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Immunology

Cytotoxic activity of a recombinant fusion protein between interleukin 4 and *Pseudomonas* exotoxin

(lymphokine/growth factor/immunology/bone marrow)

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ABSTRACT **A recombinant chimeric toxin in which the cell binding domain of *Pseudomonas* extotoxin (PE) was replaced by murine interleukin 4 (IL-4) was produced in *Escherichia coli*. This chimeric protein, IL-4–PE40, was cytotoxic to murine IL-4 receptor-bearing cell lines but had little effect on human cell lines lacking receptors capable of binding murine IL-4. A mutant form of IL-4–PE40 (termed IL-4–PE40 asp^{553}) with very low ADP-ribosylating activity displayed mitogenic activity similar to that of IL-4 rather than cytotoxic activity. Because the cytotoxic effects of IL-4–PE40 were blocked by excess IL-4 or by neutralizing antibody to IL-4 (11B11), we conclude that the cytotoxic effect of IL-4–PE40 is specifically mediated through IL-4 receptors. IL-4–PE40 could be a useful reagent for specific elimination of cells bearing IL-4 receptors.**

Bacterial and plant toxins have been attached to growth factors (1–5) and antibodies (6, 7) to make cytotoxic reagents specific for certain types of eukaryotic cells. Our laboratory has used *Pseudomonas* exotoxin (PE) for these studies. PE is composed of three structural domains (8): domain Ia for cell recognition, domain II for translocation across membranes, and domain III for ADP-ribosylation of elongation factor 2, the step actually responsible for cell death (9). We have produced a noncytotoxic mutant form of PE, termed PE40, which lacks domain Ia (the cell binding domain) but retains translocation and ADP-ribosylating activity (9). By fusing the gene encoding PE40 to genes encoding transforming growth factor α (1), interleukin 2 (IL-2) (2), interleukin 6 (4), and CD4 (10), we have developed chimeric proteins specifically toxic to cells expressing receptors or binding proteins for these.

Interleukin 4 (IL-4, also called BSF-1) is a 20,000-Da protein produced by activated T lymphocytes and was first described as a growth factor for B lymphocytes (11). IL-4 also increases the expression of class II major histocompatibility complex (MHC) molecules (12, 13) and enhances the production of IgG1 and IgE (14–17). Moreover, IL-4 has been shown to act on certain non-B cells; IL-4 enhances the growth and/or differentiation of T cells (18–21), activates macrophages (22, 23), and increases the growth of granulocytes, mast cell, and erythrocyte colonies (23–25). IL-4 seems to have a wide range of biological activities, including the proliferation and differentiation of lymphocytes and hemopoietic cells. Though the cells of various lineages have been reported to have IL-4 receptors, activated T and B lymphocytes and certain tumor cell lines derived from B lymphomas, T leukemias, and mastocytomas (26–28) have relatively high numbers of IL-4 receptors. Depletion of cells bearing high numbers of IL-4 receptors by the chimeric protein IL-4–PE40 may have some value in studying the biological function of the IL-4/IL-4 receptor system and also in treating some immunological disorders or tumors.

In this report, we describe the construction of a chimeric gene in which the gene encoding PE40 was fused to a cDNA encoding murine IL-4. The fusion gene product, IL-4–PE40, was found to be highly toxic to a murine T-cell line (CT.4R) bearing about 15,000 IL-4 receptors (29) but to have no effect on human cell lines lacking receptors for murine IL-4. In addition, a chimeric protein composed of a mutant form of PE40 that had very low ADP-ribosylating activity (PE40 asp^{553}) was also prepared. IL-4–PE40 asp^{553} displayed mitogenic activity similar to that of IL-4 rather than cytotoxic activity, showing that ADP-ribosylating activity was essential for cytotoxicity and that IL-4–PE40 asp^{553} bound specifically to the IL-4 receptor. Because the cytotoxic effects of IL-4–PE40 were blocked by excess IL-4 or by a neutralizing monoclonal antibody to IL-4 (11B11), we conclude that the cytotoxic effect of IL-4–PE40 is specifically mediated through IL-4 receptors.

