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The CXC-chemokine, H174: expression in the central nervous system

Yi Luo[1], Robert Kim[1], Dana Gabuzda[2], Sha Mi[3], Lisa A Collins-Racie[3], Zhijian Lu[3], Kenneth A Jacobs[3] and Martin E Dorf[1]

[1]Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115; [2]Department of Neurology, Harvard Medical School and Dana Farber Cancer Institute, Boston, Massachusetts 02115 and [3]Genetics Institute, Cambridge, Massachusetts 02140, USA

H174 is a new member of the CXC-chemokine family. A cDNA probe containing the entire H174 coding region recognized a predominant inducible transcript of approximately 1.5 kb expressed in interferon (IFN) activated astrocytoma and monocytic cell lines. H174 message can be induced following IFN-α, IFN-β, or IFN-γ stimulation. H174 message was also detected in IFN treated cultures of primary human astrocytes, but was absent in unstimulated astrocytes. H174, like IP10 and Mig, lacks the ELR sequence associated with the neutrophil specificity characteristic of most CXC-chemokines. Preliminary experiments suggest H174, IP10 and Mig are independently regulated. Recombinant H174 is a weak chemoattractant for monocyte-like cells. H174 can also stimulate calcium flux responses. The data support the classification of H174 as a member of a subfamily of interferon-γ inducible non-ELR CXC-chemokines. Brain tissues were obtained at autopsy from one patient with AIDS dementia, one patient with multiple sclerosis, and two normal control patients. H174 and Mig were detected by RT–PCR in brain tissue cDNA derived from the patients with pathological conditions associated with activated astrocytes but not in cDNA from control specimens.

Keywords: AIDS dementia; astrocytes; chemoattractant; interferon; multiple sclerosis

Introduction

Chemokines (for *chemo*tactic cyto*kines*) are a family of small, inducible protein or glycoprotein molecules that play an essential role in evoking inflammatory responses. Perhaps the most important attribute of chemokines is their ability to act as potent selective chemoattractants for monocytes, lymphocytes, eosinophils, and/or neutrophils (Luster, 1998; Schluger and Rom, 1997). Chemokines can also initiate proinflammatory processes in these inflammatory cells, e.g. granule exocytosis and respiratory burst (Baggiolini *et al*, 1994).

Chemokines and chemokine receptors appear to play important roles in the pathogenesis of many diseases, including inflammatory diseases such as psoriasis and rheumatoid arthritis, and infectious diseases like malaria (Koch *et al*, 1992; Gillitzer *et al*, 1993; Horuk *et al*, 1993). However, chemokines have raised the greatest interest in the study of HIV-1 infection where selected chemokine receptors serve as co-receptors for HIV-1 and the chemokine ligands can block HIV-1 infection (Alkhatib *et al*, 1996; Bleul *et al*, 1996a).

The two major families of chemokines (termed CC- and CXC-chemokines) are defined by homologies in the spacing of between the first two cysteine residues. These structural features generally correlate with distinctive biological activities. CC-chemokines act principally on monocytes but not neutrophils, whereas CXC-chemokines primarily attract neutrophils, not monocytes. However, there are notable exceptions to these findings. Among CXC-chemokines the tripeptide motif–glutamic acid-leucine-arginine (called the ELR motif)–located immediately before the first cysteine residue is essential, though not sufficient, for activity on and binding to neutrophils (Clark-Lewis *et al*, 1993, 1994). The observation that CXC-chemokines (SDF-1, PF4, IP10 and Mig) that do not possess the ELR motif are unable to activate or attract neutrophils supports this conclusion (Taub *et al*, 1993; Liao *et al*, 1995; Bleul *et al*, 1996b). The

Correspondence: ME Dorf
The first two authors contributed equally to this work.
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CXC-chemokines lacking the ELR motif attract mononuclear cells, a trait not typical of the other CXC-chemokines. Two of these non-ELR containing CXC-chemokines, IP10 and Mig, are induced by IFN-γ and bind a common receptor on activated T-lymphocytes (Luster and Ravetch, 1987; Farber, 1990; Loetscher *et al*, 1996). Non-ELR CXC-chemokines also appear to inhibit angiogenesis while ELR-containing CXC-chemokines promote angiogenesis (Strieter *et al*, 1995). These data suggest subdivision within the group of CXC-chemokines into the ELR-containing and the non-ELR subgroups.

The H174 cDNA was cloned from a library prepared from alloantigen activated human peripheral blood leukocytes (Jacobs *et al*, 1997). H174 is the newest member of the non-ELR subgroup of CXC-chemokines. Here we review our knowledge of this novel chemokine and report the expression of H174 in cells of the central nervous system.

Results

Classification of H174

A 997 bp cDNA sequence isolated from a library generated from human leukocytes stimulated by the presence of allogeneic cells was cloned by a signal sequence trap technique as detailed previously (Jacobs *et al*, 1997). This sequence, termed H174, included an open reading frame of 285 bp that encoded a 94 amino acid protein. The predicted signal peptide cleavage site after the 21st amino acid predicts that the mature H174 molecule is a 73 amino acid secreted protein. H174 contains four cysteine residues at positions characteristic of CXC-chemokines. The mature form of H174 contains no potential sites for N-linked glycosylation.

Protein sequence comparison showed the closest homologs to H174 were the human CXC-chemokines Mig and IP10. H174 is 38% identical to human Mig and 36% identical to human IP10 over the 73 amino acids of mature protein that are comparable (Farber, 1997). The sequences of these molecules and their murine counterparts are compared in Figure 1. Sequence comparisons between human and murine Mig or IP10 with H174 identified 15 identical residues dispersed along these molecules plus highly conserved amino acids at 11 additional positions (Figure 1). Generally, the areas of greatest homology appear to be clustered adjacent to the invariant cysteine residues at positions 36–46 and 53–66.

