Antitumor Effects of Interleukin 6-*Pseudomonas* Exotoxin Chimeric Molecules against the Human Hepatocellular Carcinoma, PLC/PRF/5 in Mice

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ABSTRACT

IL6-PE40 and IL6-PE66Glu are chimeric molecules composed of interleukin 6 (IL6) fused to a truncated form (PE40) or a full-length mutated form (PE66Glu) of *Pseudomonas* exotoxin. Both forms of IL6-*Pseudomonas* exotoxin are cytotoxic to IL6 receptor-bearing tumor cell lines derived from patients with multiple myeloma (1-4). IL6 receptors have also been identified on the surface of hepatocellular carcinoma and prostate carcinoma cell lines (5, 6). We have previously reported that tumor cells which express IL6 receptors could be targeted and killed by chimeric proteins composed of IL6 and various forms of *Pseudomonas* exotoxin (5-8). Two of these chimeric toxins, IL6-PE40 and IL6-PE66Glu, are composed of interleukin 6 fused either to a truncated M, 40,000 form of PE devoid of its cell-binding domain (PE40), or a mutated form of PE (PE66Glu) in which four point mutations have been introduced in the binding domain such that binding to PE receptors is eliminated.

PE is composed of three structural domains (9, 10). Domain I encodes the cell recognition function of PE (11, 12). Domain II serves as a substrate for cell-mediated proteolysis and is, in part, responsible for translocation of the toxin into the cytosol (13-15). Domain III contains the enzymatic subunit responsible for cell killing, which functions by ADP ribosylation elongation factor 2 and thereby halts protein synthesis. Domain III, by virtue of specific residues at its C-terminus, also contributes to the translocation of ADP ribosylation activity (16). By mutating the cell-binding domain of PE and fusing IL6 to the toxin, we can direct the cytotoxic action of PE preferentially to cells that display IL6 receptors.

Hepatocellular carcinoma is one of the most common causes of death due to cancer worldwide (17, 18). Only 25% of patients with primary hepatocellular carcinoma have resectable tumors. Chemotherapy does not improve the long-term survival of patients with inoperable hepatocellular carcinoma. Targeting hepatoma cells with cytotoxic proteins would be a novel way to treat this disease. We have previously demonstrated that hepatoma cells could be killed in culture with either IL6-PE40 or IL6-PE66Glu (5, 8). One of these is the hepatoma cell line PLC/PRF/5, which has served as a model for this disease (19). We have used this cell line to generate tumors in nude mice. In this report, we demonstrate, in vivo, the antitumor activities of two chimeric toxins, IL6-PE40 and IL6-PE66Glu, against this human hepatocellular carcinoma. Our results indicate that despite the fact that IL6 receptors are present on several normal cell types, it is possible to achieve an antitumor effect by targeting the IL6 receptor of tumor cells.

MATERIALS AND METHODS

Animals, Cell Lines, and Bacterial Strains. For serum level, toxicity, and antitumor assays, 6- to 8-week-old nude mice weighing 16-20 g were used (strain BALB/c, Frederick Cancer Research Facility). For WBC assays, 6- to 8-week-old immunocompetent mice were used (strain BALB/c). PLC/PRF/5 hepatoma cells and U266 myeloma cells were purchased from American Type Culture Collection (Rockville, MD). Plasmids were propagated in *Escherichia coli* strain HB101 and expressed in *E. coli* strain BL21 (ADE3).

Plasmids. The plasmids pCS68 and pCS64G, encoding IL6-PE40 and IL6-PE66Glu were previously described (5-8). Plasmid pCS64GD encodes IL6 fused to a mutant form of *Pseudomonas* exotoxin which has a deletion of amino acid 553 and has no ADP ribosylation activity.

Expression and Purification of Chimeric Toxins. Fusion proteins were expressed in *E. coli* BL21 (ADE3) under control of the T7 late promoter (20). The isolation and purification of the IL6 fusion toxins were as previously described (8). The protein concentration of the purified chimeric toxins was estimated by Bradford analysis.

Cytotoxicity of IL6 Toxins. The cell-killing activity of IL6-PE40 and IL6-PE66Glu was assessed against PLC/PRF/5 hepatocellular carcinoma cells. The chimeric proteins or their respective ADP ribosylation-deficient controls were added in various amounts to the cells and incubated at 37°C for 2 h. Incorporation of [3H]leucine into cellular protein was measured.