MATERIALS AND METHODS

Reagents. Chemicals and enzymes were described previously (2). For polymerase chain reaction (PCR), we used a DNA amplification reagent kit (no. N801-0043) from Perkin–Elmer/Cetus. Anti-IL-4 monoclonal antibody, 11B11 (30), was purchased from Tex Star Monoclonals (Dallas). Recombinant murine IL-4 was a gift from W. E. Paul (National Institutes of Health).

Plasmids, Bacterial Strains, and Cell Lines. Plasmid pVC8f(+)T, which carries domains II and III of the PE gene (PE40) from pVC8 (1), a T7 transcription terminator at the end of PE40 gene, and a f1 origin of replication, was constructed in this laboratory (V.K.C., unpublished). The plasmid carrying murine IL-4 cDNA was a gift from S. Gillis (Immunex, Seattle). Plasmid pVC45M, which carries a gene for PE with an asp^{553} mutation (ADP-ribosylating mutant), was described previously (31, 32). CT.4R is an IL-4-dependent murine T-cell line expressing about 15,000 IL-4 receptors (29). HUT 102 is a human T-cell leukemia and a gift from T. A. Waldmann (National Cancer Institute). CTLL is an IL-2-dependent murine T-cell line. P815 is a murine mastocytoma cell line. P3X63-Ag8.653 is a clone derived from murine plasmacytoma, MOPC-21, and obtained from the American Type Culture Collection. NIH 3T3, Swiss 3T3, and L929 are murine fibroblast cell lines. A431 and KB are human epidermoid carcinoma cell lines.

Plasmid Construction. Plasmid DNA was prepared and oligonucleotides were synthesized as described (2). The chimeric gene encoding IL-4–PE40 under the control of the T7 promoter was constructed as summarized in Fig. 1. First, we created an *Nde* I site at the 5′ and 3′ ends of the IL-4

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Abbreviations: IL-4, interleukin 4; IL-2, interleukin 2; PE, *Pseudomonas* exotoxin; MHC, major histocompatibility complex; PCR, polymerase chain reaction.

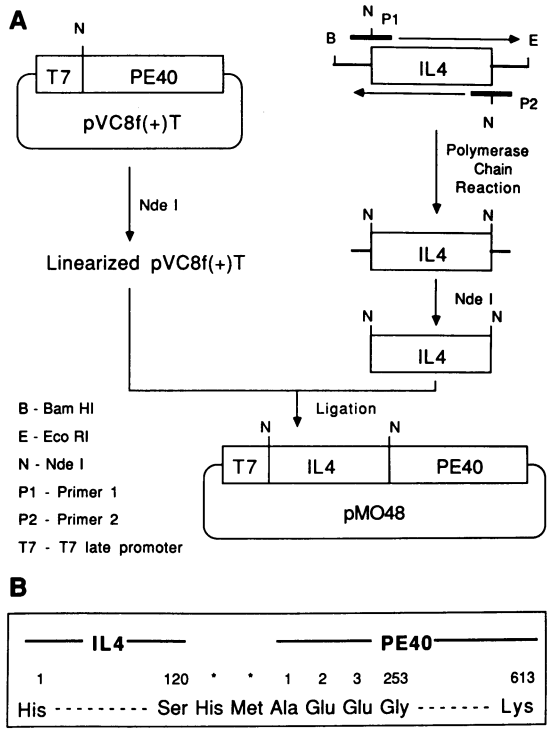

FIG. 1. Scheme for construction of expression plasmid pMO48, encoding IL-4–PE40.

