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Immunology

Cytotoxic activity of an interleukin 6–*Pseudomonas* exotoxin fusion protein on human myeloma cells

(cancer/chemotherapy/lymphokines/growth factors)

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ABSTRACT **A chimeric toxin composed of human interleukin 6 (IL-6) attached to a portion of *Pseudomonas* exotoxin (PE) devoid of its own cell recognition domain has been produced in *Escherichia coli*. The fusion protein (IL-6–PE40) is cytotoxic to a human myeloma cell line expressing IL-6 receptors but has no effect on IL-6 receptor-negative cells. The specificity of IL-6–PE40 cytotoxicity was demonstrated through competition with excess IL-6 and neutralization with an antibody to IL-6. IL-6–PE40 may be useful in the selective elimination of myeloma cells and other cells with high numbers of IL-6 receptors.**

The bacterial toxin *Pseudomonas* exotoxin A (PE) is composed of three structural domains with distinct functions (1). Domain I binds to the PE receptor on the surface of target cells, domain II is responsible for the translocation of the toxin into the cell cytoplasm, and domain III ADP-ribosylates elongation factor 2, which arrests protein synthesis and results in cell death. By removing the cell-recognition portion of PE (domain I), we have produced a noncytotoxic molecule termed "PE40" (also PE_{40}) that retains full ADP-ribosylation activity (2). The cytotoxic activity of PE40 has been restored either by fusing the gene for PE40 to genes encoding growth factors and cell-recognition proteins such as transforming growth factor α (3), interleukin 2 (4), and CD4 (5) and expressing the chimeric genes in *Escherichia coli* or by chemically coupling the PE40 protein to monoclonal antibodies.

Interleukin 6 (IL-6), also termed B-cell stimulatory factor 2, has been shown to act directly on activated B cells to induce immunoglobulin production (6). IL-6 also has been shown to be required for the growth of certain hybridomas or plasmacytomas (7), to induce neuronal differentiation in PC-12 cells (8), and to function as a hepatocyte-stimulating factor by inducing liver cells to synthesize acute-phase proteins (9). Aberrant production of IL-6 has been demonstrated in several tumor cells such as cardiac myxoma and cervical cancer cells (10). It has been shown that IL-6 receptors are expressed in high numbers on certain tumor cell lines including human myelomas, histiocytomas, and promyelocytic leukemia cells (11). It also has been found that the *in vitro* growth of human myelomas can be inhibited by anti-IL-6 antibody (12).

In this report we describe the construction of a chimeric gene through the fusion of a synthetic gene encoding IL-6 and a DNA fragment encoding PE40. The fusion gene product (IL-6–PE40) was expressed in *E. coli*, and the resulting hybrid protein was found to be highly toxic to human myeloma (U266B1; referred to as U266) cells while having no effect on IL-6 receptor-negative cells. In addition, a chimeric protein composed of IL-6 attached to an inactive mutant form of PE40 that is defective in ADP-ribosylating activity ([Asp^{553}]PE40) was also prepared (13). Because IL-6–[Asp^{553}]PE40 did not have a cytotoxic effect on U266 cells and because the cytotoxic effect of IL-6–PE40 was blocked by excess IL-6 or antibody to IL-6, we conclude that IL-6–PE40 is specifically cytotoxic to cells bearing IL-6 receptors.

MATERIALS AND METHODS

Enzymes and chemicals were purchased from standard chemical sources (4). IL-6 antibody was purchased from Genzyme (Norwalk, CT). CHO cell supernatant containing recombinant IL-6 was a gift from Steve Clark (Genetics Institute). Protein concentration and ADP-ribosylation activity was measured as described (4).

