IP-10, a -C-X-C- Chemokine, Elicits a Potent Thymus-dependent Antitumor Response In Vivo

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Summary

IP-10 is a member of the -C-X-C- chemokine superfamily of proinflammatory cytokines whose secretion is induced by interferon γ (IFN-γ) and lipopolysaccharide (LPS). To date no function has been described for IP-10. We have genetically engineered tumor cells to secrete high levels of murine IP-10 and demonstrate that while IP-10 has no effect on the growth of these tumor cells in culture, it elicits a powerful host-mediated antitumor effect in vivo. The IP-10 antitumor response is T lymphocyte dependent, non–cell autonomous, and appears to be mediated by the recruitment of an inflammatory infiltrate composed of lymphocytes, neutrophils, and monocytes. These results document an important biologic property of IP-10 and raise the possibility that some of the T cell–directed effects of IFN-γ and LPS may be mediated by this chemokine.

The observation that some cancer patients who developed concurrent bacterial infections also experienced remissions of their malignant disease led Coley over 100 years ago to treat cancer patients with a preparation of heat-killed Gram-negative and Gram-positive bacteria, now commonly referred to as "Coley's toxins" (1, 2). The study of this phenomenon in animal models led to the identification of LPS as an active component of the toxins that induces a cytokine-mediated antitumor response in vivo (3). As IP-10 is a cytokine that is dramatically induced by LPS and IFN-γ, we sought to determine if IP-10 could elicit an antitumor effect in vivo.

IP-10 was originally isolated as a predominant mRNA induced by IFN-γ (4) or LPS (5, 6) in monocytes, and its expression has been detected in vivo during the development of a delayed-type hypersensitivity cellular immune response by keratinocytes, endothelial cells, and infiltrating mononuclear cells (7). In addition, IP-10 expression has been seen in the epidermis and dermis in cutaneous lesions of psoriasis (8), tuberculoid leprosy (7), and fixed DRUG eruptions (9).

IP-10 is a member of the chemokine superfamily and is ~30% homologous to IL-8 and platelet factor (PF4).[1] The chemokines are secreted proteins induced by inflammatory stimuli and are involved in orchestrating the selective migration, diapedesis, and activation of blood-borne leukocytes that mediate the inflammatory response (10, 11). This superfamily has been subdivided into two classes based on the positions of the four invariant cysteine residues found in the primary structure of these molecules. IP-10 belongs to the so-called α, or -C-X-C-, class of chemokines that map to a cluster

on human chromosome 4 (q12-21), and includes IL-8, GRO, and the platelet α-granule basic proteins PF4 and β-thromboglobulin (βTG). The β, or -C-C-, class maps to human chromosome 17 (q11-32), and includes monocyte chemotactic protein 1 (MCP-1), RANTES, macrophage inflammatory protein 1β (MIP-1β), and I-309. The chemokines seem to affect different, yet overlapping, leukocytes subsets. For example, IL-8 is specific for neutrophils (12) and CD45RO$^+$ memory T cells (13, 14), RANTES is specific for monocytes, eosinophils (15), and a subset of CD4$^+$ T cells (16); MIP-1β is specific for monocytes and CD8$^+$ T cells (17); and MIP-1α is specific for monocytes, basophils, and mast cells (18). Although IP-10 was one of the first chemokines to be identified, its physiologic function remains unknown. We therefore undertook the present study to gain insight into the biological activities of IP-10 by analyzing the cells that respond to IP-10 in vivo and to determine if IP-10 possesses an antitumor effect in vivo.

Materials and Methods

Cell Lines and Cell Culture. J558L is a heavy chain loss variant of the BALB/c plasmacytoma line J558 (19). The K485 mammary adenocarcinoma line was derived from a mammary tumor in a transgenic mouse carrying an activated c-myc oncogene (20). The J558L cell line was grown in RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 57 μM 2-ME. K485 and RAW 264.7 cell lines were maintained in DME supplemented with 10% FCS and penicillin and streptomycin in the concentrations noted above.

Construction of Ig-IP10 and Moloney (Mo)LTR-IP10 Expression Vectors. The murine (m)IP-10 cDNA was cloned from the murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD). RNA was isolated from RAW 264.7 cells that were treated for 3 h with 200 U/ml rmIFN-γ (Genentec, Inc.,

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[1] *Abbreviations used in this paper:* M, murine; MCP-1, monocyte chemotactic protein 1; MIP-1β, macrophage inflammatory protein 1β; PF4, platelet factor 4.

San Francisco, CA). This RNA was then used as a template for first-strand cDNA synthesis. The complete mIP-10 coding sequence was cloned from this cDNA using Taq DNA polymerase, standard PCR conditions, a 5' sense oligonucleotide AAGCGCTTCATC-CACCG, and a 3' antisense oligonucleotide GCGTGGCTTCTC-TCCAG based on sequences 1–17 and 362–379, respectively, from the sequence of mIP-10 (5). The PCR product was purified by agarose gel electrophoresis, blunt-ended with Klenow, phosphorylated with T4 DNA kinase, and then blunt-end ligated into the XhoI site of pIgTE/N and the EcoRI site of a MoLTR-SV40 I/pA expression vectors that had been flushed with Klenow. Sequence analysis of the cloned PCR product confirmed the sequence of mIP-10. The Ig control sequences used in the generation of the plasmid pIgTE/N were derived from the plasmid pTARY (22), placed 5' of the Ig promoter at the unique XbaI site in pBluescript (Stratagene, La Jolla, CA). Both vectors contain an SV40 intron and splice and polyadenylation signals (23) after the cDNA cloning site. The MoLTR was isolated as a 0.6-kb ClaI-XmaI fragment of pZip-NeoSV (X)-1 plasmid (24), which includes the 3' LTR of the Moloney murine leukemia virus.