(*A*) Scheme for construction of pMO48: Primer 1, 5′–CTTTCTCGAATGTACCCATATG CATATCCACGGATGCGACAAAAATC–3′: Primer 2, 5′–GTTAAAGCATGGTGGCTCACAT ATGCGAGTAATCCATTTGCATG-3′

The bases underlined were changed from that of IL-4 to create an *Nde* I site. (*B*) Abbreviated amino acid sequence of IL-4–PE40. Two amino acids (*) were added between IL-4 and PE40 to create an *Nde* I site.

coding sequence by PCR (33, 34) using primers with recognition sites for *Nde* I. As shown in the legend of Fig. 1, we synthesized two oligonucleotide primers: primer 1 is complementary to the 3′ region of antisense strand of IL-4 cDNA and primer 2 is complementary to the 3′ region of sense strand. In both primers, five bases were changed to create an *Nde* I site. After 25 cycles of PCR using primer 1 and primer 2 (1 μM) and a 0.55-kilobase (kb) *Bam*HI–*Eco*RI DNA fragment (0.1 ng per reaction) that contained the IL-4 coding sequence as the template, a 0.406-kb DNA fragment was amplified. After separation on a low-melting-point agarose gel, the 0.406-kb DNA was eluted and cut with *Nde* I, and then a 0.366-kb fragment was separated. The 0.366-kb *Nde* I fragment was subcloned into the *Nde* I site of pVC8f(+)T. The resulting plasmid had a 0.366-kb IL-4 coding gene in two orientations at the 5′ end of the PE40 gene. After restriction analysis, a plasmid with IL-4 in the proper orientation with respect to the PE40 gene was identified (pMO48). Plasmid pMO48M (IL-4–PE40 asp^{553}) was constructed by cleaving pMO48 with *Bam*HI and *Eco*RI and replacing the 0.46-kb fragment with a similar *Bam*HI–*Eco*RI fragment from pVC45M (30).

Expression and Localization of IL-4–PE40. *Escherichia coli* BL21 (λDE3) cells were transformed with the plasmid pMO48, cultured in 1 liter of LB broth with ampicillin (100 μg/ml) up to OD_{650} = 0.5, and induced with 1 mM isopropyl β-D-thiogalactoside for 90 min. The separation of different compartments of cells was described previously (4).

Gel Electrophoresis and Immunoblotting. SDS/PAGE on 10% gels was performed as described by Laemmli (35). The gels were stained with Coomassie blue. For immunoblotting, electrophoresed samples were transferred to nitrocellulose paper and processed by using rabbit anti-PE antiserum as described (9).

Purification of IL-4–PE40. The purification protocol for IL-4–PE40 was essentially the same as described (4). Briefly, the pellet containing inclusion bodies was prepared by centrifugating the sonicated spheroplasts. This pellet, containing IL-4–PE40, was denatured in extraction buffer (7 M guanidine hydrochloride/100 mM Tris·HCl, pH 8.0/5 mM EDTA). After centrifugation at 40,000 rpm for 15 min, the supernatant containing denatured protein was rapidly diluted in 80 vol of buffer (150 mM NaCl/10 mM sodium phosphate, pH 7.4) and allowed to sit for 16 hr at 4°C for renaturation. After dialyzing against 20 mM Tris (pH 8.0), the samples were applied onto a Mono Q column (10 × 100 mm) and eluted with a 200-ml linear gradient of NaCl (0–500 mM in 20 mM Tris, pH 8.0); 4-ml fractions were collected and the absorbance at 280 nm was monitored. The fraction containing the peak cytotoxic activity (fraction 33) was applied onto a TSK G3000 (7.8 × 300 mm) gel filtration column and eluted with 0.2 M sodium phosphate (pH 7.0) containing 1 mM EDTA. The chimeric mutant protein, IL-4–PE40 asp^{553}, was expressed and purified in the same way as IL-4–PE40. It also was shown to have the same molecular mass as determined by SDS/PAGE (data not shown).

