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Antitumor activity in mice of an immunotoxin made with anti-transferrin receptor and a recombinant form of *Pseudomonas* exotoxin

(cancer/chemotherapy/toxin/nude mice)

JANENDRA K. BATRA, YOSHIHIRO JINNO, VIJAY K. CHAUDHARY, TOSHIHIKO KONDO, MARK C. WILLINGHAM, DAVID J. FITZGERALD, AND IRA PASTAN

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, 37/4E16, Bethesda, MD 20892

Contributed by Ira Pastan, August 4, 1989

ABSTRACT **LysPE40 is a modified form of *Pseudomonas* exotoxin that lacks the cell-binding domain and has a chemically reactive lysine residue near the amino terminus. LysPE40 is made in *Escherichia coli* and secreted into the medium from which it is readily purified. Two immunotoxins were constructed by coupling LysPE40 to an antibody to the human transferrin receptor (TFR) or to an antibody to the human interleukin-2 receptor. These immunotoxins were selectively cytotoxic to receptor-bearing cells in tissue culture. Anti-TFR-LysPE40 given intraperitoneally to mice appeared rapidly in the blood and caused regression of A431 tumors growing as subcutaneous xenografts. These results show that it is possible to cause regression of a solid carcinoma by an immunotoxin if proper targeting can be achieved.**

One new approach to cancer treatment that has received considerable attention in the past few years is to direct cytotoxic agents to cancer cells by using tumor-specific antibodies (1, 2). Cytotoxic molecules delivered by this method include bacterial and plant toxins, conventional chemotherapeutic agents such as adriamycin and methotrexate, and radioisotopes (1, 3–5). We have been investigating the utility of immunotoxins in which antibodies are attached to *Pseudomonas* exotoxin (PE). In early studies, we coupled native PE to an antibody specific for the human transferrin receptor (TFR) and showed that this type of conjugate (anti-TFR-PE) selectively killed human cancer cells in tissue culture (6). It was then tested in mice and, when given i.p., was shown to greatly prolong the survival of mice with a lethal human ovarian cancer growing in their peritoneal cavity (7). However, anti-TFR-PE was not effective when administered either i.v. or when given i.p. to animals with subcutaneous tumors because liver damage produced by the immunotoxin limited the amount of drug that could be given. Only by giving the immunotoxin into the same body compartment as the tumor could tumor cells be killed without unacceptable side effects. Subsequently, PE was attached to the monoclonal antibody OVB3, which specifically binds to human ovarian cancer cells. This immunotoxin (OVB3-PE) has been shown to kill human ovarian cancer cells in cell culture, prolong the life of nude mice bearing a lethal human ovarian tumor (8), and is currently in a clinical trial. However, OVB3-PE, like anti-TFR-PE, is effective primarily when given i.p. against i.p. tumors (8).

PE is a M_r 66,000 single-chain protein that is composed of three domains (9). The liver toxicity and toxicity to other cell types produced by immunotoxins made with native PE are principally due to the binding of domain I of PE to liver cells (unpublished observations). Various recombinant forms of PE have been made in *Escherichia coli*, including one with a M_r of 40,000 (PE40) that lacks domain I and has very low liver toxicity because it no longer binds to the PE receptor present on liver cells. PE40 was chemically coupled to several antibodies, including one that reacts with the human TFR (10). The anti-TFR-PE40 conjugate had the expected decrease in liver toxicity, but unexpectedly it was also much less active than anti-TFR-PE against human cells containing TFR (10). In searching for an explanation for this loss of activity, we noted that PE40 was poorly reactive with the heterobifunctional cross-linking reagent succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) used to make the immunotoxin. A 25-fold excess of cross-linker was needed to derivatize half of the PE40 molecules. Also we noted that the three "target" lysine residues were located at the carboxyl end of PE40.

