Anti-tumor activities of immunotoxins made of monoclonal antibody B3 and various forms of Pseudomonas exotoxin

(chemotherapy/epidermoid carcinoma/adenocarcinoma)

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ABSTRACT B3 is a monoclonal antibody that reacts with a carbohydrate epitope present on a variety of proteins located on the surface of many cancer cells and a limited number of normal tissues. We evaluated the cytotoxic activity of immunotoxins composed of monoclonal antibody B3 coupled to native Pseudomonas exotoxin (PE) or two recombinant forms of Pseudomonas exotoxin, PE<sub>Δ62</sub> or LysPE40, a form of PE with a deletion of the cell binding domain. All three conjugates were cytotoxic to human cell lines expressing the B3 antigen on their surface. The survival of each of the three immunotoxins in the circulation of mice was determined by immunohistochemistry. The half-life in blood of B3-PE and B3-PE<sub>Δ62</sub> was 20 hr, whereas the half-life of B3-LysPE40 was 4 hr. The short half-life of B3-LysPE40 may be due to the absence of domain I of PE. To determine the therapeutic effects of the three immunotoxins, they were given intraperitoneally to nude mice bearing subcutaneous A431 tumors. All three immunotoxins caused complete regression of 50-mm<sup>3</sup> tumors with no toxic effects to the animals at therapeutic doses. Furthermore, substantial regression was also noted with much larger tumors. Our data indicate that the monoclonal antibody B3, when coupled to PE or recombinant forms of PE, may be useful for the treatment of tumors expressing B3 antigen. The therapeutic window was largest with B3-LysPE40, which can be administered in higher doses because it lacks sequences in domain I of PE that enable PE to bind to nontarget cells.

Immunotoxins are potent cell killing agents that are emerging as chemotherapeutic agents for the treatment of cancer, acquired immune deficiency syndrome (AIDS), and some immunological disorders (1–5). We have been developing an alternate approach to conventional cancer chemotherapy using immunotoxins made from Pseudomonas exotoxin (PE) or genetically modified forms of PE coupled to antibodies reactive with antigens present on the surface of solid tumors. Initially, we showed that immunotoxins containing antibodies to the human transferrin receptor were very effective cell-killing agents in vitro and in vivo (6). However, these agents have limited clinical utility, because they react with many vital normal human tissues. We isolated a monoclonal antibody reactive with many ovarian cancers and coupled this antibody (OV3) to PE. This immunotoxin, OVB3-PE, was shown to kill human ovarian cancer cells in culture and to prolong the life of nude mice bearing human ovarian tumors (7). However, this agent was poorly tolerated in a phase I trial in women because of the reactivity of OVB3 with some cells in normal human cerebellum (unpublished data). Therefore, it was necessary to obtain a more tumor-specific antibody with no cross reactivity to vital human tissues. We have isolated a murine monoclonal antibody, termed B3, that shows strong reactivity with many mucin-producing tumors of the ovary, stomach, and colon, as well as strong reactivity with nonmucinous carcinomas of the colon and stomach (I.P., L.H.P., E. T. Lovelace, M. G. Gallo, A. V. Rutherford, J. L. Magnani, and M.C.W., unpublished data). Peroxidase immunohistochemistry using frozen sections of normal tissue demonstrated that B3 reacts with cells of the stomach, with the differentiated cell layer of the esophagus, with the epithelia of the tonsil, trachea, and urinary bladder, and with small bowel in whom. Similar reactivity was found in normal monkey tissues. Biochemical studies have shown that B3 reacts with a carbohydrate epitope present on many different cell surface proteins (unpublished data). The tissue and tumor reactivity of B3 is quite similar to that of monoclonal antibody BR96 (8). However, several differences in reactivity exist that show that B3 reacts with an epitope different from that of BR96.