Protein Synthesis Inhibition Assays. The cytotoxic activity of IL-4–PE40 was tested on CT.4R cells. CT.4R cells were maintained in the RPMI 1640 medium containing 5% fetal calf serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 50 units of penicillin per ml, 50 μg of streptomycin per ml, 50 μg of gentamycin per ml, and 500 units of murine IL-4 per ml. For assay, cells were washed to remove IL-4 and plated in 96-well tissue culture plates at 8 × 10^3 cells in 100 μl, and various concentrations of IL-4–PE40 were added. Five hundred units of IL-2 per ml was also added to keep the cells growing during the assay period. For blocking or neutralizing experiments, IL-4–PE40 was premixed with competitors or neutralizing antibodies and then added to the cells. After a 2-day incubation at 37°C, cells were cultured with 2 μCi (1 Ci = 37 GBq) of [^{3}H]leucine for 4 hr, and the radioactivity incorporated into cells was measured as described (36). When IL-4–PE40 was tested on CTLL cells, the same protocol was used. For other cell lines, we used culture medium without IL-2 and different numbers of cells (HUT 102, and P3X63-Ag8.653, 1 × 10^4 cells per well; NIH 3T3, Swiss 3T3, L929, A431, and KB, 1.6 × 10^4 cells per well; P815, 8 × 10^3 cells per well).

Mitogenic Assay. CT.4R cells (5 × 10^3 cells in 200 μl) were cultured in the absence or presence of various amounts of recombinant IL-4 or IL-4–PE40 asp^{553}. After a 40-hr incubation, cells were incubated with [^{3}H]thymidine (0.5 μCi) for 6 hr, and then the radioactivity incorporated into the cells was measured.

RESULTS

Construction of Expression Plasmid Encoding IL-4–PE40. A DNA fragment encoding murine IL-4 was subcloned into the *Nde* I site of pVC8f(+)T (Fig. 1*A*). To do this, we first created *Nde* I sites at the 5′ and 3′ ends of the IL-4 coding sequence by PCR using primers with recognition sites for *Nde* I. After 25 cycles of PCR with these primers and a DNA template that contained the IL-4 coding sequence, the amplified DNA fragment acquired *Nde* I sites at both ends.

The resulting plasmid, pMO48, was expressed under the control of bacteriophage T7 late promoter. The chimeric protein, IL-4–PE40, is composed of 486 amino acids, in which a native IL-4 sequence of 120 amino acids is followed

by amino acids histidine, methionine, and 1–3 and 253–613 of PE (Fig. 1*B*).

Expression of IL-4–PE40. To express IL-4–PE40, *E. coli* BL21 (λDE3) cells were transformed with plasmid pMO48. After induction with isopropyl β-D-thiogalactoside, the cells were collected and processed as described in *Materials and Methods*. The new protein, migrating at 53 kDa, was readily detectable on SDS/PAGE of the total cell pellet (Fig. 2*A*, lane 2). The size of this protein corresponds to the expected size for IL-4–PE40, and immunoblotting analysis showed this protein reacted with *anti-PE* antibody (Fig. 2*B*, lane 2). The culture supernatant or the periplasm had negligible amounts of this protein. Separation of sonicated spheroplasts into cytoplasm and a pellet that contained inclusion bodies showed that the 53-kDa protein was mostly retained in the pellet (Fig. 2 *A* and *B*, lanes 4).

Purification of IL-4–PE40. Because a 53-kDa protein of the size expected for IL-4–PE40 was produced and because this protein reacted with antibodies to PE, we tentatively concluded that the 53-kDa protein was IL-4–PE40 and purified this protein. To do this, the inclusion bodies were denatured in 7 M guanidine and then renatured as described in *Materials and Methods*. The renatured protein was applied to a Mono Q ion-exchange column (Fig. 3*A*). Fraction 33 from the Mono Q column showed high cytotoxic activity on IL-4 receptor-bearing cells and was applied to a TSK G3000 gel filtration column (Fig. 3*B*). Most of the cytotoxic activity came in fractions 19 and 20 (Fig. 3*B*). As shown in Fig. 3*C*, fraction 19 of the TSK G3000 column contained a 53-kDa protein that was about 90% pure and this protein reacted with anti-PE antibody (Fig. 3*D*). This protein also had almost the same ADP-ribosyltransferase activity as an equal amount of PE40 (data not shown).