The current paper describes the properties of a modified form of PE40 termed LysPE40 in which a lysine molecule is included near the amino end. This molecule is readily coupled to antibodies. The immunotoxin produced by coupling LysPE40 to anti-TFR (anti-TFR-LysPE40) has low nonspecific toxicity in mice, so that high blood levels can be obtained. Furthermore, anti-TFR-LysPE40 injected i.p. caused complete regression of small subcutaneous tumors and very marked destruction of large subcutaneous tumors. These experiments show that, if proper targeting can be achieved, an immunotoxin containing LysPE40 can have striking therapeutic effects on a rapidly growing solid carcinoma.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strains HB101 and BL21 (λDE3) have been described (11). The plasmid pJY85L was made from pVC45 (12), codons for amino acids 6–251 were deleted, and glutamic acid at position 252 was changed to lysine by site-directed mutagenesis.

Expression of LysPE40. BL21 (λDE3) cells were transformed with plasmid pJY85L and grown in LB medium containing 100 μg of ampicillin per ml at 37°C. At A_{650} = 0.6, isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. Cells were harvested 90 min later. The culture medium was used as the source of LysPE40 because most of the protein was secreted into the medium.

Purification of LysPE40. Twenty-five liters of clarified culture medium containing LysPE40 was diluted 1:4 with chilled deionized water and applied to a 10 × 5.5 cm silica-based anion-exchange column (QMA, Waters) at a flow rate of 100 ml/min. The column was washed with 0.05 M sodium

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Abbreviations: PE, *Pseudomonas* exotoxin; TFR, transferrin receptor; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; IL-2, interleukin-2.

phosphate buffer (pH 7.0), and proteins were eluted with 2 liters of 0.25 M NaCl in the equilibration buffer. LysPE40 from the QMA column was concentrated further by using Amicon YM30 membranes to 150 ml, dialyzed for 12–16 hr against 0.02 M Tris·HCl (pH 7.6), centrifuged at 10,000 × *g* for 20 min, and applied to a 2.5 × 14 cm Q-Sepharose column equilibrated with 0.02 M Tris·HCl (pH 7.6). Proteins were eluted with a 500-ml linear gradient of 0–0.5 M NaCl; LysPE40-containing fractions were detected by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), pooled, diluted 1:10 with 0.02 M Mes (pH 5.5), and loaded onto a fast protein liquid chromatography (FPLC) Mono S 16/10 column equilibrated with 0.02 M Mes (pH 5.5). LysPE40 bound to the column and was eluted by a 100-ml linear gradient of 0–0.5 M NaCl. It was further purified on TSK250 (22.5 × 600 mm) column (Bio-Rad) with 0.2 M sodium phosphate (pH 7.0) containing 1 mM EDTA.

Construction of Immunoconjugates. LysPE40 (5–10 mg/ml) in 0.2 M sodium phosphate (pH 7.0) containing 1 mM EDTA was mixed with a 3-fold molar excess of SMCC and incubated at room temperature for 30 min. Protein was separated from the unreacted cross-linker on a PD10 column. Monoclonal antibody (HB21, anti-Tac, MOPC 21) (5–10 mg/ml) was mixed with a 3-fold molar excess of 2-iminothiolane·HCl in 0.2 M sodium phosphate (pH 8.0) containing 1 mM EDTA and incubated at 37°C for 1 hr. The derivatized antibody was separated from the reactants on a PD10 column. Derivatized LysPE40 and derivatized antibody were mixed in a 4:1 molar ratio and incubated at room temperature for 16–20 hr. Immunotoxin was then purified by successive chromatography on Mono Q and TSK 250 columns (10). Immunotoxins were also made with PE as described (10). SDS/PAGE was done as described (10).

Protein Synthesis-Inhibition Assay. Activities of the conjugates were tested on A431, KB, HUT 102, HT29, OVCAR-2, OVCAR-3, OVCAR-4, CEM, and MOLT-4 cells by measuring [^{3}H]leucine incorporation (10). HUT 102 cells were washed twice with RPM 1640 and used immediately. Immunotoxins were diluted with 0.2% human serum albumin in phosphate-buffered saline prior to addition to cells.

Assay of Blood Levels of Anti-TFR-LysPE40 in Mice. Female BALB/c mice or immunodeficient (*nu/nu*) mice bearing A431 subcutaneous tumors were injected with 100 μg or 50 μg of immunotoxin i.p. Blood was drawn at different times, and the level of the immunotoxin was assayed by incubating serum with A431 cells and measuring its effect on protein synthesis. A standard curve was made with anti-TFR-LysPE40.

