Brief Definitive Report

Human Interferon-inducible Protein 10: Expression and Purification of Recombinant Protein Demonstrate Inhibition of Early Human Hematopoietic Progenitors

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Summary

Human interferon-inducible protein 10 (IP-10), a member of the family of the small secreted proteins called intercrine cytokines or chemokines, is secreted by interferon γ–stimulated T cells, monocytes, endothelial cells, and keratinocytes. We have begun to explore the biological properties of IP-10 by cloning and overexpression in baculovirus and in bacterial protein expression systems. A 9.9-kD protein was secreted by infected insect cells, which on sodium dodecyl sulfate–polyacrilamide gel electrophoresis comigrated with keratinocyte IP-10 and with f(22-98), a bacterial recombinant fragment lacking the signal sequence but containing all other residues of IP-10. All three reacted with antibodies recognizing residues 10-98 (αIP-10) and 77-98 of IP-10 (α22), demonstrating that it is secreted by keratinocytes and insect cells after removal of the signal sequence but without proteolysis of the COOH-terminal end. Purified rIP-10 suppresses in vitro colony formation by early human bone marrow progenitor cells which need r-steel factor (rSLF) and rGM-CSF or rSLF and r-erythropoeitin (rEPO). The inhibition is dose dependent, is complete at concentrations ≥50 ng/ml, is prevented by preincubation of rIP-10 with αIP-10, but not by α22, and is seen with highly purified $CD34^+$ cells, suggesting direct effect of rIP-10 on the progenitors. Combination of rIP-10 and other chemokines at inactive concentrations inhibited colony formation in a synergistic manner. rIP-10 did not affect colony formation in the absence of any growth factors or in the presence of rEPO or rGM-CSF but in absence of rSLF. The effects of IP-10 may be relevant to normal marrow function and might be harnessed to protect human hematopoietic progenitors from the cytotoxic effects of chemotherapy.

We have previously cloned human interferon–inducible protein 10 (IP-10), a protein secreted by keratinocytes, monocytes, and human endothelial cells after stimulation by rIFN-γ (1, 2). DNA sequence analysis has demonstrated that IP-10 belongs to the intercrine cytokine or chemokine family of proteins (3, 4), so named for their chemotactic activity towards neutrophils, monocytes, T cells, basophils, and fibroblasts. Some chemokines inhibit early subsets of bone marrow progenitors (5–11). This family is divided in two subgroups based on the arrangement of the first two of four conserved cysteines: the α-subfamily with the C-X-C motif which is located on human chromosome 4 (q12-21) and includes GRO-α, β-thromboglobulin, macrophage inflammatory protein (MIP)-2α, MIP-2β, IL-8, neutrophil-activating peptide (NAP)-2, and IP-10; and the β-subfamily with the C-C motif which is located on human chromosome 17(q11-32) and includes MIP-1α, MIP-1β, macrophage-chemotactic and activating factor (MCAF), and RANTES (3, 4).

We have expressed IP-10 in bacterial and baculovirus vectors and used the purified recombinant protein to investigate its biological activity. We now clarify the model of IP-10 biosynthesis, and demonstrate that it is secreted by keratinocytes and by baculovirus-infected cells after the removal of the signal peptide, but without any detectable processing of the COOH-terminal end. Purified rIP-10 directly inhibits early subsets of granulocyte/macrophage progenitor cells (CFU-GM), multipotential granulocyte-erythroid-macrophage-megakaryocytic progenitors (CFU-GEMM) and erythroid burst-forming

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/09/1127/06 $2.00
Volume 178 September 1993 1127–1132

unit progenitors (BFU-E) that depend on steel factor (rSLF) in addition to rGM-CSF or rEPO, respectively. Inactive concentrations of rIP-10 combined with inactive concentrations of rMIP-1α, rMIP-2α, platelet factor 4 (PF4), rIL-8, or rMCAF result in synergistic inhibition of early progenitors.