Protein Synthesis Inhibition by IL-4–PE40. To test the cytotoxic activity of IL-4–PE40, we used a murine T-cell line, CT.4R, that expresses around 15,000 IL-4 receptors. This cell line can grow in the presence of either IL-4 or IL-2 and was maintained in culture medium containing recombinant murine IL-4.

CT.4R cells were treated with various amounts of IL-4–PE40. IL-2 (500 units/ml) was added to the culture to keep CT.4R cells growing during the assay period. After 2 days, the level of protein synthesis was determined. As shown in Fig. 4*A*, IL-4–PE40 inhibited protein synthesis in CT.4R cells in a concentration-dependent manner. The concentration of IL-4–PE40 giving a 50% reduction of protein synthesis (ID_{50}) was 17 ng/ml. Conversely, the nonchimeric protein, PE40, which cannot bind to the IL-4 receptor, had little or no effect on protein synthesis (ID_{50} > 1000 ng/ml).

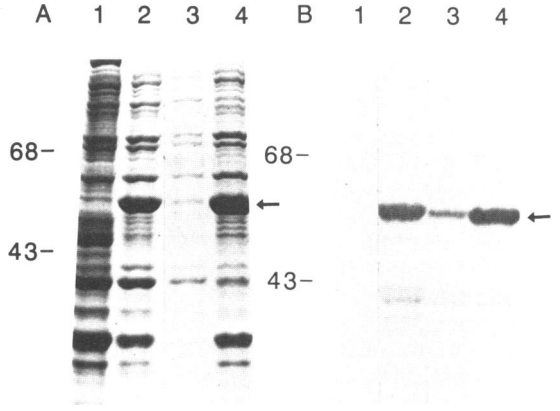

FIG. 2. Localization of IL-4–PE40 in *E. coli* BL21 (λDE3). *E. coli* BL21 (λDE3) cells were transformed by plasmid pMO48 and were processed as described in the text. (*A*) Coomassie blue-stained gel. (*B*) Immunoblotting with rabbit anti-PE antibody. Lanes 1, total cell pellet (nontransformed cells); lanes 2, total cell pellet (transformed cells); lanes 3, cytoplasm; lanes 4, 100,000 × *g* pellet of sonicated spheroplasts. Molecular masses are indicated in kDa. The arrow shows the new protein migrating at 53 kDa.

