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# The human cytokine I-309 is a monocyte chemoattractant

(T cells/cytokine superfamily/chemotaxis/monocyte/cytoplasmic free calcium)

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**ABSTRACT** **The human cytokine I-309 is a small glycoprotein secreted by activated T lymphocytes and structurally related to a number of inflammatory cytokines. To investigate the biological activities of I-309 protein, we produced a stable Chinese hamster ovary cell transfectant, CDI.10, which constitutively secretes I-309 protein into culture supernatant. Affinity chromatography on a heparin-Sepharose matrix followed by reverse-phase HPLC was used to purify to homogeneity a glycoprotein doublet of 15–16 kDa from culture supernatant. Biochemical analysis showed the purified recombinant I-309 glycoprotein to be indistinguishable from the natural I-309 glycoprotein constitutively secreted by the T-cell line IDP2. Purified recombinant I-309 stimulated migration of human monocytes but not neutrophils when tested by *in vitro* chemotaxis assay. Furthermore, the purified protein transiently increased cytoplasmic free calcium concentration in human peripheral blood monocytes but did not do so in lymphocytes or neutrophils. These results demonstrate that the I-309 gene encodes an inflammatory mediator that specifically stimulates human monocytes.**

Searches for genes expressed specifically in leukocyte subsets and in activated cells have recently converged with studies of proteins involved in chemotaxis and activation of leukocytes to define a superfamily of inflammatory cytokines. Variously called the sis family (1), the sig family (2), the platelet factor 4 superfamily (3), or the intercrine superfamily (4), this array of cytokines includes platelet factor 4, β-thromboglobulin, neutrophil activating peptide 1 (NAP-1)/interleukin 8 (IL-8), gro, IP-10, mig, macrophage inflammatory proteins 1α and 1β, monocyte chemoattractant protein 1 (MCP-1), RANTES, HC-14, C10, and I-309. All of these molecules are small secreted proteins that are structurally related and that display one of two characteristic cysteine motifs. Some of these cytokines are produced by a wide variety of cells, whereas others display a much more restricted pattern of expression. For example, IL-8 is produced by monocytes, T cells, fibroblasts, endothelial cells, keratinocytes, neutrophils, hepatocytes, and chondrocytes; platelet factor 4, on the other hand, is only produced by platelets (4). In general, these cytokines are produced in response to activating stimuli; however, RANTES expression is inhibited after stimulation of T lymphocyte clones (5).

These cytokines can induce a wide variety of cellular responses, but one hallmark of members of this family is the ability to induce chemotactic migration of specific cells such as neutrophils, monocytes, T lymphocytes, basophils, and fibroblasts (4). In neutrophils and monocytes, some cytokines can also induce respiratory burst, changes in adhesiveness, and other generally proinflammatory responses. Additional targets include hematopoietic stem cells, keratinocytes, endothelial cells, and chondrocytes; and these targets display diverse responses that range from changes in proliferation rate to changes in expression of specific cell-surface proteins and inflammatory mediators (4). Thus these cytokines have pleiotropic effects.

The human gene for protein I-309, initially identified by virtue of its expression only in T cells, is a member of the C-C [intercrine β (4) or RANTES/SIS (6)] branch of the cytokine superfamily (7). Previously, we demonstrated that the I-309 gene is expressed specifically by activated T cells upon secondary stimulation and that the product of the I-309 gene is a secreted glycoprotein doublet of 15–16 kDa, but no information was available concerning the function of I-309 protein. We now report the expression and purification of recombinant I-309 protein (rI-309) for use in studies of its biological function. We demonstrate that this protein is chemotactic for human monocytes but not for neutrophils and similarly that the protein stimulates a transient increase in the concentration of cytoplasmic free calcium in monocytes but not in other cell types. Thus, I-309 protein has properties *in vitro* consistent with its putative function as an inflammatory mediator *in vivo*.

## MATERIALS AND METHODS

**Cell Culture and Transfections.** Chinese hamster ovary (CHO) DUKX BII cells (8) were maintained in minimal essential medium $\alpha^-$ (GIBCO/BRL) as described (9). Subconfluent cultures were cotransfected with the I-309 expression plasmid CDI-309 [a derivative of CDM8 (10)] and the dihydrofolate reductase expression plasmid pMT2 (11) by using the *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid/$CaPO_4$ procedure (12). Stable transfectants were amplified with methotrexate (Pfizer, Groton, CT) (9). The I-309-expressing clone CDI.10 was maintained in 80 nM methotrexate in minimal essential medium $\alpha^-$.