Administration of Chimeric Toxins. Mice were treated by single or multiple i.p. injections, or by continuous infusion into the peritoneal cavity. For the latter mode of administration, Alza Model 1007D micro pumps (Palo Alto, CA), loaded with 96 µl of chimeric toxin, were placed in the peritoneal cavity of each animal through a single incision in the skin and peritoneal layers. During the 1- to 2-min procedure, the mice were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Inc., Washington Crossing, NJ). A single suture and staple were sufficient to close the peritoneum and abdominal wall, respectively, and in 2 to 4 minutes, the mice recovered with no complications of the surgery. Once implanted, the pumps released 12 µl of drug/day continuously for 7 days. The chimeric toxins were diluted in sterile phosphate-buffered saline, and when placed in pumps contained 1 mM NAD+.

Blood Level Assays of IL6-PE66Glu in Mice. Six female nude mice (BALB/c) were given IL6-PE66Glu 5 µg/day by continuous infusion pump. From day 0 to day 9, each of the six mice was bled 3 times (100 µl per bleeding), so that on each of the 9 days, two serum levels were obtained and averaged. The serum level was determined by incubating serum with U266 myeloma cells and measuring [3H]leucine incorporation into protein. The inhibition of protein synthesis was compared to a standard curve made with known amounts of the chimeric toxin on the same cell line. Serum levels were also assessed after a single 15-µg i.p. injection, given to each of four mice. At multiple...
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time points after injection, two of the four mice were bled and the two serum levels for each time point were averaged.

Lethality of IL6-Toxins in Mice. Groups of five or six mice were used to assess toxicity of IL6-PE664Glu, which was administered i.p. either by injection or by continuous infusion pump. The animals were observed for 14 days. IL6-PE40 was given only by single or multiple i.p. injections.

Temperature Stability Assay. The chimeric toxin was incubated at a concentration of 0.83 μg/ml in phosphate-buffered saline (equivalent to the protein concentration in an osmotic pump which releases 5 μg/day), at 37°C in the presence and absence of 1 mM NAD⁺. Samples were taken every 24 h for 7 days and the concentration of active material was determined as described above for the blood level assays.

Antitumor Activity of IL6-PE40 and IL6-PE664Glu in Nude Mice Bearing a Human Hepatocellular Carcinoma. Female nude mice were given injections s.c. on day 0 of PLC/PRF/5 cells (1 × 10⁷) and treated by either i.p. injections (as per dose schedule) or by continuous infusion i.p. Each treatment group consisted of 5 animals and tumors were measured with calipers every day starting on day 14; [tumor volume in cubic mm was calculated by using the following formula: L × (w)² × 0.4).

WBC Analysis. Immunocompetent (Balb/c) mice were treated with IL6-PE664Glu 5 μg/day i.p. for 7 days by continuous infusion. Whole blood was removed from two mice on days 0, 4, and 7. Blood was diluted 20-fold with 3% acetic acid. WBC were counted from each mouse in quadruplicate by using a hemocytometer.

RESULTS

Cytotoxicity of IL6-PE40 and IL6-PE664Glu. The data in Fig. 1 show the cytotoxic effect of IL6-PE40 and IL6-PE664Glu on PLC/PRF/5 cells. The cells were incubated with the chimeric toxin for 20 h and then pulsed with [³H]leucine for 2 h. The amount of IL6-PE40 or IL6-PE664Glu needed to inhibit protein synthesis by 50% in a 24-h assay is 5 and 1.5 ng/ml (8 × 10⁻¹¹ M and 1.7 × 10⁻¹¹ M), respectively. Mutant forms of IL6-PE40 and IL6-PE664Glu, which have no ADP ribosylation activity, were not cytotoxic to PLC/PRF/5 cells.