Transfection of Tumor Cell Lines. Transfection of J558L cells was performed by electroporation (25). 20 μg of linearized Ig-IP10 or MoLTR-IP10 expression vector plasmid DNA and 1 μg of linearized neomycin resistance plasmid pSV7Neo (26) were used per transfection of 5×10^6 cells. After 48 h in RPMI, cells were centrifuged and resuspended in selective media containing 0.8 mg/ml of G418 (as calculated for 100% antibiotic activity; Genticin; GIBCO BRL, Gaithersburg, MD) and plated in serial dilutions into 288 wells (6.4-mm diameter) to clone by limiting dilution. G418-resistant cells from single wells were expanded, and a second round of cloning by limiting dilution in selective media was performed to ensure clonality. Transfection of K485 cells was performed by electroporation using 30 μg of linearized MoLTR-IP10 expression vector DNA and 1.5 μg of pSV7Neo DNA per 100-mm plate containing 6×10^5 cells. 24 h after transfection, cells were split 1:3 in DME; G418 at a concentration 0.4 mg/ml was added after an additional 24 h. Individual G418-resistant colonies were picked after 10–12 d, and then to insure clonality, single cell clones were then isolated by limiting dilution in selective media. In vitro doubling times were determined by serial cell counts using a hemocytometer over 96 h.

RNA Blots. RNA was isolated by lysing cells in guanidine hydrochloride and pelleting the RNA through a 5.7-M CsCl₂ cushion (27). RNA was fractionated on a 1.2% agarose gel containing 0.2 M formaldehyde and then transferred to gene screen (DuPont Co., Wilmington, DE) and hybridized with [³²P]dCTP Klenow-labeled mIP-10 cDNA or a [³²P]dCTP Klenow-labeled plasmid containing a 279-bp XhoII-DraI fragment encoding the ribosomal protein rp L32 (28) as a control for RNA loading.

Protein Expression and Purification. rmIP-10 beginning with the putative mature NH₂-terminal Ile and terminating with the COOH-terminal Pro was expressed in the *Escherichia coli* strain M15 using the Qiaexpress vectors pQE12 and pQE8 (Qiagen Inc., Chatsworth, CA). Expression of IP-10 in pQE12 results in a fusion protein containing a six-histidine COOH-terminal tag, and expression of IP-10 in pQE8 results in a fusion protein containing an NH₂-terminal six-histidine tag. IP-10 was purified to apparent homogeneity (a single peak on HPLC) by sedimentation of inclusion bodies through sucrose, affinity chromatography on nickel agarose (Qiagen Inc.), and then FPLC on the cation exchange resin Mono-S (Pharmacia Biotech Inc., Piscataway, NJ) eluting with a NaCl gradient or reverse-phase HPLC (Waters Associates, Milford, MA) eluting with an acetonitrile gradient. The concentration of purified protein was determined using a Bradford assay (Bio-Rad Laboratories, Richmond, CA) with IgG or bovine gamma globulin as the known standard.

Antibody Preparation. For immunizations, IP-10 was purified as above except that the eluent from the nickel-agarose chromatography column was separated on a denaturing SDS-polyacrylamide gel. The region of the gel containing IP-10 was then emulsified with CFA for the primary immunization and with IFA for subsequent immunizations. Approximately 200 μg of the COOH-terminal-tagged protein (IP-10-[His]₆) was injected subcutaneously into each of three, 8-wk-old, female New Zealand white rabbits. The rabbits were boosted twice, at 1-mo intervals, with 100 μg of the NH₂-terminal-tagged protein ([His]₆-IP-10) per rabbit to ensure the generation of antibodies recognizing the native NH₂ and COOH-termini of IP-10. 10 d after the second boost, the three rabbits were bled and serum was isolated and pooled for further studies.

Immunoprecipitation. 10^7 cells were metabolically labeled in 1 ml for suspension cells and 2 ml for adherent cells of methionine-free DME supplemented with 5% dialyzed serum and 0.5 mCi of [³⁵S]methionine for 6 h at 37°C. The media were collected, centrifuged at 1,000 g, and the protease inhibitors (Boehringer Mannheim Biochemicals, Indianapolis, IN) at the following concentrations were added to the supernatant: leupeptin (0.3 ng/ml), aprotinin (10 ng/ml), PMSF (20 ng/ml), and pepstatin (0.8 ng/ml). The supernatant was then centrifuged at 10,000 g for 30 min at 4°C and then precleared for 2 h at 4°C with 10 μl of pooled normal rabbit serum and 100 μl of a 1:1 slurry of protein A–Sepharose (PAS; Pharmacia Fine Chemicals, Piscataway, NJ). The PAS was then spun out at 10,000 g in eppendorf tubes, the supernatant was split and transferred to new tubes, and then 2 μl of pooled immune serum or preimmune serum was added to each sample for 3 h at 4°C. 50 μl of a 1:1 slurry of PAS was added to each sample, incubated another 4 h at 4°C, and then centrifuged at 10,000 g for 10 min. The PAS beads were then washed three times with 1 ml of RIPA buffer (0.15 M NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 0.05 M Tris, pH 8.0) transferred to new tubes and then boiled for 3 min in 50 μl of sample buffer containing 0.3 M 2-ME, 4% SDS, and 0.01% Pyronin Y. 15 μl was then analyzed on a 15% SDS-polyacrylamide gel using a Tris/Tricine buffer system (29) that has good resolution in the low molecular weight region.