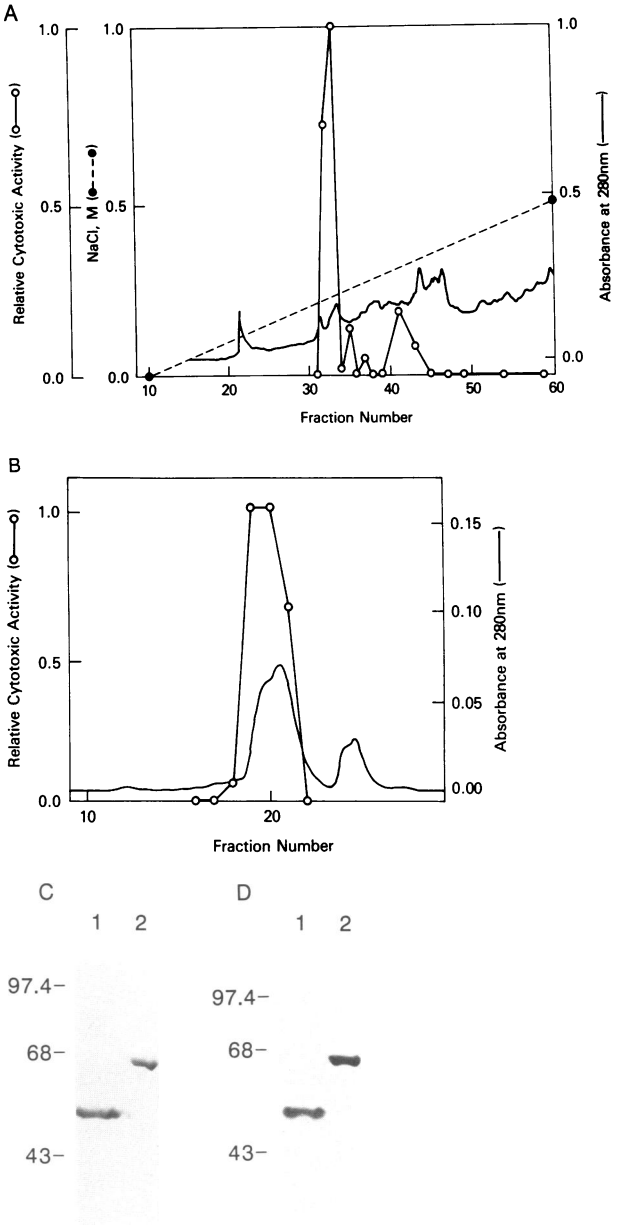

FIG. 3. Purification of IL-4–PE40. (*A*) Mono Q ion-exchange chromatography of IL-4–PE40; material from a 750-ml culture of *E. coli* BL21 (λDE3) was applied to a Mono Q column. (*B*) TSK G3000 gel filtration chromatography; fraction 33 of the Mono Q column (220 μg of protein) was applied to a TSK G3000 column. In *A* and *B*, cytotoxic activity is expressed as the relative ratio of peak activities from protein synthesis inhibition assays using CT.4R cells. Absorbance at 280 nm was measured. (*C* and *D*) SDS/PAGE of purified IL-4–PE40. (*C*) Coomassie blue-stained gel. (*D*) Immunoblotting with rabbit anti-PE antibody. Lanes 1, fraction 19 of TSK G3000 column; lanes 2, PE. Molecular masses are indicated in kDa.

ADP-Ribosylation Activity Is Essential for the Cytotoxic Effect of IL-4–PE40. The nature of the cytotoxic effect of IL-4–PE40 was further investigated by determining the cytotoxicity of IL-4–PE40 asp^{553}, a mutant form of this chimeric protein that has very low ADP-ribosylation activity (data not shown). As shown in Fig. 4*B*, IL-4–PE40 asp^{553} was found not to have any cytotoxic effect up to a concentration of 1000 ng/ml. Rather than inhibiting the synthesis of protein by CT.4R cells, IL-4–PE40 asp^{553} displayed a mitogenic activity similar to that of IL-4 (Fig. 5). Although the mitogenic