Plasmids, Bacterial Strains, and Cell Lines. Plasmid pVC8, which carries domains II and III of the gene for PE (i.e., carries the gene for PE40) under control of the bacteriophage T7 late promoter, has been described (14). Plasmid pIL-6 was purchased from British Biotechnology Limited and carries a synthetic gene for IL-6 inserted between the *Hin*dIII and *Eco*RI sites in the polylinker of pUC18. Plasmid pVC45M, which carries a gene for PE with an Asp-553 mutation (ADP-ribosylating mutant), has been described (15). *E. coli* strain HB101 was used for transformation and amplification of plasmids (16). The plasmids carrying fusion genes were expressed by isopropyl β-D-thiogalactoside induction in *E. coli* BL21(λDE3) as described (17). U266 (human myeloma) and HTB 10 (neuroblastoma; SK-N-MC) cells were from American Type Culture Collection. T24 bladder carcinoma cells were from E. Scolnick (Merck), and CEM (T) cells were from T. Waldmann (National Cancer Institute).

Nucleic Acid Preparations. Plasmid DNA was prepared and oligonucleotides were synthesized as described (4). The chimeric gene coding for IL-6–PE40 under control of the phage T7 promoter was constructed as shown in Fig. 1, starting from pVC8 [3.66 kilobases (kb)], which carries the DNA encoding the translocation and ADP-ribosylating domains of PE (14), and from pIL-6, which carries a synthetic gene for IL-6 (0.522 kb) as a *Hin*dIII–*Eco*RI fragment in pUC18. The intermediate plasmid, pCS8S (3.72 kb), was constructed by ligating *Nde* I-linearized pVC8 to a synthetic oligonucleotide containing IL-6 sequences. The oligonucleotide carried the IL-6 *Bst*XI and *Bsu*36I sites, which are located at the 5′ and 3′ ends of IL-6, respectively, and a central *Kpn* I site. Plasmid pCS8S was cleaved with the enzymes *Bst*XI and *Bsu*36I, and the large fragment was ligated to the *Bst*XI/*Bsu*36I IL-6 fragment from pIL-6. The resulting plasmid, pCS68 (4.21 kb), was identified by restriction enzyme analysis and by its expression of the IL-6–PE40 fusion protein in BL21(λDE3) cells. Plasmid pCS68M (IL-6–[Asp^{553}]PE40) was constructed by cleaving plasmid pCS68

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Abbreviations: IL-6, interleukin 6; PE, *Pseudomonas* exotoxin A.