Materials and Methods

All chemicals were reagent grade. Ficoll-Hypaque, protein A–Sepharose, and S-Sepharose were from Pharmacia (Piscataway, NJ). EX-CELL-400 was from JR Scientific (Woodland, CA). The 4.6 × 150-mm C4 reverse phase column was from Vydac (Hesperia, CA). The silver staining kit was from ICN (Cleveland, OH). rIFN-γ was a gift from Dr. G. Garotta (Hoffman-LaRoche, Basel, Switzerland). Human rMIP-1α and rMIP-2α were gifts from Dr. B. Sherry (Picower Institute, Manhasset, NY). Human rGM-CSF, human rIL-3 and human rSLF were gifts of the Immunex Corp. (Seattle, WA). We purchased human rEPO from Amgen Biologicals (Thousand Oaks, CA), human PF4 and rIL-8 from Sigma Chemical Co. (St. Louis, MO), and human rMCAF from Repro Tech Inc. (Rocky Hills, NJ).

The cloning of IP-10 cDNA and the affinity purification of Abs against residues 10-98 (αIP-10), and 77-98 (α22) or IP-10 have been described (1, 2).

Wild-type baculovirus, Sf9 insect cells, and transfer vector pVL1392 (12) were provided by MD Summers (Texas AM University, College Station, TX). Transfer vector pAcYM1 (13) was provided by D. Bishop (NERC Institute of Virology, Oxford, UK). The PstI fragment of the IP-10 cDNA was cloned in the PstI site of pVL1392, and yielded recombinant baculoviruses 8555 and 9094. For elimination of its 5′ untranslated sequences, the IP-10 cDNA was digested with Nla3, the 375-nucleotide fragment was purified, ligated to GATCCATG, restricted with BamHI, and cloned into the BamHI site of pAcYM1, generating recombinant baculoviruses A213 and A221. We used standard techniques for isolation of recombinant baculoviruses (12, 14) and sequenced (15) all junctions between recombinant transfer vectors and IP-10 cDNA to exclude cloning artifacts. For production of rIP-10, Sf9 cells were infected with A221 (20 PFU/cell), 6-d supernatants were cleared at 100,000 *g* for 1 h at 4°C, dialyzed against 40 mM Na phosphate, pH 7.2, and loaded on a 15-ml Sepharose-S fast performance liquid chromatography (FPLC) column. Proteins were eluted with a linear gradient of 0.0–2.0 M NaCl in 40 mM Na phosphate, pH 7.2 (150 ml), fractions containing rIP-10 were identified with a dot blot immunoassay (16) using αIP-10, and were loaded on a reverse-phase C4 HPLC column. Adsorbed rIP-10 was eluted with a gradient of 25% acetonitrile, 50% propanol, 25% H_2O in 0.1% TFA, lyophilized, and resuspended in endotoxin-free PBS.

With A (GGATCCATGGTACCTCTCTCTAGAACC) as 5′ and B (GGATCCATGGTTAAGGAGATCTTTTAGA) as 3′ primer we amplified (17) a cDNA coding for f(22-98), a fragment lacking the signal peptide and extending from valine 22 to proline 98 of IP-10. With C(GGATCCATGGTTATGGATTCAGACATCTCTT) as 3′ and A as 5′ primer we amplified a cDNA coding for f(22-77), a fragment extending from valine 22 to proline 77 and lacking the signal peptide and the last 21 residues of IP-10, but retaining all four cysteine residues and approximating the previously reported secreted form of IP-10 (2). The PCR products were restricted with NcoI and cloned in the NcoI site of pET-3d (provided by F. Studier, Brookhaven National Laboratories, Brookhaven, NY) eliminating all amino acid residues of ϕ10 (18), and expressing the rIP-10 fragments as nonfusion proteins with an added methionine at the NH_2 terminus. The regions adjacent to and including the IP-10 cDNA were sequenced (15) to exclude PCR and cloning errors. Lysogens BL21(DE3) transformed with recombinant plasmids were induced with 0.4 mM isopropylthiogalactoside (18), recombinant f(22-98) was purified (19) from refractile bodies (20), dialyzed against Ca/Mg-free PBS, and stored in 1-ml aliquots at −70°C. Protein concentration was measured by dye binding (21). Antiserum AS522 was raised against purified recombinant f(22-98) as described (2). Natural human IP-10 was concentrated by TCA precipitation of serum-free medium from primary human keratinocytes after 24 h induction by rIFN-γ (2).

