Brief Definitive Report

Recombinant Human Interferon-inducible Protein 10 Is a Chemoattractant for Human Monocytes and T Lymphocytes and Promotes T Cell Adhesion to Endothelial Cells

By Dennis D. Taub,* Andrew R. Lloyd,* Kevin Conlon,‡ Ji Ming Wang,* John R. Ortaldo,‡ Akihisha Harada,§ Kouji Matsushima,§ David J. Kelvin, and Joost J. Oppenheim*

*From the Laboratories of *Molecular Immunoregulation and ‡Experimental Immunology, Biological Response Modifier Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702; and the §Department of Pharmacology, Cancer Research Institute, Kanazawa University, Ishikawa 920, Japan*

Summary

The human cytokine interferon-inducible protein 10 (IP-10) is a small glycoprotein secreted by activated T cells, monocytes, endothelial cells, and keratinocytes, and is structurally related to a family of chemotactic cytokines called chemokines. Although this protein is present in sites of delayed-type hypersensitivity reactions and lepromatous leprosy lesions, the biological activity of IP-10 remains unknown. We report here that recombinant human IP-10 stimulated significant in vitro chemotaxis of human peripheral blood monocytes but not neutrophils. Recombinant human IP-10 also stimulated chemotaxis of stimulated, but not unstimulated, human peripheral blood T lymphocytes. Phenotypic analysis of the stimulated T cell population responsive to IP-10 demonstrated that stimulated $CD4^+$ and $CD29^+$ T cells migrated in response to IP-10. This resembles the biological activity of the previously described T cell chemoattractant RANTES. Using an endothelial cell adhesion assay, we demonstrated that stimulated T cells pretreated with optimal doses of IP-10 exhibited a greatly enhanced ability to bind to an interleukin 1–treated endothelial cell monolayer. These results demonstrate that the IP-10 gene encodes for an inflammatory mediator that specifically stimulates the directional migration of T cells and monocytes as well as potentiates T cell adhesion to endothelium.

The accumulation of mononuclear cells at sites of antigenic challenge appears to be mediated by the local production and secretion of chemotactic cytokines. Several recently cloned cytokines constitute a family of chemotactic cytokines (chemokines) related by a conserved motif containing four cysteine residues (1). IL-8, β-thromboglobulin (β-TG), platelet factor 4 (PF-4), GRO, ENA-78, and IFN-inducible protein 10 (IP-10) are members of the α (or c-x-c) chemokine family, while macrophage inflammatory protein 1 (MIP-1), RANTES, macrophage chemotactic and activating factor (MCAF), and I-309 are included in the β (or c-c) chemokine part of the family. All but one of these cytokines have been found to induce the directional migration of various cell types, including neutrophils, monocytes, T lymphocytes, basophils, or fibroblasts, hence the recently proposed nomenclature "chemokines" for this cytokine family. Many of the α chemokine subfamily members, namely IL-8, GRO/melanoma growth-stimulating activity (MGSA), neutrophil-attracting peptide 2 (NAP-2), a cleavage product of β-TG, and ENA-78, have been shown to chemoattract and activate neutrophils (1, 2). In contrast, many of the β chemokine subfamily members, such as MCAF/macrophage chemotactic protein 1 (MCP-1), MIP-1α and MIP-1β, RANTES, and I-309, chemoattract and activate monocytes while RANTES and IL-8 also chemoattract T lymphocytes (3–5). In addition, we have recently demonstrated that MIP-1α and MIP-1β are potent T cell chemoattractants (6).

The human gene for IP-10 was initially identified by its expression in IFN-treated monocytes (7). Although it has been several years since the molecular cloning of the IP-10 gene (8, 9), no biological function has yet been reported. The expression of IP-10 in lepromatous leprosy lesions (10) and in delayed-type hypersensitivity responses (11) suggests that this chemokine may play an important role in the generation of an immune response. To gain insight into the physiological importance of this chemokine, we have tested purified

recombinant human (rh)IP-10 for T cell, monocyte, and neutrophil chemotactic activity. The results demonstrate that rhIP-10 is chemotactic for both monocytes and stimulated T cells, but unlike many of the other α chemokine subfamily members, has only weak chemotactic activity for neutrophils. In addition, stimulated, but not unstimulated, $CD4^+$ and $CD29^+$ T cells are chemoattracted by rhIP-10. Purified T cells treated with rhIP-10 also have an increased capacity to adhere to IL-1-treated human umbilical vein endothelial cells (HUVEC). Our results suggest that rhIP-10 may play an important role in regulating migration of T lymphocytes and monocytes towards sites of antigenic challenge.

