Antitumor Effects of B3-PE and B3-LysPE40 in a Nude Mouse Model of Human Breast Cancer and the Evaluation of B3-PE Toxicity in Monkeys

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ABSTRACT

B3 is a tumor-reactive monoclonal antibody (mAb) that binds to a limited number of normal tissues. Immunotoxins made with B3 coupled to either Pseudomonas exotoxin (PE) or recombinant forms of PE with a deletion of the cell-binding domain (LysPE40) have been shown to cause complete tumor regression in nude mice bearing a rapidly growing A431 (L. H. Pai et al., Proc. Natl. Acad. Sci. USA, 88: 3358–3362, 1991) human epidermoid carcinoma. In this study we show that an immunotoxin composed of mAb B3 when chemically coupled to LysPE40 (B3-LysPE40) led to complete regression of a slowly growing breast cancer, MCF-7, in nude mice when given i.v. every other day for five doses. mAb B3 coupled to native PE also produced significant regression of the MCF-7 tumor. The reactivity of mAb B3 was evaluated using an immunohistochemical method on the two responsive tumors, MCF-7 and A431, and compared with a typical human colon carcinoma specimen that has B3 antigen on its surface. The results showed that both A431 and MCF-7 xenograft tumors have similar reactivity to B3 when compared with the human colon carcinoma specimen. To evaluate the toxicity of B3-PE in primates, Cynomolgus monkeys received escalating doses of B3-PE i.v. on Days 1, 3, and 5. Based on antibody localization studies using frozen sections of normal human and monkey tissue, gastric, trachea, and bladder mucosal injury could have occurred. However, no clinical signs of injury or histological damage to these organs were seen at the doses administered. Chemical hepatitis due to PE was transient and well tolerated at doses up to 50 μg/kg for three doses. The lethal dose was about 100 μg/kg, and the cause of death was liver necrosis, as shown by necropsy. We conclude that mAb B3, when coupled to PE or PE, can produce strong antitumor activity in vivo. The similar level of reactivity of the B3 antibody in our tumor models with a surgical specimen of a human colon carcinoma and the toxicity study in monkeys indicate that therapeutic doses of B3-PE and B3-LysPE40 can be delivered without causing toxicity to normal organs that express B3 antigen. Although both B3-PE and B3-LysPE40 have antitumor activity in nude mice bearing a human xenograft, B3-LysPE40 is better tolerated and should be further evaluated as a therapeutic agent for cancer patients.

INTRODUCTION

In the past few decades monoclonal antibodies against human cancer-associated antigens have allowed specific tumor targeting to be explored as a new therapeutic modality for cancer. Radioactive nuclides, chemotherapeutic agents, and bacterial toxins can be covalently coupled to monoclonal antibodies to produce immunotoxins. These immunotoxins react with the stomach, trachea, and bladder mucosal tissues of mice, however, with no clinical signs of injury or histological damage to these organs seen at the doses administered. Both B3-PE and B3-LysPE40 have antitumor activity in nude mice bearing a human xenograft, B3-LysPE40 is better tolerated and should be further evaluated as a therapeutic agent for cancer patients.

MATERIALS AND METHODS

Monoclonal Antibody B3. The properties of mAb B3 (IgG1c) have been previously described (8). The mAb used for these experiments was purified from serum-free culture medium by ammonium sulfate precipitation and chromatography on Mono Q and TSK-250 gel filtration columns. Its purity was established by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Monoclonal antibody MPC-21 (Sigma Chemical Co.) was used as control antibody in our experiments.

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3 The abbreviations used are: PE, Pseudomonas exotoxin A; LysPE40, recombinant form of PE with a deletion of the cell-binding domain; mAb, monoclonal antibody; IgG1c, immunoglobulin G1; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HSA, human serum albumin; LD50, 50% lethal dose; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; ID50, 50% inhibitory dose.

* Unpublished data.
Pseudomonas aeruginosa, was purchased from Swiss Serum (Berne, Switzerland).