Proteins were analyzed on 10–20% gradient PAGE with 0.75 M Tris-HCl, pH 8.45, in the stacking gel; 1.0 M Tris-HCl, pH 8.45, in the separating gel; 0.2 M Tris HCl, pH 8.9, in the anode reservoir; 0.1 M Tris-0.1 M Tricine in the cathode reservoir; and 0.1% SDS in all buffers (22). They were quantitated by densitometry after silver staining (23). Western blots were performed on Immunobilon-P (16, 24). rIP-10 was immunoprecipitated from [^{35}S]methionine-labeled Sf9 cells after boiling in 500 μl of 0.2% SDS-50 mM Tris-HCl, pH 7.5, followed by the sequential addition of NP-40 (final concentration 1%), affinity-purified Abs (final concentration 5 μg/ml), and protein A beads (25). The relative molecular mass of IP-10 was estimated from the mobilities of marker proteins of 43–14.3 kD. NH_2-terminal sequencing was performed as described (26, 27).

Human bone marrow CFU-GM, BFU-E, and CFU-GEMM of healthy volunteers were assayed as described with added rEPO, rGM-CSF, rSLF (50 ng/ml), and other recombinant or natural chemokines as indicated (6, 7, 11). Highly purified CD34$^+$ cells (6, 11) were plated (500 cell/ml) in the presence of rEPO (1 U/ml), rSLF (50 ng/ml), rGM-CSF (100 U/ml), and rIL-3 (200 U/ml), and yielded 126 ± 10, 60 ± 10, and 17 ± 5 CFU-GM, BFU-E, and CFU-GEMM per 500 plated cells, respectively. For reversal of inhibition, we preincubated chemokines with fourfold molar excess of affinity-purified Abs (αIP-10 or α22) or with excess whole serum (AS522) in McCoy's medium at room temperature for 1 h. The mixture was added to human bone marrow cells and CFU-GM were assayed in McCoy's medium supplemented with FCS, rSLF (50 ng/ml), and rGM-CSF (100 U/ml). For control, an equal volume of similarly incubated McCoy's medium was added to cells. Statistical significance was determined with the Student's two-tailed test.

Results

Recombinant baculoviruses 8555, 9094, A213, and A221 express rIP-10 as a nonfusion protein. In 8555, the initiating ATG of the polyhedrin gene is mutated to ATT, and protein synthesis starts 118 nucleotides downstream, at the initiating ATG of the IP-10 cDNA. In 9094, the IP-10 was inserted in the same location but in reverse orientation. In A221 and A213, the initiating ATG of the polyhedrin gene is destroyed by deletion of nucleotides 2-751 of its coding region, and protein synthesis starts eight nucleotides downstream at the initiating ATG of IP-10.

Analysis of protein synthesis after infection by 8555 and A221 demonstrated a major new band of 9.9 kD, similar in size to IP-10 without the signal sequence (10.0 kD), and a minor band of 11.9 kD, similar in size to IP-10 with the signal sequence (12.4 kD). Neither band was detected in uninfected cells or in cells infected with 9094 or with wild-type virus (Fig. 1 *A*). The 9.9-kD but not the 11.9-kD band was de-

tected in supernatants of infected cells by autoradiography (data not shown). Nonimmune serum did not precipitate any proteins from cells infected with A221, but αIP-10 and α22 precipitated both bands (Fig. 1 *B*). Western blotting with αIP-10 and α22 detected both bands in cells, but only the 9.9-kD band in the medium (data not shown).

To precisely define the molecular weight of baculovirus IP-10, we used f(22-98) and f(22-77) as molecular weight markers and demonstrated that f(22-98) and IP-10 derived from keratinocytes or baculovirus comigrated at 10.2 kD, and were recognized by αIP-10 and α22. By contrast, f(22-77) migrated with a relative molecular mass of 6.2 kD, and was recognized by αIP-10 but not by α22 (Fig. 2). The reactions of AS522 and αIP-10 in immunoprecipitations and Western blots were identical (results not shown).

Levels of rIP-10 were 5–10 times higher after infection with A213 and A221 than after infection with 8555, and were not affected by FCS reaching 9% of the total protein in the supernatant of cells grown in EXCELL-400 (data not shown). With αIP-10 and a dot blot immunoassay, we purified rIP-10 from supernatants of infected cells, and obtained a major peak on HPLC (Fig. 3 *A*) which was a single band on SDS-PAGE (Fig. 3 *B*, lanes *S*, *F*, and *H*) comigrating with purified bacterial f(22-98) (Fig. 3 *B*, lane *E*). The faint bands near the top of the gels correspond in size to keratins, and were seen in unloaded lanes or in lanes loaded only with sample buffer. Western blotting confirmed that the purified band represented rIP-10, because it reacted with αIP-10 and α22 during all stages of purification (Fig. 2).

