Interleukin-4 Receptor Expression on AIDS-Associated Kaposi's Sarcoma Cells and Their Targeting by a Chimeric Protein Comprised of Circularly Permuted Interleukin-4 and Pseudomonas Exotoxin

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ABSTRACT

Background: AIDS-associated Kaposi's sarcoma (AIDS-KS) represents one of the most common malignancies associated with human immunodeficiency virus infection. To target effective therapeutic agents to AIDS-KS, we have identified a new target in the form of interleukin-4 receptors (IL-4R).

Materials and Methods: The expression of IL-4R on AIDS-KS cells and their subunit structure was determined by radioligand receptor binding, cross-linking, and Northern and RT-PCR analyses. The in vitro effect of IL-4 and recombinant fusion protein made up of circularly permuted IL-4 and a mutated form of Pseudomonas exotoxin, IL-4(38-37)-PE38KDEL, was examined by clonogenic and protein synthesis inhibition assays.

Results: Five AIDS-KS cell lines expressed high-affinity IL-4R with a K_d of 23.5–219 pM. IL-4 appeared to cross-link to one major protein corresponding to 140 kDa and a broad band corresponding to 60–70 kDa. Both cross-linked proteins were immunoprecipitated with an antibody to human IL-4Rβ chain. AIDS-KS cells exhibited IL-4Rβ-specific mRNA. IL-4 caused a modest inhibition (31–34%) of colony formation in two AIDS-KS cell lines tested. IL-4(38-37)-PE38KDEL was found to be highly effective in inhibiting the protein synthesis in all five AIDS-KS examined. The IC_{50} ranged from 32 to 1225 pM. The cytotoxic action of IL-4 toxin was blocked by an excess of IL-4, exhibiting the specificity of IL-4(38-37)-PE38KDEL. The cytotoxicity of IL-4 toxin observed by a clonogenic assay corroborated well with the IC_{50} obtained by protein synthesis inhibition assay. Normal human endothelial cells expressed a negligible number of IL-4R (<50 sites/cell) and were less sensitive or not sensitive to IL-4(38-37)-PE38KDEL.

Conclusion: The presence of a new plasma membrane protein in the form of IL-4R on AIDS-KS cells may be targeted by IL-4(38-37)-PE38KDEL for its potential implication in the treatment of AIDS-KS.

INTRODUCTION

Kaposi's sarcoma (KS) is an invasive and intensely angiogenic multifocal tumor characterized by proliferation of mesenchymal cells. KS is more prevalent in HIV-infected individuals (1,2). AIDS-associated KS (AIDS-KS) lesions are composed of spindle-like cells, mixed with endothelial cells, fibroblasts, and vascular smooth muscles (3–5). Several lines of evidence suggest that AIDS-KS is a cytokine-mediated disease, and many growth factors play a role in the pathogenesis of AIDS-KS (6,7). AIDS-KS cells express ba-

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sic and acidic fibroblast growth factor (8), interleukin-1 and -6 (8,9), platelet-derived growth factor (10,11), granulocyte/macrophage colony-stimulating factor (8,9,12), and oncostatin M (13). Development of AIDS-KS lesions has been reported to be caused by autocrine production of several diverse cytokines and growth factors (14).

Interleukin-4 (IL-4) is a product of activated T lymphocytes and mast cells, and exerts a variety of effects on different cell types (15–17). Our group and others have shown that IL-4 inhibits the growth of different tumor types, including human melanoma, ovarian, breast, and multiple myeloma, colon and renal cell carcinoma, and hematologic malignancies (18–22). IL-4 has also been implicated as playing a controversial role in the progression of AIDS (23). A wide variety of solid tumor cells and other cells have been reported to overexpress high-affinity IL-4R (17–19,24,25). A low level of IL-4R is also expressed in normal immune cells, eosinophils, basophils, fibroblasts, and endothelial cells (15–17). Like other cytokines, IL-4 mediates its biological activity through its cell surface receptors, which appear to consist of three binding proteins of different sizes, e.g., IL-4Rα (p70), IL-4Rp140 (termed here IL-4Rβ), and common γ chain (p63) (26). Because of the association of IL-4R in various malignancies, we decided to determine whether AIDS-KS cells express IL-4R and if so, to determine the structure of IL-4R on such cells. We observed that all AIDS-KS cells examined in this study expressed high-affinity IL-4R.

The presence of IL-4R on AIDS-KS cells was exploited by targeting them with chimeric protein composed of circularly permuted IL-4 and a mutated form of Pseudomonas exotoxin (27,28). In our earlier reports, IL-4R-bearing tumor cells had been found to be extremely sensitive to chimeric fusion toxin (28–33). Our results demonstrate that circularly permuted interleukin-4 toxin, IL-4(38-37)-PE38KDEL, is highly and specifically cytotoxic to these IL-4R-positive AIDS-KS cells. In contrast, human endothelial and normal cells expressing a low level of IL-4R were less or not at all sensitive to IL-4 toxin.

MATERIALS AND METHODS

Cell Culture and Reagents

AIDS-KS-derived cells (KS248, NCB-59, KS54A, KS220B, and ARL-13) were generated from biopsy samples obtained from AIDS-KS patients (34). These cells were grown in 1% gelatin-coated flasks in RPMI culture medium supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, essential and nonessential amino acids (1 mM each), 1% nutridoma-HU (Boehringer Mannheim, Indianapolis, IN), penicillin (100 U/ml), and streptomycin (100 μg/ml; Biowhittaker, Walkersville, MD). The human umbilical vein endothelial cells (HUVEC) and human umbilical artery endothelial cells (HUAEC) were obtained from Clonetics (San Diego, CA) and cultured in endothelial cell growth medium (Clonetics) supplemented with 10% FBS and 12 μg/ml bovine brain extract (endothelial cell growth factor), 1.0 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 50 μg/ml gentamicin sulphate, and 50 ng/ml amphotericin B.

Recombinant human IL-4 was a gift from Schering Corp. (Kenilworth, NJ). IL-13 was expressed in *Escherichia coli* and purified as previously described (35). A polyclonal rabbit antibody to the p140 form of human IL-4R (P7) was a kind gift from Immunex Corp. (Seattle, WA). Rabbit polyclonal antibody against IL-2Rγ (anti-γc) was purchased from Santa Cruz Biotechnology (Lake Placid, NY).

IL-4 Receptor Binding Studies

Recombinant human IL-4 was labeled with ^{125}I (Amersham) by using IODO-GEN reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. The specific activity of radiolabeled IL-4 ranged from 31.5 to 179 μCi/μg of protein. The IL-4 equilibrium binding studies were carried out as described elsewhere (18). Briefly, 0.5 $\times$ 10^6 AIDS-KS cells in 100 μl of binding buffer were incubated at 4°C for 2 hr with ^{125}I-IL-4 (500 pM) in the absence and presence of increasing concentrations (10 pM–200 nM) of unlabeled IL-4. The duplicate samples of the cells associated with ^{125}I-IL-4 were separated from free ^{125}I-IL-4 by centrifugation through cushion of phthalate oils. The cell pellets were counted in a Gamma-counter (Wallac, Gaithersburg, MD). The data were analyzed with the LIGAND program (39) to calculate the number of receptors and binding affinities.