Human peripheral blood mononuclear cells and neutrophils were isolated as described (13) and resuspended in Hanks' balanced salt solution/0.5% bovine serum albumin or in RPMI 1640 medium/10% fetal bovine serum (GIBCO/BRL).

**Generation and Purification of rI-309.** CDI.10 cells were grown for 2–3 days in serum-free Opti-MEM (GIBCO/BRL). Culture supernatants were applied to a heparin-Sepharose CL-6B column (Pharmacia LKB). The beads were washed with NET (50 mM Tris·HCl, pH 8.0/150 mM NaCl/5 mM EDTA), and bound proteins were eluted with NET supplemented to 0.65 M NaCl. The eluate was dialyzed against 0.1

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Abbreviations: fMLP, *N*-formylmethionylleucylphenylalanine; IL-8, interleukin 8; rI-309, recombinant I-309 protein; MCP-1, monocyte chemoattractant protein 1; NAP-1, neutrophil activating peptide 1.

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M $NH_4OAc$, pH 5.6, at 4°C in SpectraPor 3 membranes (Spectrum Medical Industries) and was loaded onto a 10 × 250-mm RP-318 reverse-phase column (Bio-Rad); an Applied Biosystems HPLC system was used. Bound proteins were eluted with a gradient of acetonitrile in 0.05% trifluoroacetic acid (Fig. 1). Fractions containing rI-309 were pooled and dialyzed against phosphate-buffered saline with a system 500 microdialyzer (Pierce) and SpectraPor 3 dialysis membranes. Concentration of rI-309 was determined by amino acid analysis, and endotoxin content was assayed with a quantitative colorimetric limulus amoebocyte assay (QCL-1000, Whittaker Bioproducts). Automated Edman degradation was done by using an Applied Biosystems 477A gas-phase protein sequencer with a 120A on-line phenylthiohydantoin–amino acid analyzer (Harvard Microchemistry Facility, Cambridge, MA).

**Radiolabeling, Immunoprecipitation, and SDS/PAGE.** IDP2 and CDI.10 cells were labeled by using Expre$^{35}$S$^{35}$S (New England Nuclear). Immunoprecipitation was done by using a rabbit antiserum raised against purified I-309 protein. Cell supernatants were precleared with preimmune serum and Pansorbin (Calbiochem), and I-309 proteins were immunoprecipitated with immune serum and protein A-Sepharose CL-4B (Pharmacia/LKB). Beads were washed five times with ice-cold NET supplemented to 0.65 M NaCl. Immunoprecipitated proteins were eluted into SDS/PAGE sample buffer or into digestion buffer for N-glycanase as specified by the manufacturer (Genzyme) and were recovered by precipitation with trichloroacetic acid. Proteins were electrophoresed through reducing 15% Laemmli gels (14) or 16.5% Tris/tricine gels (15) and were visualized by fluorography (16) or by silver staining (17). Either $^{14}$C-labeled (GIBCO/BRL) or unlabeled (Bio-Rad) protein standards were used as molecular mass markers.

**Density Gradient Centrifugation.** Culture supernatants (0.1 ml) were layered on 4.8-ml linear gradients of 2%–20% (vol/vol) glycerol in NET. Gradients were centrifuged at 40,000 rpm for 24 hr by using an SW50.1 rotor (Beckman) at 0°C. Fractions (220 μl) were collected with the aid of a peristaltic pump.

**Chemotaxis Assays.** Chemotaxis was assayed by using a 48-well microchemotaxis chamber with polyvinylpyrrolidone-free polycarbonate Nuclepore membranes (Neuro Probe, Cabin John, MD) essentially as described (18). Cells and peptides were resuspended in Hanks' balanced salt solution/0.5% bovine serum albumin, and assays were done at 37°C. Monocyte chemotaxis was assayed by using cells at 2.5 × $10^6$ cells per ml and filters with 5-μm pores for 90 min. Neutrophils were assayed at $10^6$ cells per ml by using 3-μm pores for 45 min. *N*-Formylmethionylleucylphenylalanine (fMLP; Sigma) at $10^{-8}$ M was used as a positive control, and all points were assayed in duplicate. After the nonmigrating cells were washed from the upper surface of filters, migrating cells were fixed in methanol and stained with Diff-Quick (Baxter Scientific Products, McGaw Park, IL), and the number of cells in five randomly selected high-power (×1000) fields was counted for each well.