Construction of Plasmid-expressing LysPE40. Fig. 1 shows the plasmid pJ88L1 f+t used for the expression of LysPE40 in Escherichia coli. pJ88L1 carries a LysPE40 gene under control of a T7 promoter and also contains a T7 transcriptional terminator and an f1 phage origin. Earlier, for LysPE40 expression we used the plasmid pJY85L in which the LysPE40 gene is preceded by an OmpA signal sequence. OmpA is cleaved upon secretion of LysPE40, leaving a three-amino acid extension Ala–Asn–Leu at the amino terminus of LysPE40 (10, 11). To construct pJ88L1 f+t, a 180-base pair fragment was amplified by PCR using two oligonucleotides and pJY85L as the template. One oligonucleotide was the same as the 5' end of DNA encoding for LysPE40 because most of the expressed protein was secreted into the periplasm. After purification the PCR fragment was ligated with PCR amplified fragment was restricted by Ndel and SalI and purified by electrophoresis. After purification the PCR fragment was ligated with a 4.8-kilobase pair dephosphorylated Ndel–SalI fragment of pVC 8 f+t. pVC 8 f+t is a plasmid containing a gene for PE40 under the control of T7 promoter.

Expression and Purification of LysPE40. BL21 (DE3) cells were transformed with the plasmid pJ88L1 f+t. The cells were grown in superbroth containing 100 μg/ml of Ampicillin at 37°C. At an A650 of 1.0, cells were induced with 1 mM isopropyl-B-D-thiogalactopyranoside. The cells were harvested 1 h later, and protein was localized as described elsewhere (10). The periplasm was used as the source of LysPE40 because most of the expressed protein was secreted into the periplasm. After filtration through a 0.45-μm filter, periplasm was applied onto an 8-ml Q-Sepharose column. Proteins were eluted by a linear gradient described for the Q-Sepharose column. Further purification was achieved by gel filtration chromatography on a TSK 250 column. The N-terminal sequence of LysPE40 was found to be Ala–Asn–Leu–Ala–Glu–Glu–Ala–Phe–Lys–Gly–Gly–Ser–Leu.

Construction of Immunconjugates. The antibody and toxin were linked by a thioether bond. PE and LysPE40 were incubated with 3-fold molar excess of succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce Chemical Co., Rockford, IL) at room temperature for 30 min. Monoclonal antibody B3 and MOPC-21 were incubated with 2-iminothiolane hydrochloride (Pierce Chemical Co., Rockford, IL) at 37°C for 1 h. Details of conjugation and purification methods have been described elsewhere (9).

Protein Synthesis Assay. The cytotoxic activity of the immunotoxins was evaluated in cultured tumor cell lines by measuring [3H]leucine incorporation as previously described (9). The mean values of triplicates were expressed as a percentage of controls that did not receive immunotoxin.

LD₅₀. Regarding toxicity of B3-PE and B3-LysPE40 in mice, groups of female BALB/c mice were given injections i.p. with escalating doses of immunotoxin diluted in 200 μl of PBS-HSA (0.2%). Animals were observed for 72 h for toxicity and death. The LD₅₀ is the dose of immunotoxin that kills 50% of the animals in 48 h.

Antitumor Activity of B3-PE and B3-LysPE40 in Nude Mice Bearing a Human Breast Carcinoma. Human MCF-7 cells were grown as subcutaneous tumors in female athymic nude mice. On Day 0 of each experiment, subcutaneous tumors were surgically removed from carrier mice and cut in pieces of approximately 1 mm in size. The tumor was implanted in the right flank using a sterile trochar. A 17β-estradiol pellet (0.72 mg/pellet, 60-day release) from Innovative Research of America, Toledo, OH, was placed in the posterior cervical region to maintain tumor growth. Treatment of mice with B3-PE or B3-LysPE40 started 13 to 15 days after tumor implantation, when the tumor had reached 5 x 5 mm in size (50 mm³). Control groups received MOPC-PE, mAb B3, or PBS containing 0.2% HSA. Each treatment group consisted of six to eight animals. Tumors were measured with a caliper every 4 days, and the volume of the tumor was calculated by using the following formula: tumor volume (mm³) = length x (width)² x 0.4.