Stability of IL6-PE664Glu at 37°C. Since we planned to administer the chimeric toxin via osmotic pumps implanted in the peritoneal cavity of mice, we needed to assess its stability at 37°C. To do this, we incubated the toxin at 37°C in the presence and absence of 1 mM NAD⁺. Another chimeric toxin, IL2-PE40, was previously found to be stabilized by the addition of NAD⁺ when incubated at 37°C (21). The results were determined by comparing the dose-dependent cytotoxicity of IL6-

PE664Glu incubated over a 7-day period to a standard cytotoxicity assay performed with fresh IL6-PE664Glu on the human myeloma cell line U266 (Fig. 2). U266 cells were used because they are easy to culture and are very sensitive to the chimeric toxin (5, 8). The bioactivity of IL6-PE664Glu in the presence of NAD⁺ decreases gradually from 100% (day 0) to 55% (day 5) (Fig. 2). On days 6 and 7, the bioactivity of the chimeric toxin drops to 25 and 24%, respectively. The bioactivity of IL6-PE664Glu incubated in the absence of NAD⁺ was significantly lower (Fig. 2B).

Serum Level of IL6-PE664Glu in Mice. We administered the toxin to mice by different routes and determined serum levels. As shown in Fig. 3, a single i.p. injection of 15 μg results in a peak of 12 μg/ml detected in 1 h. The level then rapidly fell so that 0.91 μg/ml, 0.1 μg/ml, and less than 0.002 μg/ml remained after 2, 4, and 8 h, respectively. Using an osmotic pump which

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Fig. 1. Cytotoxic activity of IL6-PE40 and IL6-PE664Glu. The chimeric toxins were added to PLC/PRF/5 hepatoma cells, incubated for 20 h and pulsed with [³H]leucine for 2 h. Incorporation into cellular protein was measured.

Fig. 2. Temperature stability of IL6-PE664Glu over 7 days. Bioactive protein amounts in the presence and absence of NAD⁺ were determined by cytotoxicity assay on U266 myeloma cells and calculated relative to the sample prior to incubation at 37°C (day 0). (A) Protein synthesis inhibition curves, day 0, day 2, day 6. (B) Bar graph representation of the bioactive material remaining after incubation periods. For details see "Materials and Methods."
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continuously infused the chimeric toxin over a 7-day period (5 μg/day), the level of IL6-PE664Glu (measured daily) ranged from 22 to 50 ng/ml. We were unable to detect any IL6-PE664Glu on day 8.

Schedule Dependence of IL6-Pseudomonas Toxin. To determine the most successful schedule with which to administer IL6-toxin to tumor-bearing mice, we assessed the lethality of IL6-PE40 and IL6-PE664Glu in various treatment schedules. Table 1 lists the different schedules of administration. IL6-PE40, administered by i.p. injection, was lethal to 40% of injected mice at a single dose of 1 mg/kg; if that total dose was given on 2 consecutive days (0.5 mg/kg/day for 2 days) it was not lethal. However, 0.5 mg/kg/day for 4 days was lethal to more than 50% of treated mice. Treatment over longer periods of time with 0.25 mg/kg/days of IL6-PE40 was lethal to more than 50% of treated mice. Treatment over longer periods of time with 0.25 mg/kg/days of IL6-PE664Glu was assessed by both i.p. injection and i.p. continuous infusion. A single injection of 0.5 mg/kg was lethal to 50% of the mice treated. Two injections of 0.5 mg/kg given 1 day apart were lethal to all of the mice treated. One or two doses of IL6-PE664Glu at 0.35 mg/kg per injection was not lethal to the mice but two 0.375-mg/kg injections were lethal to 50%. At 0.25 mg/kg, 4 doses administered daily were not lethal, whereas five doses were lethal to 20% of the animals. Thus, the maximum total dose of IL6-PE664Glu which could be injected i.p. over a 4-day period was 1.0 mg/kg. Using osmotic pumps implanted i.p., we assessed the lethality of IL6-PE664Glu continuously released for 7 days at 0.25, 0.35, 0.375, and 0.4 mg/kg/day. No lethality was observed at doses of up to 0.35 mg/kg/day (total calculated dose, 1.75 to 2.45 mg/kg), while 0.375 or 0.4 mg/kg/day was lethal to 20 and 80% of the animals, respectively.