Antitumor Activity of Anti-TFR-LysPE40 in Nude Mice Bearing a Human Epidermoid Carcinoma. A431 cells (3×10^6) were injected subcutaneously on day 0 into female nude mice, and tumors about 5 × 5 mm in size developed in all mice by day 5. Treatment of mice was started either 2 days, 5 days, or 9 days after tumor implantation. Each treatment group consisted of four to six animals. Tumors were measured with a caliper every fourth day, and the volume of the tumor was calculated by using the formula: tumor volume in mm^3 = length × $(width)^2$ × 0.4.

RESULTS

Previously, we described the properties of immunotoxins made with PE40. The activities of these immunotoxins were lower than anticipated, and the yields were poor (10). Cross-linking of the toxin appeared to be through one of three lysine residues present at the carboxyl end of PE40, which were poorly reactive with SMCC or similar heterobifunctional cross-linking reagents used in the construction of immunotoxins (10). In contrast, native PE is readily conjugated to antibodies. Since in native PE there are 12 lysine residues in domain I, some of which are very reactive with SMCC, we thought a useful immunotoxin could be made by modifying PE40 so that conjugation would proceed through a readily accessible lysine residue that did not play an important role in the activity of the protein. To accomplish this, the amino end of PE40 was altered so that it contained a lysine residue and an OmpA signal sequence. The OmpA signal sequence was added to direct the export of LysPE40 to the growth medium. The structure of LysPE40 and the plasmid encoding LysPE40, which is under the control of a phage T7 promoter, is shown in Fig. 1. LysPE40 is secreted into the culture medium in large amounts.

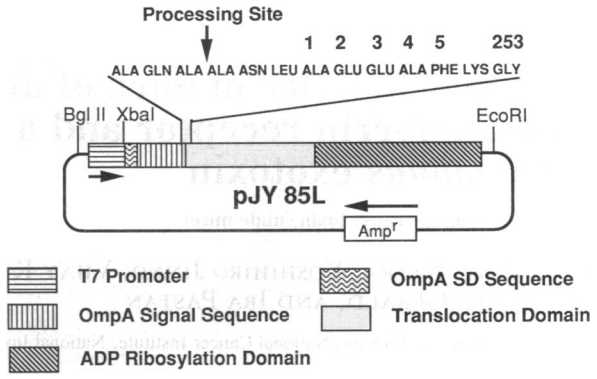

FIG. 1. Structure of plasmid pJY85L showing the presence of a phage T7 promoter, an OmpA signal sequence, and domains II (translocation domain) and III (ADP ribosylation domain) of PE.

The purification of LysPE40 is described in *Materials and Methods*. The purity of the protein at each purification step is shown in Fig. 2*A*. Material from the TSK 250 column, which was used as the final step, appeared to be homogeneous when analyzed by SDS/PAGE (Fig. 2*A*, lane 5) as well as by immunoblotting with an antibody to PE (data not shown). Typically, 2–3 mg of pure LysPE40 was obtained from 1 liter of culture induced at an absorbance at 650 nm of 0.6.

The amino-terminal sequence of the purified protein was found to be Ala-Asn-Leu-Ala-Glu-Glu-Ala-Phe-Lys-Gly-Gly-Ser-Leu. This is the amino acid sequence expected from the DNA sequence with processing occurring within the OmpA sequence (Fig. 1). The amino-terminal sequence of PE40 is Ala-Asn-Leu-Ala-Glu-Glu-Gly-Gly (10).