Materials and Methods

Cells. PBMC were obtained from normal donors by leukapheresis. The donors had given informed consent. T cells and T cell subsets were purified using previously described techniques (12). Briefly, PBMC were passaged over Ficoll-Hypaque to remove erythrocytes, granulocytes, and cellular debris. Small lymphocytes were obtained by sequential depletion of adherent cells in plastic flasks, B lymphocytes on nylon wool columns, and large granular lymphocytes by Percoll gradient centrifugation. This isolation procedure typically yielded >90% $CD3^+CD16^-$ lymphocytes. For T cell subset isolation, lymphocyte preparations were washed twice in cold PBS and resuspended at 2–3 $\times$ 10^7 cells/ml. Saturating concentrations of anti-CD4, anti-CD8, anti-CD45RA, or anti-CD45RO were added to the T cell suspension for overnight incubation at 4°C. Purified $CD4^+$, $CD8^+$, $CD45RA^+$, and $CD29^+$ lymphocytes were obtained through negative selection using goat anti-mouse-specific Fc beads (Advanced Magnetics, Cambridge, MA) to selectively deplete the unfractionated T cell populations of particular T cell subsets as described previously (12). This procedure routinely yielded a >94% purified T cell subpopulation. For antibody activation of T cells or T cell subsets, the T cell preparations were adjusted to a concentration of 5 $\times$ 10^6 cells/ml and cultured on tissue culture plates previously coated with a 10-μg/ml suspension of anti-CD3 mAb. The T cells were cultured for 6–8 h, harvested, and used in T cell chemotaxis assays.

Chemokines. Purified rhIP-10 was expressed in *Escherichia coli* and purified by K. Matsushima (Kanazawa University). rhRANTES was also expressed in *E. coli* and was purchased from Pepro Tech Inc. (Rocky Hill, NJ).

Chemotaxis Assay. Cell migration was evaluated by using a 48-well microchemotaxis chamber technique (13) with polycarbonate filters (5-μm pore size; Nucleopore Corp., Pleasanton, CA). In the monocyte and neutrophil chemotactic assays, cells and chemokines were resuspended in RPMI 1640 containing 1% BSA at 10^6 cells/ml, and 50 μl/well was applied to the upper chamber. *N*-FMLP (Sigma Chemical Co., St. Louis, MO) at 10^{-7} M was used as a positive control, and all responses and filters were assayed in triplicate. Migration was allowed to continue at 37°C for 30–60 min. After the nonmigrating cells were washed from the upper surface of the filters, migrating cells were fixed in methanol and stained with Diff-Quik, and the numbers of migrating cells in five high-powered fields were counted for each well. The results represent the average number of migrating cells per high-powered field ($\pm$ SD).

T lymphocyte migration was quantitated by a modification of the Boyden chamber technique. Briefly, T cells were suspended at 10^7 cells/ml in RPMI 1640 plus 0.5% FCS, and were placed in the top wells of a 48-well microchemotaxis chamber. The upper and lower wells were separated by a 5-μm pore size polycarbonate filter that separated the cells from the control and experimental samples in the bottom wells. All polycarbonate filters used in these experiments were coated with collagen type IV 1–2 h before use in the assay (6, 14). The chambers were incubated for 4 h at 37°C in a 5% CO_2 moist atmosphere, and the filters were prepared as already outlined. It should be noted that large clumps of T lymphocytes were observed migrating through the filter making enumeration of migrating T cells difficult. The data are expressed as the number of countable cells per high-power field; however, this may represent a considerable underestimate of the total number of cells that migrated into or through the filter. The statistical significance of cell migration in response to various concentrations of chemokines was determined using a repeated measure one-way analysis of variance (ANOVA). Results of these calculations are listed in the legends of each figure.