The competition of ^{125}I-IL-4 binding with varying concentrations (up to 1000 nM) of IL-4 or IL-13 was performed in a similar manner as described above.

Affinity Cross-linking Studies

Cells (4 $\times$ 10^6) were labeled with ^{125}I-IL-4 (500 pM) in the absence or presence of 200-fold

molar excess of unlabeled IL-4 or IL-13 for 2 hr at 4°C. The bound ^{125}I-IL-4 was cross-linked to its receptor with disuccinimidyl suberate (Pierce) at a final concentration of 2 mM for 45 min. Cells were then lysed at 4°C in a buffer containing 1% Triton X-100, leupeptin (0.02 mM), trypsin inhibitor (5 μM), benzamidine HCl (10 mM), phenanthroline (1 mM), iodoacetamide (20 mM), ε-amino caproic acid (50 mM), pepstatin (10 μg/ml), and aminoethyl benzenesulfonyl fluoride HCl (1 mM). The resulting cell lysates were cleared by being boiled in sample buffer containing 2-mercaptoethanol and were analyzed by electrophoresis through 8% SDS-PAGE gel as described earlier (35). In another experiment, the IL-4/IL-4R complex was immunoprecipitated from the lysate overnight at 4°C through incubation with protein-A sepharose beads that had been preincubated with anti-hIL-4R antibody (P7). The resulting conjugates were washed twice with solubilizing buffer, diluted with reducing buffer, boiled for 5 min, and analyzed by SDS-PAGE as described above. The gels were dried and autoradiographed.

Northern Analysis

AIDS-KS cells were harvested by trypsinization, and extensively washed, and total cellular RNA was extracted with TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA (10 μg) for each cell line was electrophoresed through formaldehyde-denaturing gel containing 1.0% agarose and transferred to nylon membrane (S&S Nytran, Scheicher and Schuell, Keene, NH) by capillary action. The nucleic acid on membrane was immobilized by ultraviolet (UV) cross-linking (Stratalinker, Stratagene, La Jolla, CA). The membrane was allowed to prehybridize for 4 hr at 42°C and hybridized overnight with ^{32}P-labeled cDNA probes of human IL-4Rβ insert cDNA (0.8 Kb) or IL-2R γ chain (~1.5 Kb). The membrane was washed and subsequently exposed to X-ray film for autoradiographs.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA of AIDS-KS cells was extracted using TRIzol reagent. The reverse transcription was performed in 50 μl reaction buffer containing 2.5 μg total RNA, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 1 mM of each dNTP, RNAse inhibitor (1 U/μl), Random hexamer (2.5 μM), and 2.5 U/μl of Moloney murine leukemia virus–reverse transcriptase (RT) (Perkin Elmer). The mixture was incubated for 10 min at room temperature and then for 15 min at 42°C. The enzyme was heat inactivated at 99°C for 5 min and chilled to 5°C for 5 min with the Perkin Elmer Cetus 9600 PCR System.

An aliquot of 10 μl of synthesized cDNA was amplified by polymerase chain reaction (PCR) in a 50-μl final volume of mixture containing 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 1.25 U of Ampli*Taq* DNA polymerase (Perkin-Elmer), and 0.1 μg of the following primer pairs: IL-4Rβ (+598) 5′-GACCTGGAG CAACCCGTATC-3′/(+913) 5′-CATAGCACAAC AGGCAGACG-3′ or IL-2R γ (+419) 5′-ACGG GAACCCAGGAGACAGG-3′/(+674) 5′-AGCG GCTCCGAACACGAAAC-3′ (36). The amplification procedure involved an initial denaturation step at 95°C for 2 min, 35 cycles at 95°C for 15 sec, and 60°C for 30 sec, respectively. Both PCR products were run on 2% Nusieve 3:1 agarose (FMC BioProducts, Rockland, ME) for UV analysis.

Protein Synthesis Inhibition Assay

Protein synthesis was determined by the incorporation of [^{3}H]leucine into AIDS-KS tumor cells. The cytotoxicity of the chimeric toxin IL-4(38-37)-PE38KDEL on various KS cells was tested as previously described (27,28,31). In brief, 1×10^4 cells per well were cultured overnight in 96-well, flat-bottomed microtiter plates. Medium was aspirated and replaced by 200 μl leucine-free medium (Biofluids, Rockville, MD) with or without various concentrations of IL-4(38-37)-PE38KDEL. For blocking experiments, cells were preincubated with IL-4 or IL-13 for 45 min at 37°C prior to the addition of IL-4 toxin to the cells. Cells were further incubated for 20–24 hr at 37°C and then 1 μCi of [^{3}H]leucine (NEN, Boston, MA) was added to each well and cultured for an additional 4 hr. The cells were washed and harvested on fiberglass filtermat and the cell-associated radioactivity was measured with a Beta Plate Counter (Wallac, Gaithersburg, MD). The data were obtained from the average of quadruplicates and the assays were repeated several times. The concentration of IL-4(38-37)-PE38KDEL at which 50% inhibition of protein synthesis (IC_{50}) occurred was calculated.

Clonogenic Assay

The effect of IL-4 and IL-4(38-37)-PE38KDEL on AIDS-KS cells was examined by colony-forming