ELISA. Conditioned medium was collected for 48 h from 2 × 10^6 cells/ml in serum-free medium and 100 μl was then adsorbed in duplicate to microtiter wells (3911 Micro Test III; Falcon Labware, Oxnard, CA) for 4 h. Nonspecific binding sites were blocked for 2 h at room temperature with 200 μl/well of a PBS solution containing 2% goat serum and 2% BSA (blocking solution). This was followed by 100 μl/well of anti-IP-10 antiserum at a 1:1,000 dilution in blocking solution for 2 h at room temperature. The plates were then washed four times with water, incubated for 1 h at room temperature with a 1:10,000 dilution of an alkaline phosphatase–conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), washed four more times with water, and treated with 100 μl/well of the substrate p-nitrophenyl phosphate in diethanolamine (Pierce, Rockford, IL). The v_{max} mOD₄₀₅/min was measured on a v_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA).

Animal Studies. Tumor cell injections were carried out using freshly prepared suspensions at a concentration of 10^7 cells/ml in PBS. The total number of tumor cells injected was 2×10^6, except in mixed tumor transplantation experiments, in which 2 × 10^6 cells of each type were mixed and injected. All injections were performed subcutaneously in the right lower abdominal quadrant

via 25- or 27-gauge needles. BALB/c, BALB/c *nu/nu*, Swiss *nu/nu*, and FVB mice were obtained from Taconic Farms (Germantown, NY) and were cared for according to the guidelines provided by the Harvard Medical Area Animal Research Center.

Histology. Tissue at the site of tumor cell inoculation was fixed in Optimal Fix (American Histology Reagent Co., Stockton, CA). Standard paraffin-embedding, sectioning, and staining with hematoxylin and eosin was performed by the Transgenic Pathology Laboratory (University of California, Davis, CA).

Results

Generation of IP-10-expressing Tumor Lines. To establish tumor cell lines expressing mIP-10, the mIP-10 cDNA was cloned by PCR based on the mouse sequence and the coding sequence was ligated into two expression vectors (Fig. 1 *a*). One vector, Ig-IP10, places IP-10 under the control of the human and mouse Ig heavy chain promoter/enhancer (EμPμ); the other, MoLTR–IP-10, places IP-10 under the control of the murine Moloney leukemia virus LTR. The MoLTR-IP10 vector was used with a neomycin resistance plasmid to cotrans-

fect the J558L plasmacytoma cell line and the K485 mammary adenocarcinoma cell line. The J558L cell line is a heavy chain loss variant of the BALB/c-derived plasmacytoma cell line J558. The K485 cell line was derived from a breast tumor that arose in a transgenic mouse harboring an activated *c-myc* oncogene. Because of its lymphoid-specific expression properties, the Ig-IP10 vector was used with a neomycin resistance plasmid to cotransfect only the J558L cell line.