Adhesion Assay. HUVEC were prepared by collagenase treatment of umbilical cords as previously described (15). Endothelial cells at passage three or earlier were plated at 1.5 $\times$ 10^5 cells/well onto gelatin-coated 24-well plates (Costar, Cambridge, MA) and cultured to confluence over 48 h. Before the adhesion assays HUVEC were washed once with RPMI 1640/5% FCS and then incubated in this medium for 6 h at 37°C with or without the addition of 10 ng/ml of rhIL-1α.

The measurement of T cell adhesion was based upon the binding of ^{51}Cr-labeled T cells as previously described (16). Briefly, after preincubation, HUVEC were washed twice with medium (RPMI 1640/5% FCS) and 500,000 ^{51}Cr-labeled T cells were added to each well in a final volume of 500 μl. The plates were incubated at 37°C for 60 min and then washed three times with prewarmed medium using a standardized wash procedure. The contents of each well were lysed with 300 μl of 1% Triton X-100 and γ emissions counted. Wells were inspected before lysis to ensure that the integrity of the HUVEC monolayer had been maintained. Total counts added and spontaneously released were determined for each variable in the assays.

Percentage adhesion was calculated according to the following formula: percentage adhesion = 100$\times$ [measured cpm/(total cpm − spontaneously released cpm)]. The statistical significance of these results was determined using an unpaired student's *t* test.

Results

Purified rhIP-10 Is Chemotactic for Stimulated but Not Unstimulated Human T Lymphocytes. The results (Fig. 1) demonstrate that stimulated T lymphocytes consistently responded to concentrations of IP-10 ranging from 1 to 10 ng/ml. Higher concentrations ($\geq$25 ng/ml) demonstrated little or no reproducible activity on T cells, thus yielding the typical "bell-shaped" chemotaxis dose-response curve. RANTES induced significantly higher T cell migration at similar concentrations. Unstimulated T cells only exhibited migratory activity to RANTES but not to IP-10.

The Effect of Human IP-10 on the Chemotactic Response of Subsets of Stimulated $CD4^+$, $CD8^+$, $CD29^+$, and $CD45RA^+$ T Lymphocytes. Since RANTES has been reported to be a selective chemoattractant for memory $CD4^+$ T cells (5), the effects of rhIP-10 on subsets of T lymphocytes were investigated. The phenotypic characteristics of chemotactic T lymphocytes were determined through the use of negative selection to enrich for T cell subpopulations. The results (Fig.

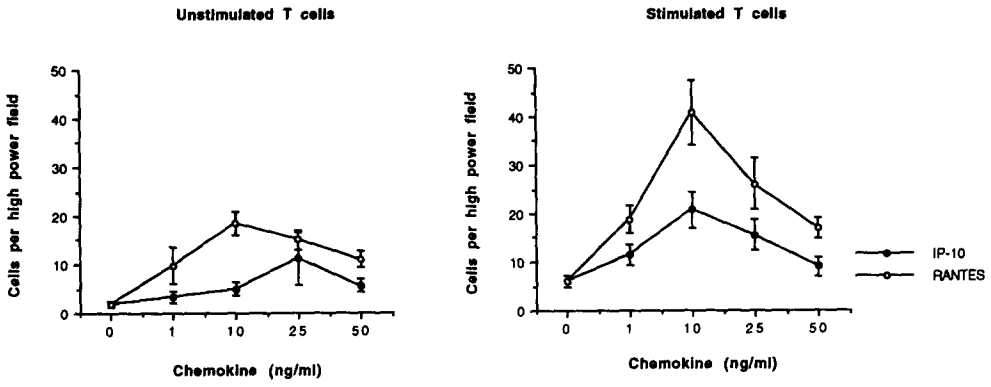

Figure 1. Human peripheral blood T lymphocyte migration in response to RANTES and IP-10. Human lymphocytes were isolated from venous blood and either placed in culture (*unstimulated*) or stimulated on anti-CD3-coated plates (*stimulated*) for 6–8 h. Results determined as described in Materials and Methods are expressed as the mean number of migrating cells per high-power field (±SE) of three replicate experiments. The dose-response effects of chemokines on T cells were determined to be significant for unstimulated T cells/RANTES ($p < 0.04$ [$F = 7.3$]), stimulated T cells/RANTES ($p < 0.0002$ [$F = 9.1$]), and stimulated T cells/IP-10 ($p < 0.0025$ [$F = 5.6$]) using a repeated measure ANOVA.