Preliminary examination of the genomic organization of H174 with a series of PCR primers indicates the locus encoding H174 is approximately 1.6 kb. There are at least three introns within the coding region. The first intron is approximately 600 bp and is located between residues 124 and 174 based on the cDNA sequence (GenBank accession #AF002985). The second intron is about 130 bp and is localized between 251 and 292 bp, and the third intron (about 400 bp) is between nucleic acids 304 and 531. This genetic organization is similar to that reported with other CXC chemokines including IP10 and IL8 (Baggiolini *et al*, 1994).

```
           1                                                  50
     H174  PPMFKRGRCL CIGPGVKAVK VADIEKASIM YPSNNCDKIE VIITLKENKG
     HIP10 VPLSRTVRCT CISISNQPVN PRSLEKLEII PASQFCPRVE IIATMKKKGE
     MIP10 IPLARTVRCN CIHIDDGPVR MRAIGKLEII PASLSCPRVE IIATMKKNDE
     HMIG  TPVVRKGRCS CISTNQGTIH LQSLKDLKQF APSPSCEKIE IIATLK.NGV
     MMIG  TLVIRNARCS CISTSRGTIH YKSLKDLKQF APSPNCNKTE IIATLK.NGD

Consensus  TP--RT-RCS CIS-S-G-VH -RSL-KL-I- -PSP-CPK-E IIATLKKNGE

           51                                                 100
     H174  QRCLNPKSKQ ARLIIKKVER KNF*~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
     HIP10 KRCLNPESKA IKNLLKAVSK EMSKRSP~~~ ~~~~~~~~~~ ~~~~~~~~~~
     MIP10 QRCLNPESKT IKNLMKAFSQ KRSKRAP~~~ ~~~~~~~~~~ ~~~~~~~~~~
     HMIG  QTCLNPDSAD VKELIKKWEK QVSQKKKQKN GKKHQKKKVL KVRKSQRSRQ
     MMIG  QTCLDPDSAN VKKLMKEWEK KINQKKKQKR GKKHQKNMKN RKPKTPQSRR

Consensus  QRCLNP-SK- -KNL-K--EK K-S--K-QK- GKKHQK---- ---K---SR-

           101
     H174  ~~~~~~
     HIP10 ~~~~~~
     MIP10 ~~~~~~
     HMIG  KKTT~~
     MMIG  RSRKTT

Consensus  ------
```

Comparisons to H174 Protein Sequence

Chemokine	% A.A.Identity	% A.A.Homology
HuIP10	36	53
MuIP10	36	49
HuMig	38	50
MuMig	34	44

Figure 1 Alignment of H174 with other non-ELR CXC-chemokines. Top panel: Amino acid sequence comparisons to the human and mouse CXC-chemokines Mig and IP10. Stippling marks conserved residues and a consensus sequence for this chemokine subfamily is presented. Bottom panel: Comparisons of H174 and human and mouse IP10 and Mig amino acid sequences based on the Wisconsin Sequence Analysis Package, GCG gap program.

Expression of H174 mRNA

Since the genes most homologous to H174 encode chemokines that are inducible following treatment with IFN-γ, this method of activation was used to follow gene expression. Using a 453 bp probe which included the entire coding region for H174, the induction of a major 1.5 kb and a minor 4.0 kb RNA transcript was noted in the THP-1 monocytic cell line following 8 h treatment with IFN-γ (1000 U/ml). No H174 message was detected in unstimulated cells (Figure 2A). IFN-γ stimulated U-937 monocyte-like and U-373 astrocytoma cell lines also expressed a major 1.5 kb band, but the weak 4.0 kb band was not detectable in U-373 RNA (data not shown). The inability of resting astrocyte cell lines to produce H174 was confirmed by RT–PCR. A band at the expected size (439 bp) was amplified from IFN-γ-treated CCF, U-373, and SW 1783

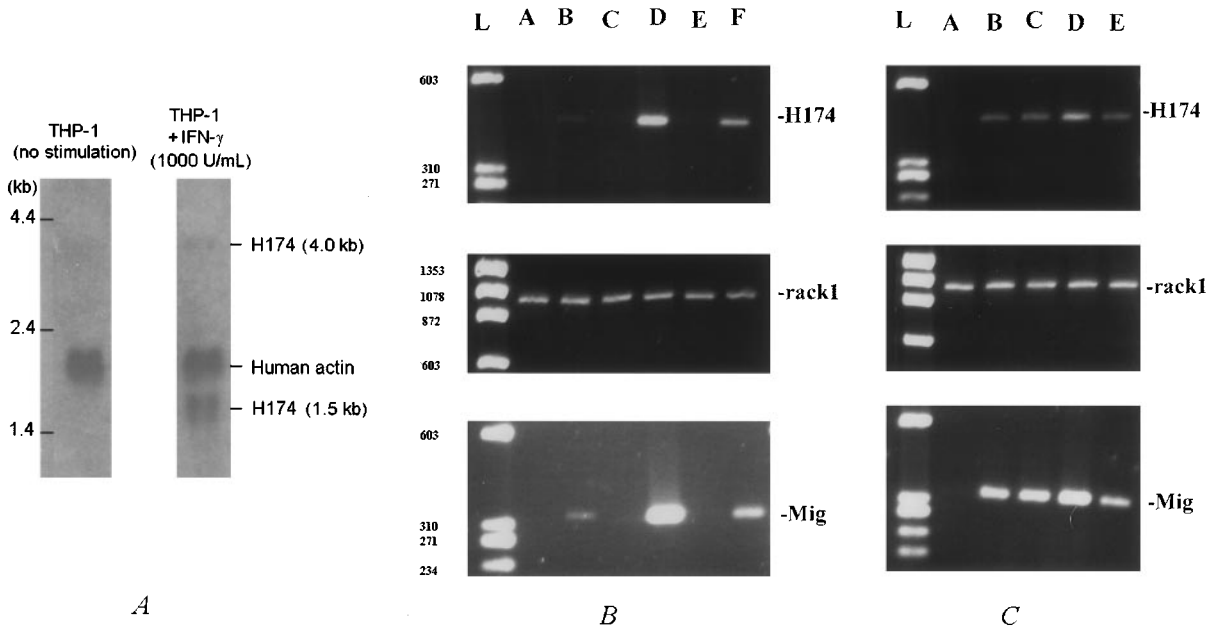

Figure 2 Induction of H174 in astrocytes. (**A**) Total RNA was prepared from unstimulated or IFN-γ stimulated (8 h with 1000 U/ml) THP-1 monocytic cells. Twenty μg RNA were electrophoresed on a 1.5% agarose-formaldehyde gel and blotted onto a nylon membrane which was probed with a 453 bp cDNA radiolabeled probe consisting of the entire H174 coding region. A major 1.5 kb species and a minor 4.0 kb band were identified in cells stimulated with IFN-γ. Subsequently the blot was reprobed with a human actin probe. (**B**) CCF (lanes A and B), U-373 (lanes C and D), and SW1783 (lanes E and E) astrocytoma cells were cultured in either medium (lanes A, C and E) or 1000 U/ml IFN-γ for 8 h (lanes B, D and F). RNA samples were reverse transcribed and amplified with PCR primers for H174 (439 bp), rack1 (1093 bp), or Mig (325 bp) by RT–PCR. The housekeeping gene rack1 was used as a positive control for cDNA integrity. (**C**) Cultured human fetal astrocytes were treated with 100 U/ml IFN-γ for 0, 4, 18, 24 or 48 h (lanes A, B, C, D and E, respectively). The cells were examined for H174, rack1, and Mig gene expression by RT–PCR. Lane L contains the ladder of size markers corresponding to the indicated sizes (**B**).