Antitumor Activity. To produce tumors, 1 x 10⁶ hepatocellular carcinoma (PLC/PRF/5) cells were injected s.c. in a total volume of 200 μl into the dorsal-lateral surface of the mice as previously described (22). Tumors formed in 89% (17 of 19) of the nontreated mice. To maximize tumor take, the cells were split at a ratio of 1:10, 48 h prior to harvesting and injection into animals. The frequency of tumor take was reduced if the cultured cells were not actively dividing prior to harvesting (data not shown). Tumors were first evident on day 14 and grew to 1–2 cm³ in size before breaking the surface of the skin and bleeding.

In the first experiment, we assessed the ability of IL6-PE40 to inhibit the growth of PLC/PRF/5 cells in mice at a dose of 0.25 mg/kg injected i.p. every 12 h for eight doses (total dose, 4.0 mg/kg). Treatment was initiated 24 h after the tumor cells were implanted, and the size of the tumors was measured starting on day 14. In the control (nontreated) group, five of six animals developed tumors that grew rapidly, and the animals were sacrificed on day 29 with large tumors. In the IL6-PE40-treated group, five of six animals developed tumors that grew slowly until day 22, and then rapid growth ensued (Fig. 4). This experiment was repeated two additional times with similar results (data not shown).

Since IL6-PE664Glu was more cytotoxic than IL6-PE40 toward PLC/PRF/5 cells in culture, we also determined whether it would have increased antitumor activity in mice. To do this, we used a dose schedule of 250 μg/kg injected into the peritoneal cavity every 24 h for four doses (total dose, 1.0 mg/kg). In this experiment, five of five untreated animals developed tumors that rapidly grew to a large size. In the treated group, all five animals developed tumors but these grew slowly until day 20 and then began to grow rapidly (Fig. 5A). This result was confirmed in two other experiments.

Table 1 Schedule-dependent toxicity of IL6-Pseudomonas exotoxin A in nude mice (~20 g)

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<tr>
<th>Dose (mg/kg)</th>
<th>Treatment schedule</th>
<th>Total dose (mg/kg)</th>
<th>Toxicity (%) (dead mice/total mice treated) (N = 5–6)</th>
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<td>Injections i.p.</td>
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<td>5 μg (0.25)</td>
<td>Every 12 h for 8 doses</td>
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Fig. 4. Antitumor activity of IL6-PE40. Nude mice, given injections s.c. of 5 x 10^5 PLC/PRF/5 cells on day 0, were either treated starting on day 1 with IL6-PE40 0.25 mg/kg i.p. every 12 h for 8 doses, or were observed. Tumor volume in each group of animals, including only those which formed tumors, were averaged.

Fig. 5. Antitumor activity of IL6-PE664Glu. Nude mice, given injections s.c. of 1 x 10^7 PLC/PRF/5 cells on day 0 were either treated starting on day 1 with IL6-PE664Glu 0.25 mg/kg i.p. daily for 4 days, 0.25 mg/day for 7 days by continuous infusion, phosphate buffered saline (PBS) for 7 days by continuous infusion, or were not treated. Tumor volumes are the average from five mice. Another set of animals received IL6-PE664Glu by continuous infusion at 0.25 mg/kg/day for 7 days (total dose, 1.75 mg/kg) starting 24 h after the tumor cells were implanted. Four of five animals developed tumors (Fig. 5B) and the antitumor activity was greater than with repeated i.p. injections (Fig. 5A). The total dose administered was 75% greater in the continuously infused group as compared to the i.p. injected group (1.75 versus 1.0 mg/kg). Also, 5 mice were implanted with osmotic pumps which released only the diluent, phosphate-buffered saline; 4 of 5 animals developed tumors and these grew in a fashion similar to that of nontreated mice (Fig. 5B).

We next performed experiments in which tumors were allowed to grow for 14 days to a measurable size before administration of IL6-PE664Glu. We chose to give the agent by continuous infusion (0.35 mg/kg/day for 7 days) starting 24 h after implantation. Two such experiments are shown in Fig. 6. In both experiments, IL6-PE664Glu showed a pronounced antitumor effect even when the treatment was delayed. In Group A (Fig. 6), we compared 5 nontreated animals to 5 animals treated on days 14-20 with IL6-PE664Glu. In Group B, we also included 5 animals which were treated on days 14-20 with the ADP-ribosylating mutant and thereby nontoxic form of the chimeric toxin, IL6-PE664GluD553. In all of these experiments, only animals which had measurable tumors on day 14 were included. In no case did the tumor disappear following therapy with IL6-PE664Glu, although the rate of tumor growth was considerably retarded (Fig. 6).