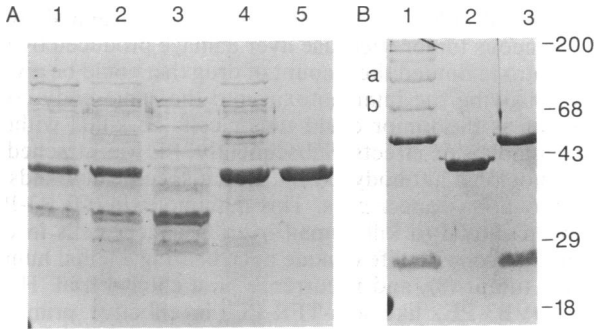

FIG. 2. (*A*) SDS/PAGE analysis of LysPE40 pools at various stages of purification; 12.5% gels were stained with Coomassie blue. Lanes: 1, QMA-concentrated culture medium proteins; 2, Q-Sepharose pool; 3, flow-through material from Mono S; 4, Mono S pool; 5, TSK 250 pool. The positions of molecular weight standards are indicated in kDa. (*B*) SDS/PAGE analysis of anti-TFR-LysPE40. Samples were applied on a 12.5% reducing polyacrylamide gel. Lanes: 1, anti-TFR-LysPE40 conjugate, with heavy chain (a) and light chain (b); 2, LysPE40; 3, anti-TFR. Gels were stained with Coomassie blue.

Table 1. Activity of anti-TFR-LysPE40 on various human cell lines

Cells	ID_{50}*, ng/ml	
	Anti-TFR-LysPE40	LysPE40
A431	4.0	>2000
KB	14.0	>2000
HT29	6.6	>2000
HUT 102	21.0	>2000
CEM	22.5	>2000
OVCAR-2	135.0	ND
OVCAR-3	280.0	ND
OVCAR-4	30.0	ND
MOLT-4	32.0	ND

*ID_{50} is described as the concentration of the immunotoxin needed for 50% inhibition of protein synthesis. ND, not done.

Construction of Immunotoxins with LysPE40. LysPE40 was chemically coupled to two different monoclonal antibodies—HB21, which binds to the human TFR (anti-TFR) (13), and anti-Tac, which binds to the 55-kDa subunit of human interleukin-2 (IL-2) receptor (14). After the conjugation, the immunotoxins were purified on Mono Q and TSK 250 columns. Purified conjugates were found to contain LysPE40 coupled to both the light and heavy chain of the antibody and did not contain any free LysPE40 (Fig. 2*B*, lane 1).

Activity of Immunotoxins Made with LysPE40. The activity of anti-TFR-LysPE40 was assayed on a variety of human cell lines; it inhibited protein synthesis in all of the human cell lines studied (Table 1). Anti-TFR-LysPE40 was most active on A431 cells with an ID_{50} of 4.0 ng/ml. Specificity was demonstrated by showing that excess unconjugated antibody prevented the cytotoxic effect of anti-TFR-LysPE40 (Fig. 3*A*). Anti-TFR-LysPE40 was not cytotoxic to murine Swiss 3T3 cells even at 2 μg/ml (data not shown).

To determine if LysPE40 could be coupled to other antibodies to yield an active conjugate, the anti-Tac antibody was chosen. This antibody reacts with the p55 subunit of the IL-2 receptor. The cytotoxic activity of anti-Tac-LysPE40 was determined on HUT 102 cells, a human T-cell leukemia line containing IL-2 receptors. Anti-Tac-LysPE40 inhibited protein synthesis in HUT 102 cells with an ID_{50} of 2.5 ng/ml, and excess anti-Tac blocked this effect, demonstrating the specificity of the immunotoxin (Fig. 3*B*). Anti-Tac-LysPE40 did not inhibit protein synthesis on IL-2 receptor-negative cells, e.g., CEM and KB, even at 2000 ng/ml, further showing the specificity of the immunotoxin.

Blood Levels of Anti-TFR-LysPE40 in Mice. BALB/c mice were injected i.p. with a single dose of 100 μg of anti-TFR-LysPE40, and blood was drawn at different times after the injection to assay for immunotoxin activity. A peak blood level of 78 μg/ml was obtained 4 hr after the injection, and a level of 10 μg/ml was still present 24 hr after the injection (Fig. 4). A similar experiment was performed in athymic mice bearing A431 tumors. After injecting 50 μg of anti-TFR-LysPE40, a blood level of 27 μg/ml was detected 4 hr after injection and 8 μg/ml after 24 hr (Fig. 4).