**Measurement of Cytoplasmic Free Calcium Concentration.** Cells ($10^7$ cells per ml) in RPMI 1640 medium/10% fetal bovine serum were incubated with indo-1 acetoxymethyl (AM) ester at 10 μg/ml (Molecular Probes) (19) for 1 hr at 37°C. Loaded cells were washed, resuspended at 2 × $10^6$ cells per ml, and 0.5-ml aliquots were analyzed for fluorescence emission at 405 nm and 485 nm by using an EPICS 723 flow cytometer (Coulter).

## RESULTS

**Purification of rI-309 from Transfected CHO Cell Line.** Previous studies identified a human T cell line, IDP2, that constitutively transcribes the I-309 gene. However, these cells are IL-2 dependent and secrete relatively small amounts of I-309 protein (7, 20). Therefore, we generated a CHO cell transfectant, designated CDI.10, which stably expresses the transfected I-309 cDNA. CDI.10 cells were found to constitutively secrete proteins that could be immunoprecipitated with an anti-peptide serum specific for the I-309 protein (7) (data not shown). To purify the secreted I-309 protein, cells were cultured for up to 3 days in serum-free medium. Serum-free culture supernatant was passed over a heparin-Sepharose column, and bound proteins were eluted with high salt buffer. Proteins in the eluate were resolved by reverse-phase HPLC. rI-309 eluted from the reverse-phase column as a single peak which, when analyzed by SDS/PAGE, contained a doublet of ≈15–16 kDa (Fig. 1 *A* and *B*). To verify that the purified proteins were products of the I-309 gene,

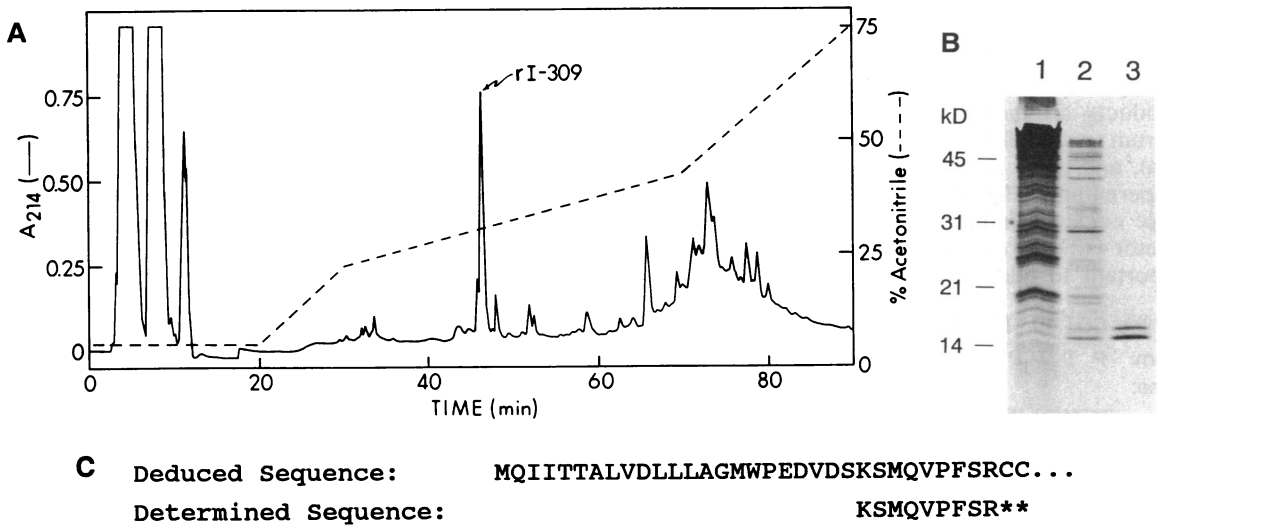


FIG. 1. Purification of rI-309. (*A*) Reverse-phase HPLC chromatogram. Heparin-Sepharose eluates were loaded on a Bio-Rad RP-318 column and eluted by using a gradient of acetonitrile in 0.05% trifluoroacetic acid as shown; the rI-309 peak is indicated. (*B*) SDS/PAGE of rI-309 during purification. Proteins were electrophoresed through a 15% Laemmli gel and were detected by silver staining. Lanes: 1, 0.1% of proteins in starting CDI.10 serum-free culture supernatant; 2, 0.25% of proteins in dialyzed eluate of heparin-Sepharose column; 3, 0.8% of peak from reverse-phase HPLC. kD, kDa. (*C*) Comparison of N-terminal sequence of purified rI-309 with sequence deduced from the I-309 cDNA (7). *, No amino acid detected.