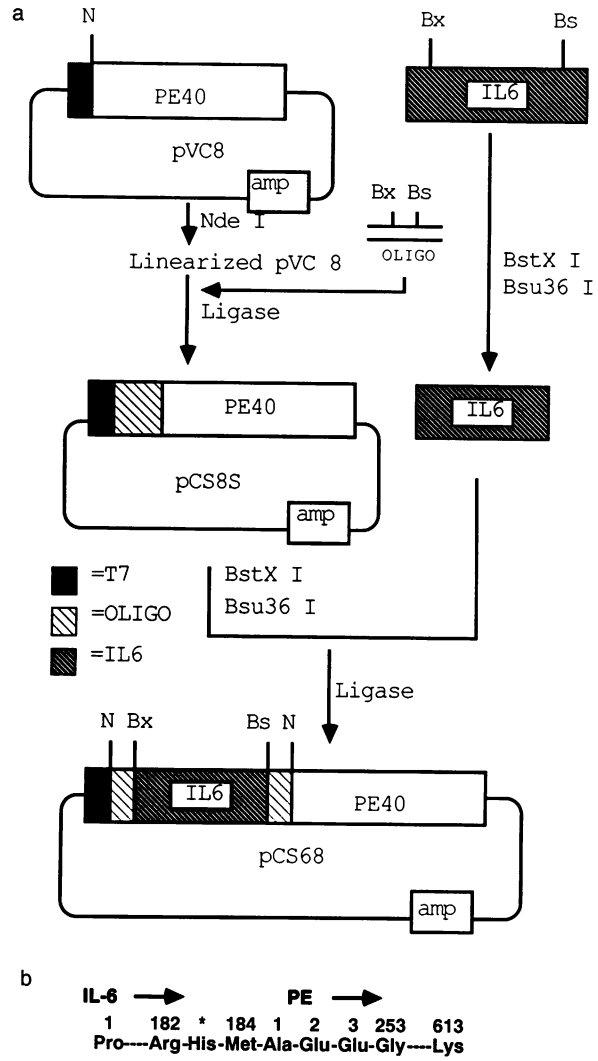

FIG. 1. (*a*) Scheme for construction of plasmid pCS68 encoding the IL-6–PE40 fusion protein. N, *Nde* I; Bx, *Bst*XI; Bs, *Bsu*36I. Sequence of the oligonucleotide linker is: 5′-TATGCCAGTAC-CCCCAGGAGAAGATTCCAAAGATGTGGGGTACCCCT-GAGGGCTCTTCGGCA ACGGTCATGGGGGTCCTCTTCTAAG-GTTTCTACTCCCCATGGGGACTCCCGAGAAGCCGTAT-5′. (*b*) Abbreviated amino acid sequence of IL-6–PE40. The asterisk indicates the amino acid at position 183 that was changed from glutamine to histidine to create an *Nde* I site.

with *Bam*HI and *Eco*RI and replacing the 0.46-kb fragment with a similarly cleaved fragment from pVC45M (15).

Expression and Localization of Recombinant Proteins. To express and localize the chimeric protein IL-6–PE40, *E. coli* BL21(λDE3) cells were transformed with the plasmid pCS68 and cultured in 250 ml of LB broth with ampicillin (50 μg/ml) for 90 min and with 1 mM isopropyl β-D-thiogalactoside when the OD_{650} value reached 0.45. The induced cells were centrifuged at 3000 rpm (Sorvall GSA rotor) for 10 min, and the total cell pellet was resuspended in 10 ml of sucrose buffer (20% sucrose/30 mM Tris·HCl, pH 7.5/1 mM EDTA). After 10 min at 4°C, the cell suspension was centrifuged at 6000 rpm (Sorvall SS-34 rotor) for 10 min, and the resulting pellet was resuspended in 10 ml of ice-cold H_2O. After 10 min at 4°C, the cell suspension was centrifuged at 8000 rpm for 10 min, and the supernatant (periplasm) was saved. The spheroplasts (pellet) were resuspended in TE buffer (50 mM Tris, pH 8.0/1 mM EDTA), sonicated three times (30 sec each), and centrifuged at 40,000 rpm (Beckman 70.1 Ti rotor) for 45 min. This pellet containing inclusion bodies was resuspended in extraction buffer (7 M guanidine hydrochloride/100 mM Tris·HCl, pH 7.0/5 mM EDTA/1 mM dithiothreitol), sonicated four times (20 sec each), and stirred at 4°C for 1 hr. The resuspended pellet was centrifuged at 40,000 rpm for 15 min, and the supernatant was retained. A small aliquot of each of the cellular fractions was retained for assay.

Purification of IL-6–PE40. The guanidine hydrochloride-extracted supernatant of inclusion bodies was rapidly diluted in 80 volumes of phosphate-buffered saline, allowed to sit for 16 hr at 4°C, and dialyzed against 50 volumes of 20 mM Tris (pH 7.4) for 6 hr with two changes. The solution of refolded protein was centrifuged at 10,000 rpm for 10 min, filtered, and applied onto a Mono Q column attached to a Pharmacia fast protein liquid chromatograph at a flow rate of 1 ml/min. After the column was washed with 5 ml of 20 mM Tris (pH 7.4), the proteins were eluted with a 25-ml linear gradient of 0–500 mM NaCl in 20 mM Tris (pH 7.4); absorbance was monitored at 254 nm. The fraction containing the peak cytotoxic activity (fraction 20) was applied onto a TSK-250 gel filtration column and eluted with 0.2 M sodium phosphate, pH 7.0/1 mM EDTA at a flow rate of 0.5 ml/min. The chimeric mutant protein, IL-6–[Asp^{553}]PE40, was expressed, localized, and purified through the Mono Q step exactly as performed for IL-6–PE40.