astrocytoma cells but not from unstimulated cells (Figure 2B). The amount of H174 PCR product varied among astrocytoma cell lines; U-373 cDNA consistently yielded the most H174 PCR product while CCF cells produced minimal levels (Figure 2B). To establish the presence of H174 in primary astrocytes the kinetics of H174 expression were evaluated on cultured human fetal astrocytes. H174 expression was noted after a 4–48 h treatment with IFN-γ, with maximal expression at 24 h (Figure 2C). Again, without IFN-γ treatment no H174 PCR products were detected. All samples expressed comparable levels of the control housekeeping gene, rack1, at the predicted size of 1093 bp (Figure 2B and C).

The same cDNA samples were examined for expression of another IFN-γ inducible non-ELR chemokine, Mig, by RT–PCR. H174 and Mig were both induced by IFN-γ in the limited series of samples tested (Figure 2B and C). To further compare the regulation of H174 and Mig or IP10 expression we treated cells with type I (IFN-α and IFN-β) or type II (IFN-γ) interferons. SW 1783 astrocytoma cells were stimulated with graded doses of IFN-α, IFN-β, or IFN-γ. As shown in Figure 3, H174 PCR products were detected after activation with $\geqslant$100 U/ml IFN-γ, $\geqslant$100 U/ml IFN-α or $\geqslant$1000 IFN-β. In addition to the rIFN-αA used in the above experiment rIFN-αD and rIFN-αA/D stimulate H174 RNA production with similar efficacy (data not shown). Similarly, native IFN-β was substituted for the rIFN-β used in the above experiments with identical results (data not shown). Message for another non-ELR CXC chemokine, Mig, was detected in these cells after treatment with $\geqslant$100 U/ml IFN-γ, but was not induced by treatment with up to 5000 U/ml IFN-α or IFN-β. IP-10 was not expressed in resting SW 1783 cells but was inducible following stimulation with all types of interferon. The above experiment was repeated with U-373 astrocytoma and U-937 monocytic cells with similar results, although H174 was inducible with as little as 1.0 U/ml IFN-γ in the latter cell lines (data not shown). The combined data indicate that H174 and IP-10 are inducible with all types of interferon. In contrast, Mig expression is strictly IFN-γ dependent. Recent studies demonstrated IP10 message is constitutively expressed by CaSki cervical carcinoma cells while message for H174 and Mig are not detected without IFN-γ treatment (data not shown). Thus, the combined data suggest that these structurally related interferon-inducible non-ELR CXC-chemokines are differentially expressed and regulated.

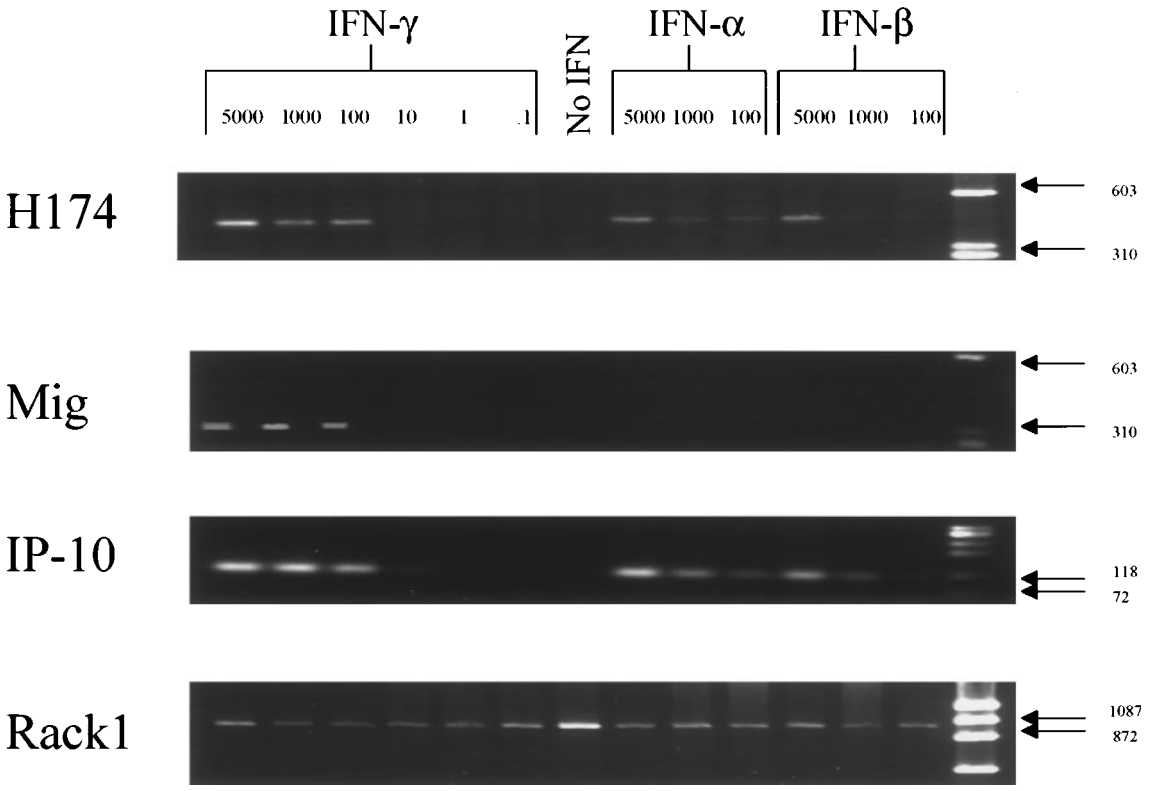

Figure 3 Comparison of non-ELR CXC chemokine induction by interferons. SW1783 astrocytoma cells were treated with the indicated concentrations (U/ml) of either rINF-γ, rINF-αA, or rINF-β for 18 h at 37°C. The cells were harvested and RNA samples were reverse transcribed and amplified with PCR primers for H174, Mig, IP-10, and rack1.