≈200 pmol of material was subjected to 11 cycles of automated Edman degradation. The determined amino acid sequence was identical to that deduced from the nucleotide sequence of the I-309 cDNA (Fig. 1*C*). This result confirms the identity of the purified protein and establishes the amino terminus of the mature I-309 protein. Furthermore, because the sequence displayed <1% contamination, we conclude that the material is highly pure and that both bands of the doublet are likely to have the same amino terminus. The yield of purified protein was typically 50–75 ng per ml of starting culture supernatant. The concentration of I-309 in our working stock solution was $1.8 \times 10^{-6}$ M, and endotoxin contamination was determined to be 0.28 endotoxin units per ml (≈3 pg/μg of protein).

**rI-309 Is Biochemically Indistinguishable from Natural I-309 Protein.** Purified rI-309 was used to raise a rabbit antiserum that specifically immunoprecipitated metabolically labeled I-309 protein from culture supernatants of CDI.10 cells and of the γδ T cell line IDP2 (20) (Fig. 2). The doublet of 15–16 kDa immunoprecipitated from CDI.10 supernatants precisely comigrates in SDS/PAGE with the doublet immunoprecipitated from IDP2 culture supernatants. In the Tris/tricine gel system shown, the doublet migrates slightly faster than in Laemmli gels and appears to be ≈11–12 kDa. Given the N terminus established above, the I-309 cDNA sequence predicts a protein backbone of 8 kDa with a single potential site for asparagine-linked glycosylation. Enzymatic deglycosylation of the natural protein and rI-309 with N-glycanase yielded apparently identical core peptides of ≈6.5 kDa (or 8 kDa on 15% Laemmli gels; data not shown). Further, although in each case the glycosylated material migrated as a doublet on SDS/PAGE, the deglycosylated material migrated as a single species. This result argues that the difference in mobility between the two glycosylated species results from the presence of distinct asparagine-linked glycans. This glycosylation heterogeneity is not simply the result of differential sialylation because a doublet is still detected after digestion with neuraminidase (data not shown). Taken together, our results indicate that natural protein and rI-309 have indistinguishable protein backbones and carbohydrate moieties.

CDI.10 supernatants also contained I-309-related peptides distinct from the glycosylated species described above. One, of ≈8 kDa, appears to be unglycosylated I-309 protein; this band can also be seen in IDP2 supernatants after longer gel exposure (Fig. 2 and data not shown). The others migrate as two larger doublets and probably represent products of aberrantly spliced, translated, or processed I-309 mRNA. These products are not immunoprecipitated by an anti-peptide serum specific for a C-terminal I-309 peptide (7) (data not shown), and these products are not detectable in IDP2 culture supernatants (Fig. 2).

Available evidence indicates that both IL-8 (21, 22) and platelet factor 4 (23, 24) exist primarily as multimers. Thus we felt it important to address whether natural I-309 protein behaves as a monomer or a multimer in solution and whether rI-309 displays the same quaternary structure. Proteins in supernatants of metabolically labeled IDP2 and CDI.10 cells were fractionated by using glycerol-gradient sedimentation, and fractions were analyzed by immunoprecipitation and SDS/PAGE (Fig. 3). The I-309 doublet produced by CDI.10 cells precisely cosedimented with the doublet produced by IDP2 cells, indicating that the quaternary structure of the natural and recombinant forms of the protein are the same. Furthermore, sedimentation of I-309 protein was essentially identical to that of bovine pancreatic trypsin inhibitor (molecular mass, 6.2 kDa). Assuming that I-309 protein is globular, these data argue that I-309 protein probably exists primarily as a monomer in solution. The sedimentation behavior of purified rI-309 was indistinguishable from that of the material in crude IDP2 or CDI.10 cell supernatants (data not shown).