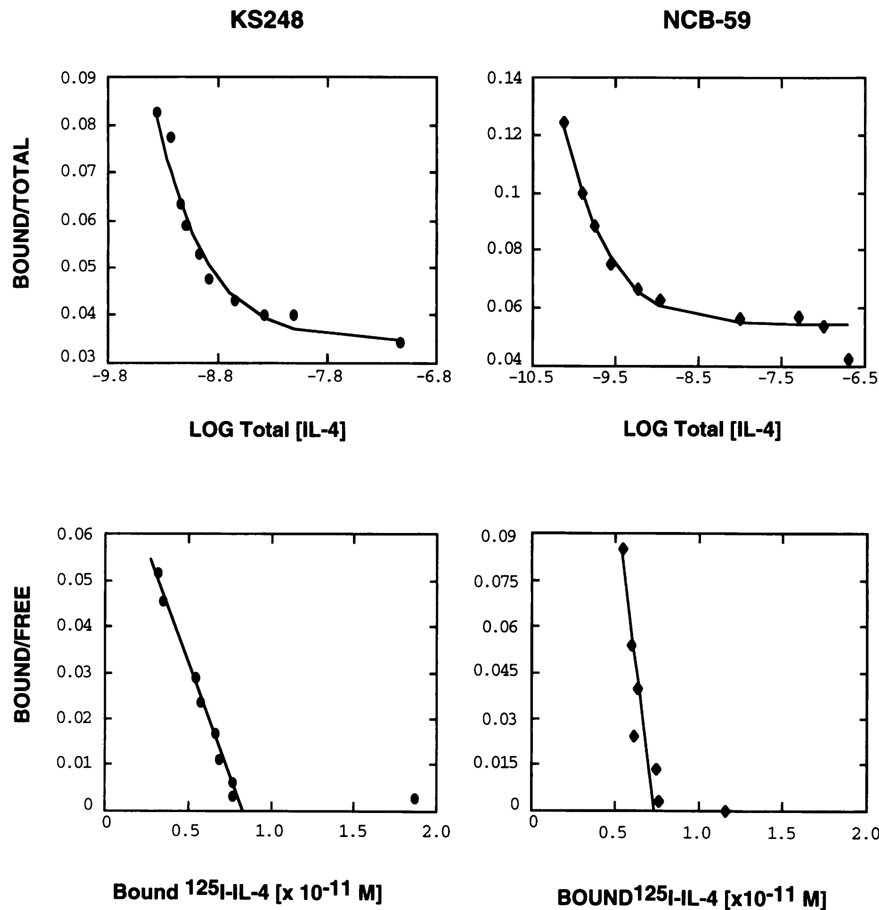

FIG. 1. Equilibrium binding of ^{125}I-IL-4 to AIDS-KS cells

Cells were surface labeled with ^{125}I-IL-4 as described in Materials and Methods. The displacement curve (top panel) and Scatchard analysis (lower panel) for KS248 and NCB-59 are representative of the several experiments performed for each AIDS-KS cell line. The average binding sites/cell and receptor affinity for each cell line are tabulated in Table 1. The binding data were analyzed with LIGAND software program (39).

assay (37,38). AIDS-KS (500 cells) and HUVEC (200 cells) were plated in triplicate in 100-mm and 60-mm petri dishes, respectively. The next day, the cells were exposed to either IL-4 (1–1000 ng/ml) or IL-4(38-37)-PE38KDEL (1–200 ng/ml) or control medium. The plates were incubated for 10 days at 37°C in a humidified CO_2 incubator. The cells were washed, fixed, and stained with crystal violet (0.25% in 25% alcohol). The colonies consisting of more than 50 cells were scored. The percent ratio of colony survival was calculated from the colonies formed in control and treated groups.

RESULTS

Expression of High-Affinity IL-4R on AIDS-KS

AIDS-KS cell lines KS248 and NCB-59 expressed 2643 ± 30 and 2567 ± 35 high affinity IL-4R molecules/cell with a dissociation constant (K_d) of 62.8 ± 10.4 and 23.5 ± 4.7 pM, respectively (Fig. 1 and Table 1). Three other AIDS-KS

TABLE 1. IL-4 receptor expression on AIDS-KS cells determined by ^{125}I-IL-4 binding studies

Cell type	IL-4 binding sites/cell[a]	K_d (pM)
AIDS-KS		
KS248	2643 ± 30	62.8 ± 10.4
NCB-59	2567 ± 35	23.5 ± 4.7
Normal endothelial cells		
HUVEC	<50	76.2 ± 12.4
HUAEC	<50	—[b]

[a]Binding sites and receptor affinity (K_d) for each cell type were calculated by the LIGAND program with (39) the data obtained from radioreceptor binding as described in Materials and Methods. The data are presented as the mean no. of molecules/cell ± S.D. of two to four experiments performed in duplicate.

[b]Could not be calculated.

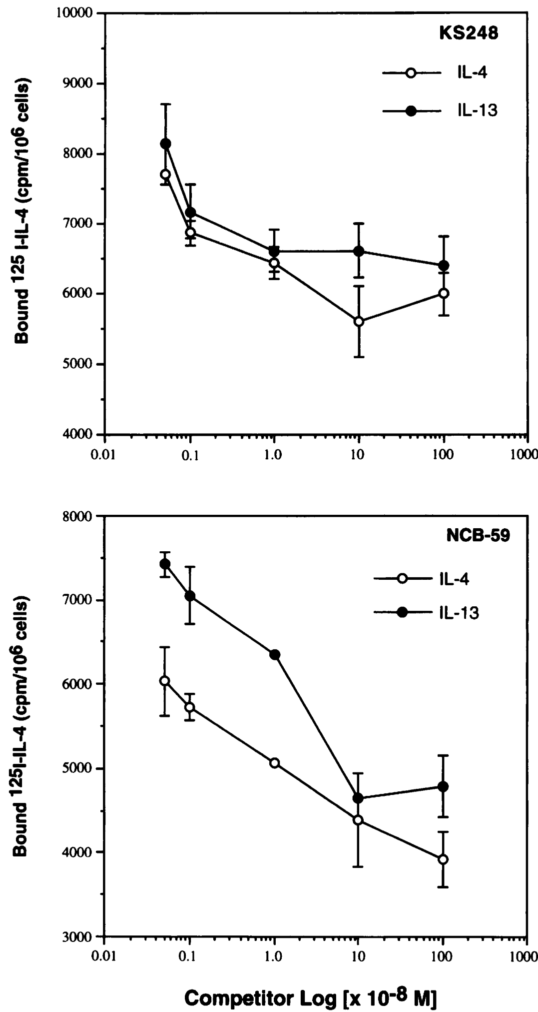

FIG. 2. IL-13 competes for the binding of ^{125}I-IL-4 on AIDS-KS cells

Data presented for KS248 and NCB-59 are the actual ^{125}I-IL-4 bound to 10^6 cells in the presence of varying concentrations of either unlabeled IL-4 or IL-13. KS248 and NCB-59 cells incorporated 9296 ± 236 (cpm ± S.D.) and 11,009 ± 366 (cpm ± S.D.), respectively, in the absence of competitor. The error bars represent the standard deviation of duplicate determinations.

(KS220B, ARL-13, KS54A) cell lines also expressed IL-4R, but with a relatively lower affinity (K_d ranging between 202 and 219 pM). In contrast, HUVECs and HUAECs, the possible precursor of Kaposi's sarcoma, expressed barely detectable levels of IL-4R (<50 sites/cell) (Table 1). It has been recently proposed that IL-4R may interact with IL-13 (35,40,41). To examine the interaction between IL-4R with IL-13, we compared the ^{125}I-IL-4 binding in the presence of either unlabeled IL-4 or IL-13 in KS248 and NCB-59 cell lines. Both

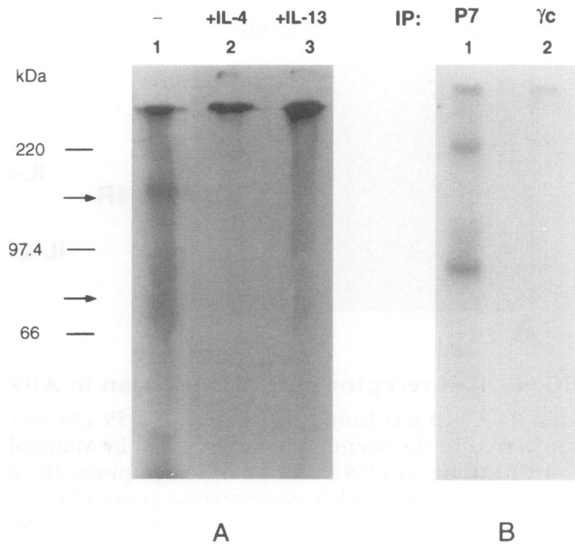

FIG. 3. Characterization of IL-4 receptor by affinity cross-linking

Cells (4 × 10^6) were incubated with ^{125}I-IL-4 in the absence (lane 1) and presence of either unlabeled IL-4 (lane 2) or IL-13 (lane 3) for 2 hr at 4°C (A) as described in Materials and Methods. (B) IL-4/IL-4R complex was immunoprecipitated with anti-hIL-4R antibody, P7 (lane 1) or anti-γc antibody (lane 2) overnight at 4°C. The cell lysates were electrophoresed on 8% SDS-PAGE gel and autoradiographed. The arrows on the left indicate the specific IL-4R bands. Lane 1 (A) is from the same gel and has been realigned for presentation.