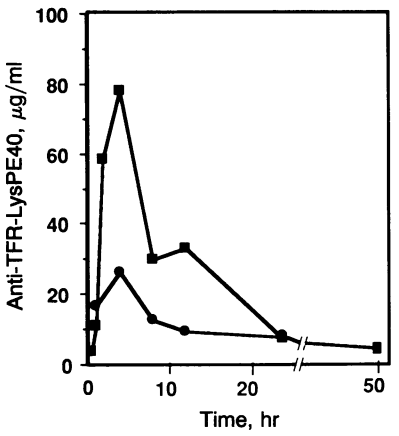

FIG. 4. Blood levels of anti-TFR-LysPE40. BALB/c mice (■) or nude mice with A431 tumors (●) were injected i.p. with 100 μg or 50 μg of anti-TFR-LysPE40. Immunotoxin levels were measured in serum at different time periods. Results are the average of two experiments.

Antitumor Activity of Anti-TFR-LysPE40. Anti-TFR-LysPE40 was assayed for its ability to inhibit the growth of A431 cells as subcutaneous xenografts in nude mice. To produce tumors, 3×10^6 A431 cells were injected subcutaneously on day 0. In the control group, treated only with diluent, the tumors grew rapidly, and the animals were sacrificed on day 19 with large tumors that were penetrating the skin. (Figs. 5 and 6). In a group that received anti-TFR-LysPE40 (50 μg per injection) on days 2, 4, 6, and 8, no tumors were evident on day 24 when the experiment was terminated (Figs. 5*A* and 6). In another group of animals, treatment was delayed until the tumors were about 125 mm^3 in volume and was given on days 9, 11, 13, and 15. As soon as the treatment was initiated, the tumors stopped increasing in size (Figs. 5*A* and 6) and then developed soft centers. Histological examination showed that almost all of the cells in the center of the tumor were nonviable (Fig. 7). However, at the rim of the tumor, viable cells were observed.

To determine if the antitumor effect was dose-related, treatment with various amounts of anti-TFR-LysPE40 was begun on day 5, when small tumors were evident (Fig. 5*B*). Even the 5-μg dose produced a delay in tumor growth, but after the treatment was stopped on day 11, the tumors began to grow rapidly. As the dose was increased to 20 or 50 μg, some or all of the tumors became undetectable by day 11. However, with time, some tumors began to reappear and grow (Table 2). One group of animals received a single dose on day 5 of 150 μg, which is close to the LD_{50}. Two animals died, but in the other three animals the tumor regressed and did not reappear (Table

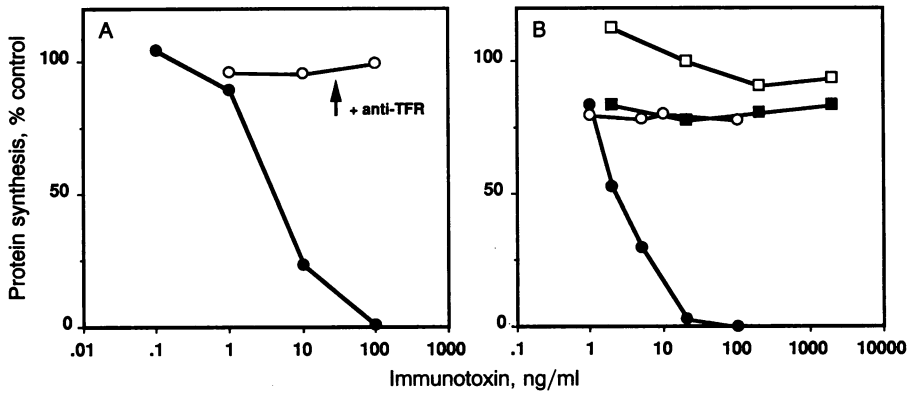

FIG. 3. (*A*) Inhibition of protein synthesis in A431 cells by anti-TFR-LysPE40. Immunotoxin was added to the cells in the absence (●) and presence (○) of excess anti-TFR (50 μg/ml) for 18–24 hr at 37°C. (*B*) Cytotoxic activity of anti-Tac-LysPE40 on HUT 102 cells. Anti-Tac-LysPE40 was added at various concentrations to HUT 102 (●), CEM (■), and KB (□) cells. For the competition experiment, 70 μg of anti-Tac per ml was added before the addition of the immunotoxin to HUT 102 cells (○). [^{3}H]Leucine incorporation was measured as described.