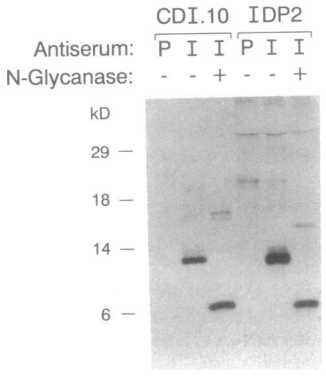


FIG. 2. SDS/PAGE of natural protein and rI-309. $^{35}$S-labeled proteins immunoprecipitated with either preimmune (P) or anti-I-309 immune (I) rabbit serum were digested with N-glycanase or were mock incubated. Products were resolved on a 16.5% Tris/tricine gel and detected by fluorography.

**Purified rI-309 Is Chemotactic for Human Monocytes but Not for Human Neutrophils.** Because cytokines related to I-309 can function as chemoattractants, we used an *in vitro* chemotaxis assay to ask whether I-309 protein would stimulate the migration of human peripheral blood leukocytes. Results of a typical experiment are presented in Fig. 4. Purified rI-309 stimulated the migration of human mononuclear cells in a dose-dependent manner; maximal migration occurred at a concentration of $1–3 \times 10^{-7}$ M, and half-maximal migration occurred at $1–3 \times 10^{-8}$ M. Microscopic analysis revealed that the migrating cells were predominantly monocytes (data not shown). By contrast, rI-309 failed to stimulate migration of neutrophils at any concentration tested. fMLP, on the other hand, was chemotactic for both neutrophils and monocytes. Optimal migration of monocytes in response to rI-309 was less than the migration in response to fMLP (Fig. 4); about 35% of input monocytes migrated in response to $10^{-7}$ M rI-309, whereas ≈50% migrated in response to $10^{-8}$ M fMLP.

To distinguish between chemotaxis and chemokinesis, a monocyte migration assay was done in which rI-309 was added to equal concentrations on both sides of the polycarbonate membrane. Under these conditions migration was substantially reduced (Table 1). Thus, I-309-stimulated migration requires a concentration gradient and reflects chemotaxis rather than chemokinesis.

**Monocytes Respond to rI-309 with Increase in Cytoplasmic Free Calcium.** Most chemoattractants stimulate a transient rise in the cytoplasmic free calcium concentration in responsive cells (25). We, therefore, examined the effects of rI-309 and fMLP on cytoplasmic free calcium levels in indo-1-loaded mononuclear cells and neutrophils by using flow cytometry. For analysis of the mononuclear cells, forward and side light-scattering properties were used to include only the monocyte or lymphocyte population. Fig. 5 shows that rI-309 at $2 \times 10^{-7}$ M elicited a transient increase in cytoplas-

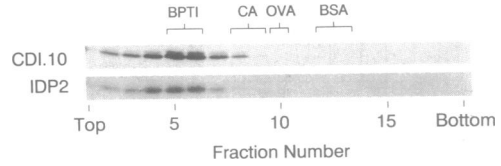


FIG. 3. Glycerol density-gradient sedimentation of natural protein and rI-309. Culture supernatants from $^{35}$S-labeled CDI.10 or IDP2 cells were fractionated on glycerol gradients, and I-309 proteins in gradient fractions were immunoprecipitated, resolved on 15% Laemmli gels, and detected by fluorography. Marker proteins [bovine pancreatic trypsin inhibitor (BPTI, 6.2 kDa), carbonic anhydrase (CA, 29 kDa), ovalbumin (OVA, 45 kDa), and bovine serum albumin (BSA, 67 kDa)] were centrifuged through a parallel gradient and detected by silver staining of 15% Laemmli gels; peak fraction(s) are indicated.