PE is a 66-kDa protein composed of three domains: an amino-terminal cell receptor binding domain (domain I), a middle translocation domain (domain II), and a carboxyl-terminal activity domain (domain III). Domain III catalyzes the ADP-ribosylation and inactivation of elongation factor 2, which inhibits protein synthesis and leads to cell death (9, 10). Immunotoxins made with native PE are often very active but can produce liver toxicity due to the presence of domain I of PE (amino acids 1–252), which binds to PE receptors found on many normal cells. To overcome liver toxicity, various recombinant forms of PE have been made in Escherichia coli. Lysine-57 (domain I) was shown to be involved in the cellular binding of PE (11), and when mutated to arginine, its cytoxic activity on mouse L929 cells fell about 5-fold (unpublished data). LysPE40 is a 40-kDa recombinant form of PE that lacks all of domain I and contains an extra lysine residue near its amino end to facilitate coupling to antibodies (6).

The current report describes the characteristics and activity of three immunotoxins in which B3 was coupled to native PE to make B3-PE, to PE<sub>Δ62</sub> to make B3-PE<sub>Δ62</sub>, or to LysPE40 to make B3-LysPE40.

MATERIALS AND METHODS

Monoclonal Antibody B3. The isolation and characterization of B3 will be described in detail elsewhere. Briefly, BALB/c mice were tolerized with normal human kidney membranes and immunized with trypsin-treated MCF-7 cells. Spleens from immunized mice were removed and the suspended cells were fused with AG8 mouse myeloma cells. The antibodies produced by the resulting clones were screened 2 weeks later using rhodamine indirect immunofluorescence on living MCF-7 cells. Screening was carried out using a ScreenFast (Life Technologies, Gaithersburg, MD) large-scale screening chamber (12). Selected clones were secondarily screened using peroxidase immunohistochemistry on cryostat sections of human tumors and nor-

Abbreviation: PE, Pseudomonas exotoxin.

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Table 1. Cytotoxicity of B3-PE, B3-PEArg57, and B3-Lys PE40 on various tumor cell lines

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cell line</th>
<th>ID50, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B3-PE</td>
<td>B3-PEArg57</td>
</tr>
<tr>
<td>Epidermoid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vulva</td>
<td>A431</td>
<td>0.1</td>
</tr>
<tr>
<td>Breast</td>
<td>MCF-7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>HTB-19</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>HTB-30</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>CRL-1500</td>
<td>20</td>
</tr>
<tr>
<td>Gastric</td>
<td>HTB103</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CRL 1793</td>
<td>0.1</td>
</tr>
<tr>
<td>Prostate</td>
<td>LnCAP</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>PC-3</td>
<td>30</td>
</tr>
<tr>
<td>Bladder</td>
<td>RT-4</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 1. A431 at 5 x 10⁶ cells per 1.5 ml was incubated with various concentrations of free antibody (○) or immunotoxins B3-PEArg57 (●) or B3-Lys PE40 (□), and their competition for the binding of ¹²⁵I-labeled B3 was measured.

Fig. 2. Inhibition of protein synthesis in A431 cells by B3 conjugates. Immunotoxins were added to the cells in the absence (●), or in the presence (△, ○) of excess B3 (25 µg/ml) for 18–24 hr at 37°C. • and □, B3-PE; △ and ○, B3-PEArg57; ● and ○, B3-LysPE40.

mal tissues. One clone was retained that reacted with human colon cancers and gastric cancers but not with normal human liver, kidney, or colon. It was doubly subcloned and its isotype was determined to be IgG1k. The antibody was purified from serum-free culture medium by ammonium sulfate precipitation and chromatography on a TSK-250 gel-filtration column (21.5 x 600 mm). Its purity was established by SDS/PAGE. Monoclonal antibody MOPC-21 is a mouse myeloma antibody (IgG1) with no known reactivity with mouse or human tissues and was purchased from Sigma.

**PE, PEArg57, and LysPE40.** PE purified from the culture medium of *Pseudomonas aeruginosa* was purchased from Swiss Serum (Bern, Switzerland). PEArg57 was prepared from the periplasm of *E. coli* as described for other PE constructs using plasmid pJYS57R (Y. Jinno, V. Chaudhary, and I.P., unpublished data). LysPE40 was prepared from the medium as described (6).