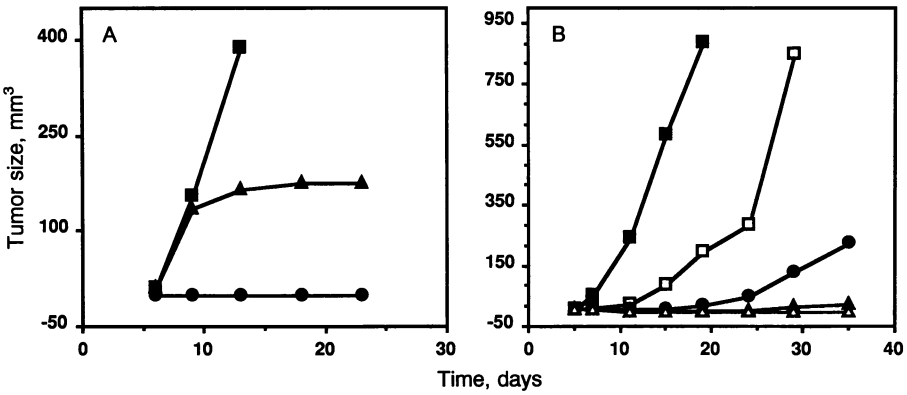

FIG. 5. Effect of anti-TFR-LysPE40 on the growth of A431 tumors in nude mice. Mice were injected with 3×10^6 A431 cells and treated i.p. with the immunotoxin as indicated. (*A*) Mice were given four doses of 50 μg each on days 2, 4, 6, and 8 (●), or on days 9, 11, 13, and 15 (▲) or were given no treatment (■). (*B*) Mice were given no treatment (■) or were given four doses on days 5, 7, 9, and 11 of 5 μg (□), 20 μg (●), or 50 μg (▲) or a single dose of 150 μg on day 5 (△).

2). When antibody alone or an immunotoxin composed of an antibody that did not react with the tumor (MOPC21-LysPE40) was administered at the 50-μg dose level, an antitumor response was not observed (data not shown).

DISCUSSION

We describe here a modified form of PE termed LysPE40 that can be efficiently coupled to antibodies yielding an immunotoxin with high cytotoxic activity against cultured cell lines bearing the appropriate antigen and no detectable cytotoxicity against cultured cells to which the antibody does not bind. Furthermore, an immunotoxin composed of an antibody to the human TFR coupled to LysPE40 (anti-TFR-LysPE40) could be administered safely in large amounts to mice and caused regression of a rapidly growing human epidermoid carcinoma implanted subcutaneously.

When administered i.p. in mice, anti-TFR-LysPE40 appeared rapidly in the blood. A dose of 50 μg gave a peak blood level 4 hr after injection of about 30 μg/ml, and blood levels were still 8 μg/ml at 24 hr (Fig. 4), indicating that this immunotoxin has a relatively long half-life. A single dose of 100 μg gave a peak blood level of 80 μg/ml. Since a 20-g mouse has a blood volume of about 1 ml, almost all of the immunotoxin is found in the blood 4 hr after i.p. administration.

In most of the treatment protocols used in this study, the tumor cells were allowed to grow to form a detectable solid tumor before treatment was initiated. Under these conditions, a treatment consisting of four injections given over 8 days caused obvious tumor regression (Figs. 5–7). At the lower dose levels with small tumors, or even at the high dose level with large tumors, viable tumor cells remained. Perhaps by continuing the treatment for longer periods, a larger antitumor effect could be achieved. Nevertheless, we have been able to cause marked regression by an immunotoxin of a solid cancer that is resistant to standard chemotherapy.