2) show significant migration of anti-CD3-stimulated but not unstimulated CD4$^+$ T cells to rhIP-10. However, RANTES induced significantly more migration of CD4$^+$ T cells regardless of the activation state.

Further fractionation of T lymphocytes into naive (CD45RA$^+$) and memory (CD29$^+$) T cell subsets revealed that both stimulated CD45RA$^+$ and CD29$^+$ cells migrated to RANTES, while only stimulated CD29$^+$ T cells migrated significantly in response to IP-10 (Fig. 3). Unstimulated naive and memory T cell populations failed to respond to IP-10, but both subsets were chemoattracted by RANTES. RANTES was previously reported to show a preferential migration of memory CD4$^+$ CD45RO$^+$, but not naive CD4$^+$ CD45RA$^+$ or CD8$^+$ T lymphocytes (2, 5). However, in our hands, RANTES chemoattracted stimulated naive and memory T cells (6). This discrepancy may be based on the use of collagen-coated chemotaxis filters, which in our experience provide a more sensitive reproducible assay of T lymphocyte chemotaxis than the uncoated nitrocellulose filters used by Schall et al. (5). These results suggest that both the activation status and phenotypic properties of a T cell population determine whether they respond to IP-10 and RANTES.

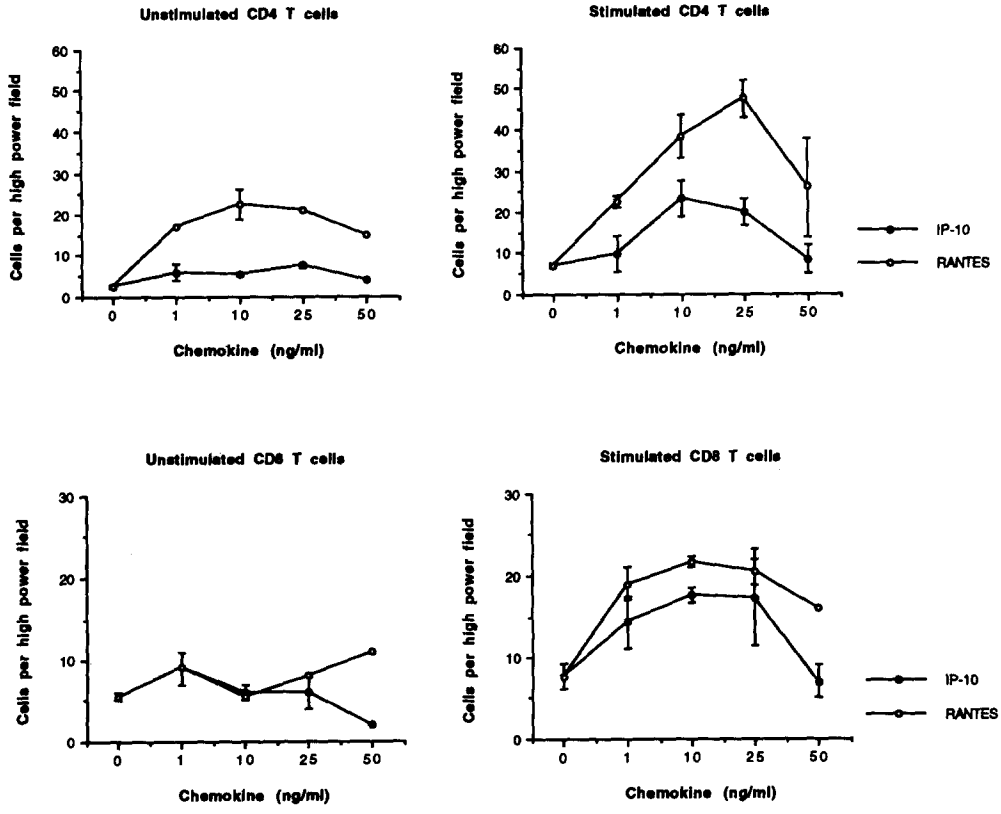