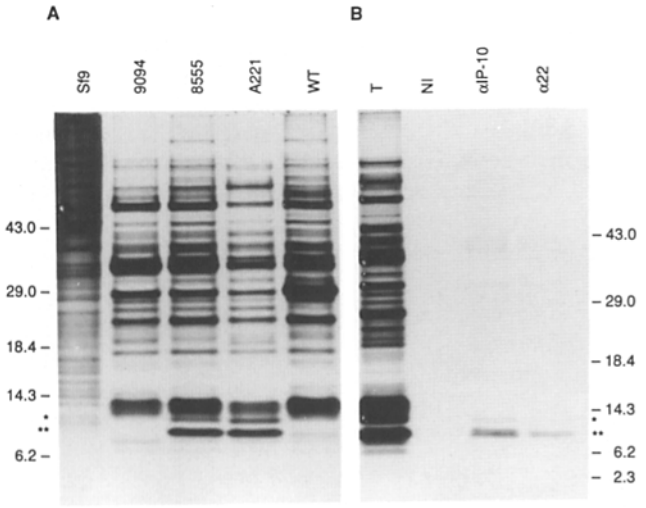

Figure 1. (*A*) Protein synthesis by wild-type and recombinant baculoviruses. Cells were pulsed with [^{35}S]methionine 60–66 h after infection, washed, boiled in sample buffer, and analyzed by SDS-PAGE. The top of each lane designates the infecting baculovirus. (*WT*) Wild-type baculovirus; (Sf9) uninfected cells. (*B*) Immunoprecipitations of cell-associated rIP-10. Sf9 cells were infected with A221, pulsed with [^{35}S]methionine 60–66 h after infection, boiled in SDS (*T*), and immunoprecipitates of an equal number of cells by nonimmune serum (*NI*), αIP-1, or α22 were analyzed with SDS-PAGE followed by fluorography, as designated at the top of the corresponding lanes. (*) The 11.9-kD form, and (**) the 9.9-kD form of rIP-10.

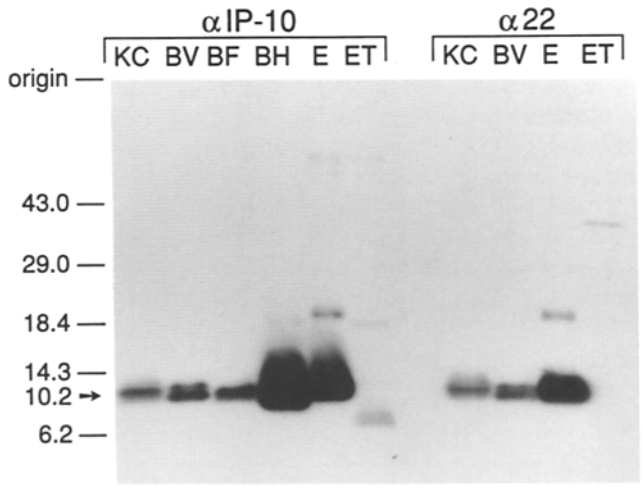

Figure 2. Western blot analysis of natural and rIP-10. Keratinocyte IP-10 (*KC*), supernatants from A221-infected Sf9 cells (*BV*), and representative fractions from Sepharose-S FPLC (*BF*) or C4 reverse phase HPLC (*BH*) were analyzed by SDS-PAGE along with f(22-98) (*E*) or f(22-77) (*ET*). Duplicate membranes were stained with αIP-10 or α22.

NH_2-terminal sequencing of baculovirus rIP-10 demonstrated a major NH_2-terminal sequence of VPLSRTVRØT (66%) and a minor sequence of RTVRØT (34%), both matching the sequence of IP-10 secreted by keratinocytes (2). Sequencing of f(22-98) demonstrated a single sequence of MVPLSRTVRØTØISISNQPVN matching the sequence of secreted IP-10 (2) with an additional NH_2-terminal methionine. The yield of purified rIP-10 was 0.5 μg/ml of supernatant in the baculovirus system and 5 μg/ml of bacterial culture.