Inhibition of Protein Synthesis Assay. The cytotoxic activity of IL-6–PE40 was tested on U266 human myeloma cells. The cells were washed three times with RPMI 1640 medium to remove secreted IL-6 and were placed in 24-well tissue culture dishes at 5×10^5 cells per well. IL-6–PE40 was added in various concentrations (diluted in phosphate-buffered saline containing 0.2% human serum albumin), and the cells were incubated at 37°C for 18–24 hr, after which the rate of [^{3}H]leucine incorporation was measured as described (3). The biological activity on T24, CEM, and HTB 10 cells was tested in a similar fashion, except that the T24 and HTB 10 cells were seeded at 1×10^5 cells per well.

Competition assays were performed by the addition of various amounts of either IL-6–[Asp^{553}]PE40 or IL-6 prior to addition of IL-6–PE40 to U266 cells. Neutralization experiments were done by incubation of various amounts of IL-6 antibody (Genzyme) with IL-6–PE40 for 30 min at 37°C prior to addition to U266 cells.

RESULTS

Construction of IL-6–PE40. A synthetic gene encoding human IL-6 was inserted between the T7 promoter and the 5′ end of a gene encoding PE40 (domains II and III of PE) derived from the plasmid pVC8 (Fig. 1*a*). The resulting plasmid, pCS68, was expressed under control of the bacteriophage T7 late promoter. The chimeric protein, IL-6–PE40, is composed of amino acids 1–184 of mature IL-6 (except that amino acid 183 was changed from a glutamine to histidine) fused to amino acids 1–3 and 253–613 of PE (Fig. 1*b*). The molecular weight of IL-6–PE40 is 60 kDa. The expected IL-6 gene sequence was confirmed by DNA sequence analysis (data not shown).

Expression and Localization of IL-6–PE40. To express the chimeric protein IL-6–PE40, plasmid pCS68 was transformed into *E. coli* BL21(λDE3) cells, which were induced with isopropyl β-D-thiogalactoside. To discern the location of the chimeric protein, the induced *E. coli* cells were collected by centrifugation, and the components were isolated as described in *Materials and Methods*. Fig. 2*A* shows some of the cellular fractions used to localize IL-6–PE40. The largest proportion of a new protein, migrating at 60 kDa, was found in the total cell pellet. The size of this protein corresponds to the expected size for IL-6–PE40. The periplasm and medium contained negligible amounts of the 60-kDa protein, indicating that IL-6–PE40 is not secreted. The spheroplast fraction (lane 2) contained most of the expressed fusion protein.

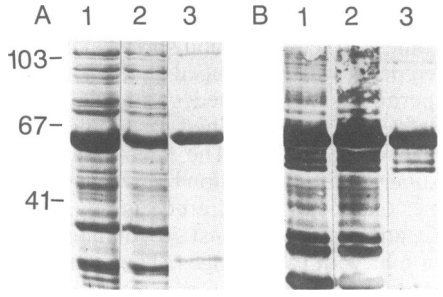

FIG. 2. NaDodSO$_4$/PAGE of expressed IL-6–PE40 localized in *E. coli* BL21(λDE3). (*A*) Coomassie blue-stained protein gel. (*B*) Immunoblot with the antibody to PE. Lanes in *A* and *B*: 1, total cell pellet; 2, spheroplast; 3, inclusion bodies. Molecular masses of the standards are indicated in kDa.

Separation into cytoplasm and the pellet that contained inclusion bodies (lane 3) showed that IL-6–PE40 was retained in the inclusion bodies. The immunoblot analysis of the various fractions shows that the 60-kDa fusion protein retained in the inclusion bodies (Fig. 2*B*, lane 3) reacts with anti-PE antibodies. Because a new protein of the size expected for IL-6–PE40 was produced and because the protein reacted with antibodies to PE, we tentatively concluded we had produced IL-6–PE40.