Figure 2. rhIP-10 attracts stimulated CD4$^+$ T lymphocytes. Purified unstimulated T cells were separated by negative selection into purified CD4$^+$ and CD8$^+$ T cell populations (12). Results determined as described in Materials and Methods are expressed as the mean number of migrating cells per high-power field (±SE) of five replicate experiments. The dose-response effects of chemokines on T cell subsets were determined to be significant for unstimulated CD4$^+$ T cells/RANTES ($p < 0.04$ [$F = 4.3$]), stimulated CD4$^+$ T cells/RANTES ($p < 0.004$ [$F = 9.3$]), stimulated CD8$^+$ T cells/RANTES ($p < 0.007$ [$F = 19.8$]), and stimulated CD4$^+$ T cells/IP-10 ($p < 0.04$ [$F = 4.3$] using a repeated measure ANOVA.

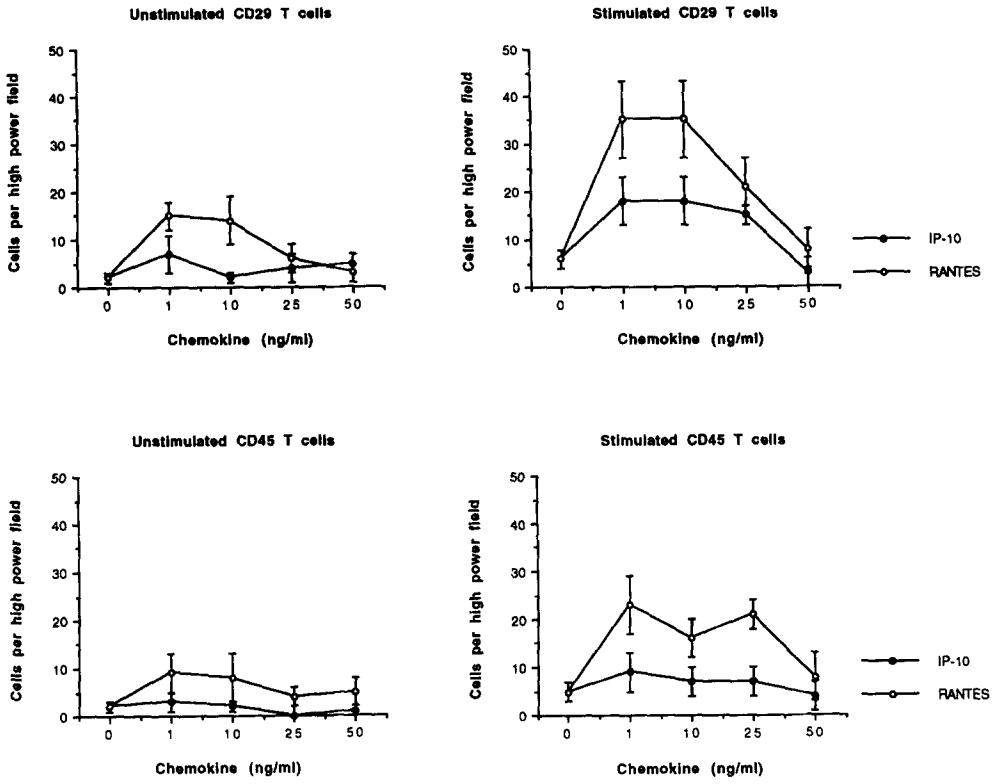

Figure 3. rhIP-10 attracts stimulated CD29$^+$ T lymphocytes. Using similar purification protocols as above, highly enriched CD45RA$^+$ and CD29$^+$ T cell populations were either stimulated on anti-CD3-coated plates or cultured in medium for 6 h. After incubation, the cells were harvested and tested for chemotactic responses to various concentrations of RANTES and IP-10 as described in Materials and Methods. Results are expressed as the mean number of migrating cells per high-power field (±SD) of one of three representative experiments. The dose-response effects of chemokines on T cell subsets were determined to be significant for unstimulated CD29$^+$ T cells/RANTES (p <0.004 [F 26.0]), stimulated CD45RA$^+$ T cells/RANTES (p <0.04 [F = 4.3]), stimulated CD29$^+$ T cells/RANTES (p <0.004 [F = 25.4]), and stimulated CD29$^+$ T cells/IP-10 (p <0.004 [F = 26.0]) using a repeated measure ANOVA.