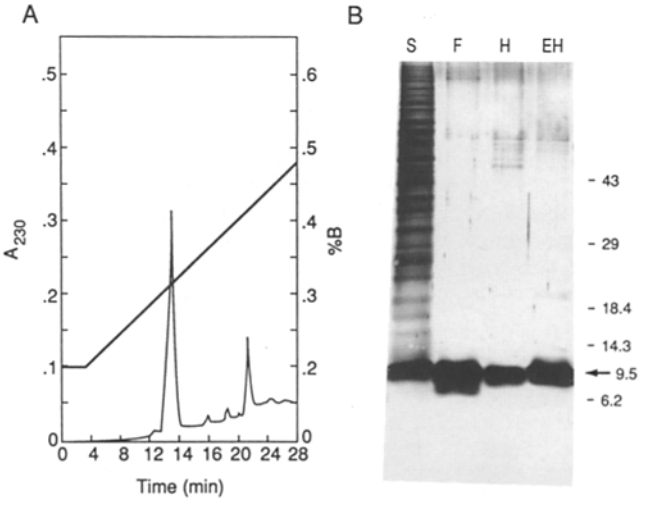

Figure 3. Purification of rIP-10 from baculovirus-infected cells. (*A*) Reverse-phase HPLC or rIP-10 purified from Sf9 cells infected with A221. (A_{230}) OD of the eluate at 230 nm; (%*B*) gradient of propanol-acetonitrile-TFA. (*B*) Analysis of the purification of rIP-10 by SDS-PAGE. (*S*) supernatants of A221-infected Sf9 cells; (*F*) peak fraction of S-Sepharose FPLC; (*H*) peak fraction of the reverse-phase HPLC analysis shown in (*A*); and (*E*) purified f(22-98) from *Escherichia coli*.

We evaluated the effect of rIP-10 on colony formation by CFU-GM, BFU-E, and CFU-GEMM. rIP-10 (50 and 500 ng/ml) did not affect colony formation by marrow cells plated in medium alone or in the presence of single growth factors (rEPO or rGM-CSF, data not shown). However, rIP-10 suppressed colony formation of CFU-GM stimulated by rGM-CSF and rSLF, and BFU-E and CFU-GEMM stimulated by rEPO and rSLF. Concentrations of 1–10 ng/ml were inactive, but there was a dose-dependent inhibition between 25 and 50 ng/ml. Maximal inhibition (50–60%) was seen at 50–500 ng/ml of rIP-10 (Fig. 4), representing complete suppression of the additional CFU-GM and BFU-E or CFU-GEMM colonies generated by the respective addition of rSLF to rGM-CSF or rEPO (data not shown). The dose–response was similar to that of rMIP-1α, rMIP-2α, PF4, rIL-8, or rMCAF (7, 11) which were assessed in the same assays. Whereas individual chemokines were inactive at concentrations <10 ng/ml, significant suppression of colony formation ($p < 0.01$) was seen when 0.01 ng/ml of rIP-10 was combined with 0.01 ng/ml of rMIP-α, rMIP-2α, PF4, rIL-8, or rMCAF. Combination of 0.1 ng/ml of rIP-10 with 0.1 ng/ml of any of these chemokines resulted in a 50–60% inhibition of colony formation by CFU-GM, BFU-E, and CFU-GEMM ($p < 0.001$). This represented complete inhibition of the rSLF-dependent colonies, and could not be suppressed further with a combination of rIP-10, rMIP-1α, rMIP-2, PF4, rIL-8, and rMCAF each at 50 ng/ml (Fig. 4). In the presence of rIP-10 (100 ng/ml), colony formation by $CD34^+$ cells was inhibited by 77% for CFU-GM ($p < 0.01$), by 58% for BFU-E ($p < 0.05$), and by 82% for CFU-GEMM ($p < 0.05$).

The inhibitory activity of rIP-10 was neutralized by Abs raised against whole IP-10 (αIP-10 and AS522) but not by Abs raised against the 22 COOH-terminal residues of IP-10 (α22). None of these Abs neutralized the inhibitory activity of rMIP-1α or PF4, or affected the number of colonies grown in the absence of rIP-10 (Table 1).