Several investigators have reported partial suppression of tumor growth in various animal models by specific immunotoxins made with ricin A chain (15, 16), diphtheria toxin A chain (17), or gelonin (18). In most of these studies, the *in vivo* cytotoxic effect was observed when the treatment was started on the same day or 1 day after the tumor implantation. Immunotoxins made with ricin A chain are rapidly eliminated from the blood. The clearance of i.v.-injected immunotoxins has been shown to be biphasic, with an initial rapid α phase followed by

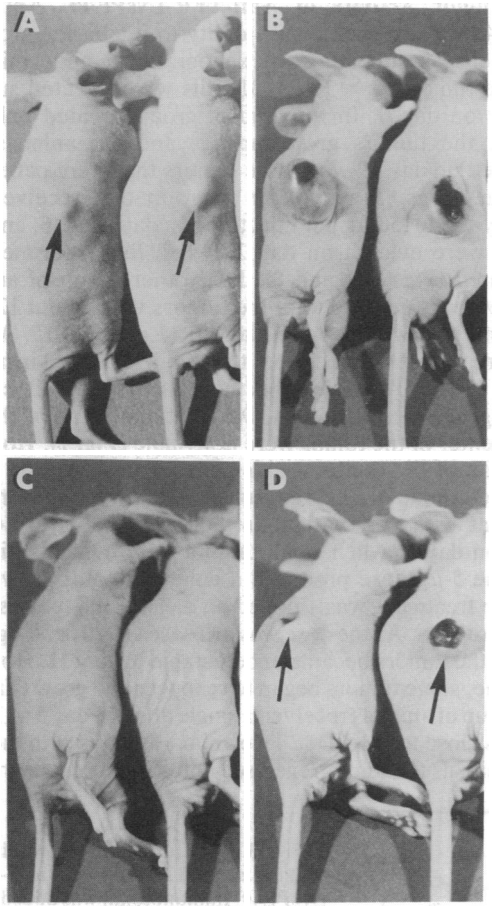

FIG. 6. Gross appearance of subcutaneous A431 tumors in nude mice with and without treatment with anti-TFR-LysPE40. Nude mice were injected subcutaneously with 3×10^6 A431 cells on day 0. The tumors were apparent as small bumps under the skin on day 5 (arrows in *A*), and by day 15 tumors were large, often erupting through the skin surface (*B*). Mice treated with anti-TFR-LysPE40 on days 2, 4, 6, and 8 usually showed no development of tumor, demonstrated here as the absence of gross tumor on day 26 (*C*). When mice with large tumors were treated with anti-TFR-LysPE40 (on days 9, 11, 13, and 15), the tumors began to shrink, collapsing inward on their necrotic centers (arrows in *D*).

Table 2. Incidence of tumors in nude mice treated with anti-TFR-LysPE40

	Animals on postinjection day, no. with tumors/no. injected					
Anti-TFR-LysPE40	11	15	19	24	29	35
None	5/5	5/5	5/5	—	—	—
5 μg	5/6	6/6	6/6	6/6	6/6	—
20 μg	1/5	1/5	3/5	3/5	3/5	4/5
50 μg	0/5	0/5	1/5	1/5	1/5	2/5
150 μg*	0/3	0/3	0/3	0/3	0/3	0/3

Athymic mice were injected subcutaneously with 3×10^6 A431 cells. On days 5, 7, 9, and 11, the animals were injected i.p. with anti-TFR-LysPE40 at the indicated dose. The number of animals with tumors/total animals injected is shown on different days after tumor transplantation.

*Single dose on day 5. Two animals died on day 7.