The Chemotactic Effect of Purified rhIP-10 for Human Neutrophils and Monocytes. We next examined the effects of IP-10 on monocyte and neutrophil migration. The results shown in Fig. 4 demonstrate that both human monocytes and neutrophils migrate in response to lower concentrations of rhIP-10. Monocytes exhibited a modest but significant migration to IP-10 at concentrations of ≥50 ng/ml. Neutrophils also demonstrated significant migratory activity in response to rhIP-10 at concentrations of ≥50 ng/ml, however, the magnitude of this response in comparison with monocytes is extremely weak and did not exhibit a typical bell-shaped curve. Checkerboard analysis of rhIP-10 activity on human monocytes demonstrated that IP-10-induced migration is chemotactic rather than chemokinetic (data not shown).

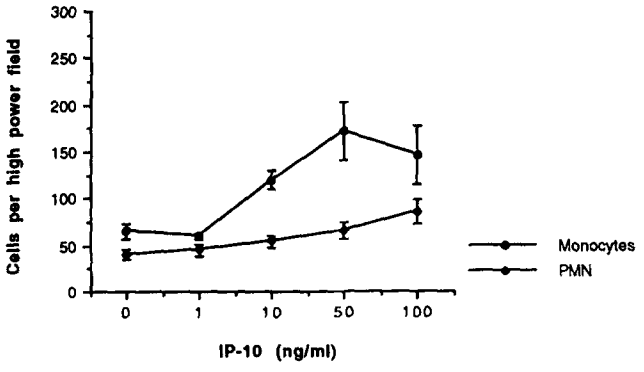

Figure 4. Human peripheral blood monocytes but not neutrophils migrate in response to IP-10. Human monocytes and neutrophils were isolated from venous blood and tested for chemotactic activity using 10^6 cells/ml suspended in RPMI 1640 containing 1% BSA. Results are expressed as the mean number of migrating cells per high-power field (±SE) of three experiments performed. The dose-response effects were significant for both monocytes (p <0.006 [F = 6.63]) and neutrophils (p <0.02 [F = 9.5]) as determined by repeated measure ANOVA.

Effect of rhIP-10 on T Cell Adhesion to IL-1-treated HUVEC. Peripheral blood T cells were pretreated with various concentrations of rhIP-10 and RANTES at 37°C for 6 h on uncoated or anti-CD3 antibody–coated plates. After washing, lymphocytes were incubated with human umbilical vein endothelium that has been pretreated with an optimal concentration of rhIL-1α for the binding assay. The results shown in Fig. 5 demonstrate that rhIP-10 and rhRANTES at a concentration of 10 ng/ml were equipotent in augmenting adhesion of activated but not resting T lymphocytes to IL-1-treated endothelium.

Discussion

Our findings that rhIP-10 is chemotactic for stimulated but not unstimulated T lymphocytes are novel. Our data demonstrate that rhIP-10, like RANTES, is also a monocyte chemoattractant. Both chemokines attract monocytes at similar dose ranges and possess little activity on neutrophils. Although compared with rhRANTES the effect on T cell chemotaxis by IP-10 is modest, the more potent effects of rhIP-10 on T cell adhesion may reflect the fact that endothelial cells are IP-10 producers and suggest that it may play an important