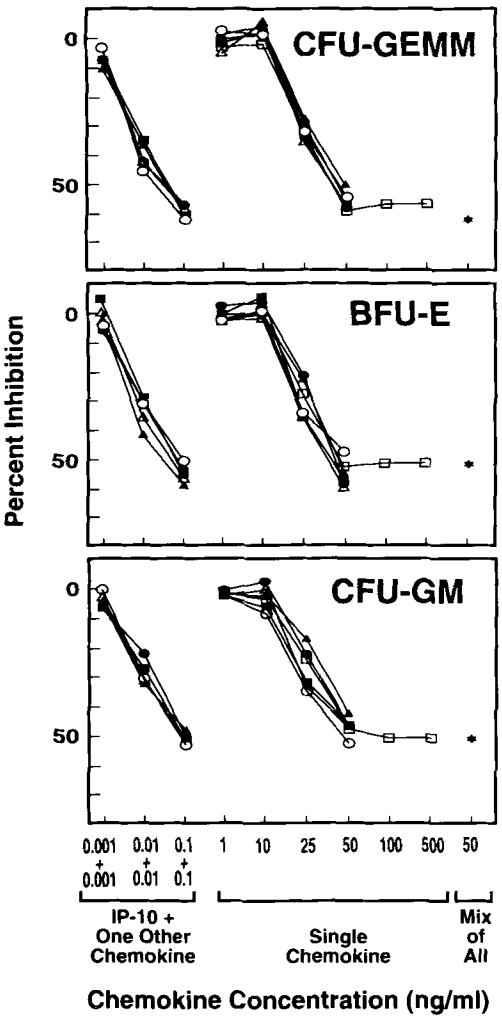

Figure 4. Influence of rIP-10 on colony formation. CFU-GM were plated in the presence of rGM-CSF (100 U/ml) and rSLF (50 ng/ml). CFU-GEMM and BFU-E were plated in the presence of rEPO (1 U/ml) and rSLF (50 ng/ml). Percent inhibition is the mean of three separate experiments with rIP-10, and two to three experiments for the other chemokines. Symbols in the graphs are smaller than the SEM, which was always ≤12% of the mean percent change. Inhibition was based on control colony numbers for CFU-GM (59 ± 2 to 106 ± 7), BFU-E (58 ± 4 to 105 ± 2), and CFU-GEMM (37 ± 68 ± 1). (□) rIP-10; (▲) rMIP-1α; (■) rMIP-2α; (○) PF4; (●) rIL-8; (△) rM-CAF. (*) Combination of all chemokines each at 50 ng/ml.

Discussion

We had previously suggested that IP-10 was secreted as a 6–7 kD-polypeptide after cotranslational removal of an NH_2-terminal signal peptide, and posttranslational proteolysis of the COOH-terminal end. This conclusion was based on two observations, the first being an estimated relative molecular mass of 6–7 kD (2) with SDS-PAGE on polyacrylamide looser than used here (acrylamide/bisacrylamide 30/0.27 vs 30/0.80). Keratinocyte and baculovirus rIP-10 comigrated on tricine SDS-PAGE and had a relative molecular mass of 10 kD, suggesting no posttranslational processing of the COOH terminus. However, IP-10 migrates in SDS-PAGE in an area where the mobility of marker proteins is not linearly related to the logarithm of their M_r, and consequently its estimated size could be inaccurate. Keratinocyte and baculovirus IP-10 comigrated with f(22-98), a fragment lacking the signal sequence but retaining all other residues of IP-10, but were larger than f(22-77), which approximates the previously described secreted form of IP-10 without the signal peptide and the last 21 amino acids (2). Protein sequencing demonstrated that the NH_2 terminus of baculovirus IP-10 started at valine 22 of the predicted sequence, and confirmed that Sf9 cells correctly remove the signal peptide.