IL-4 and IL-13 competed for the binding of ^{125}I-IL-4 in both cell lines examined (Fig. 2). IL-4 was better at displacing ^{125}I-IL-4 binding than IL-13, which was less efficient in the displacement of IL-4 binding. These data suggest that IL-4R also interacts with IL-13 in AIDS-KS cells and that it may share some components of IL-13R.

Subunit Structure and Characterization of IL-4R by Affinity Cross-linking

The molecular nature of IL-4R on AIDS-KS cells was also investigated by cross-linking studies using ^{125}I-IL-4 as a ligand. Figure 3A shows the SDS-PAGE analysis of ^{125}I-IL-4/IL-4R complex electrophoresed under reducing conditions. One main band of 155 kDa and a broad band of 75–85 kDa were cross-linked to ^{125}I-IL-4 on NCB-59 cells (lane 1). After subtracting 15 kDa, the molecular mass of IL-4, the size of IL-4 cross-linked proteins corresponded to 140 kDa and 60–70 kDa, respectively. The broad 60–70 kDa protein appeared to consist of two proteins of 63

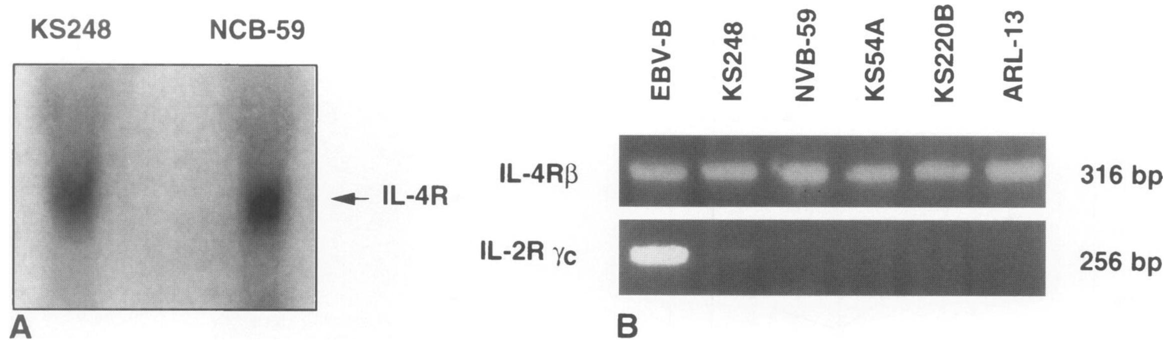

FIG. 4. IL-4 receptor mRNA expression in AIDS-KS cells

Total RNA (10 μg) from KS248 and NCB-59 (A) were subjected to Northern analysis by using 1% agarose gel and transferred to the membrane as discussed in Materials and Methods. The membrane was probed with ^{32}P-labeled IL-4R p140 insert cDNA and autoradiographed. (B) RT-PCR analysis of products amplified with IL-4R-specific primers. AIDS-KS mRNA were reverse transcribed to cDNA and subjected to PCR analysis using specific IL-4Rβ and IL-2R γc primers as outlined in Materials and Methods. The PCR products were run on Nusieve 3:1 gel and stained with ethidium bromide for UV analysis. EBV-immortalized B (EBV-B) cell cDNA was used as a positive control for both PCRs.

and 70 kDa. The size of each of these three proteins is similar to that of IL-4R p140 (IL-4Rβ), p70 (IL-4Rα), and IL-2R γc, respectively, as previously suggested (26,42–46). As expected, these cross-linked proteins were not observed in the presence of 200-fold molar excess of unlabeled IL-4 (lane 2) indicating that these bands are specific IL-4R binding proteins. Similar to the binding studies, unlabeled IL-13 could also partially displace radiolabeled IL-4 from ^{125}I-IL-4/IL-4R complex (lane 3), which is consistent with previous reports (35,41,47).

To further investigate the immunoreactivity of putative IL-4R, ^{125}I-IL-4/IL-4R cross-linked complex was immunoprecipitated with a rabbit polyclonal antibody against human IL-4R p140 (P7), which has previously been used to immunoprecipitate IL-4R subunits (35,46). The pattern of bands in Fig. 3B (lane 1) was similar to that observed when lysates were analyzed without immunoprecipitation (Fig. 3A, lane 1), except that a sharp band at about 60 kDa in addition to 140 kDa was observed. ^{125}I-IL-4/IL-4R complex was also immunoprecipitated with anti-γc antibody. We and others have previously reported that antibody to IL-2R γc can co-immunoprecipitate IL-4R p140 and IL-7R along with γc in immune cells (35,46,48). However, in KS cells, as shown in Fig. 3B, lane 2, no protein was immunoprecipitated with anti-γc antibody. The inability of anti-γc antibody to immunoprecipitate cross-linked IL-4R suggests that γc may not be associated with the IL-4R on AIDS-KS cells.

AIDS-KS Cells Express IL-4Rβ mRNA but not IL-2Rγ Chain mRNA

We next investigated the expression of IL-4R p140 (IL-4Rβ) and IL-2R γ-chain mRNA in AIDS-KS cells. Total cellular RNA was isolated from AIDS-KS cells and subjected to Northern blot analysis. RNA from KS248 and NCB-59 exhibited a 3.9-Kb species of IL-4R mRNA (Fig. 4A); however, γc mRNA expression was not detected in these two cell lines (data not shown).

Even though cross-linking and Northern analysis did not demonstrate the expression of γc protein or mRNA, respectively, in AIDS-KS cells, we next utilized a very sensitive technique, RT-PCR, to examine the expression of these mRNAs. As shown in Fig. 4B, all five AIDS-KS cells examined in this study expressed IL-4Rβ chain (upper lanes). Epstein-Barr virus (EBV)-immortalized B cells served as a positive control and expressed this mRNA. However, as observed with cross-linking and Northern analysis, γc mRNA was either very low or it was not expressed at all in all five AIDS-KS cell lines tested. KS248 cells showed a weak ethidium bromide staining. Thus, it is possible that a low level of γc mRNA was expressed in these cells as detected by a sensitive technique or it was a technical artifact due to nonspecific binding of template cDNA. Further studies continue to address the issue of expression of γc by AIDS-KS cells. EBV-immortalized B cells again served as a positive control and exhibited a strong band for IL-2R γc, as expected.