Purification of IL-6–PE40 Fusion Protein. Before testing the cytotoxic action of the fusion protein on U266 cells, which bear IL-6 receptors, it was necessary to extract the IL-6–PE40. To do this, the protein was dissolved in guanidine and renatured as described in *Materials and Methods*. The renatured protein was purified by anion-exchange chromatography (Mono Q column) and gel filtration (TSK-250 column) (Fig. 3). Fractions 19 and 20 from the Mono Q column contained most of the cytotoxic activity as judged by protein inhibition assays (Fig. 3 *Upper*). Fraction 20, which mainly contained a M_r 60,000 band (Fig. 4*A*, lane 1) that reacted with antibodies to PE and IL-6 (Fig. 4 *B* and *C*, lanes 1), was then subjected to gel filtration. Fraction 18 from the TSK-250 column had most of the cytotoxic activity (Fig. 3 *Lower*). The fraction contained material that ran as a single 60,000-Da band, and this band reacted with antibodies to PE and IL-6 (Fig. 4 *B* and *C*, lane 1). Active material from both the Mono Q and TSK-250 columns was also identified by measuring ADP-ribosylation activity (19) (data not shown).

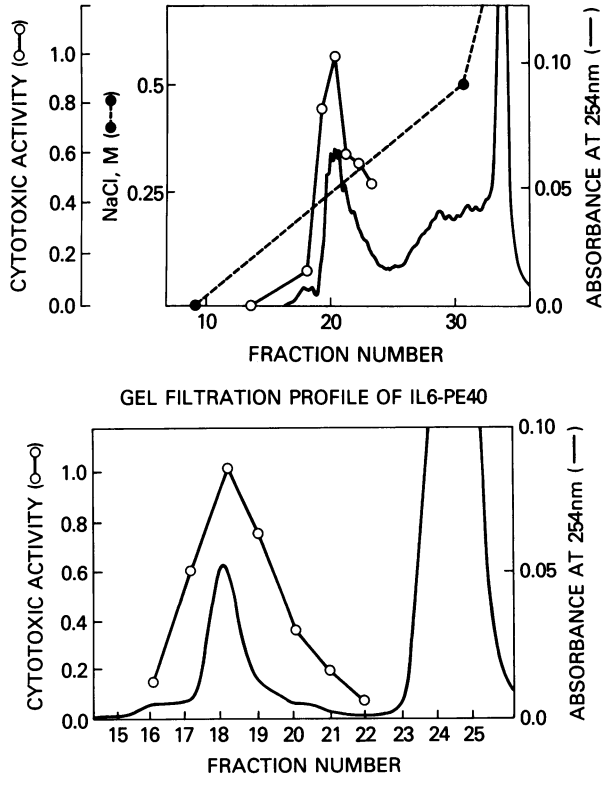

FIG. 3. Purification of IL-6–PE40 fusion protein. (*Upper*) Mono Q ion-exchange chromatography of IL-6–PE40; 5 mg of protein was applied, and 1-ml fractions were collected. (*B*) TSK-250 gel filtration chromatography of fraction 20 from the Mono Q column; 1 mg of protein was applied, and 0.5-ml fractions were collected. Cytotoxic activity is expressed as a percentage of peak activity from assays measuring inhibition of protein synthesis. Absorbance at 254 nm was monitored.

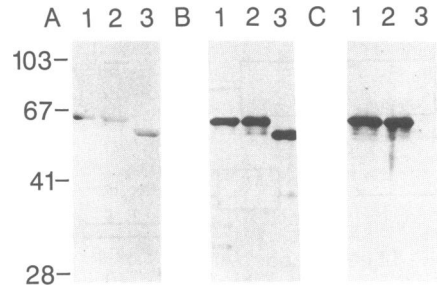

FIG. 4. NaDodSO$_4$/PAGE of IL-6–PE40 purified from Mono Q or TSK-250 columns. (*A*) Coomassie blue-stained protein gel. (*B* and *C*) Immunoblots with antibody to PE (*B*) and to IL-6 (*C*). Lanes in *A–C*: 1, fraction 18 from the TSK-250 column; 2, fraction 20 from the Mono Q column; 3, IL-2–PE40 (54.4 kDa) (ref. 4). Molecular mass standards are in kDa.

Cytotoxic Activity of IL-6–PE40. To characterize the cytotoxic activity of IL-6–PE40, we used the human myeloma cell line U266, which express 11,000 IL-6 receptors per cell (11). We assayed the cytotoxic activity by measuring the ability of various concentrations of IL-6–PE40 to inhibit protein synthesis on U266 cells. U266 cells were very sensitive to IL-6–PE40 (Fig. 5). An ID_{50} of 8 ng/ml was calculated. Conversely, IL-6–[Asp^{553}]PE40 was found not to have any cytotoxic effect on U266 cells up to a concentration of 250 ng/ml (Fig. 5).