H174 expression in disease

Specimens of white matter from the brain of one patient with AIDS dementia and HIV-1 encephalitis (case #2648) and one patient with multiple sclerosis (case #2684) were obtained at autopsy. Specimens that exhibited pathological lesions were selected. The neuropathology was verified by histologic examination of adjacent tissue sections. The tissues from both patients revealed the presence of diffuse reactive astrocytes (i.e. gliosis), perivascular mononuclear cell infiltrates, and demyelination (data not shown). Control samples were obtained from autopsies of two normal control patients without evidence of reactive astrocytes or other brain pathology.

cDNA was prepared from each autopsy sample and examined for expression of the housekeeping gene rack1 by RT–PCR. There were indications of RNA degradation in all samples, therefore fourfold excess cDNA was used in the PCR reactions. As shown in Figure 4 each sample expressed the housekeeping gene, rack1, although the level of the PCR product varied among samples. H174 and Mig PCR products were detected in AIDS and multiple sclerosis brain specimens, but not in specimens from controls. The level of H174 expression in the multiple sclerosis specimen appeared low; however, RNA degradation as evidenced by the low levels of the rack1 control product contributed to this result. Rack1 was amplified from both control samples but both specimens failed to display the H174 or Mig PCR products.

Purification of H174

The amino acid sequence of H174 predicts a secreted, mature protein of 73 amino acids with a molecular weight of 8.3 kDa. Partial purification of this protein from supernatants of H174-transfected Sf9 cells was achieved by elution from a heparin-agarose column with 10 mM HEPES/2.0 M NaCl. However, this one-step process left several contaminating proteins, as evidenced by the multiple bands observed on SDS–PAGE (Figure 5A). Thus the heparin eluates were next applied, after dialysis against 10 mM HEPES, to a reverse-phase C18 column and fractionated by elution with a gradient of 0 to 70% acetonitrile in 0.1% trifluoroacetic acid. Two proteins typically co-eluted from the reverse-phase column as a major peak over a range of 59 to 62% acetonitrile. Elution at 60% acetonitrile produced the highest concentration of these proteins (Figure 5).

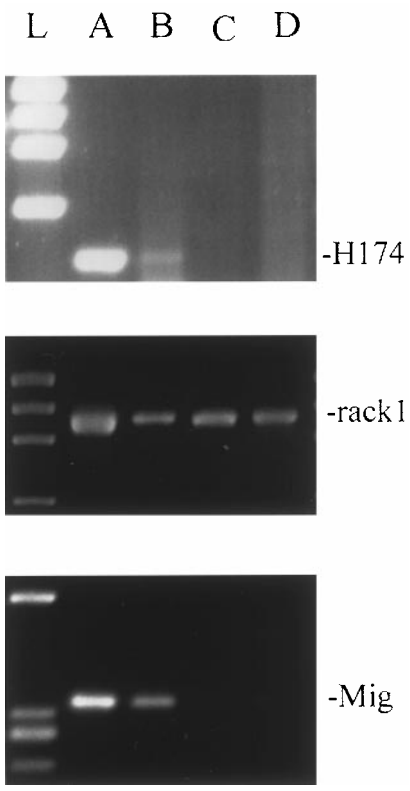

Figure 4 Analysis of H174 and Mig expression in brain tissues. White matter from patients with AIDS dementia and severe HIV-1 encephalitis (lane A), multiple sclerosis (lane B) and two normal control patients without CNS pathology (lanes C and D) was examined for H174, rack1, and Mig expression by RT–PCR. The size markers are the same as those described for Figure 2.

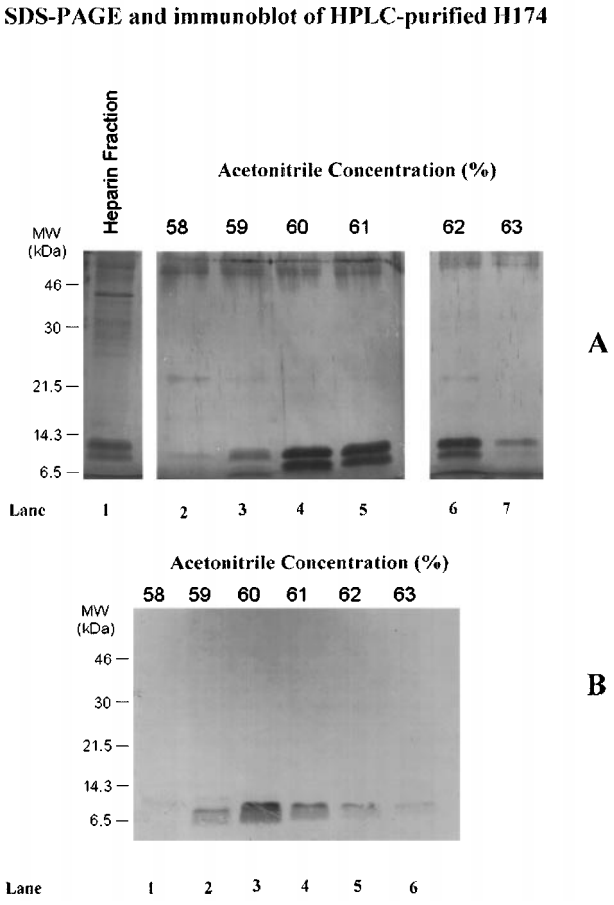

Figure 5 Purification of recombinant H174 protein (**A**). SDS–PAGE analysis of HPLC-purified H174. Two milliliters of heparin-purified H174 (lane 1) were applied to a Super Pac pep-S C18 column subjected to reverse-phase chromatography. H174 was typically eluted from the column over a range of 59 to 62% acetonitrile in 0.1% trifluoroacetic acid (lane 2). After elution from the HPLC column fractions were subjected to 15% SDS–PAGE. Silver-stain visualization indicated the highest concentration of H174 eluted at 60% acetonitrile. (**B**) Immunoblot of H174 purification. Supernatants from H174 baculovirus-infected Sf9 cells were purified as above. Selected HPLC fractions (58–63% acetonitrile) were run on 15% SDS–PAGE then transferred to a nitrocellulose membrane and stained with antisera from hamsters immunized with a 28 amino acid carboxy-terminus peptide of H174. Staining of HPLC-purified H174 fractions revealed two bands that reacted to differing degrees or a broad band that correlated with the visualization of H174 on silver-stained gels, with the highest concentration of H174 having eluted from the reverse-phase column at 60% acetonitrile (lane 3).