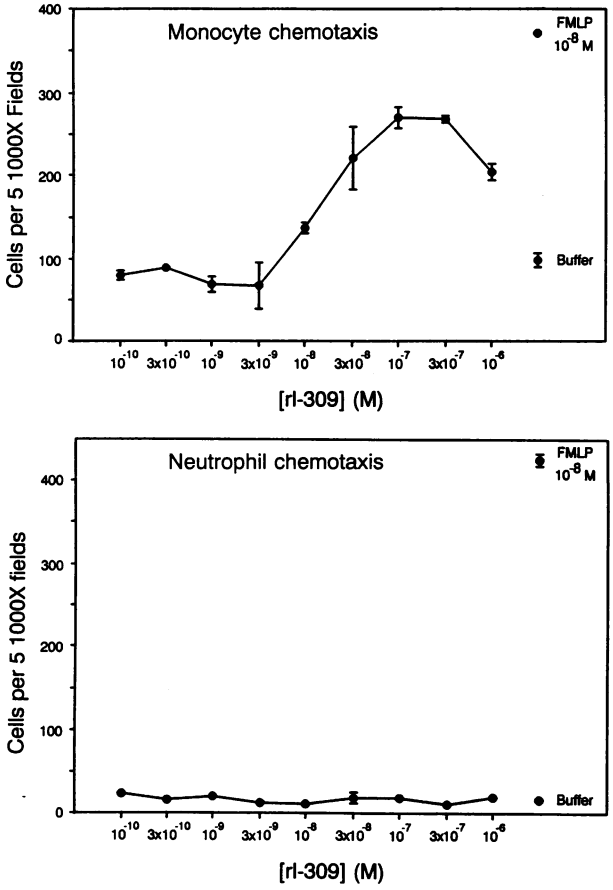


FIG. 4. Purified rI-309 stimulates *in vitro* migration of monocytes but not *in vitro* migration of neutrophils. Human peripheral blood mononuclear cells and neutrophils were assayed for chemotaxis to rI-309 and fMLP as described. For each well, number of migrating cells in five ×1000 fields was determined. Results represent the average of duplicate determinations.

mic free calcium in monocytes. By contrast, neutrophils failed to respond to rI-309 at $2 \times 10^{-7}$ M, although they did respond to fMLP. Similarly, lymphocytes showed no response to rI-309 in this assay. These results reflect the cell specificity seen in chemotaxis assays and argue that among leukocyte subsets, rI-309 acts specifically on monocytes.

## DISCUSSION

We have used CHO cells stably expressing a full-length I-309 cDNA as a source of secreted I-309 protein and have purified

Table 1. rI-309 stimulates chemotaxis but does not stimulate chemokinesis

| | Migration index* | |
|---|---|---|
| [rI-309] (M) | rI-309 in lower wells | rI-309 in upper and lower wells |
| $3 \times 10^{-7}$ | 4.4 | 1.3 |
| $10^{-7}$ | 3.7 | 1.5 |
| $3 \times 10^{-8}$ | 3.1 | 1.5 |
| $10^{-8}$ | 1.8 | 1.8 |
| $3 \times 10^{-9}$ | 1.6 | 1.0 |

*Migration index is defined as number of cells counted in five ×1000 fields for test solution divided by number of cells counted in five ×1000 fields for negative control. In this experiment, background migration was 56 ± 2 cells per five ×1000 fields (migration index = 1.0), and migration in response to $10^{-8}$ M fMLP was 336 ± 34 cells per five ×1000 fields (migration index = 6.0). Results represent the average of duplicate determinations.

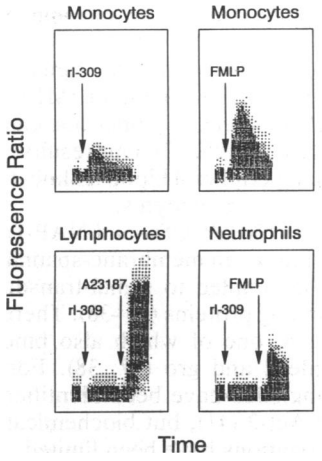


FIG. 5. Purified rI-309 stimulates a transient increase in cytoplasmic free calcium in monocytes but does not do so in neutrophils or lymphocytes. indo-1-loaded cells were monitored for changes in cytoplasmic free calcium by flow cytometry. After a baseline was established, test solutions were added to cells, and the fluorescence ratio was monitored for at least 2 min. rI-309 was tested at $2 \times 10^{-7}$ M, fMLP was tested at $10^{-6}$ M, and calcium ionophore A23187 was tested at 1 μg/ml.

the rI-309 to homogeneity. To the best of our knowledge, rI-309 is identical to the natural I-309 protein constitutively secreted by a human T cell line. Functional analysis indicated that rI-309 stimulates chemotaxis and a transient increase in cytoplasmic free calcium levels in human monocytes but does not do so in neutrophils or lymphocytes. Thus rI-309 functions *in vitro* as a monocyte-specific chemoattractant.