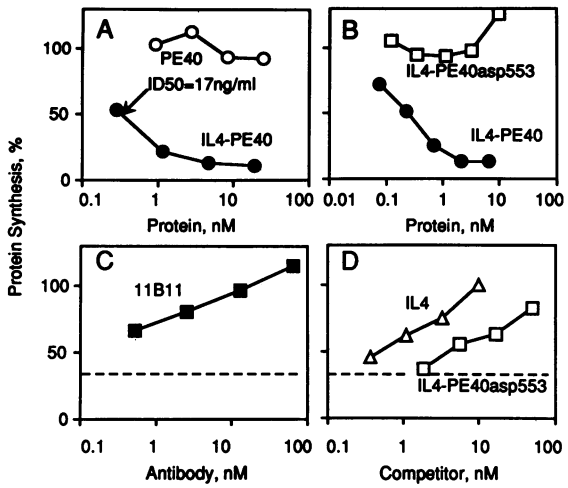

FIG. 4. Protein synthesis inhibition by IL-4–PE40. CT.4R cells (8×10^3 in 200 μl) were incubated in the culture medium containing IL-2 (50 units/ml). (*A* and *B*) Various amounts of IL-4–PE40, PE40, or IL-4–PE40 asp^{553} were added to the culture. (*C* and *D*) IL-4–PE40 (1.9 nM) and various amounts of anti-IL-4 antibody (11B11), IL-4, or IL-4–PE40 asp^{553} were added to the culture. After a 20-hr (*C* and *D*) or 40-hr (*A* and *B*) incubation, protein synthesis was measured. Results are expressed as the % of the value of cells incubated without toxin. The dotted line (*C* and *D*) shows the protein synthesis level without antibody or competitor (36%).

activity of IL-4–PE40 asp^{553} was less than that of IL-4 by a factor of $\approx$10, this result clearly showed that the IL-4 toxin retained substantial binding activity toward IL-4 receptors.

IL-4 Receptor-Mediated Cytotoxicity by IL-4–PE40. To demonstrate further that the cytotoxic activity of IL-4–PE40 was mediated by the IL-4 receptor, we used two other approaches. First, we examined the neutralizing effect of anti-IL-4 antibody, 11B11, on IL-4–PE40. 11B11 is a monoclonal antibody that can bind to IL-4 and inhibit IL-4 binding to the IL-4 receptor (26). As shown in Fig. 4*C*, 11B11 neutralized the cytotoxic effect of IL-4–PE40.

The second approach involved competition of the cytotoxic activity of IL-4–PE40 by either IL-4 or IL-4–PE40 asp^{553}. Both IL-4 and IL-4–PE40 asp^{553} blocked the cytotoxic effect of IL-4–PE40 (Fig. 4*D*). IL-4 was about 10-fold more effective in blocking the cytotoxic effect of IL-4–PE40 than IL-4–PE40 asp^{553}. These results clearly showed that the binding of IL-4–PE40 to IL-4 receptors is essential for its cytotoxic effect.

Cytotoxic Effect of IL-4–PE40 on Various Cell Lines. The effect of IL-4–PE40 on cell lines lacking receptors for murine IL-4 was examined to demonstrate further the specificity of the cytotoxic effect. HUT 102 is a human T-cell leukemia cell line. It is well known that murine IL-4 does not bind to human

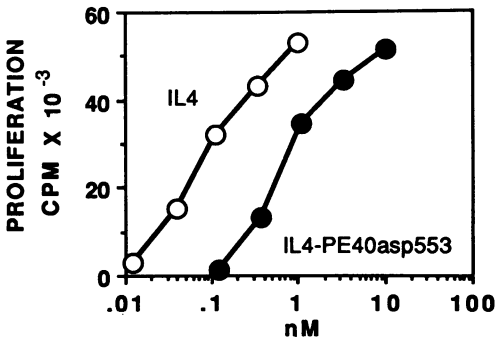

FIG. 5. Mitogenic effect of IL-4–PE40 asp^{553} on CT.4R cells. CT.4R cells (5×10^3 cells in 200 μl) were incubated in the presence of various amounts of recombinant IL-4 or IL-4–PE40 asp^{553}. After a 40-hr incubation, [^{3}H]thymidine incorporation was measured.

Table 1. Cytotoxic activity of IL-4–PE40

Cell line	ID_{50}, ng/ml		
	IL-4–PE40	IL-4–PE40 asp^{553}	PE40
CT.4R	17	>1000	>1000
CTLL	250	>1000	1000
P3X63-Ag8.653	12	>1000	>1000
P815	20	>1000	>1000
NIH 3T3	>1000	>1000	>1000
Swiss 3T3	420	>1000	>1000
L929	350	>1000	>1000
HUT 102	>1000	ND	ND
A431	>1000	ND	ND
KB	>1000	ND	ND

Protein synthesis level was measured after 2 days of incubation with toxin. ID_{50} is the protein concentration required to inhibit protein synthesis by 50%. ND, not done.