IL-4R on AIDS-KS Cells Are Highly Sensitive to IL-4(38-37)PE38KDEL

PROTEIN SYNTHESIS INHIBITION ASSAY. We have recently demonstrated that circularly permuted recombinant IL-4 toxin is highly cytotoxic to IL-4R-bearing tumor cells in vitro (27–29) and in vivo (27, S.R. Husain et al., unpublished results). Because AIDS-KS cells express a high number of IL-4R on their surface, it was of interest to examine the cytotoxicity of IL-4 toxin on these cells. IL-4(38-37)-PE38KDEL was constructed by fusing two proteins in which a truncated form of Pseudomonas exotoxin is fused to the new carboxy terminus of a circularly permuted form of IL-4 (28,29). We examined the cytotoxicity of circularly permuted IL-4 toxin on five different AIDS-KS cells by measuring the inhibition of protein synthesis determined by the incorporation of [^{3}H]leucine. The IC_{50}, the toxin concentration necessary for 50% inhibition of protein synthesis, ranged from 1.7 to 65.0 ng/ml (32 to 1225 pM). We found that KS248 and NCB-59 were the most sensitive cell lines; other AIDS-KS cells were also highly sensitive to the cytotoxic effect of IL-4(38-37)-PE38KDEL (Table 2). HUAECs that expressed low levels of IL-4R (<50 sites/cell) were not sensitive to IL-4 toxin (IC_{50} = >1000 ng/ml). The protein synthesis inhibitory effect of IL-4 toxin was blocked by an excess of IL-4 (2 μg/ml) and IL-13 (2 μg/ml) in KS248 and NCB-59 cells, although IL-4 was better at blocking cytotoxicity than IL-13 (Fig. 5). These findings suggest that IL-4(38-37)-PE38KDEL exerts its cytotoxic effect specifically through IL-4R, and that IL-4R interacts with IL-13; this has also been shown by binding and cross-linking studies (Figs. 2 and 3A).

CLONOGENIC ASSAY. Since the AIDS-KS cells studied were not tumorigenic in immunosuppressed mice, the cytotoxic activity of IL-4(38-37)-PE38KDEL on two of the AIDS-KS cells (KS248 and NCB-59) was tested by clonogenic assay (37,38). Clonogenic assays in vitro correlates well with in vivo malignant phenotype in xenografts (49). Five hundred AIDS-KS cells were plated on 100-mm petri dishes and treated with various concentrations of either IL-4 or IL-4(38-37)-PE38KDEL. After 10 days of incubation, the percent ratios of colonies formed in control and treated groups were compared. Both IL-4 and IL-4-toxin inhibited colony formation in a concentration-dependent manner in both AIDS-KS cells studied (Table 3). IL-4 induced modest inhibition (27–34%) in colony formation in both AIDS-KS (KS248 and NCB-59) cells. HUVECs were also found to be sensitive to IL-4 and had modest growth inhibitory effects. Similar to the cytotoxicity of toxin assessed by protein synthesis inhibition assay, IL-4(38-37)-PE38KDEL exhibited a marked inhibition in colony formation in a concentration-dependent manner. IL-4-toxin completely suppressed colony formation at 100 ng/ml in both AIDS-KS cells, as shown in Fig. 6. The IC_{50} of IL-4(38-37)-PE38KDEL on KS248 and NCB-59 were found to be as low as 3.5 and 4.9 ng/ml, respectively, which are in very close agreement with the IC_{50}s obtained in protein synthesis inhibition assay (Table 2). However, HUVECs that express few IL-4R (<50 sites/cell) were 40- to 60-fold less susceptible to toxin, with <50% cell death even at a very high concentration of toxin (200 ng/ml). These results suggest that IL-4(38-37)-PE38KDEL has a low toxicity on human endothelial cells, a possible precursor to AIDS-KS cells, because there is low-level expression of IL-4R on these cells.

TABLE 2. Cytotoxicity of IL-4(38-37)-PE38KDEL in AIDS-KS cells by protein synthesis inhibition assay

Cell type	IC_{50} (ng/ml) Mean ± S.D.[a]
AIDS-KS	
KS248	1.7 ± 0.5
NCB-59	6.2 ± 3.5
KS54A	41[b]
KS220B	42.5 ± 17.5
ARL-13	64.5 ± 6.0
Normal endothelial cells	
HUAEC	>1000[c]

For cytotoxic assay, 10^4 cells/well were cultured in 96-well microtiter plates with different concentrations (0.1 to 1000 ng/ml) of IL-4(38-37)-PE38KDEL, as detailed in Materials and Methods.

[a] IC_{50}, the concentration of IL-4 toxin at which 50% inhibition of protein synthesis occurred, was calculated from the graphs generated from the experimental data explained in Materials and Methods. The data are shown as the mean ± S.D. of two to five experiments done for each cell type.

[b] IC_{50} was determined from a single experiment.

[c] IC_{50} could not be achieved even at 1000 ng/ml concentration of IL-4 toxin.

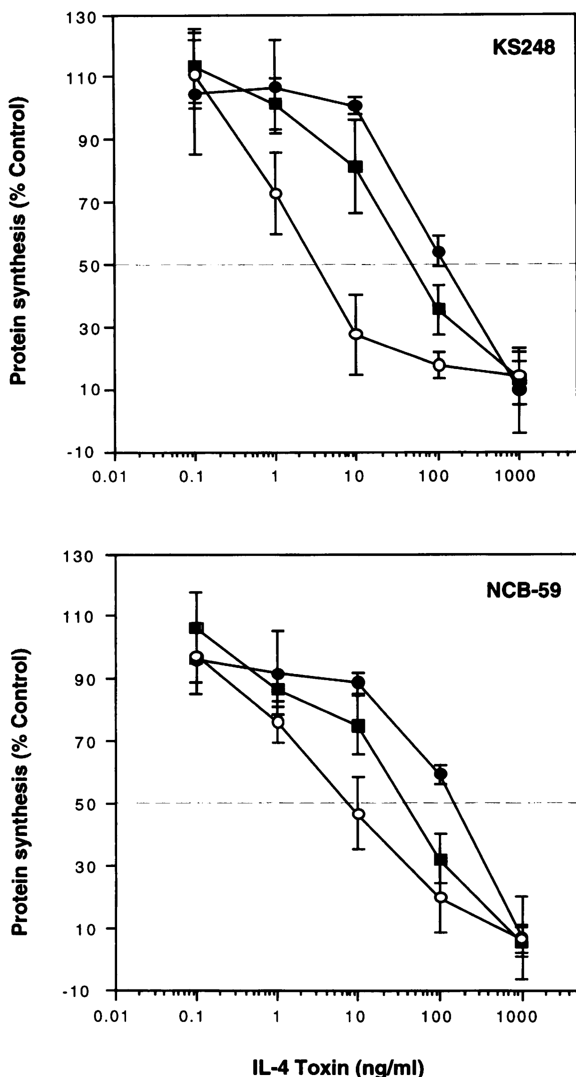

FIG. 5. Cytotoxicity of IL-4(38-37)-PE38KDEL on AIDS-KS cells

KS248 and NCB-59 cells (10^4 cells of each) were cultured in 96-well microtiter plates with the indicated concentration of IL-4 toxin in the absence (○) and presence of 2 μg/ml of either IL-4 (●) or IL-13 (■). After 20 hr, cells were pulsed with [^{3}H]leucine (1 μCi per well) for 4 hr and harvested to determine the inhibition in the protein synthesis. Error bars represent the S.D. of quadruplicate determination.