Amino acid sequence analysis of purified I-309 indicated that the signal peptide is cleaved between Ser-23 and Lys-24 to generate a mature I-309 protein backbone of 73 amino acids. This serine residue is conserved in all human and murine members of the C-C family except MCP-1/JE and is known to be the site of signal peptide cleavage in murine macrophage inflammatory proteins 1α and -β (7, 26, 27). Signal peptide cleavage for MCP-1 apparently occurs at the homologous site (28). In contrast to NAP-1/IL-8, there is, as yet, no evidence for N-terminal sequence variation among C-C family members (29, 30).

Glycerol density-gradient sedimentation argues that I-309 protein exists as a monomer in solution. Two members of the C-X-C family, NAP-1/IL-8 and platelet factor 4, have been determined both by NMR spectroscopy and x-ray crystallography to form predominantly dimers and tetramers, respectively (21–24). No quaternary structures of C-C family members have been published, but MCP-1 has been predicted to form dimers (31). If the quaternary structure of I-309 is atypical, it may be from the location of the attached carbohydrate moiety. By analogy with MCP-1 (31), the N-linked sugar on I-309 would lie in the β strand that forms the dimer interface, and its presence may discourage dimerization. An alternative possibility is that other structural differences, most notably the presence of a pair of cysteine residues in I-309 not present in most other family members (7), may alter the tertiary structure so that dimerization is unfavorable.

Most members of the C-C cytokine family, including I-309, were initially identified due to their selective expression in activated cells, and studies of their biological functions have only recently been initiated. Among the human members of the C-C family, functions have been ascribed to MCP-1/JE and RANTES, both of which have monocyte chemoattractant activity in addition to other properties (6). Our results extend this analysis by demonstrating that I-309 encodes a cytokine that is chemotactic for monocytes but not for neutrophils. In agreement with this finding, rI-309 stimulates

an increase in cytoplasmic free calcium concentration in monocytes but not in neutrophils or lymphocytes. This observation is consistent with the signaling properties of other chemotactic agonists, including fMLP, C5a, and NAP-1/IL-8, which also increase cytoplasmic calcium concentration in responsive cells (25, 32). Our results, therefore, argue that among human peripheral blood leukocytes, the effects of I-309 are restricted to monocytes.

The receptors for fMLP, C5a, and NAP-1/IL-8 are related proteins containing seven membrane-spanning segments and are thought to be coupled to signal-transduction pathways through GTP-binding proteins (33–38). There are at least two receptors for IL-8, one of which also binds to neutrophil activating peptide 2 and gro (37, 38). For C-C subfamily members, binding sites have been identified for MCP-1/JE (39, 40) and for Act-2 (41), but biochemical and pharmacological characterizations have been limited. It will be important in future studies to assess whether I-309 interacts with receptors that are identical to or distinct from those of other C-C family cytokines and to define the I-309 receptor in molecular terms. Our results suggest that the expression of the I-309 receptor may be restricted to monocytes. Alternatively, the receptor may be more broadly distributed but may be differentially coupled to second-messenger pathways in different cell types. Evidence for IL-8 receptors on apparently IL-8-unresponsive cells has already been presented (42).

The murine homolog of I-309, TCA-3, has also been expressed in CHO cells (43). Like I-309, the expressed TCA-3 cDNA directs the synthesis of a 16-kDa secreted protein carrying a single N-linked glycan, and deglycosylation of this material yields a protein core of ≈8 kDa. When injected into mouse footpads, partially purified recombinant TCA-3 generates an inflammatory response which, after 2 hr, consists primarily of a neutrophilic infiltrate. Thus, there is an apparent discrepancy between the observed biological activities of these homologous proteins. Because recombinant TCA-3 has only been tested *in vivo*, this discrepancy could arise from differences in experimental protocols and potential indirect effects *in vivo*. Our results provide direct *in vitro* evidence that I-309 protein has no chemotactic activity for neutrophils. Additional experiments designed to test recombinant TCA-3 functions *in vitro* and rI-309 functions *in vivo* will be required to clarify these apparent differences.

Known monocyte chemoattractants display widely differing potencies. For example, the optimal concentrations of I-309, MCP-1/JE, and transforming growth factor $\beta$ for monocyte chemotaxis are ≈100 nM, 1 nM, and 10 fM, respectively (44, 45). All of these mediators could be produced at the same site. Because both suboptimal and supraoptimal concentrations of individual chemoattractants are ineffective for stimulating migration, combinations of chemotactic cytokines that function cooperatively may be required to recruit monocytes to inflammatory sites. This model should be amenable to testing *in vitro*.

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