Reversal of the Cytotoxic Activity of IL-6–PE40. To demonstrate that the cytotoxic activity of IL-6–PE40 on U266 cells was mediated by IL-6 receptors, we used IL-6 and IL-6–[Asp^{553}]PE40 as competitors for binding. Each 1 ml (5×10^5 cells) of U266 cells was incubated with 50 ng of IL-6–PE40 and various amounts of competitor (Fig. 6). Both IL-6 derived from CHO cell supernatant and IL-6–[Asp^{553}]PE40

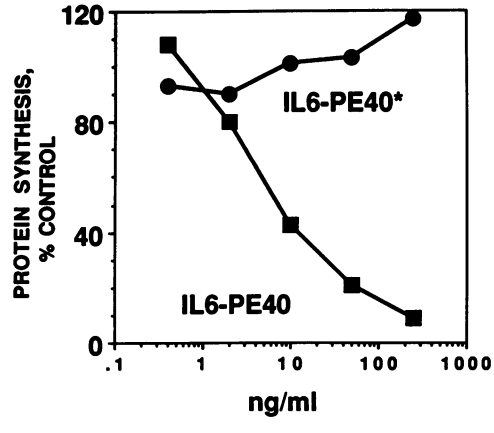

FIG. 5. Cytotoxic activity of IL-6–PE40 (■) and IL-6–[Asp^{553}]PE40 (IL-6–PE40*) (●) fusion proteins on U266 cells. IL-6–PE40 or IL-6–PE40* was added at various concentrations to U266 cells, and [^{3}H]leucine incorporation into cellular protein was measured.

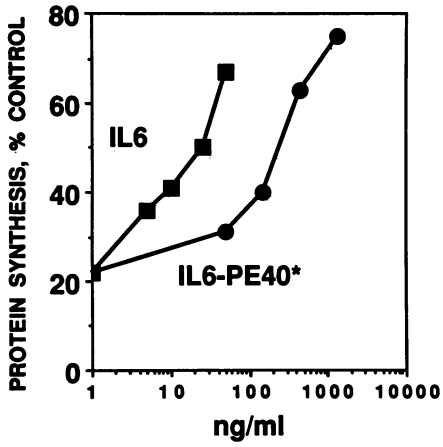

FIG. 6. Inhibition of cytotoxic activity of IL-6–PE40 on U266 cells. IL-6–PE40 (50 ng/ml) competed with various amounts of IL-6 from CHO cell supernatant (■) or with IL-6–[Asp^{553}]PE40 (IL-6–PE40*) (●). Results are expressed as the percentage of protein synthesis of control cells not treated with IL-6–PE40.

were effective in blocking the cytotoxic effect of IL-6–PE40. It is of interest that IL-6 was only about 3-fold more effective at competing against IL-6–PE40 than was IL-6–[Asp^{553}]-PE40. With no competitor, 50 ng of IL-6–PE40 caused a 76% inhibition of protein synthesis. This was reversed to 50% inhibition of protein synthesis by 10–12 ng of IL-6 or 100 ng of IL-6–[Asp^{553}]PE40. Since IL-6–[Asp^{553}]PE40 has a M_r of 60,000 and IL-6 has a M_r of 20,000, these results suggest that the affinity of IL-6–[Asp^{553}]PE40 for IL-6 receptors is no less than one-third that of IL-6 itself. Since IL-6–PE40 and IL-6–[Asp^{553}]PE40 are identical except for the amino acid at position 553, we assume that IL-6–PE40 and IL-6–[Asp^{553}]-PE40 have similar affinities for IL-6 receptors.