Antisera prepared against the 28 amino acid carboxy-terminus peptide of H174 enabled the specific identification of H174 throughout the various purification steps. Immunoblots indicated H174 remained bound to heparin-agarose beads following a wash with 10 mM HEPES/250 mM NaCl buffer, but eluted completely from them with a 10 mM HEPES/2.0 M NaCl solution (data not shown). H174 appeared as a peak over a range of 59 to 62% acetonitrile on elution from the reverse-phase column. The anti-H174 serum failed to react with proteins isolated from mock transfected Sf9 cells (data not shown).

SDS–PAGE and silver staining of the HPLC-purified H174 revealed two distinct bands that ran at an apparent molecular weight of 8.8 and 10 kDa (Figure 5A). However, immunoblotting with antisera to the C-terminal peptide revealed a broad band, the lower portion of this band was usually faint or smeared so that a distinct second band could not be clearly distinguished by Western blotting (Figure 5B). While the predicted H174 sequence lacks the required motif for N-linked glycosylation, O-linked glycosylation remained a possibility. Therefore, an O-glycosidase digest was performed to evaluate the presence of O-linked sugars. One μg H174 was incubated with 2.5 mU of neuraminidase (New England Biolabs, Beverly, MA, USA) for 2 h at 37°C to remove potential sialic acid residues prior to addition of 1 mU of O-glycosidase for an additional 18 h 37°C incubation. Fetuin, a control protein that contains O-linked sugars, was treated simultaneously. While fetuin underwent

glycosylation cleavages resulting in molecular weight shifts on SDS–PAGE, H174 demonstrated no change whatsoever in either of the bands previously isolated (data not shown). Thus, H174 appears to be a non-glycosylated protein.

H174 stimulates chemotaxis

Mononuclear leukocytes are among the targets of non-ELR chemokines. Therefore, the ability of rH174 to induce migration of U937 monocyte-like cells was evaluated in a 48-well Boyden microchamber. U937 cells exhibited optimal migratory responses with 1 ng/ml H174; a control CC-chemokine, MIP-1β, demonstrated responses of comparable magnitude under these experimental conditions (Figure 6). In contrast, H174 did not induce significant migration of human neutrophils (data not shown). Experiments to evaluate the activity of H174 on lymphocytes were inconclusive.

The chemotactic responses to H174 were completely inhibited by pretreatment of cells for 1 h with 100 ng/ml pertussis toxin (Figure 6). The sensitivity of H174-induced chemotaxis to pertussis toxin suggests that H174, like most chemokines, acts through Gαi protein-coupled receptors.

H174 induces a calcium flux

Chemokine receptors are seven-transmembrane spanning $G\alpha_i$ protein-coupled receptors that, upon binding their appropriate chemokine, provoke a transient rise in intracellular calcium levels. The calcium-sensitive fluorescent dye fura-2 AM was loaded into promyelocytic HL-60 cells and their responses to H174 were assayed in a fluorospectrophotometer. HL-60 cells experienced a transient calcium flux in response to H174 at concentrations of 1 μg/ml and 100 ng/ml (approximately 10^{-7} M and 10^{-8} M, respectively), but not to 10^{-5} M fMLP (Figure 7A–C). The magnitude of the calcium flux mediated by H174 was comparable to that previously noted with the chemokine SDF-1α on transfected target cells (Heesen *et al*, 1997). The calcium response was abolished in HL-60 cells that had been differentiated toward a neutrophil phenotype by culture with 1.25% DMSO for 2 days (Figure 7D).

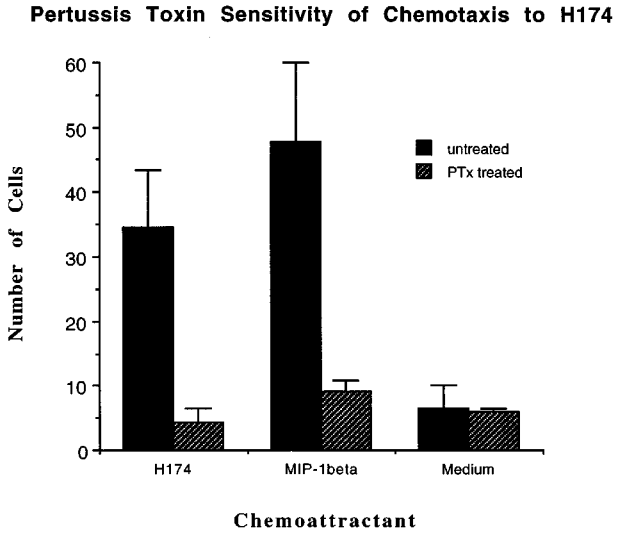

Figure 6 Migration of U-937 monoytic cells. U-937 cells were incubated for 1 h with or without 100 ng/ml of pertussis toxin and then assayed for chemotaxis toward H174 or MIP-1β. HPLC-purified rH174 or MIP-1β were evaluated at a concentration of 1 ng/ml and 10 ng/ml, respectively (approximately 0.1 and 1 nM, respectively). Migratory responses (cells per high power field) were assayed in triplicate. The data are an average from three experiments, with error bars indicating the s.e.m. Background migration without addition of chemokine is also presented.

Discussion

H174 is a new member of the CXC-chemokine family. The four cysteine residues characteristic of all CXC-chemokines are conserved in H174. The N-terminus of most CXC-chemokines encodes an ELR motif that is critical for neutrophil specificity (Clark-Lewis *et al*, 1993, 1994). The ELR sequence is missing in H174. The closest homologs of H174 are Mig and IP10, both of which also lack the ELR motif suggesting that these chemokines may form a subgroup of IFN-inducible non-ELR CXC-chemokines. The structural homologies among these molecules indicate a clustering of conserved residues with the group. However, this non-ELR CXC subgroup does not have a readily identifiable contiguous motif at the N terminus. Nonetheless, a few interesting features are notable in their N-terminal sequences. Most CXC chemokines have variable numbers of residues preceding the first cysteine. In contrast, H174, IP10 and Mig consistently display only eight amino acids in this region of which only residues three, five and eight are highly conserved. A truncated form of IP10 lacking the first three amino acid residues was nonfunctional and failed to bind to receptor bearing cells (Piali *et al*, 1998). Mutational analysis will be required to establish the critical residues involved in H174 binding and function.