The second observation supporting the original model was the inability of α22 to immunoprecipitate keratinocyte IP-10 when nonionic detergent was added first, followed by SDS, Ab, and protein A beads (2). However, under these conditions, Ag–Ab–protein A interactions are not affected, because free SDS does not reach denaturing levels (28). When IP-10 was first boiled in SDS followed by the addition of nonionic detergent in amounts sufficient to sequester SDS and permit

Table 1. *Antibody against IP-10 Neutralizes Its Ability to Suppress CFU-GM In Vitro*

	Colony formation after preincubation with			
	Medium	αIP-10	AS522	α22
Medium	61 ± 1	64 ± 1 (+2)	61 ± 1 (0)	63 ± 2 (+3)
rIP-10	34 ± 3 (−44)*	62 ± 1 (+2)	61 ± 2 (0)	32 ± 2 (−48)*
rMIP-1α	33 ± 2 (−46)*	32 ± 3 (−48)*	37 ± 2 (−39)*	33 ± 2 (−46)*
PF4	31 ± 1 (−49)*	34 ± 2 (−44)*	36 ± 1 (−41)*	33 ± 2 (−48)*

CFU-GM grown with rGM-CSF (100 U/ml) and rSLF (50 ng/ml) are expressed as mean ± 1 SEM per 10^5 plated cells. Chemokines were used at 50 ng/ml. Purified f(22-98) was the source of rIP-10. Values in parentheses designate percent inhibition from control.
* Designates significant decrease ($p < 0.001$) from control.

Ag–Ab interactions, it was immunoprecipitated by αIP-10 and α22. Western blotting confirmed these results. The reactions with antisera, the identity of size, and NH_2-terminal sequences of keratinocyte, baculovirus, and f(22-98), demonstrate that IP-10 is secreted without significant processing of its COOH-terminal end.

We demonstrated that rIP-10 is renatured during purification and has similar biological activity with rMIP-1α, rMIP-2α, PF4, rIL-8, or rMCAF (6, 7, 10, 11), abolishing colony formation by early bone marrow progenitors which require rSLF and rGM-CSF or rSLF and rEPO. Maximally effective concentrations did not abolish all colony formation, because colonies that formed in the absence of rSLF, and that presumably represent later progenitors, were not inhibited by rIP-10. The inhibition of colony formation by highly purified $CD34^+$ cells is consistent with a direct effect of rIP-10 on early progenitors (11). The combination of rIP-10 and any one of rMIP-1α, rMIP-2α, PF4, rIL-8, or rMCAF synergistically inhibited colony formation at concentrations 2,500 times lower than required with single chemokines (Fig. 4), but its molecular basis remains undefined.

The inhibitory activity is intrinsic to purified rIP-10, because it was specifically neutralized by Abs against whole IP-10 (αIP-10 and AS522), but not by α22, that recognizes IP-10 only after SDS denaturation. The results of the immunoprecipitations of rIP-10 agree with the neutralization of its inhibitory activity, and suggest that the COOH-terminal end is buried in the quaternary structure of the native protein. Quantities of natural IP-10 are too small for purification of amounts sufficient for investigation of its biological activity. The demonstration that rIP-10 has the same specific activity as the other chemokines in the colony suppression assay, supports the notion that bacteria and insect cells generate a protein with the correct disulfide arrangements, and that the purification of rIP-10 results in a completely renatured product. Therefore, the inability of rIP-10 to activate neutrophils (Wirthmueller, U., A. Sarris, and M. Baggiolini, unpublished observations and 29), or to stimulate the growth of melanoma cells (Richmont, A., and A. Sarris, unpublished observations), probably reflect properties of the naturally occurring protein.

Since intravenous administration of rMIP-1α protected murine hematopoietic progenitors from hydroxyurea and cytosine arabinoside (8, 9), rIP-10 may also be protective, and may become clinically useful by allowing repetitive chemotherapy administration without stem cell depletion.

We thank Drs. Michael Lanzer and Rena Feinman for many suggestions and helpful discussions. We acknowledge Scott Geromanos of the Microchemistry Facility at Memorial Sloan-Kettering Cancer Center for protein sequencing and amino acid analysis, and Jeffrey Krane of The Rockefeller University with help with keratinocyte cultures.

A. H. Sarris was supported by the Mortimer Lacher Fellowship Fund, and U. Wirthmueller by the Swiss National Science Foundation (grant 82BE-29193). This work was supported by a National Institutes of Health to J. V. Ravetch, Memorial Society Fund 7141 to A. H. Sarris, and by U.S. Public Health Service grants R01HL49202, R37CA36464, and R01HL46549 to H. E. Broxmeyer.

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Received for publication 13 April 1993 and in revised form 17 June 1993.

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