Immunohistochemistry. A frozen sample from a human colon carcinoma specimen and a MCF-7 subcutaneous tumor resected from nude mice 20 days after tumor implantation were cryostat sectioned and processed for peroxidase immunohistochemistry as previously described (12). To minimize cross-reaction with endogenous mouse IgG in the tumor transplant, the initial immunoreactive step utilized a B3-PE conjugate, followed by rabbit anti-PE, than an anti-rabbit-horseradish peroxidase conjugate.

Immunotoxins made from monoclonal antibody B3 have been shown to be cytotoxic for various human cancer cell lines that express the B3 antigen on their surface (9). B3-PE is a potent immunotoxin active against human epidermoid carcinoma, A431, and breast carcinoma, MCF-7; the ID₅₀ for inhibition of protein synthesis for these cell lines was consistently between 0.1 and 0.5 ng/ml (9). B3-LysPE40 is also active.
To determine if tumors growing in B3-PE-treated mice failed to fully respond because they had become "antigen-negative," tumor was removed from a mouse treated with 1.5 µg of B3-PE. By immunohistochemistry, these tumor cells had a positive pattern of reactivity with B3 that is similar to cells maintained in tissue culture (results not shown). We conclude that the failure of B3-PE to cure the MCF-7 tumor was not due to the emergence of antigen-negative clones, but due to the presence of antigen-positive cells which might have been killed if larger amounts of immunotoxin could have been delivered to the tumor.

To determine if the MCF-7 tumors represent a model that is relevant for the treatment of human cancers, we examined the reactivity of B3 with a MCF-7 tumor removed from a nude mouse 20 days after tumor implantation and an A431 tumor removed 10 days after implantation, and compared their reactivity with that of a human colon carcinoma sample. Immunohistochemical studies showed that the colon tumor reacted with a stronger intensity than did the MCF-7 or A431 tumor growing in mice (Fig. 4). Therefore we conclude that the model is relevant for the study of human cancers.

To determine whether the specific (monoclonal antibody B3 related) or the nonspecific (PE related) toxicities would be dose limiting, B3-PE was administered to Cynomolgus monkeys. In addition, we wished to determine the maximum tolerated dose in primates. These issues were especially relevant because monkey tissues react in a similar manner and intensity as do human tissues with the B3 antibody (8). Eight Cynomolgus monkeys, two in each group, received i.v. injections of B3-PE at dose levels of 10, 30, 50, and 100 µg/kg on Days 1, 3, and 5. No major clinical abnormalities were noted in the animals that received 10, 30, and 50 µg/kg except transient loss of appetite and mild weight loss (less than 8% of the initial body weight) during the first week. Elevation of SGOT, SGPT, alkaline phosphatase, and lactate dehydrogenase occurred in all animals. The magnitude of the enzyme elevation ranged from 3- to 15-fold from the pretreatment level. Fig. 5 shows the SGOT, SGPT, and alkaline phosphatase levels of one animal from each group. Elevation occurred by Day 3, generally peaking by Day 5, and then returning to pretreatment values by the end of 2 wk.
in the animals that received 10, 30, and 50 $\mu$g/kg of B3-PE. Bilirubin remained normal in these groups. There were no significant changes in the other blood chemistries (electrolytes, blood urea nitrogen, creatinine) or cell blood count. Serum amylase levels remained normal. Urinalysis showed no evidence of bladder injury during therapy. However, occult blood was noted occasionally in the urine of female monkeys due to menses contamination. There were no occult blood found in the stool.

Monkeys that received 100 $\mu$g/kg developed massive liver failure. Both animals died on Day 5 of the study. Liver enzymes were elevated more than 100-fold from pretreatment levels, and serum bilirubin ranged from 4 to 5 mg/dl on Day 5. At necropsy, the liver was found to be very pale in both animals. Histological examination of the liver showed diffuse hepatic necrosis characterized by cleared, vacuolated cytoplasm in most hepatocytes with condensed, pyknotic nuclei and perivascular acute inflammatory infiltrates. Histological examination revealed no significant abnormalities in the heart, kidney, trachea, spleen, lymph node, pancreas, stomach, urinary bladder, or lungs.