Although H174, Mig and IP10 are all inducible following IFN-γ stimulation, type I interferons only stimulate expression of H174 and IP10. The observation that some cervical carcinoma cell lines constitutively express IP10 but not H174 or Mig suggests that each of these non-ELR CXC chemokines is selectively expressed and regulated. A better understanding of the process controlling chemokine expression may be obtained by future comparisons of the relevant promoter regions (Wright and Farber, 1991).

Undifferentiated HL-60 promyelocytic cells are targets for H174 while HL-60 cells differentiated toward the neutrophil lineage are not responsive to

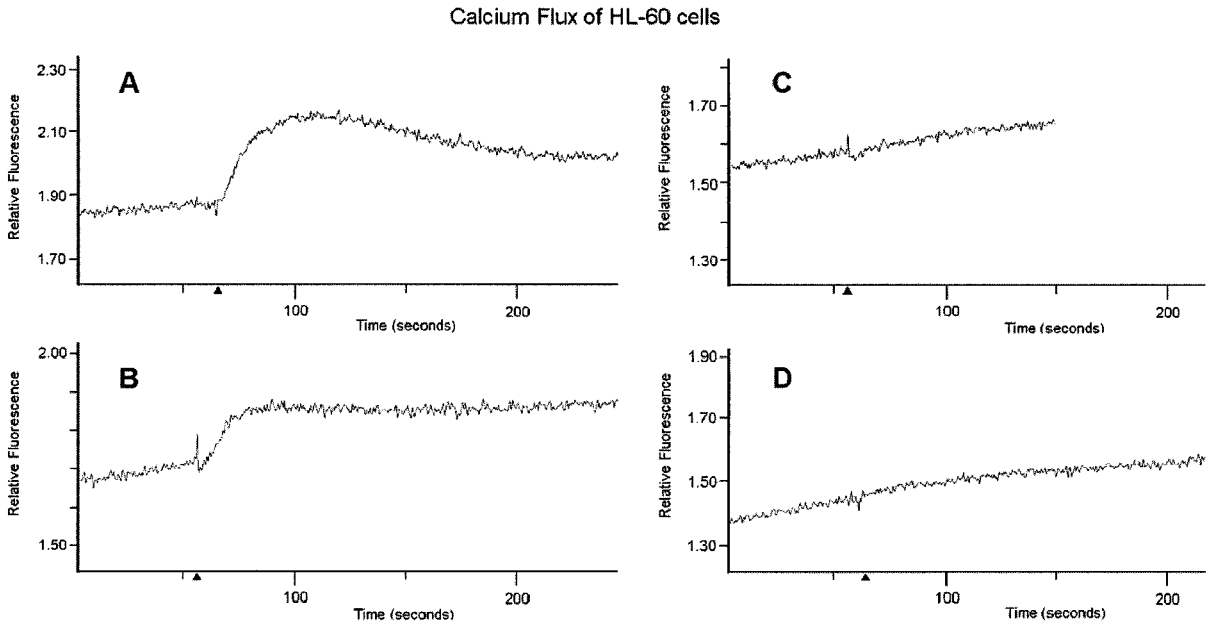

Figure 7 Calcium mobilization in HL-60 cells. HL-60 promyelocytic cells were loaded with fura-2 dye and assayed for elevations in $[Ca^{2+}]_i$ in a fluorospectrophotometer. (**A**) H174 induced a calcium increase in 2×10^6 undifferentiated HL-60 cells at concentrations of 1000 ng/ml (approx. 10^{-7} M). (**B**) Stimulation of 2×10^6 undifferentiated HL-60 cells with 100 ng/ml H174 (approximately 10^{-8} M). (**C**) 2×10^6 undifferentiated HL-60 cells fail to mobilize calcium in response to 10^{-5} M fMLP. (**D**) 2×10^6 HL-60 cells treated with 1.25% v/v DMSO for 2 days to cause differentiation to a neutrophil phenotype do not display a calcium flux in response to 1000 ng/ml H174. The time of H174 addition is indicated with a closed triangle (▲).

H174. These findings mirror other reports, demonstrating that IP10 inhibits colony formation of human bone marrow progenitor cells but neutrophils fail to respond (Dewald *et al*, 1992; Sarris *et al*, 1993).

However, the major cellular target of IP10 and Mig are activated T lymphocytes (Loetscher *et al*, 1996). Characterization of lymphocyte responses to H174 remains incomplete. CXCR3 is the T cell receptor for both IP10 and Mig and CXCR3 transfected cells migrate in response to IP10 or Mig (Loetscher *et al*, 1996). However, CXCR3 is apparently absent on monocytes, U-937, and HL-60 cells (Loetscher *et al*, 1996). Therefore, an alternative receptor molecule on U-937 and HL-60 cells is presumably involved in H174 responsiveness. We noted that rH174 possessed chemoattractant activity at subnanomolar concentrations suggesting a high affinity receptor (see Addendum). The complete inhibition of the migratory response by pretreatment of cells with pertussis toxin is consistent with previous observations that suggest chemokine receptors are coupled to $G\alpha_i$ proteins (Kuang *et al*, 1996) as is the finding that recombinant H174 protein induces calcium mobilization in target cells (Figure 7).

RNA transcripts for H174 are inducible by IFN in monocytic and astrocytoma cell lines. In contrast, treatment with bacterial lipopolysaccharide induces only minimal H174 expression (data not shown). This implies that H174 will be preferentially produced by astrocytes following activation of cellular responses particularly those involving NK, Th1 and/or CD8 cells which release IFN-γ.

A deduced protein sequence with 93.5% homology to H174 was initially reported by Rani *et al* (1996). These investigators identified a partial nucleic acid sequence from astrocytes stimulated with IFN-β but not IFN-α. In contrast, IFN-α and IFN-β both stimulate H174 production (Figure 3). We cannot understand the basis for these disparities, although the possibility of alternative spliced products remain.