Another set of animals received IL6-PE664Glu by continuous infusion at 0.25 mg/kg/day for 7 days (total dose, 1.75 mg/kg) starting 24 h after the tumor cells were implanted. Four of five animals developed tumors (Fig. 5B) and the antitumor activity was greater than with repeated i.p. injections (Fig. 5A). The total dose administered was 75% greater in the continuously infused group as compared to the i.p. injected group (1.75 versus 1.0 mg/kg). Also, 5 mice were implanted with osmotic pumps which released only the diluent, phosphate-buffered saline; 4 of 5 animals developed tumors and these grew in a fashion similar to that of nontreated mice (Fig. 5B).

WBC Analysis. Because IL6 receptors are present on lymphocytes (1), monocytes and hepatocytes (23), we determined the effect of IL6-PE664Glu on bone marrow-derived cells, some of which have IL6 receptors, by measuring the number of WBC in mice that were treated for 4 or 7 days with the cytotoxic agent. In these experiments, osmotic pumps which infused 5 μg/day for 7 days were surgically implanted i.p. We found that there was no difference in the number of WBC on day 0 as compared to days 4 or 7 after treatment with IL6-PE664Glu. The number of WBC measured was 11.2 x 10^6 cells/ml, 11.2 x 10^6 cells/ml, and 11.7 x 10^6 cells/ml on days 0, 4, and 7, respectively. We also measured the percentage of eosinophils, neutrophils, lymphocytes, and monocytes which comprise the total WBC in two mice treated for 4 and 7 days with IL6-PE664Glu. The differential cell counts were assessed by microscopic evaluation of Wright-stained smears of peripheral blood from the mice. We found no difference in the mice which were treated with IL6-PE664Glu versus untreated mice (Table 2).

DISCUSSION

We have investigated the effects of IL6-PE40 and IL6-PE664Glu, two chimeric toxins shown to kill tissue culture cell lines with IL6 receptors, on the growth of the PLC/PRF/5 hepatoma in nude mice. We have found that both IL6-PE40 and IL6-PE664Glu have antitumor activity against this carcinoma (Figs. 4 and 5). To carry out these experiments, we initially determined the maximum dose that could be given by repeated injections or continuous infusion. We then showed that both routes of administration were effective, but that continuous infusion of IL6-toxin for a 7-day period had a greater antitumor effect than multiple i.p. injections when

Table 2 WBC analysis

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treatment began 24 h after the tumor cells were implanted into mice. Apparently, constant low blood levels of IL6-toxin produced by continuous infusion are more effective than transiently higher blood levels of the toxin resulting from single or multiple injections. For subsequent experiments, treatment was delayed until tumors appeared and IL6-PE66^Glu^ was able to retard the growth of these tumors when administered 14 days after implantation in nude mice. However, it did not shrink tumor masses that were already present (Fig. 6).

To use pumps implanted in the peritoneal cavity, it is necessary for the chimeric toxin to be free of proteases and to be stable. We have demonstrated that IL6-PE66^Glu^ is quite stable and bioactive for 7 days when incubated at 37°C. The addition of NAD^+ (1 mM) increased its stability by blocking the inactivation of the enzyme portion of the toxin (21). After 5 and 7 days, 70 and 33% (respectively) of the activity remained (Fig. 2). Therefore, the toxin in the pump which is placed in the peritoneal cavity of a mouse should remain bioactive during the 7-day infusion. We confirmed this by showing that sera taken from animals during the infusion retained cytotoxicity (Fig. 3). The IL6-toxins are made in E. coli which contain many proteases and are purified to near homogeneity by a few purification steps (7). Although not specifically designed to remove proteases, these steps are apparently quite effective in doing so.

Measurement of IL6-PE66^Glu^ in the serum showed that after i.p. injection about 80% of the chimeric toxin appeared in the serum 1 h after administration, but the level fell very rapidly and within 8 h, the IL6-toxin was undetectable (Fig. 3). In contrast, continuous infusion from osmotic pumps implanted in the peritoneal cavity produced a relatively constant level that ranged from 22 to 50 ng/ml when infused at 5 μg/day (Fig. 3).