Between 15 and 20 individual G418 resistant clones from each transfection experiment were analyzed for IP-10 expression by Northern blot analysis. High-level IP-10-expressing clones were subcloned by limiting dilution to ensure clonality, and the subclone from each of the transfection experiments that secreted the most IP-10, as determined by ELISA, was selected for further studies. These cell lines are referred to as J558L-MoLTR-IP10, J558L-Ig-IP10, and K485-MoLTR-IP10. A Northern blot demonstrating the level of IP-10 mRNA in these transfected lines compared with their parental lines is shown in Fig. 1 *b*. The IP-10/SV40 transcript
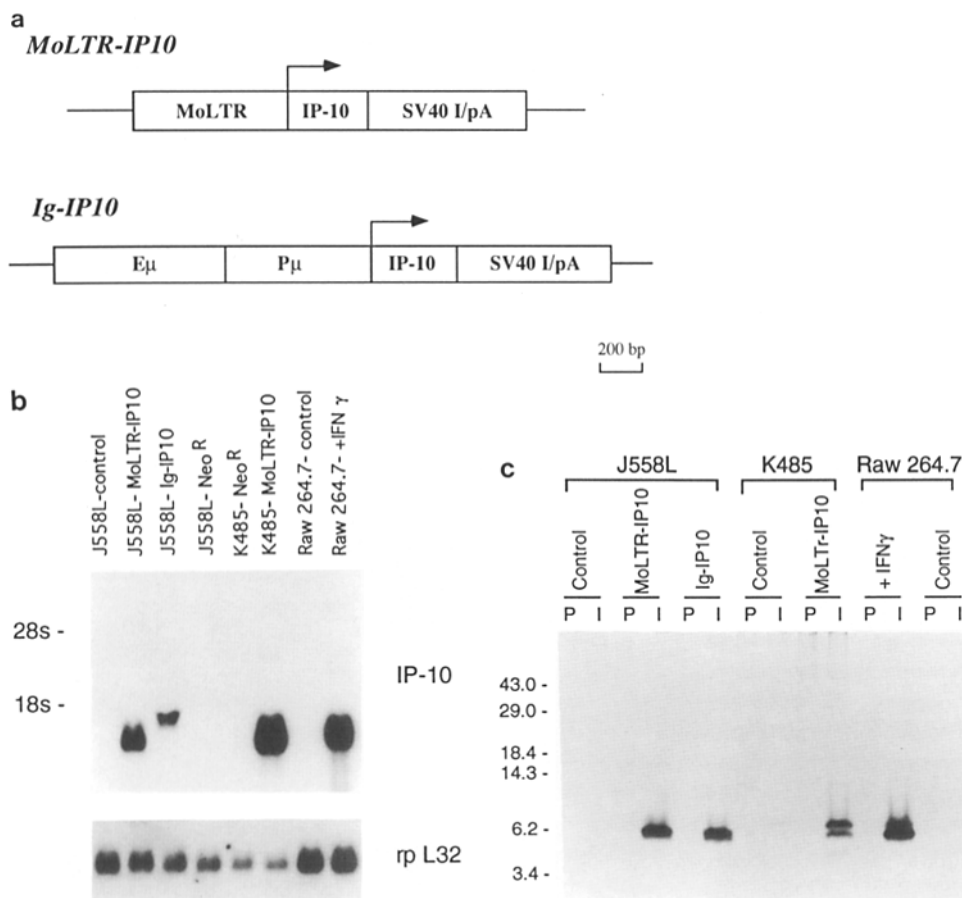
Figure 1. Expression and secretion of IP-10 in transfected tumor lines. (*A*) Diagram of the IP-10 expression vectors used in the generation of IP-10-expressing tumor cell lines. (*Eμ*) 1.0-kb fragment of the mouse Ig heavy chain enhancer; (*Pμ*) 0.6-kb fragment of the human Ig heavy chain promoter. MoLTR was isolated as a 0.6-kb fragment of the 3' LTR from Moloney murine leukemia virus. (*IP-10*) The entire mIP-10 coding sequence; (*SV40 I/pA*) a fragment of the SV40 genome that contains an intron and polyadenylation signal. (*Arrows*) The direction of transcription. (*b*) Northern blot analysis of transfected and control tumor cell lines. 10 μg of total RNA isolated from the parental J558L plasmacytoma cell line (*J558L-control*), J558L transfected with the neomycin resistance gene alone (*J558L-NeoR*), J558L cotransfected with the neomycin resistance gene plus the MoLTR-IP10 or Ig-IP10 expression vectors (*J558L-MoLTR-IP10* or *J558L-Ig-IP10*, respectively), K485 mammary adenocarcinoma cells transfected with the neomycin resistance gene alone (*K485-NeoR*), or with the neomycin resistance gene plus the MoLTR-IP10 expression vector (*K485-MoLTR-IP10*), RAW 264.7 macrophage cell line unstimulated (*control*), or stimulated with 200 U/ml of rmIFN-γ for 3 h. The blot labeled IP-10 was hybridized with a ^{32}P-labeled mIP-10 cDNA probe and

exposed for 2 h at −70°C with a Lightening Plus intensifying screen (DuPont Co.), and the lower blot, labeled rp L32, was hybridized with a ^{32}P-labeled ribosomal protein L32 probe and exposed for 1 h at room temperature as a control for RNA loading. The positions of 18S and 28S ribosomal RNAs are indicated on the upper blot. (*c*) Immunoprecipitation of secreted IP-10. Control J558L and K485 tumor lines and those lines transfected with either the MoLTR-IP10 or Ig-IP10 expression vectors, and control and IFN-γ-stimulated (3 h, 200 U/ml) RAW 264.7 cells, were biosynthetically labeled with [35S]methionine for 6 h. The media were then immunoprecipitated with either preimmune rabbit serum (*P*), or immune serum (*I*) isolated from rabbits immunized with purified rmIP-10. The immunoprecipitates were fractionated by SDS-PAGE on a 15% gel. The gel was then treated with EN³HANCE (DuPont Co./NEN Research Products, Boston, MA), dried, and then exposed at −70°C with an intensifying screen for 18 h. Molecular mass markers in kilodaltons are indicated.

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present in cells expressing the transfected Ig-IP10 gene is larger than the transcript seen in cells expressing the transfected MoLTR-IP10 gene. This is presumably due to a more proximal site of RNA transcription initiation as determined by the different promotors.

To ensure that these clones secreted authentic IP-10 protein, secreted IP-10 was visualized by immunoprecipitation and SDS-PAGE (Fig. 1 c). Since the previously characterized rabbit anti–human IP-10 antiserum (30) does not recognize the murine homologue, mIP-10 was expressed and purified from E. coli and used to generate a monospecific polyclonal rabbit antiserum. Various control and IP-10-expressing J558L and K485 cell lines were metabolically labeled with [^{35}S]methionine, and IP-10 was immunoprecipitated from the media using this rabbit anti–murine IP-10 antiserum. The protein immunoprecipitated from the transfected tumor cell lines and from the IFN-γ-treated murine macrophage cell line, RAW 264.7, used as a positive control, migrated as a doublet between 6 and 7 kD under reducing conditions (Fig. 1 c). A doublet of the same size had been seen when human IP-10 was immunoprecipitated from endothelial cells, keratinocytes, and monocytes (30). This size heterogeneity probably reflects proteolytic processing. In fact, IP-10 purified from a stimulated tumor line revealed heterogeneity at the NH$_2$ terminus (31). This type of NH$_2$-terminal processing is a characteristic feature of the α chemokines. Several NH$_2$-terminally processed forms of IL-8 (32) and βTG (33) have been identified that differ in their biological activities. The IP-10 protein immunoprecipitated from the transfected tumor cell lines comigrated with the protein immunoprecipitated from IFN-γ-stimulated RAW 264.7 cells (Fig. 1 c). This confirms that the transfected tumor cells are constitutively secreting authentic IP-10.