Blood samples were obtained approximately 10 min after dosing on Days 1, 3, and 5. Serum levels of B3-PE were measured by bioassay, and results from each individual monkey are shown on Table 1. Results are consistent with the amount of immunotoxin administered. The serum level of B3-PE increased after each dose. Monkeys that received 10, 30, and 50 $\mu$g/kg on Days 1, 3, and 5 achieved serum levels as high as 27 ng/ml, 130 ng/ml, and 384 ng/ml by Day 5, respectively. These animals had liver injury that was reversible, as shown by normalization of liver enzymes. The two monkeys that received 100 $\mu$g/kg on Days 1 and 3 achieved blood levels as high as 909 and 806 ng/ml of B3-PE after the second dose. The third

### Table 1 Serum level of B3-PE in monkeys

<table>
<thead>
<tr>
<th>Dose ($\mu$g/kg)</th>
<th>Monkey</th>
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<tr>
<td></td>
<td>Day 1</td>
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<td>833</td>
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*Third dose omitted because of toxicity.*
dose was omitted in this group due to toxicity. Plasma levels obtained prior to necropsy were 227 and 83 ng/ml.

DISCUSSION

In the present study, monoclonal antibody B3 was linked to native PE or LysPE40, a recombinant form of PE, and shown to have a significant antitumor effect in nude mice bearing an established human breast carcinoma, MCF-7. B3-PE was able to cause substantial tumor regression and delay of growth at a dose level of 1.5 μg every other day for 4 days. B3-LysPE40 at a dose level of 75 μg every other day for 4 days caused complete regression of MCF-7 tumor. We have previously shown that both immunotoxins were capable of causing complete disappearance of A431 tumors in nude mice at a lower dose level. Because both A431 and MCF-7 cells in vitro have a similar ID50, 0.1 to 0.3 ng/ml for B3-PE and 1 to 4 ng/ml for B3-LysPE40, this difference in tumor response in mice may be due to the difference in the growth rate of the tumors in mice or the ability of the immunotoxin to penetrate into the tumor (9). A431 tumors grow very rapidly in mice, taking only 7 to 10 days to achieve 0.5 cm³, while it takes approximately 4 wk for MCF-7 tumors to achieve a similar volume. Because PE kills cells by inhibiting protein synthesis, cells that are actively growing may be more sensitive to these immunotoxins.

When compared with the reactivity of a human colon carcinoma specimen removed directly from a patient, the MCF-7 tumor and the A431 tumor grown in mice had a lower dose required for complete regression with the B3 antibody as shown by immunohistochemical analysis. Since the intensity of staining of the colon tumor is typical of many human carcinomas studies (8), we believe that similar or greater antitumor effects can be achieved in patients with tumors reactive to B3 antibody.

To determine if B3 linked to PE or PE40 could be developed for use in humans, we next evaluated the toxicity of B3-PE in Cynomolgus monkeys. B3-PE rather than B3-LysPE40 was selected because B3-PE is about 10-fold more potent, and toxicity studies in Cynomolgus monkeys. B3-PE rather than B3-LysPE40 was selected because B3-PE is about 10-fold more potent, and toxicity was observed at the doses administered. Except for liver failure related to PE at the 100-μg/kg dose level, liver damage was the major toxicity observed.5 Because B3-LysPE40 is better tolerated than is B3-PE, we anticipate that in monkeys and humans that therapeutic levels can be achieved without significant liver toxicity. Previously we described a method of preparing LysPE40 from the bacterial culture medium (11). The toxin contained an OmpA signal sequence to promote secretion. Recently, we have found it is easier to purify LysPE40 from the periplasm where it accumulates at much higher concentrations than in the medium. When the OmpA signal sequence was removed, a large proportion of LysPE40 was found in the periplasm from which it could be readily purified.

We conclude that monoclonal B3 when coupled to PE or PE40 has strong antitumor activity in nude mice bearing xenograft tumors that express B3 antigen. The similar reactivity of mAb B3 with immunotoxin-sensitive MCF-7 and A431 tumors and a human colon carcinoma sample makes us believe that a similar effect can be achieved in patients. Toxicity studies in monkeys demonstrate that therapeutic doses of B3-PE can be delivered without injury to normal organs that express B3 antigen. These data indicate that immunotoxins composed of B3 and PE or LysPE40 warrant further evaluation as antitumor agents in cancer patients.

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REFERENCES


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5 R. Kirkman, D. FitzGerald, and I. Pastan, unpublished data.
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