In preliminary studies H174 and Mig expression were noted in brain tissue samples from two patients with clinical disease (AIDS dementia and multiple sclerosis) and histological evidence of astrocyte activation and inflammation. Presumably, in these conditions IFN-γ producing CD8, NK, and/or CD4 cells stimulate astrocyte chemokine production within CNS lesions resulting in amplification of the inflammatory response by recruitment and activation of mononuclear cells which mediate demyelination.

If chemokines such as H174, IP10 and Mig function synergistically, stimulation of multiple chemokine species may provide more effective inflammatory responses and may account for the redundancy frequently noted among chemokines. The implications from these hypotheses are that the

predominant interferons released in response to viral infection may directly influence the ability of astrocytes to recruit inflammatory cells to the CNS. Perhaps the most vigorous responses resulting in CNS demyelination require expression of all three of these non-ELR CXC chemokines. It has been reported that in the inflammatory demyelinating autoimmune disease murine experimental allergic encephalomyelitis (EAE) astrocytes are the major source of mRNAs encoding IP10 (Ransohoff *et al*, 1993). The current data demonstrate that MS patients can also produce H174 and Mig at inflammatory sites. Thus, the source of expression and the ability to attract mononuclear cells implicate H174 as a potential participant in inflammatory responses within the central nervous system in several diseases including AIDS dementia and multiple sclerosis.

In summary, H174 is a new member of the non-ELR CXC-chemokine subfamily. H174 is inducible in astrocytes and astrocytoma cell lines by treatment with IFN-γ. Recombinant H174 is chemotactic for monocytic cells and induces a transient calcium flux in the targets. H174 message was detected in the brain lesions from patients with neuropathological conditions associated with activated astrocytes.

Materials and methods

Cells and cell lines
The human HL-60 (promyelocytic leukemia), U-937 (monocyte-like), U-373 MG (glioblastoma/astrocytoma), CCF-STTG1 (astrocytoma) and SW 1783 (astrocytoma) cell lines were purchased from the American Tissue Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% fetal calf serum. Dr FW Luscinskas and Dr Keith Crawford (Harvard Medical School) generously provided human peripheral blood neutrophils and monocytes, respectively.

Cytokines
Human interferon-gamma (IFN-γ) and the CC-chemokine MIP-1β were purchased from R&D Systems (Minneapolis, MN). Recombinant human IFN-α subtypes A, D and A/D and IFN-β were purchased from Biosource International (Camarillo, CA). Native human interferon-β was purchased from Access Biomedical, San Diego, CA. Recombinant H174 protein was prepared in Sf9 insect cells and generously provided by the protein expression unit at Genetics Institute.

Isolation of astrocytes
Purified fetal astrocyte cultures were prepared from the cerebral cortex of 16 to 18 weeks fetal human brain tissue as described (Busciglio *et al*, 1993). Tissue was procured using an approved protocol in compliance with institutional and federal regulations. The tissue was minced and incubated briefly with 0.25% trypsin, dissociated by trituration, washed, and plated onto plastic culture plates (100 mm) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The confluent cells were passaged following removal of the loosely adherent microglial cells by orbital shaking (Tornatore *et al*, 1991). Experiments were conducted after the third cell passage when fetal neurons and microglia were no longer apparent in the culture. The purity of astrocyte cultures ($>99\%$) was determined by immunostaining with mouse anti-glial fibrillary acidic protein (GFAP) (1 : 100, Sigma) followed by FITC-conjugated goat anti-mouse IgG (Sigma).

Protein gel electrophoresis and silver staining
Electrophoresis of protein samples was carried out in 15% SDS–polyacrylamide mini-gels made with a Mini-Protean II gel assembly kit (Bio-Rad, Hercules, CA). Samples were boiled for 5 min in 5% β-mercaptoethanol/2% SDS prior to loading and electrophoresed at 150 V for 1.25 to 1.5 h. Acrylamide gels were fixed in 40% methanol/10% acetic acid for 30 min, followed by two 15 min fixing periods in 30% ethanol/5% acetic acid. Silver staining followed the manufacturer's protocol (Bio-Rad).

Production of anti-H174 antisera
Ten-week-old female Armenian hamsters were immunized subcutaneously with 100 μg of a 28 amino acid carboxy-terminus peptide of H174 in Complete Freund's Adjuvant (Life Technologies, Grand Island, NY) and were boosted five times at 3-week intervals with 100 μg of the peptide in Incomplete Freund's Adjuvant (Life Technologies). Anti-H174 antisera from two separately immunized hamsters were pooled for use in staining immunoblots.

Immunoblotting
Proteins separated by SDS–PAGE were transferred to a 0.22 μm pore size nitro-cellulose sheet (Bio-Rad). Sheets were then blocked for 2 h at room temperature with 3% BSA/PBS, washed three times with PBS and, if necessary, stored at -20°C. Nitrocellulose sheets were next reacted with a 1 : 1000 dilution in 3% BSA/PBS of anti-H174 antisera for 2 h at room temperature. The sheets were then washed three times for 10 min each with 3% BSA/PBS, and reacted for 2 h at room temperature with a 1 : 1000 dilution of alkaline phosphatase-conjugated goat anti-hamster Ig antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 3% BSA/PBS. The nitrocellulose sheet was finally washed three times for 5 min each with PBS and bound antibodies were visualized by incubation with nitro blue tetrazolium/bromochloroindolyl phosphate (Kirkegaard & Perry Laboratories) at

room temperature. The reaction was stopped by vigorous washing with PBS.

Chemotaxis assay
Cell migration was evaluated in 48 well Boyden microchambers (Neuroprobe, Cabin John, MD) as previously reported for macrophages (Luo *et al*, 1994). Cells were washed and resuspended in endotoxin-depleted RPMI 1640 with 1% BSA (hence called chemotaxis medium) to a concentration of 3×10^6 cell/ml. Fifty microliters of cells were added to the upper well of the Boyden chamber, which was separated from the chemokine by a polycarbonate filter with 5 μm pores (Poretics, Livermore, CA) for monocytes or 3 μm pores for neutrophils. All responses were assayed in triplicate. The chamber was incubated for 1.25 h at 37°C in a moist 5% CO_2 atmosphere. After incubation, the upper surface of the filter was scraped to remove non-migrating cells. Filters were subsequently fixed in methanol and stained with Diff-Quik (Baxter, McGaw Park, IL). The number of migrating cells per high-powered field was determined microscopically at 400 × magnification.