cells (26, 27). We first tested this cell line because it had been shown previously to be sensitive to another chimeric toxin, IL-2–PE40 (2). As shown in Table 1, IL-4–PE40 had very little or no cytotoxic effect on HUT 102 cells (ID_{50} > 1000 ng/ml). We also tested two other human cell lines, A431 and KB, and IL-4–PE40 was not cytotoxic to them. On the contrary, IL-4–PE40 was cytotoxic to murine cell lines, CTLL (a T-cell line) and P815 (a mastocytoma cell line), that had been reported to possess IL-4 receptors (26, 27). IL-4–PE40 was also cytotoxic to a murine myeloma cell line, P3X63-Ag8.653. IL-4–PE40 had weak cytotoxic effects on two murine fibroblast cell lines, Swiss 3T3 and L929, but had little or no effect on NIH 3T3. The cytotoxic activity of IL-4–PE40 to CTLL, P815, Swiss 3T3, and L929 was neutralized by anti-IL-4 antibodies (11B11) (data not shown). IL-4–PE40 asp^{553} or PE40 lacking ADP-ribosylating activity or cell binding domain, respectively, had very low effects on all cell lines listed in Table 1. These results confirm the specific cytotoxicity of IL-4–PE40.

DISCUSSION

We have produced a chimeric protein IL-4–PE40, by fusing a gene encoding murine IL-4 to a gene encoding domains II and III of PE. IL-4–PE40 was highly cytotoxic to the murine T-cell clone CT.4R, bearing about 15,000 IL-4 receptors, and was nontoxic to human cell lines lacking receptors for murine IL-4. The cytotoxic effect of IL-4–PE40 on CT.4R was mediated by IL-4 receptor because PE40, a nonchimeric protein that cannot bind to the IL-4 receptor, showed no cytotoxicity and because an excess amount of IL-4 or a neutralizing monoclonal antibody (11B11) to IL-4 blocked the cytotoxicity of IL-4–PE40. We also investigated the cytotoxicity of a chimeric protein, IL-4–PE40 asp^{553}, in which glutamic acid at position 553 was changed to aspartic acid. This change causes a large reduction of ADP-ribosylating activity to <1% of that of IL-4–PE40 (data not shown). IL-4–PE40 asp^{553} was not cytotoxic to CT.4R cells up to a concentration of 1000 ng/ml. This indicates that the ADP-ribosylating activity is essential for the cytotoxicity of IL-4–PE40. Moreover, IL-4–PE40 asp^{553} displayed mitogenic activity similar to that of IL-4, although IL-4–PE40 asp^{553} was less active than IL-4 by a factor of $\approx$10. IL-4–PE40 asp^{553} was also less active than IL-4 by a factor of $\approx$10 in blocking the cytotoxicity of IL-4–PE40. All together these results showed that the chimeric protein between IL-4 and PE40 possessed a reasonably high affinity for the IL-4 receptor and had biological activity similar to that of IL-4.

Various lineages of cells including lymphocytes and hemopoietic cells at various differentiation stages have been reported to express IL-4 receptors (26–28). However, little is known about the biological role of the cells bearing high

number of IL-4 receptors. To investigate such questions, IL-4–PE40 could be useful by depleting cells with high numbers of IL-4 receptors. Another possible use of IL-4–PE40 may be suppressing immune responses. It has been reported that activation of B and T cells with mitogen or anti-IgM antibody produces a 5- to 10-fold increase in IL-4 receptor number (26–28). IL-4–PE40 could be immunosuppressive by depleting these activated lymphocytes. It may also be possible to use IL-4–PE40 for the treatment of certain tumors because it has been reported that certain tumor cell lines derived from B lymphomas, T leukemias, and mastocytomas have relatively high number of IL-4 receptors (26–28). For these purposes, it will be important to determine how many receptors on a cell are necessary for killing by IL-4–PE40 because the sensitivity of cells to growth factor–toxin fusion proteins seems to be largely dependent on the number of receptors on a cell (36).

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