To quantify the amount of IP-10 secreted from these transfected tumor cell lines, a solid-phase ELISA was developed using the rabbit anti–murine IP-10 antiserum. The amount of IP-10 collected in serum-free media was compared with a standard curve generated using known amounts of IP-10 purified from E. coli (data not shown). The IP-10-expressing J558L and K485 clones used in this study secreted $\sim$20 and $\sim$150 ng/ml/2 × 10^6 cells per 48 h, respectively (Table 1). Comparable amounts of IP-10 are secreted from equivalent numbers of transfected tumor cells and from IFN-γ-stimulated RAW 264.7 cells (Fig. 1 c). IP-10 expression could not be detected in the parental J558L cell line by Northern blot, immunoprecipitation, or ELISA. Although the ELISA did not detect IP-10 in the medium collected from the parental K485 cell line (lower limit of detection, $\sim$5 ng/ml), low levels of baseline IP-10 mRNA could be detected by Northern blot and immunoprecipitation (at least 100-fold less than after transfection with the MoLTR-IP10 vector).

Inhibition of Tumor Growth In Vivo. Both parental cell lines chosen for this study readily form tumors when injected subcutaneously into appropriate inbred strains of mice, with a latency of 10–14 d for J558L in BALB/c and $\sim$28 d for K485 in FVB. The transfection and expression of IP-10 in the J558L and K485 cell lines did not alter their growth properties in culture as assayed by doubling time or morphology (data not

Table 1. *Quantitation of Secreted IP-10 by ELISA*

Cell line	mOD/min	Concentration
		ng/ml
J558L transfected with:		
None	3.54 ± 0.1	–
MoLTR-IP10	29.03 ± 0.5	$\sim$20
Ig-IP10	24.31 ± 1.4	$\sim$20
K485 transfected with:		
None	9.08 ± 0.8	–
MoLTR-IP10	91.01 ± 1.3	$\sim$150

The approximate concentration of secreted IP-10 from stably transfected cell lines was determined by comparing the ELISA value obtained from the media of 2 × 10^6 cells grown for 48 h in serum-free conditions to a standard curve obtained using purified rmIP-10.

shown). However, the expression of IP-10 did have a profound effect on the growth of these tumor cells in vivo. As is shown in Fig. 2 A and Table 2, the IP-10-producing J558L tumor cells failed to grow when they were injected subcutaneously into BALB/c mice. In contrast, both the parental J558L line and clones of J558L transfected with the neomycin resistance gene alone grew rapidly and formed large local masses 10–14 d after subcutaneous injection into BALB/c mice. The antitumor effect was observed with both the MoLTR-IP10 and Ig-IP10 vectors, demonstrating that the effect is not vector specific.

That the effect is not restricted to a single tumor type, or to a single inbred genetic background of mice, was shown by demonstrating IP-10's antitumor effect on the K485 mammary adenocarcinoma cell line in FVB mice. Fig. 2 B and Table 3 demonstrate the inhibition of tumor formation of a K485 clone cotransfected with MoLTR-IP10 and a neomycin resistance gene, compared with a control K485 clone trans-

Table 2. *Tumorigenicity of J558L Control and Transfectants in Syngeneic BALB/c and Nude Mice*

	Tumor occurrence	
J558L transfected with:	BALB/c	*nu/nu*
None	21/21	7/8
Neo^R alone	4/5	5/5
MoLTR-IP10	0/14	12/16
Ig-IP10	2/16	12/16

Mice were autopsied 10–14 d after 2 × 10^6 cells were injected subcutaneously into the right lower abdominal quadrant. The weight of the extirpated tumor or residual scar was determined and a tumor mass was considered positive if it weighed ≥0.5 g. This table is a compilation of seven different experiments; control and experimental animals for any given experiment were always autopsied on the same day.
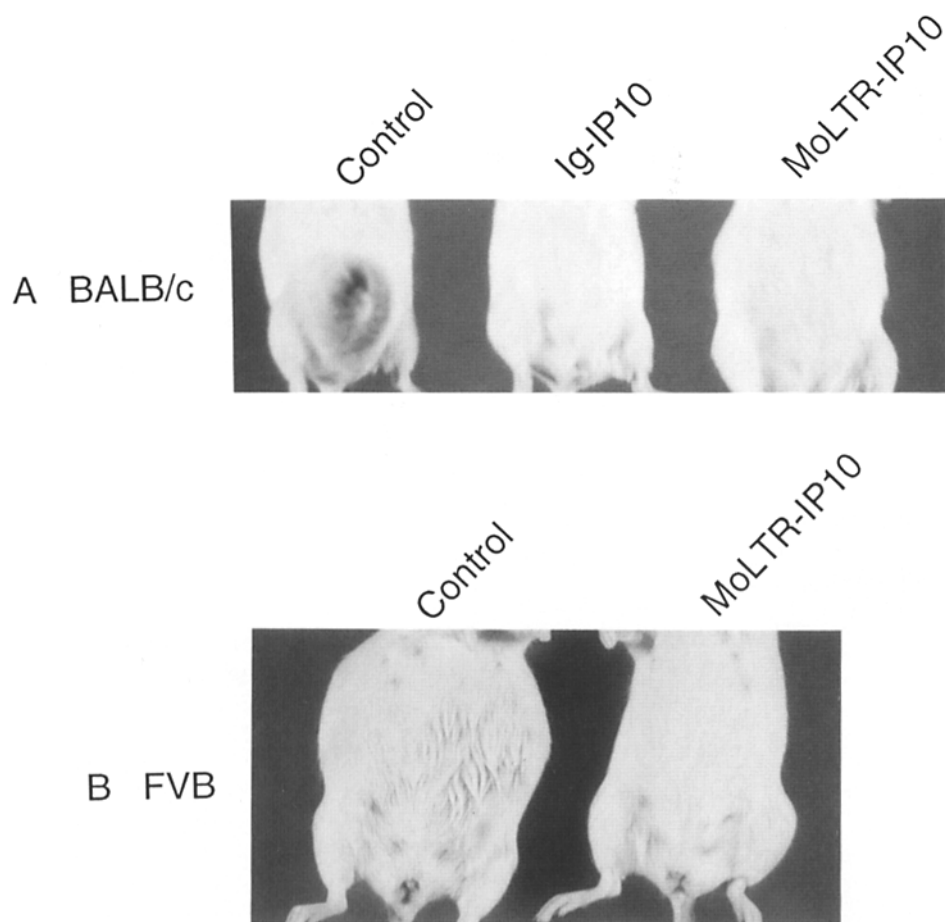
Figure 2. Mice injected with control and IP-10-secreting tumors. (A) 12 d after subcutaneous right lower quadrant injection of control (untransfected), MoLTR-IP10, and Ig-IP10 J558L tumor lines into syngeneic BALB/c mice. (B) 28 d after subcutaneous right lower quadrant injection of control (Neo[R] alone) and MoLTR-IP10 K485 tumor lines into FVB mice. 2×10^6 tumor cells were injected per animal.