TABLE 3. AIDS-KS cell growth inhibition by IL-4 and IL-4(38-37)-PE38KDEL in clonogenic assay

	Colonies ± S.D. (% of control)		
	KS-248 (AIDS-KS)	NCB-59 (AIDS-KS)	HUVEC (normal endothelial)
IL-4 (ng/ml)			
1	82 ± 1.9	89 ± 1.9	89 ± 5.5
10	79 ± 1.9	79 ± 3.6	79 ± 6.1
100	78 ± 5.7	75 ± 2.4	82 ± 2.5
1000	73 ± 4.1	66 ± 1.5	80 ± 1.5
IL-4(38-37)PE38KDEL (ng/ml)			
1	71 ± 5.3	65 ± 3.0	97 ± 7.7
5	32 ± 2.3	49 ± 2.8	74 ± 6.0
50	5 ± 1.2	10 ± 0.6	65 ± 3.3
100	0	0	66 ± 7.2
200	0	0	51 ± 7.1

KS248 or NCB-59 (500 cells of each) and HUVECs (200 cells) were plated in triplicate in 100-mm or 60-mm petri dishes, respectively, and treated with either IL-4 or IL-4 toxin on the subsequent day. The culture dishes were incubated for 8 to 10 days and then washed with PBS and colonies stained with crystal violet. The colonies consisting of >50 cells were scored. The results are presented as a percentage of the colony counts in control and treated groups.

DISCUSSION

In this study, we have demonstrated that AIDS-KS-derived spindle cells express high-affinity IL-4R on the cell surface and at the mRNA level. These receptors are functional, since IL-4 inhibited the cell growth, albeit modestly, as measured by clonogenic assay. The growth of tumor cells was inhibited in a dose-dependent manner with a maximum of 34% inhibition in NCB-59 cells (Table 3). Similarly, IL-4 also inhibited colony formation of HUVECs. We next attempted to characterize the subunit structure of IL-4R in AIDS-KS cells. The IL-4R on these cells appeared to be mainly composed of two major proteins migrating at 140 and 60–70 kDa. All IL-4 cross-linked bands disappeared when cross-linking was performed in the presence of an excess of IL-4, indicating that these proteins are specific IL-4 binding proteins. The immunoprecipitation of IL-4R proteins revealed the same pattern of bands as observed in direct cross-linking. The predominant 140 kDa IL-4R protein has been well characterized (50) and designated as IL-4Rβ (26); however, the identity of the 60–70 kDa protein is still ambiguous. The 70 kDa protein was previously reported to be a proteolytically degraded subunit of a larger, 130 kDa protein (42,51). However, later studies suggest that p70 is a distinct protein and not a degradation product of p140 (52). Galizzi et al. (44) observed the

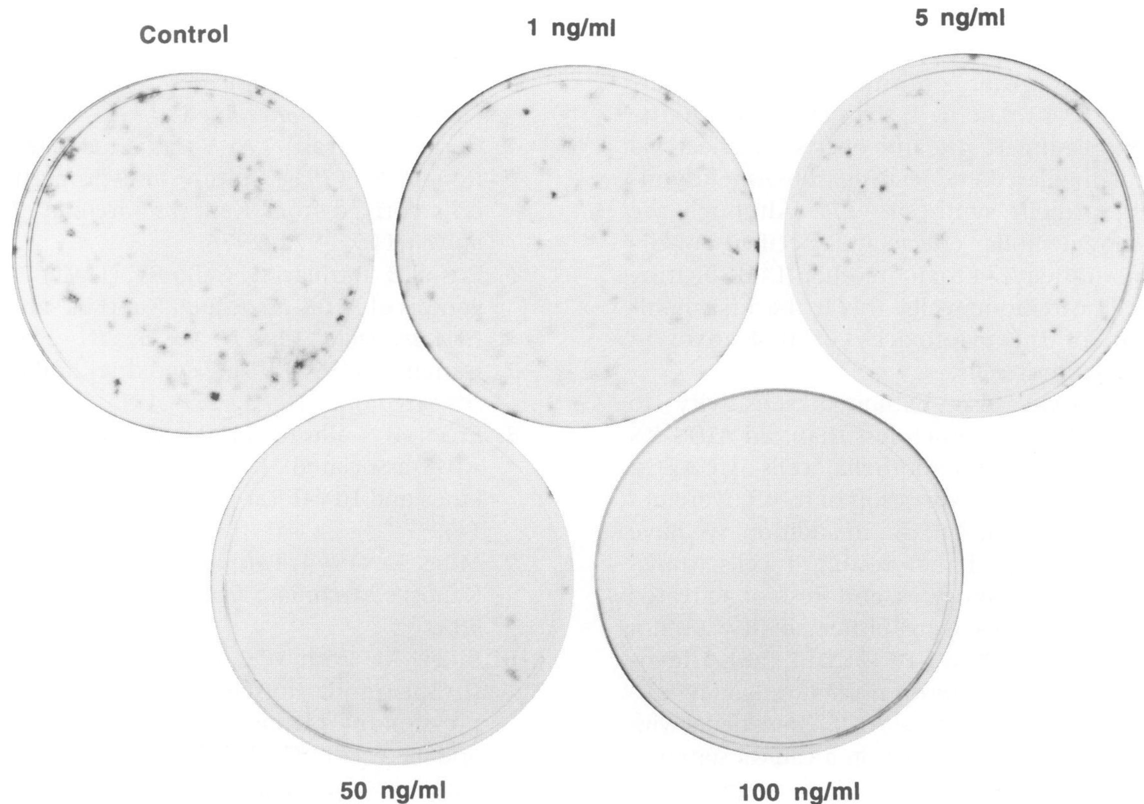

FIG. 6. Inhibition of colony formation of AIDS-KS cells by IL-4(38-37)-PE38KDEL.
KS248 cells (500) were plated in 100-mm petri dishes and exposed with the indicated concentration of IL-4(38-37)-PE38KDEL as performed for Table 3. The control cells formed 218 ± 8 (mean ± S.D.) colonies with the cloning efficiency of 43.6%. Similar results were obtained for NCB-59 cells (not shown). For illustration purposes, one out of three plates is chosen for each concentration.

expression of a 70 kDa subunit along with a 140 kDa protein in B cells. We have also reported the presence of a 70 kDa protein as a subunit of IL-4R in human renal cell carcinoma (53), colon carcinoma (26), and B-lymphoblastoid cells (54). We considered the possibility that the 60–70 kDa protein includes IL-2R γ chain because it has been shown to be a component of the IL-4R system in immune cells (26,46). However, we were unable to show γc expression by cross-linking and Northern analyses. RT-PCR analysis showed a very faint band that may be an experimental artifact. These data suggest that γc is not a predominant component of the IL-4R complex in AIDS-KS cells. The identity of all proteins in the 60–70 kDa band is still not clear. Further studies will delineate the composition of the IL-4R complex in AIDS-KS cells.

It is interesting to note that IL-13, a related cytokine to IL-4, also partially inhibited the binding as well as the cross-linking of ^{125}I-IL-4 to AIDS-KS cells (Figs. 2 and 3). These data agree with our recent report in which IL-13 competed for the binding of IL-4 to its receptors on renal cell carcinoma, colon carcinoma, and brain tumor cells (26,35,41). Our results are also consistent with another report of the displacement of IL-4 binding by IL-13 in TF-1 cells, suggesting that IL-4R and IL-13R share a novel component(s) that functions in signal transduction (40).