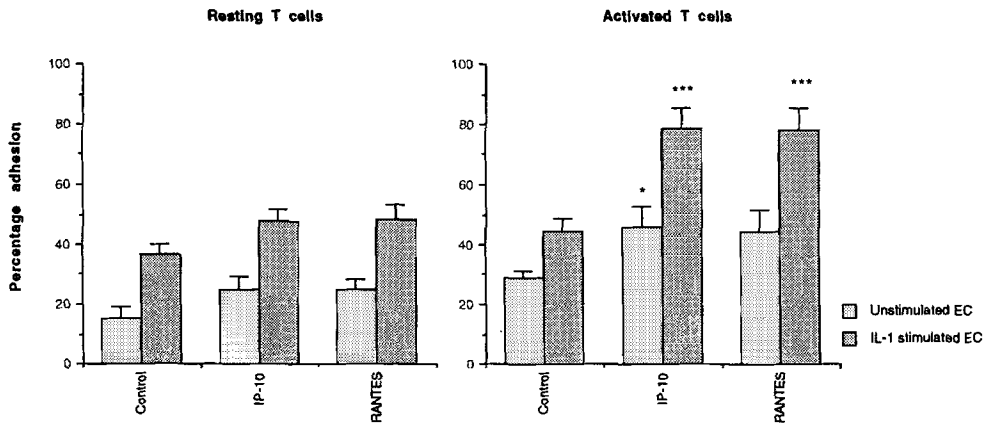

Figure 5. rhIP-10 induces significant adhesion of stimulated T cells to IL-1-treated human umbilical vein endothelium. Lymphocytes were labeled with ^{51}Cr and preincubated on anti-CD3 mAb–coated or uncoated plates with optimal concentrations of rhIP-10 and RANTES for 6 h at 37°C. Results are data (mean ± SD) from a single representative experiment of five performed. Asterisks indicate significant of pooled data points as determined by an unpaired student's *t* test. $***p < 0.005$; $*p < 0.05$.

role in the entry of T lymphocytes into inflammatory sites and sites of delayed-type hypersensitivity. IP-10, like RANTES, also appears to selectively recruit and activate specific T cell subsets. rhIP-10 induced significant migratory activity in stimulated $CD4^+$ and $CD29^+$ T lymphocytes, while stimulated and unstimulated $CD4^+$ as well as stimulated $CD8^+$, $CD45RA^+$, and $CD29^+$ T cells migrated in response to rhRANTES. The activity of IP-10 appears to be dependent on the activation state of a T cell population and, in contrast to RANTES, unstimulated T lymphocytes were not affected by rhIP-10. These results suggest that IP-10 and RANTES are not redundant in their actions but play different roles attracting T lymphocytes to inflammatory sites.

T cell adhesion to endothelial cells is necessary for the development of inflammatory and immunological responses. Immobilization of T cells on endothelial cells is required for recruitment of recirculating T cells from the vascular compartment, followed by transendothelial migration of T cells into tissues (17). Cell–cell contact is necessary for specific immunological recognition by T cells of antigen presented by endothelial cells and other accessory cells in the context of MHC molecules (17–19). Immunological mediators such as IFN-γ, TNF-α, and IL-1 have been shown to promote these functions (17–19). Our findings suggest that the T cell/monocyte chemoattractants, IP-10 as well as MIP-1α, MIP-1β, and RANTES (6), are all able to augment the T cell component of these phenomena, namely adhesion. Purified T cells triggered through the TCR complex with fixed anti-CD3 mAb in the presence of rhIP-10 or rhRANTES resulted in an increased adhesion to IL-1-treated endothelium when compared with control T cell populations. We did not detect alteration in surface expression of adhesion molecules on the T cell after IP-10 pretreatment. No alteration in the level of surface expression of LFA-1, VLA-3, VLA-4, VLA-5, VLA-6, CD44, intercellular adhesion molecule 1, vascular cell adhesion molecule, and endothelial leukocyte adhesion molecule (ELAM) was observed (data not shown). Changes in the conformational states of LFA-1 on T cells, Mac-1 on neutrophils, and ELAM-1 on endothelium have been implicated in regulatory adhesive phenomena (16–19), and raise the possibility that IP-10 may work through this or a similar mechanism. We suggest that IP-10 may have an important role in vivo in promoting endothelial cell–lymphocyte interactions and subsequent transmigration of T cells, monocytes, and possibly neutrophils in various inflammatory states. Recent data supporting this hypothesis were communicated by Dr. Andrew Luster (20), demonstrating that mice injected with tumor cells transfected with the IP-10 gene exhibited a protective antitumor effect that appeared to require the presence of T cells.