fected with the neomycin resistance gene alone. K485 control tumor cells subcutaneously injected into FVB mice initially grew as a local tumor mass that, by 1 mo after injection, locally invaded through the peritoneum and led to malignant ascites (Fig. 2 B). However, IP-10-expressing K485 tumor cells were inhibited from growing and did not invade and metastasize, even though a small (<0.5-g) tumor mass was occasionally present at 1 mo.

IP-10's Antitumor Effect Is Thymus Dependent. The in vivo antitumor effect of IP-10 was dependent on thymus-derived cells as was evidenced by the rapid growth of all tumor lines (IP-10 expressors and nonexpressors) in both BALB/c

nu/nu and Swiss *nu/nu* mice (Table 2). This implies that the antitumor response evoked by IP-10 was T cell mediated.

Coinjection of IP-10 Producers and Nonproducers Demonstrates the Non–cell Autonomous Effect of IP-10. Mixing producer and nonproducer cells and transplanting them in a 1:1 ratio into syngeneic mice demonstrated that the effect of IP-10 was non–cell autonomous (that is, did not require that IP-10 be produced by all tumor cells). As shown in Table 4, when a J558L neomycin resistant IP-10 nonproducer clone was injected subcutaneously into BALB/c mice, it formed tumors

Table 3. *Effect of IP-10 on the Tumorigenicity of Mammary Adenocarcinoma Cells in FVB Mice*

K485 transfected with:	Tumor occurrence
Neo[R] alone	5/6
MoLTR-IP10	0/8

Mice were autopsied 28 d after 2×10^6 cells were injected subcutaneously into the right lower abdominal quadrant. Tumors were associated with a tense bloody malignant ascites.

Table 4. *IP-10's Effect in Mixed Tumor Cell Transplantation*

Cell	Tumor occurrence
J558L-Neo[R] alone	4/5
1:1 mix of J558L-Neo[R] and J558L transfected with	
MoLTR-IP10	0/5
Ig-IP10	1/5