We further exploited the presence of IL-4R on AIDS-KS cells by targeting them with IL-4-fused cytotoxin, IL-4(38-37)-PE38KDEL. The IL-4 toxin was extremely to highly cytotoxic to all AIDS-KS cells, with the IC_{50} ranging from 1.7 to 65 ng/ml. The IC_{50} of IL-4(38-37)-PE38KDEL on KS248 (1.7 ng/ml) and NCB-59 (6.2 ng/ml) obtained by protein synthesis inhibition assay corroborated well with the IC_{50} observed in a clonogenic assay (3.5 and 4.9 ng/ml, respectively). The specificity of action of the toxin was well documented by the abrogation of its cytotoxicity

by the excess of IL-4. Interestingly, IL-13 also partially blocked the cytotoxicity of IL-4 toxin (Fig. 4) and further confirmed that IL-13 interacts with IL-4R. The IL-4 toxin binds only to the cells expressing IL-4R and exerts its effect via receptor-mediated endocytosis, subsequently inhibiting protein synthesis (27). Although we have shown similar effects and specificity of circular permuted IL-4 toxin in other IL-4R-bearing renal cell carcinoma cells, this is the first manifestation of the cytotoxicity of IL-4 toxin in AIDS-KS tumor cells.

IL-4 toxin also exhibited sensitivity to HUVECs, although much less than did AIDS-KS isolates. The other endothelial cells, HUAECs, which also lack the expression of IL-4R, were not at all sensitive to IL-4 toxin. In addition, we have previously reported that resting T cells, transformed B cells, monocytes, and fresh or activated bone marrow cells were either slightly or not sensitive to IL-4(38-37)-PE38KDEL (28). The insensitivity of IL-4 toxin on normal cells may provide an opportunity to treat AIDS-KS patients with a low concentration of toxin in a clinical setting.

We have shown that IL-4 receptors on AIDS-KS cells represent a new plasma membrane protein for targeting potent, Pseudomonas exotoxin–based, cytotoxic agents. Further studies are warranted to examine the expression of IL-4 receptors in fresh AIDS-KS tumors as well as to evaluate the in vivo anti-tumor activity of IL-4 toxin.

ACKNOWLEDGMENTS

We thank Drs. T. Murata and T. Eggerman for reading this manuscript, Dr. Nicholas Obiri for suggestions and providing radiolabeled IL-4, and Pam Dover for sustained technical help. We also thank Dr. T. Murata for helping us with designing RT-PCR experiments. Part of this work was done while S. R. Husain held a National Research Council-National Institute of Health, Center for Biologics Evaluation and Research, Senior Research Associateship.

REFERENCES

1. Freidman-Kien AE (1981) Disseminated Kaposi's sarcoma syndrome in young homosexual men. *J. Am. Acad. Dermatol.* **5:** 468–471.
2. Gill PS (1991) Pathogenesis of AIDS related malignancies. *Curr. Opin. Oncol.* **3:** 867–871.
3. Arms J (1989) A review of Kaposi's sarcoma. *Adv. Cancer Res.* **53:** 73–87.
4. Werner S, Hofschneider PH, Roth WK (1984) *Int. J. Cancer* **43:** 1137–1144.
5. Corbeil J, Evans LA, Vasak E, Cooper DA, Ronald P (1991) Culture and properties of cells derived from Kaposi's sarcoma. *J. Immunol.* **146:** 2972–2976.
6. Ensoli B, Barillari G, Gallo RC (1991) Pathogenesis of AIDS-associated Kaposi's sarcoma. *Hematol. Oncol. Clin. N. Am.* **5:** 281–295.
7. Ganem D (1995) Virus, cytokines and Kaposi's sarcoma. *AIDS* **5:** 469–471.
8. Ensoli B, Gallo RC (1992) Growth factors in AIDS-associated Kaposi's sarcoma: Cytokines and HIV-1 Tat protein. *AIDS Updates* **5:** 1–7.
9. Miles SA (1994) Pathogenesis of HIV-related Kaposi's sarcoma. *Curr. Opin. Oncol.* **6:** 497–502.
10. Stürzl M, Roth WK, Brockmeyer NH, Zietz C, Speiser B, Hofschneider PH (1992) Expression of platelet-derived growth factor and its receptor in AIDS-related Kaposi's sarcoma *in vivo* suggests paracrine and autocrine mechanisms of tumor maintenance. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 7046–7050.
11. Pistritto G, Ventura L, Mores N, Lacal PM, D'Onfrio C (1994) Regulation of PDGF-B and PDGF receptor expression in the pathogenesis of Kaposi's sarcoma in AIDS. In: Giraldo G, Salvatore M, Bianchi LC, Giraldo EB, (eds) *Diagn. Treat. AIDS Oncol. Adv. Tech. Res.* **46:** 73–87.
12. Hermans P, Gori A, Lemone M, Franchioly P, Clumeck N (1994) Possible role of granulocyte-macrophage colony stimulating factor (GM-CSF) on the rapid progression of AIDS-related Kaposi's sarcoma lesions in vivo. *Br. J. Haematol.* **87:** 413–414.
13. Murakami-Mori K, Taga T, Kishimoto T, Nakamura S (1995) AIDS-associated Kaposi's sarcoma (KS) cells express oncastatin-M (OM)-specific receptor but not leukemia inhibitory factor/OM receptor or interleukin-6 receptor. *J. Clin. Invest.* **96:** 1319–1327.
14. Ensoli B, Nakamura S, Salahuddin SZ, et al. (1989) AIDS-Kaposi's sarcoma-derived cells express cytokines with autocrine and paracrine growth factors. *Science* **243:** 223–226.
15. Paul WE (1991) Interleukin 4: A prototypic immunoregulatory lymphokine. *Blood* **77:** 859–1870.
16. Puri RK, Siegel JP (1993) Interleukin-4 and cancer therapy. *Cancer Invest.* **11:** 473–486.