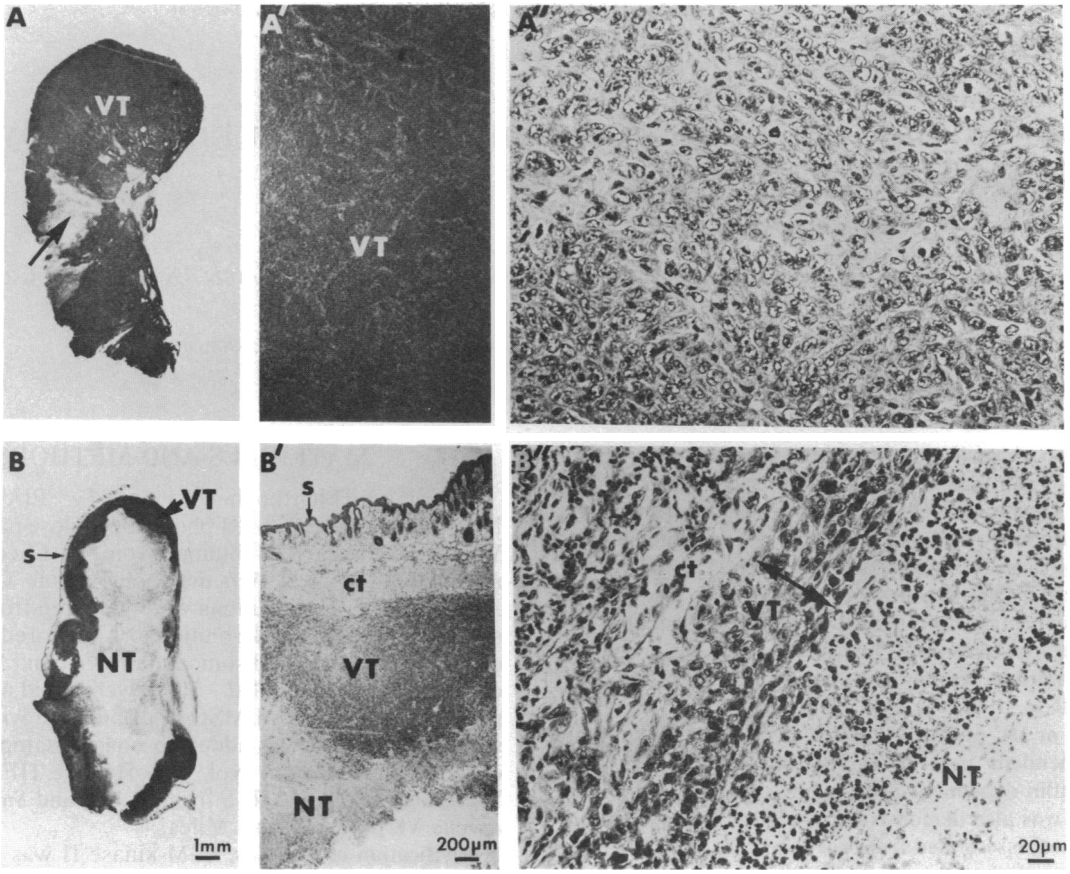

FIG. 7. Histological appearance of typical treated and control A431 subcutaneous tumors. A431 tumors were removed, fixed in formaldehyde, and processed for routine paraffin embedding and sectioning and staining with hematoxylin and eosin. (*A*) A section from a tumor removed from a mouse at day 15. (*B*) A similar section of a tumor removed from a mouse on day 19 that had been treated on days 9, 11, 13, and 15 with anti-TFR-LysPE40. *A* shows that the majority of the untreated tumor contains viable tumor cells (VT) with only small areas of necrosis (arrow). *B* shows that a large percentage of the treated tumor is necrotic (NT), with only a small rim of viable tumor (VT) remaining (s = skin). (A', A'' and B', B'') Higher magnification fields from the margins of tumors. The untreated tumor is mostly composed of homogeneous viable tumor cells (VT), whereas the treated tumor shows a rim of viable cells of variable thickness lying adjacent to a connective tissue capsule (ct). (*A* and *B* = ×3, Bar = 1 mm; A' and B' = ×17, Bar = 200 μm; *A''* and *B''* = ×170; Bar = 20 μm).

a slower β phase (19, 20). An immunotoxin made with anti-epidermal growth factor receptor and ricin A (528-rRA) has been shown to inhibit growth of A431 xenografts in nude mice when the treatment is started at the time of tumor implantation (16). The peak blood level of only 3.2% of injected conjugate was achieved 6 hr after the i.p. injection (16).

The current reagent, anti-TFR-LysPE40, is probably not useful for human studies because transferrin receptors are present on important normal tissues. Therefore, LysPE40 will need to be attached to antibodies like anti-Tac that bind antigen that are less widely distributed on normal cells to determine if any of these molecules can be developed into useful therapeutic agents.

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