Mice were autopsied 14 d after 2×10^6 of each cell type was injected subcutaneously into the right lower abdominal quadrant. Tumors were extirpated and considered positive if they weighed ≥0.5 g.
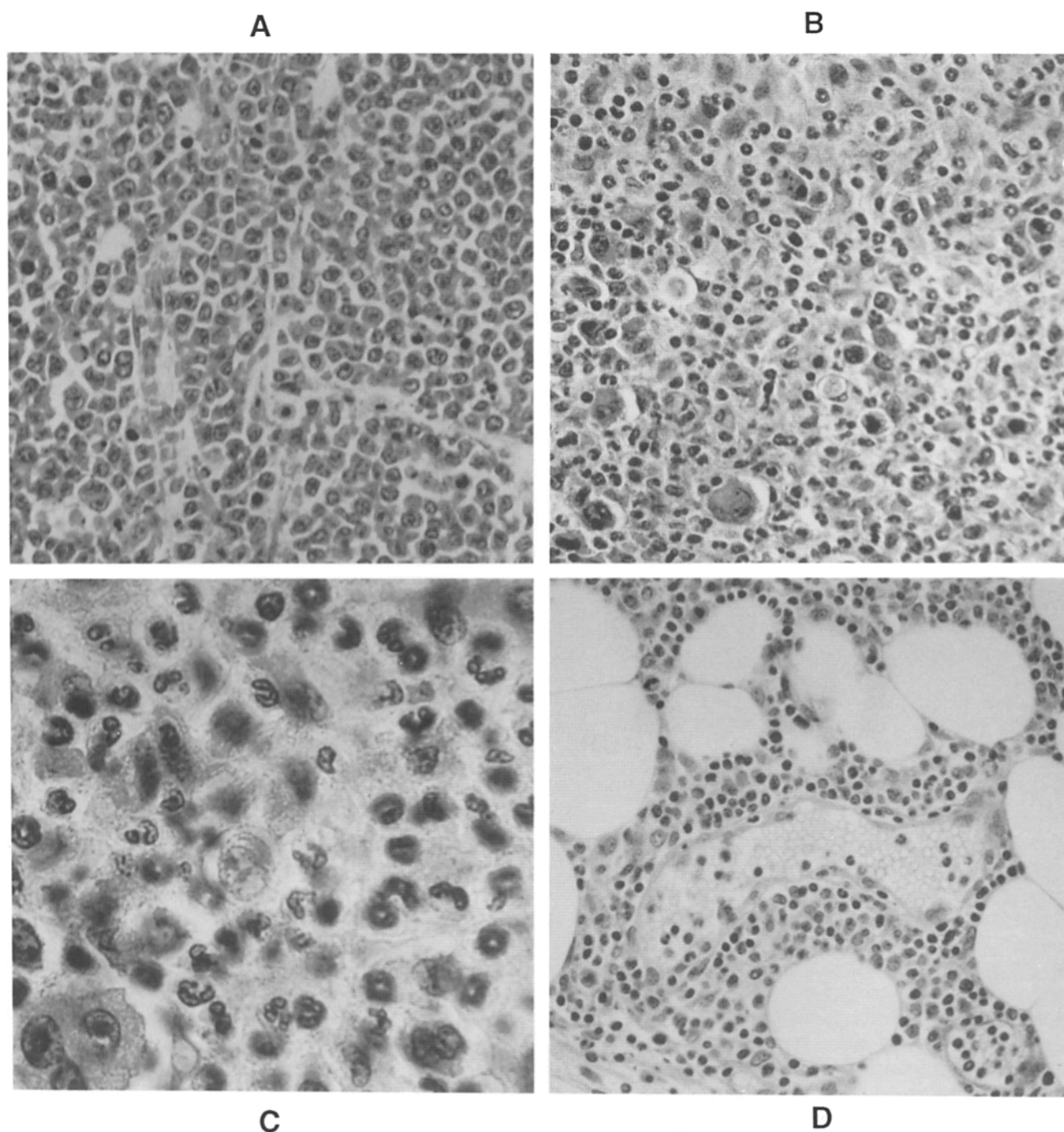
Figure 3. Histology of control and IP-10–secreting tumors. Representative hematoxylin and eosin stains of control and IP-10–secreting J558L tumors 12 d after subcutaneous injection into BALB/c mice. (*A*) A control J558L tumor, ×400; (*B*) a J558L IP-10–secreting tumor, ×400; (*C*) a J558L IP-10–secreting tumor, ×1,000; (*D*) a blood vessel in a J558L IP-10–secreting tumor, ×400. Note the characteristic appearance of the malignant plasmacytoma cells in the control with the absence of a host response (*A*). In contrast, in the IP-10–secreting tumor (*B* and *C*), note the infiltration of neutrophils, monocytes, and lymphocytes that tends to separate the tumor cells into clusters containing tumor giant cells (*B* and *C*, *bottom left*). Note the accumulation of lymphocytes and neutrophils in and around the blood vessel shown in the IP-10–secreting tumor (*D*).

comparable to the parental clone. However, when a non-producer clone was mixed in a 1:1 ratio with J558L clones that secrete IP-10 (either the J558L MoLTR-IP10 or the J558L Ig-IP10) and injected subcutaneously into BALB/c mice, both IP-10 producer and IP-10 nonproducer cells were rejected.

Histology of Tumor Rejection. Histologic analysis of the injection site at sequential time points was performed to evaluate the host cellular response induced by local IP-10 production. After the injection of the parental J558L tumor cells, histological analysis of the injection site at 1, 2, 3, 6, 10, and 14 d

revealed progressive growth of the characteristic malignant plasmacytoma cells with large pleomorphic hyperchromatic nuclei, prominent nucleoli, and abundant amphophilic cytoplasm (Fig. 3 A). Abundant mitotic figures were usually evident. An occasional peripheral neutrophil, monocyte, or thin connective tissue capsule was seen. In contrast, the injection site of IP-10-expressing J558L cells, analyzed at the same time points as the controls, revealed the infiltration of monocytes, lymphocytes, and neutrophils by day 3 after injection. At later time points, relatively more neutrophils were observed, and by day 12, this leukocytic infiltrate seemed to invade and separate the tumor cells into clusters, with a tendency of the residual tumor cells to form tumor giant cells (Fig. 3, B and C). Lymphocyte and neutrophil accumulation was seen surrounding blood vessels in the IP-10-secreting tumors, suggesting recent diapedesis (Fig. 3 D). In addition, a dense connective tissue response surrounding the infiltrated residual tumor was evident by day 12 (data not shown). In accordance with these histological changes, the gross appearance of these injection sites often consisted of a small white patch of scar tissue. At no time point were eosinophils observed, unlike the antitumor infiltrate elicited by J558L and K485 cells that were transfected with IL-4, which contained predominantly eosinophils (34). Of note, however, IL-2 induced an inflammatory infiltrate that also showed a predominance of neutrophils even though its antitumor effect in tumor cell gene transfer experiments was also T cell dependent (35).