Pertussis toxin treatment
U-937 cells were washed twice and resuspended in serum-free medium. The cells were then treated with 100 ng/ml of pertussis toxin (Sigma) for 60 min at 37°C. After treatment, the cells were washed twice and suspended in chemotaxis medium. The viability of cells before and after pertussis toxin treatment was greater than 95% as assayed by staining with trypan blue.

Cell stimulation
5×10^6 U-373, SW 1783, CCF, HL-60 cells or human fetal astroyctes were incubated at 10^6 cells/ml with or without 1000 U/ml IFN-γ (Sigma) for 8 h at 37°C in a moist 5% CO_2 atmosphere.

Measurement of intracellular calcium concentration $[Ca^{2+}]_i$
HL-60 cells (1×10^6 or 2×10^6 cells/ml) were incubated with 2.5 mM fura-2 AM (Molecular Probes, Eugene, OR) in HBSS containing 1% BSA and 1.25 mM $CaCl_2$ for 60 min at 37°C. Subsequently, the cells were washed twice and resuspended in a light-shielded tube at room temperature until use. Fluorescence measurements were performed at excitations of 340 nm and 380 nm with a fluorescence emission at 510 nm in a fluorospectrophotometer (Hitachi F-4500, Tokyo, Japan) while stirring the cell suspension at 37°C. The data is presented as the relative ratio (R) of fluorescence at 340 and 380 nm.

RNA isolation
RNA was isolated from cell suspensions following a RNA Isolation Kit protocol (Stratagene, La Jolla, CA). Briefly, $5-10 \times 10^6$ cells were lysed with a guanidinium thiocyanate solution followed by a phenol-chloroform single-step extraction. The RNA was further cleansed by precipitation followed by washing with isopropranol and 75% ethanol, respectively. RNA was finally resuspended in 50 μl of DEPC-treated water.

Probe preparation
H174 and human β-actin cDNA probes were labeled for hybridization following restriction enzyme digestion and agarose gel purification to remove vector sequences. The H174 probe, excised from the vector with *Eco*RI, was 453 bp in length and included the entire coding region. DNA was purified from agarose using JETSORB (Genomed Inc., Research Triangle Park, NC) protocol, and labeled by random oligonucleotide priming using [α^{32}P]dCTP (New England Nuclear, Boston, MA), dTTP, dATP, dGTP, and Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA).

Northern blotting
Total RNA was prepared as described above. Twenty micrograms of total RNA were subjected to electrophoresis in 1.5% agarose-formaldehyde gels and blotted onto Genescreen Plus nylon membranes (New England Nuclear). Membranes were hybridized to radiolabeled probe for 48 h at 42°C in 50% formamide. Blots were washed in SET buffer, pH 8.0 (15 mM NaCl, 0.1 mM Na_3EDTA, 3 mM Tris base) with 0.1% sodium pyrophosphate, 0.1% SDS, and 0.1 M sodium phosphate at room temperature or 55°C and exposed on X-ray film (Kodak, Rochester, NY) with a fluorescent screen at −80°C.

RT–PCR
Before cDNA synthesis, 1.5 μg RNA was treated with 1 U DNase-I (bovine pancreas; Sigma Chemical Co.) for 15 min at room temperature in 10 μl 20 mM Tris-HCl (pH 8.4) containing 2 mM $MgCl_2$ and 50 mM KCl, which was then inactivated by incubation with 2.5 mM EDTA at 65°C for 10 min. Single-stranded cDNA was synthesized from the RNA in a 20 μl reaction containing 50 ng of random hexamers, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 10 mM 1.4-DTT 50 mM KCl, 20 mM Tris-HCl (pH 8.4), and 200 U SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD) for 10 min at 25°C, followed by 50 min at 42°C. The sample was then incubated with 2 U RNase H for 20 min at 37°C. Controls included RNA samples that were not subjected to reverse transcriptase. H174 gene specific primers were 5′-GCCTTGGCTGTGATATTGTGTGC and 3′-TTTTGGTCCTTTCACCCACC. The Mig specific primers were 5′-TCATCTTGCTGGTTCTGATTG and 3′-ACGAGAACGTTGAGATTTTCG. The IP10 specific primers were 5′-GGAACCTCCAGTCTCAGCACC and 3′-

GCGTACGGTTCTAGAGAGAGGTAC. The primers for the housekeeping gene control, human rack1 (Shan *et al*, 1992), were 5′-ATGACTGAGCAGATGACCCTT and 3′-CTAGCGTGTGCCAATGGTCA). PCR was carried out in a reaction mixture containing 2 mM $MgCl_2$, 0.5 μM primers, 10 mM Tris-HCl pH 8.3, 50 mM KCl, and 0.5 U/20 μl Amplitaq DNA Polymerase™ (Perkin Elmer, Modesto, CA). The PCR program for cDNA derived from cell lines and primary astrocyte cultures was as follows: 40 ng cDNA were preincubated at 94°C for 2 min followed by addition of enzyme and amplification with 30 cycles of PCR at 94°C for 45 s annealing and 50 s 72°C extension. The annealing temperature was 55°C. For patient samples the PCR conditions were modified to include addition of 150 ng cDNA and amplification for 38 cycles with a 50°C annealing temperature. Six μl of the PCR mixtures was visualized on a 3% agarose gel. ΦX174 RF DNA/*Hae*III fragments (Life Technologies) were included as molecular weight standards.

Nucleotide sequence
The H174 nucleotide sequence has been deposited in the GenBank. The accession number is AF002985.

Addendum

Subsequent to submission of this manuscript Cole *et al* (1998) reported an IFN-γ or IFN-β inducible chemokine termed I-TAC (GenBank accession #AF030514) which has the same sequence as H174 that was previously deposited in the GenBank (Jacobs *et al*, 1997). Cole *et al* (1998) demonstrated that the synthetic H174/I-TAC peptide binds CXCR3 transfected cells with 0.3 nM affinity. However, Cole *et al* (1998) reported that synthetic H174/I-TAC peptide lacked activity on resting monocytes in both chemotaxis and calcium flux assays. The potential disparity with our data may reflect differences between recombinant and synthetic chemokine and/or differences in the sensitivities of the different target cells used in these experiments.

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