17. Puri RK (1995) Structure and functions of interleukin 4 and its receptors. In: Kurzock R, Talpaz M. (eds) *Cytokines: Interleukins and Their Receptors.* Kluwer Academic Publishers, Norwell, MA, pp. 143–185.
18. Obiri NI, Hillman GG, Haas GP, Sudha S, Puri RK (1993) Expression of high affinity interleukin-4 receptors on human renal cell carcinoma cells and inhibition of tumor cell growth in vitro by interleukin 4. *J. Clin. Invest.* **91:** 83–89.
19. Obiri NI, Siegel JP, Varricchio F, Puri RK (1994) Expression of high-affinity IL-4 receptors on human melanoma, ovarian and breast carcinoma cells. *Clin. Exp. Immunol.* **95:** 148–155.
20. Taylor CW, Crogan TM, Salmon SE (1990) Effects of IL-4 on the in vitro growth of human lymphoid and plasma cell neoplasm. *Blood* **75:** 1114–1118.
21. Herrmann F, Andreeff M, Gruss HJ, Brach MA, Lubbert M, Martelsmann R (1991) Interleukin-4 inhibits growth of multiple myeloma by suppressing IL-6 expression. *Blood* **78:** 2070–2074.
22. Toi M, Bicknell R, Harris AL (1992) Inhibition of colon and breast carcinoma cell growth by interleukin 4. *Cancer Res.* **52:** 275–279.
23. Clerici M, Hakim FT, Venzom DJ, et al. (1993) Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. *J. Clin. Invest.* **93:** 759–765.
24. Park LS, Friend D, Sassenfeld HM (1987) Characterization of human B cell stimulatory factor 1 receptor. *J. Exp. Med.* **166:** 476–488.
25. Ohara J (1989) Interleukin 4: Molecular structure and biochemical characteristics, biological function and receptor expression. In Cruse JM, Lewis Jr. RE (eds) *The Year in Immunology. Immunoregulatory Cytokines and Cell Growth.* Basil Karger, pp 126–159.
26. Murata T, Noguchi PD, Puri RK (1995) Receptors for interleukin (IL)-4 do not associate with the common γ chain, and IL-4 induces the phosphorylation of JAK2 tyrosine kinase in human colon carcinoma cells. *J. Biol. Chem.* **270:** 30829–30836.
27. Kreitman RJ, Puri RK, Pastan I (1995) Increase antitumor activity of circularly permuted interleukin 4-toxin in mice with interleukin 4 receptor-bearing human carcinoma. *Cancer Res.* **55:** 3357–3363.
28. Puri RK, Leland P, Obiri NI, et al. (1996) An improved circularly permuted interleukin 4-toxin is highly cytotoxic to human renal cell carcinoma cells. *Cell. Immunol.* **171:** 80–86.
29. Kreitman RJ, Puri RK, Pastan I (1994). A circularly permuted recombinant interleukin 4 toxin with increased activity. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 6889–6893.
30. Puri RK, Debinski W, Obiri NI, Kreitman R, Pastan I (1994) Human renal cell carcinoma are sensitive to the cytotoxic effect of a chimeric protein composed of human interleukin-4 and Pseudomonas exotoxin. *Cell. Immunol.* **154:** 369–379.
31. Puri RK, Leland P, Kreitman RJ, Pastan I (1994) Human neurological cancer cell express interleukin-4 (IL-4) receptors which are targets for the toxic effects of IL-4 Pseudomonas exotoxin chimeric protein. *Int. J. Cancer* **58:** 574–581.
32. Debinski W, Puri RK, Kreitman RJ, Pastan I (1993) A wide range of human cancers express interleukin 4 (IL-4) receptors that can be targeted with chimeric toxin composed of IL-4 and Pseudomonas exotoxin. *J. Biol. Chem.* **268:** 14065–14070.
33. Debinski W, Puri RK, Pastan I (1994) Interleukin-4 receptors expressed on tumors may serve as target for anticancer therapy using chimeric Pseudomonas exotoxin. *Int. J. Cancer* **58:** 744–748.
34. Masood R, Husain SR, Rahman A, Gill P (1993) Potentiation of cytotoxicity of Kaposi's sarcoma related to immunodeficiency syndrome (AIDS) by liposome-encapsulated doxorubicin. *AIDS Res. Hum. Retroviruses* **8:** 741–746.
35. Obiri NI, Debinski W, Leonard WJ, Puri RK (1995) Receptors for interleukin 13: Interaction with interleukin 4 by a mechanism that does not involve the common γ chain shared by receptors for interleukin 2, 4, 7, 9 and 15. *J. Biol. Chem.* **270:** 8797–8804.
36. Reinecker HC, Podolsky DK (1995) Human intestinal epithelial cells express functional cytokine receptors sharing the common gamma C chain of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. U.S.A.* **92:** 8353–8357.
37. Yoshida D, Piepmeier J, Weinstein M (1994) Estramustine sensitizes human glioblastoma cells to irradiation. *Cancer Res.* **54:** 1415–1417.
38. Puri RK, Leland P, Obiri NI, et al. (1996) Targeting of interleukin-13 receptor on human renal cell carcinoma cells by a recom-

binant chimeric protein composed of interleukin-13 and a truncated form of Pseudomonas exotoxin. *Blood* **87:** 4333–4339.
39. Munson PJ, Rodbard D (1980) Ligand: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107:** 220–239.
40. Zurawski SM, Vega F, Hughe B, Zurawski G (1993) Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that function in signal transduction. *EMBO J.* **12:** 2663–2670.
41. Obiri NI, Leland P, Murata T, Debinski W, Puri RK (1997) The interleukin 13 receptor structure differs on various cell types and may share more than one component with interleukin 4 receptor. *J. Immunol.* **158:** 756–764.
42. Keegan AD, Beckman MP, Park LS, Paul WE (1991) The IL-4 receptor: Biochemical characterization of IL-4 binding molecules in a T cell line expressing large number of receptors. *J. Immunol.* **146:** 2272–2279.
43. Galizzi JP, Zuber CE, Cabrillat H, Djossou D, Banchereau J (1989) Internalization of human interleukin-4 and transient down regulation of its receptor in the CD23 inducible Jijoye cells. *J. Biol. Chem.* **264:** 6984–6989.
44. Galizzi JP, Zuber CE, Cabrillat H, et al. (1990) Purification of 130 kDa T cell glycoprotein which binds human interleukin-4 with high affinity. *J. Biol. Chem.* **265:** 439–444.
45. Foxwell BMJ, Woerly G, Ryffel B (1989) Identification of interleukin 4 receptor-associated proteins and expression of both high and low affinity binding on human lymphoid cells. *Eur. J. Immunol.* **19:** 1637–1641.
46. Russell SM, Keegan AD, Harada N, et al. (1993) Interleukin-2 receptor gamma chain: A functional component of the interleukin-4 receptor. *Science* **262:** 1880–1883.
47. Vita N, Lefort S, Laurent P, Caput D, Ferrara P (1995) Characterization and comparison of the interleukin 13 receptor with the interleukin 4 receptor on several cell types. *J. Biol. Chem.* **270:** 3512–3517.
48. Noguchi M, Aldestein S, Cao X, Leonard W (1994) Interleukin-2 receptor gamma chain: A functional component of the interleukin-7 receptor. *Science* **263:** 1453–1454.
49. Freedman VH, Shin SI (1974) Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* **3:** 355–359.
50. Mosley B, Beckmann P, March CJ, et al. (1989) The murine interleukin-4 receptor: Molecular cloning and characterization of secreted and membrane bound forms. *Cell* **59:** 335–348.
51. Keegan AD, Nelms K, Wang L.-M, Pierce JH, Paul WE (1994) Interleukin 4 receptor: Signaling mechanism. *Immunol. Today* **15:** 423–432.
52. Harada N, Yang G, Miyajima A, Howard M (1992) Identification of an essential region for growth signal transduction in the cytoplasmic domain of the human interleukin-4 receptor. *J. Biol. Chem.* **267:** 22752–22758.
53. Obiri NI, Puri RK (1994) Characterization of interleukin-4 receptors expressed on human renal cell carcinoma cell. *Oncol. Res.* **6:** 419–427.
54. Husain SR, Leland P, Aggarwal BB, Puri RK (1996) Transcriptional regulation of interleukin 4 receptors by human immunodeficiency virus type 1*tat* gene. *AIDS Res. Hum. Retroviruses* **12:** 1349–1359.

Communicated by I. Pastan. Accepted March 26, 1997.