In marked contrast to this vigorous cellular infiltration mounted by a syngeneic immunocompetent host, histological analysis of IP-10-producing tumors in congenitally athymic *nu/nu* mice revealed essentially no host inflammatory response (data not shown). There was no appreciable difference in the histological appearance of the IP-10 producers and nonproducers. This correlated with the gross appearance of palpable tumors in these mice. In the few cases (1/26 for J558L into BALB/c, 1/6 K485 into FVB, and 1/13 J558L into nudes) in which no tumors formed after the injection of control tumor cells, no evidence of scar or histologic evidence of a host response was seen, suggesting the leakage of cells from the injection site. Likewise, in the 8 of 32 instances when IP-10 producers failed to grow in nude mice, there was no evidence suggesting rejection. While we cannot rule out a partial effect in nude mice, we feel the data are most consistent with failure to successfully implant the tumor. Contrarily, in the rare instances (total of 3/48) when IP-10 producers formed tumors >0.5 g, histologic analysis revealed one area of the tumor with a typical inflammatory infiltrate, but another area without the infiltration of host-immune cells. This may have been due to the inability of the inflammatory response to reach that part of the tumor, or to the outgrowth of IP-10 nonexpressors in that area.

Discussion

An analysis of the growth properties and immune response to transplanted tumor cells overexpressing a cytokine gene is a useful approach for exploring and biological potential of a cytokine with unknown function and unknown cell

targets, such as IP-10. This approach is also useful for studying better characterized cytokines, since their in vitro activities do not always predict their predominant in vivo effects. For example, the fact that IL-4 elicits an antitumor infiltrate that is dependent on eosinophils (36) was not predicted by in vitro studies. In this study we demonstrate that IP-10 can elicit an antitumor inflammatory response that is capable of inhibiting the growth of a plasmacytoma and mammary adenocarcinoma in immunocompetent mice. This effect was shown to be thymus dependent, providing the first indication that IP-10 might act on T lymphocytes. The neutrophil, monocyte, and lymphocyte accumulation, seen as a result of the in vivo production of IP-10 in immunocompetent mice, is not seen in *nu/nu* mice. It has previously been demonstrated in G-CSF tumor cell gene transfer experiments, however, that neutrophils from *nu/nu* mice are capable of inducing an antitumor response (37). This suggests that the neutrophil and monocyte accumulation seen after implantation of IP-10-secreting tumor cells in immunocompetent mice may be the result of secondary T cell product(s) induced by IP-10. The fact that IP-10 appears to have no direct effect on neutrophils in vivo was quite unexpected, since all other -C-X-C- chemokines have been shown to be chemotactic for neutrophils. IP-10's inability to affect neutrophils has recently been corroborated in vitro using chemically synthesized IP-10. (38)

To date, one member of the -C-X-C- family, IL-8 (13), and two members of the -C-C- family, RANTES and MIP-1β, have been shown to be chemotactic for T cells. RANTES has been shown to be specific for CD4+/CD45RO+-prestimulated memory T cells (16), and MIP-1β has been shown to attract and induce binding of CD8+ T cells to the vascular cell adhesion molecule (VCAM-1) (17). It is an intriguing possibility that, in addition to controlling the selective migration of monocytes and granulocytes, the chemokine superfamily is also involved in controlling the trafficking of T cell subsets. In this regard, the T cell–directed effects of LPS and IFN-γ may be mediated by IP-10. For example, it has been demonstrated in gene transfer tumor cell transplantation experiments that IFN-γ elicits an antitumor response that is T cell dependent (39). Furthermore, although parenteral administration of endotoxin causes hemorrhagic necrosis of tumors, complete tumor regression after endotoxin injection was shown to be indirect, T cell dependent, and limited by severe toxicities (40).

The chemokines may be useful clinically as combination anticancer agents because they seem to have different antitumor mechanisms and seem to be well tolerated at high doses. For example, intralesional injection of PF4 has been shown to inhibit the growth of the B-16 melanoma in syngeneic mice and the HCT-116 human colon carcinoma in *nu/nu* mice, without direct inhibitory activity on either cell type (41). PF4 has been shown to inhibit angiogenesis in a chorioallantoic membrane assay and inhibit proliferation of endothelial cells in culture (42). It has therefore been proposed that the in vivo antitumor mechanism for PF4 is suppression of angiogenesis. Another chemokine, MCP-1, a member of the -C-C- family, has been shown to elicit an antitumor response

with a different mechanism of action than either IP-10 or PF4. The MCP-1 antitumor response is effective in nude mice and seems to be mediated by the recruitment of an inflammatory infiltrate composed of eosinophils and monocytes, with a striking absence of neutrophils and lymphocytes (43). It is interesting to note that both MCP-1 and IP-10 are induced by LPS, an active component of Coley's toxins. Mice harboring tumor cells secreting IP-10 or MCP-1 show no signs of systemic toxicities or cachexia. This is in contrast to the cachectic state induced in mice injected with tumor cells secreting another LPS-inducible cytokine, TNF (44).

A greater understanding of cytokine control of the immune response will lead to more efficacious and better tolerated anticancer immunotherapy. One can envision combination cytokine therapy using cytokines with synergistic anticancer action to improve upon the antitumor effects of Coley's toxins but with fewer toxic side effects. The chemokines may be good candidates since they seem to lack the systemic toxicities of LPS, TNF, IL-1, IL-2, and IL-4, and have the potential of recruiting many types of leukocytes into the inflammatory process.

Note added in proof: Recently, Taub et al. (45) demonstrated that human IP-10 is a chemoattractant for human T lymphocytes and monocytes, and promotes T cell adhesion to endothelial cells.

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