Administration of IL6-PE66^Glu^ by continuous infusion allowed for treatment with higher total doses than was possible with bolus injection. IL6-toxin-induced death in nude mice was also schedule dependent (Table 1). In the case of IL6-PE66^Glu^, we were able to infuse up to 2.4 mg/kg over a 7-day period without any deaths to mice, while we could only inject as repeated bolus injections 1.0 mg/kg over 4 days without lethality. The constant low serum levels obtained with continuous infusion may not be cytotoxic to normal cell types which display very low levels of IL6 receptors (i.e., hepatocytes, monocytes, lymphocytes) (1, 23).

The antitumor activity of IL6-PE40 injected i.p. (0.25 mg/kg, every 12 h for 8 doses), was equivalent to that of IL6-PE66^Glu^ (0.25 mg/kg, daily for 4 days). This finding that IL6-PE66^Glu^ had the same tumor regression capacity as twice as much IL6-PE40 was expected from the in vitro results showing a 3-fold higher specific activity of IL6-PE66^Glu^ compared to IL6-PE40. In comparison to administration of IL6-PE66^Glu^ by continuous infusion, multiple bolus i.p. injections had considerably less antitumor activity. This result is in agreement with previous data indicating a similar trend with IL2-PE40 (21). The greater therapeutic effect of continuously infused IL6-PE66^Glu^ may be due to a prolonged maintenance of a serum level which will kill malignant cells but spare normal cells. In culture, the 50% protein synthesis inhibition of IL6-PE66^Glu^ toward PLC/PRF/S is 1.5 ng/ml. In vitro, however, the toxin concentration is relatively constant during the 24-h incubation, and access of the toxin to tumor cells is optimal. In vivo, poor access of toxin in the serum to the s.c. tumor cells may require a much higher serum level for longer periods of time. During this time, it may be necessary to avoid transiently high levels which could kill the more accessible normal cells having low receptor numbers. The dose-limiting side effect of the IL6-toxins used in this study is hepatotoxicity, as determined by autopsy of animals administered lethal levels of IL6-toxin. In these studies, we have had to administer these agents i.p. in order to maintain high blood levels for long periods of time. By this route of administration, much of the drug passes through the liver before it reaches the tumor cells. Continuous administration of the chimeric toxin through i.v. means or the production of chimeric toxins with longer survival in the blood may increase the therapeutic window.

Using the same conditions under which we obtained significant antitumor activity (IL6-PE66^Glu^, 0.25 mg/kg/day continuous infusion for 7 days), we found no effect on WBC numbers. If IL6-PE66^Glu^ had a detrimental effect on hematological cells, it might be reflected in WBC. We observed no difference in numbers of lymphocytes, neutrophils, eosinophils, or monocytes at days 0, 4, and 7 following administration of IL6-toxin (Table 2).

IL6 has been used as an antitumor agent by itself both in vitro (24) and in vivo (25). High level expression of IL6 in the blood has been found in malignant conditions, many of which appear to be regulated in an autocrine manner (2, 26, 29). By combining IL6 with a cell-killing toxin, we may be able to interrupt the IL6 autocrine loop which is functioning to promote these cancers. Here we have used a hepatocellular carcinoma cell line which displays 2300 IL6 receptors per cell in vitro as an in vivo tumor model (5). This study represents the first example of an in vivo antitumor effect by using a cytotoxic form of IL6. The drugs used here, IL6-PE40 and IL6-PE66^Glu^, might have greater antitumor activity if these agents were directed against cancer cells expressing larger numbers of IL6 receptors, such as multiple myeloma (which in some cases display over 15,000 IL6 receptors per cell). It will be important to test IL6-toxins against myeloma tumors in mice. It will also be important to measure IL6 expression in human cancers to determine which of these will be targets for IL6 receptor-targeted therapy. IL6 receptors have already been detected on prostatic carcinoma cells and on an epidermoid carcinoma (6, 8).

ACKNOWLEDGMENTS

We thank Drs. S. Epstein, M. Willingham, R. Dedrick, and R. Nordan for helpful comments, Drs. J. Batra and U. Brinkman for critical reading, and A. GADDIS for expert secretarial assistance.

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