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Address correspondence to Dennis Taub, Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, National Cancer Institute, Building 560, Room 31-19, Frederick Cancer Research and Development Center, Frederick, MD 21702.

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References

1. Oppenheim, J.J., C.O.C. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "Intercrine" cytokine family. *Annu. Rev. Immunol.* 9:617.
2. Schall, T.J. 1991. Biology of the rantes/sis cytokine family. *Cytokine.* 3:165.
3. Miller, M.D., and M.S. Krangel. 1992. The human cytokine I-309 is a monocyte chemoattractant. *Proc. Natl. Acad. Sci. USA.* 89:2950.
4. Larsen, C.G., A.O. Anderson, E. Appella, J.J. Oppenheim, and K. Matsushima. 1989. Neutrophil activating factor (NAP-1) is also chemotactic for T lymphocytes. *Science (Wash. DC).* 243:1464.
5. Schall, T.J., K. Bacon, K.I. Toy, and D.V. Goedell. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by the cytokine RANTES. *Nature (Lond.).* 347:669.
6. Taub, D.D., K. Conlon, A.R. Lloyd, J.J. Oppenheim, and D.J. Kelvin. 1993. Preferential migration of activated $CD4^+$ and $CD4^+$ T cells in response to MIP-1α and MIP-1β. *Science (Wash. DC).* 260:368.
7. Luster, A.D., J.C. Unkeless, and J.V. Ravetch. 1985. Gamma-interferon transcriptionally regulates an early response gene containing homology to platelet proteins. *Nature (Lond.).* 315:672.
8. Luster, A.D., and J.V. Ravetch. 1987. Biochemical characterization of a γ-interferon-inducible cytokine (IP-10). *J. Exp. Med.* 166:1084.
9. Luster, A.D., and J.V. Ravetch. 1987. Genomic characterization of a gamma-interferon-inducible gene (IP-10) and identification of an interferon-inducible hypersensitive site. *Mol. Cell. Biol.* 7:3723.
10. Kaplan, G., A.D. Luster, G. Hancock, and Z.A. Cohn. 1987. The expression of a γ interferon-induced protein (IP-10) in delayed-type hypersensitivity responses in skin. *J. Exp. Med.* 166:1098.
11. Enk, A.H., and S.I. Katz. 1992. Early molecular events in the induction phase of contact sensitivity. *Proc. Natl. Acad. Sci. USA.* 89:1398.
12. June, C.H., J.A. Ledbetter, M.M. Gillespie, T. Lindsten, and L.B. Thompson. 1987. T cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* 7:4472.
13. Falk, W.R., R.H. Goodwin, Jr., and E.J. Leonard. 1980. A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods.* 33:239.
14. Pilaro, A.M., T.J. Sayers, K.L. McCormick, C.W. Reynolds, and R.H. Wiltrout. 1990. An improved *in vitro* assay to quantitate chemotaxis of rat peripheral blood large granular lymphocytes (LGL). *J. Immunol. Methods.* 135:213.
15. Jaffe, E.A., R.L. Nachman, C.G. Becker, and C.R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* 52:2745.
16. Shimizu, Y., S. Shaw, N. Graber, T.V. Gopal, K.J. Horgan, G. Van Seventer, and W. Newman. 1991. Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. *Nature (Lond.).* 349:799.
17. Dustin, M.L., and T.J. Springer. 1991. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu. Rev. Immunol.* 9:27.
18. Shimizu, Y., W. Newman, Y. Tanaka, and S. Shaw. 1992. Lymphocyte interactions with endothelial cells. *Immunol. Today.* 13:106.
19. Pardi, R., L. Inverardi, and J.R. Bender. 1992. Regulatory mechanisms in leukocyte adhesion: flexible receptors for sophisticated travelers. *Immunol. Today.* 13:224.
20. Luster, A.D., and P. Leder. 1993. IP-10, a gamma-interferon inducible "intercrine," elicits a potent thymus-dependent antitumor response *in vivo*. *J. Cell. Biochem.* 17B(Suppl.):E547.