**Construction of Immunotoxins.** PE, PEArg57, or LysPE40 at 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 succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate and incubated at room temperature for 30 min. Protein was separated from the unreacted cross-linker on a PD10 column. Monoclonal antibody (B3 or MOPC-21) at 5–10 mg/ml was mixed with a 3-fold molar excess of 2-iminothiolane hydrochloride 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 antibody and PE, PEArg57, or LysPE40 were mixed and incubated at room temperature for 16–20 hr. Immunotoxin was then purified by successive chromatography on MonoQ and TSK 250 columns (13). SDS/PAGE was done as described (13). The major immunotoxin peak consisted of a 1:1 conjugate of antibody and PE or PEArg57 or LysPE40. Yields were in the range of 10–15% of the starting materials.

**Protein Synthesis Assay.** Inhibition of protein synthesis was used to measure the activity of each immunotoxin on various cell lines. Immunotoxins were diluted with 0.2% human serum albumin in phosphate-buffered saline (PBS) prior to addition to cells. Cells were seeded at 1 x 10⁶ cells per ml in 24-well plates or 96-well plates 24 hr prior to the addition of immunotoxin, incubated at 37°C for 24 hr, and then assayed for incorporation of [³⁵S]methionine (New England Nuclear; specific activity, 1408 Ci/mmol; 1 Ci = 37 GBq) as described (13). The mean values of triplicates were expressed as percentages of controls that did not receive immunotoxin.

**Antibody Binding Assay.** B3 was iodinated by the lactoperoxidase method and purified by gel filtration on a PD10 column. MCF-7 and A431 cells were seeded at 5 x 10⁵ cells per 1.5 ml in 12-well plates 24 hr prior to the assay. Cells were washed with ice-cold phosphate-buffered saline containing bovine serum albumin (2 mg/ml). Initial experiments determined that the binding of ¹²⁵I-labeled B3 (specific activity, 0.014 µCi/ng) to A431 and MCF-7 cells reached equilibrium at 2 hr. In the competition studies, various concentrations of free antibody or B3-PEArg57 or B3-LysPE40 were added to these cells and their ability to compete for the binding of ¹²⁵I-labeled B3 was determined.

**Assay of Blood Levels of B3-PE, B3-PEArg57, and B3-LysPE40 in Mice.** Four- to 6-week-old (19–20 g) female BALB/c mice or immunodeficient (nu/nu) mice bearing A431 subcutaneous tumors were injected with 1 µg of B3-PE, B3-PEArg57, or 20 µg of B3-LysPE40 in the tail vein. Blood was drawn at 2 min, 10 min, 1 hr, 4 hr, 8 hr, 12 hr, 24 hr, 48 hr, 72 hr and the level of the immunotoxin was assayed by incubating serum with A431 cells and measuring its ability to inhibit protein synthesis. A standard curve was made for each immunotoxin.

**Anti-Tumor Activity of B3-PE, B3-PEArg57, and B3-LysPE40 in Nude Mice Bearing a Human Epidermoid Carcinoma.** A431 cells (3 x 10⁶ cells) were injected subcutaneously on day 0 into female nude mice (4–6 weeks old, 18–20 g). Tumors about 5 mm x 5 mm in size developed in all mice by day 5. Treatment of mice with B3-PE, B3-PEArg57, and B3-LysPE40 was started on either day 1, day 4, or day 7 after tumor implantation. Each treatment group consisted of five or six
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 (in mm$^3$) = length $\times$ (width)$^2 \times 0.4$. All statistical comparisons were made using the Wilcoxon rank sum test.

**RESULTS**

Monoclonal antibody B3 was selected because of its reactivity with many colon, gastric, breast, and ovarian tumors. To determine if B3 could deliver toxins to target cells and kill these cells, immunotoxins were prepared consisting of B3 coupled to three forms of PE. B3-PE is an immunotoxin in which B3 is coupled to native PE. B3-PE$^{\text{Arg57}}$ is an immunotoxin in which B3 is coupled to a mutant form of PE in which lysine-57 is changed to arginine. This mutation decreases the ability of PE to bind to the PE receptor found on almost all cells; PE$^{\text{Arg57}}$ is about 5-fold less toxic than PE to 3T3 cells and to mice (unpublished data). Finally, B3 was coupled to LysPE40, a mutant form of PE that lacks domain I and is, therefore, much less toxic to cultured cells and to mice (6).