Neutralization of IL-6–PE40 with Anti-IL-6. The specificity of IL-6–PE40 was also examined by neutralization with an antibody to IL-6. Increasing amounts of antibody to IL-6 neutralized the cytotoxic effect of IL-6–PE40 for U266 cells (Fig. 7). With no addition of antibody to IL-6, the cytotoxic effect of IL-6–PE40 (14 ng/ml) produced a 67% reduction in protein synthesis. The addition of 70 ng of antibody to IL-6 per ml completely neutralized this cytotoxic effect.

Cytotoxic Activity of IL-6–PE40 on Various Cell Lines. The effect of IL-6–PE40 on several IL-6 receptor-negative cell lines (CEM, T24, and HTB10) was examined to determine further the specificity of the cytotoxic effect. Both sensitivity to IL-6–PE40 and PE were tested. The ID_{50} for IL-6 receptor-negative cell lines was greater than 1250 ng/ml; none of those cell lines were resistant to PE (Table 1). These results confirm

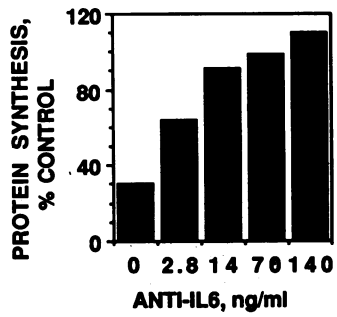

FIG. 7. Neutralization of IL-6–PE40 by antibody to IL-6. IL-6–PE40 (14 ng/ml) was incubated with various amounts of anti-IL-6 or with no antibody and added to U266 cells. Results are expressed as the percentage of untreated control cells.

Table 1. Cytotoxic activity of IL-6–PE40 and PE on various cell lines

Cell line	ID_{50}, ng/ml	
	IL-6–PE40	PE
U266	8	68
CEM	>1250	180
T24	>1250	25
HTB 10	>1250	5

ID_{50} was calculated from the inhibition of protein synthesis assay measuring [^{3}H]leucine incorporation.

that IL-6–PE40 is specifically cytotoxic to cells with IL-6 receptors.

DISCUSSION

We have constructed a chimeric protein, IL-6–PE40, by fusing a DNA segment encoding human IL-6 to a DNA segment encoding domains II and III of PE (Fig. 1); the chimeric gene was expressed in *E. coli*, and the chimeric protein was purified to near homogeneity by two chromatographic steps. IL-6–PE40 was found to selectively kill U266 cells, which express 11,000 IL-6 receptors per cell (11). IL-6–PE40 did not kill several cell lines that did not express IL-6 receptors (Table 1). This result shows that IL-6–PE40 is a very potent and specific cell-killing reagent. The cytotoxic effect of IL-6–PE40 on U266 cells was inhibited both by excess IL-6 and by an enzymatically inactive mutant form of IL-6–PE40 termed IL-6–[Asp^{553}]PE40. Competition studies showed that IL-6 was only 3-fold more effective than IL-6–[Asp^{553}]PE40 in displacing IL-6–PE40-mediated cytotoxicity. This indicates that both chimeric proteins could bind to the IL-6 receptor with a reasonably high affinity. The specificity of the cytotoxic effect was also established by neutralizing IL-6–PE40 action with antibodies to IL-6 (Fig. 7).

IL-6–PE40 may be of use in eliminating cells containing high levels of IL-6 receptors. Increased expression of IL-6 receptors and IL-6 has been found in human myeloma cells and may be responsible for their tumorigenicity (12). Other tumors that express high levels of IL-6 and may be targets for IL-6–PE40 include cardiac myxomas, cervical cancers, and bladder-cell carcinomas (20). Aberrantly high expression of IL-6 has also been observed in synovial fluid of patients with rheumatoid arthritis (18). Another use for IL-6–PE40 may be in determining if cell lines express IL-6 receptors. Currently, it is difficult to measure IL-6 receptor numbers. This difficult task can be simplified by screening cell lines with IL-6–PE40. Such cytotoxic assays should enable one to identify tumor cells with high IL-6 receptor numbers. In this report, we provide evidence that a chimeric protein, IL-6–PE40, can selectively intoxicate cells that express IL-6 receptors. IL-6–PE40 should be evaluated as an agent in the therapy of human myelomas.

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