
0	25±3	48±6	76±7	97±6	129±13
3.7×10^{-7}	17±2	24±7	50±4	67±3	82±8
7.3×10^{-7}	18±2	19±2	19±1	58±2	71±5
1.5×10^{-6}	18±1	18±1	19±2	18±1	54±2
2.2×10^{-6}	18±3	17±2	17±2	18±1	20±2

* Various concentrations of synthetic IL-4 peptide 89-122 were added as noted to the upper and/or lower compartments of modified blind-well Boyden chambers in quadruplicate, and migration of fibroblasts was quantitated.

*Table VI. Inhibition of hrIL-4, IL-4 Peptide 70-88, or Il-4 Peptide 89-122 Induced Fibroblast Chemotaxis by Each Other**

Contents of lower compartment	Fibroblast chemotaxis (mean cells per 20 OIF±SEM)
Media in upper compartment	
TGFβ1 (2×10^{-13} M)	98±12
hrIL-4 (3.5×10^{-11} M)	58±5
IL-4 Peptide 70-88 (4.1×10^{-7} M)	93±10
IL-4 Peptide 89-122 (1.8×10^{-6} M)	98±7
Media	8±1
hrIL-4 (3.5×10^{-11} M) in upper compartment	
TGFβ1 (2×10^{-13} M)	96±8
hrIL-4 (3.5×10^{-11} M)	10±1
IL-4 Peptide 70-88 (4.1×10^{-7} M)	9±1
IL-4 Peptide 89-122 (1.8×10^{-6} M)	23±3
Media	11±1
IL-4 Peptide 70-88 (4.1×10^{-7} M) in upper compartment	
TGFβ1 (2×10^{-13} M)	100±12
hrIL-4 (3.5×10^{-11} M)	16±3
IL-4 Peptide 70-88 (4.1×10^{-7} M)	8±1
IL-4 Peptide 89-122 (1.8×10^{-6} M)	44±5
Media	8±1
IL-4 Peptide 89-122 (1.8×10^{-6} M) in upper compartment	
TGFβ1 (2×10^{-13} M)	97±13
hrIL-4 (3.5×10^{-11} M)	13±2
IL-4 Peptide 70-88 (4.1×10^{-7} M)	9±1
IL-4 Peptide 89-122 (1.8×10^{-6} M)	8±1
Media	9±1

* Fibroblasts were suspended in either serum-free MEM, or MEM containing hrIL-4, IL-4 peptide 70-88, or IL-4 peptide 89-122 at the concentrations indicated, and migration of fibroblasts to TGFβ-1 and these agents was quantitated.

tissue (27, 28). The observation that IL-4 is a potent inducer of fibroblast chemotaxis in vitro suggests that it could play a role in the recruitment of fibroblasts in vivo to sites of tissue injury. IL-4 could be released from mast cells and/or T lymphocytes at sites of inflammation and promote fibrogenesis.

Mast cells are associated with a variety of human diseases characterized by excessive fibroblast accumulation and fibrosis including chronic inflammatory states such as parasitic diseases, rheumatoid arthritis synovium, psoriasis, interstitial pulmonary fibrosis, various tumors including carcinoids, keloids, hemangiomas, neurofibromas, mastocytosis, scleroderma, and scleroderma-like conditions such as toxic oil syndrome, chronic graft versus host disease, and eosinophilic fasciitis (see reference 29 for review).

IL-4 could also be released from T lymphocytes participating in immune and inflammatory reactions in vivo. Several conditions such as the early lesions of scleroderma, chronic graft versus host disease, streptococcal cell wall induced granulomatous inflammation in rats, granulomas associated with tuberculosis, and sarcoidosis have prominent infiltration of T lymphocytes (29-33). Perhaps the multinucleated giant cells associated with the latter three conditions also develop in part from IL-4, which is also known to promote monocyte polykaryon formation (34).

The mechanism(s) by which IL-4 induces fibroblast chemotaxis is unknown. Presumably it is effecting migration via an IL-4 receptor (IL-4R)-dependent or related mechanism. IL-4Rs have been described on fibroblasts as well as a variety of hematopoietic cells (13, 14). The IL-4R on human gingival fibroblasts and Raji B cells has an observed mol wt of 139,000 (14). The human IL-4R cDNA has been recently isolated and when transfected into COS-7 cells, was shown to encode a 140,000 mol wt protein (35). The predicted extracellular domain of the human IL-4R has a high degree of amino acid sequence homology with receptors for interleukin-6, erythropoietin and prolactin, and with the β subunit of the IL-2R (35). The mechanisms by which the IL-4R transduces signals is not apparent from the predicted amino acid sequence of its cytoplasmic domain, because no homologies were found with protein kinases or sequences characteristic of phosphorylation acceptor sites for protein tyrosine kinases or protein kinase C (35). Our finding in this study that specific desensitization of fibroblasts to IL-4, peptide 70-88 and 89-122 by treating fibroblasts with each of these agents would be compatible with IL-4R occupancy and signal transduction by these agents to induce chemotaxis. Additional studies will clarify this issue.

In a given inflammatory reaction, a number of different chemoattractants may be available to recruit new neighboring fibroblasts to the site. This redundancy of chemoattractants may exist to assure that tissue repair is effected contributing to survival of the host. The list of agents that induce chemotaxis of fibroblasts includes collagen types I, II, and III and their constituent α chains, hydroxyproline containing oligopeptides, fibronectin, tropoelastin and elastin peptides, platelet-derived growth factor, leukotriene B_4, an 80,000 mol wt fragment from the fifth component of complement, C5a and C5a des arg, TGF-β, tumor necrosis factor α, interferon γ, and uncharacterized factors from T lymphocytes and breast carcinoma cell lines (16, 21, 22, 25, 36–45). This would imply that stimulated T lymphocytes have the capacity to synthesize and release at least three different cytokine-lymphokine agents (TGF-β, interferon γ, and IL-4) to promote the accumulation of fibroblasts in vivo.

The synthetic IL-4 peptides 70-88 and 89-122 were much less potent than hrIL-4 in inducing fibroblast chemotaxis. Synthetic human IL-1β peptides have been reported to have less potent biological effects than hrIL-1β, and a biologically active synthetic growth hormone peptide has been reported to be less potent than intact growth hormone (46–48). This reduced potency of synthetic cytokine oligopeptides may be due to their small size and absence of other sequences required for optimal receptor binding.

There is ~ 50% homology between inferred amino acid sequences of human and murine IL-4, however, murine IL-4 does not stimulate human cells, and human IL-4 does not stimulate murine cells (17, 49). It is not apparent why the chemotactic property of human IL-4 resides in residues 70-88 and 89-122. It is interesting and perhaps significant that the most

potent chemotactic peptide (70-88) has the most amino acid homology (58%) with its murine IL-4 counterpart (calculated from sequences shown in reference 17 for murine and human IL-4). However, peptide 89-122, which was 10-fold less potent, has the least amino acid homology with its murine IL-4 counterpart (29%) than any of the six IL-4 peptides studied. Whether either of these peptides are involved in IL-4 receptor binding remains to be established.

This report characterizes another formerly unrecognized property of IL-4, namely, its ability to induce fibroblast migration. It is interesting to speculate that the fibrogenic reaction characterized by accumulation of fibroblasts and matrix components at sites of immune and inflammatory reactions in which T cells and/or mast cells play a prominent role may be due in part to the stimulation of chemotaxis and replication of fibroblasts by IL-4. Future work dealing with the mechanisms by which a fibrogenic response accompanies certain T lymphocyte- and mast cell-mediated immune and inflammatory reactions should assess the possible participation of IL-4.

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