The activities of B3-PE, B3-PE$^{\text{Arg57}}$, and B3-LysPE40 were tested on a variety of human adenocarcinoma cell lines as well as on the epidermoid carcinoma cell line A431 (Table 1). The three immunotoxins were found to be active in most of the cell lines tested; however, they were especially active on three cell lines: the A431 epidermoid carcinoma cell line, the MCF-7 breast carcinoma cell line, and the CRL 1793 gastric carcinoma cell line. On A431 and MCF-7 cells, conjugates made with both PE, PE$^{\text{Arg57}}$, and LysPE40 were extremely active with ID$_{50}$ values ranging from 0.2 to 1 ng/ml. The cytotoxic effect of these immunotoxins was shown to be specific because MOPC-PE, an immunotoxin made with an antibody that does not react with human cells, had no cytotoxic activity even at 1000 ng/ml.

To determine the relative binding activity of two of the immunotoxins, the native B3 antibody was iodinated and the ability of B3-PE$^{\text{Arg57}}$ and B3-LysPE40 to displace the iodinated antibody was determined and compared with the ability of native unlabeled antibody to displace the $^{125}$I-labeled antibody. As shown in Fig. 1, B3-PE$^{\text{Arg57}}$ and B3-LysPE40 displaced $^{125}$I-labeled B3 in a similar manner with 50% inhibition occurring at $\approx$100 pM. Based on the finding that 50% inhibition of binding by B3 occurred at 35 pM, the immunotoxins bind to cells about 20% as well as native antibodies.

Fig. 2 shows the cytotoxic activity of the three B3 conjugates on A431 cells. The activities of B3-PE and B3-PE$^{\text{Arg57}}$ are very similar and B3-LysPE40 is somewhat less active. The cytotoxic activities of all three immunotoxins are spe-
specific, because they are completely eliminated by the addition of excess B3 (25 μg/ml).

The potent cytotoxic activity of the immunotoxins containing B3 suggested that these agents might be effective against tumors bearing the B3 antigen. To determine the blood levels that could be achieved in these mice and the duration that the immunotoxins would be present in the blood, 1 μg of B3-PE, 1 μg of B3-PEArg57, or 20 μg of B3-LysPE40 were administered to groups of mice. The immunotoxins were given intravenously and serum samples were collected beginning 2 min after the i.v. injection. The data in Fig. 3 show the blood levels of the three immunotoxins. B3-PE and B3-PEArg57 decayed slowly with an apparent half-life of about 20 hr. B3-LysPE40 decayed more quickly with an apparent half-life of about 4 hr.

Because the immunotoxins were present at substantial concentrations for many hours, it seemed possible that they might exert an anti-tumor effect. Therefore, the anti-tumor activity of the immunotoxin was assessed in nude mice bearing A431 human epidermoid cancer cells. To do this, 3 × 10⁶ cells were injected subcutaneously on day 0. Treatment was started on day 4 with either PBS or immunotoxin dissolved in PBS. On day 4, the tumors measured about 5 × 5 mm and were about 50 mm³ in size. As shown in Fig. 4, in animals that were given three doses of 0.75 μg of B3-PE or B3-PEArg57 on days 4, 6, and 8, the tumors completely disappeared and remained undetectable for 3 months. The two groups, control and treated, were compared at each time point using the Wilcoxon rank test. Beginning on day 12 there was a significant anti-tumor effect evident with both B3-PE and B3-PEArg57 (P₂ < 0.005). In the animals treated on days 7, 9, and 11, the tumors stopped growing, regressed somewhat, and then resumed their growth. In animals treated on days 7, 9, 11, 13, and 15, the tumors regressed substantially leaving a hemorrhagic mass with a rim of living cancer cells. The mass then remained at about the same size for 24 days (P₂ = 0.009).

Fig. 5 A and B shows the effect of B3-PE and B3-PEArg57, respectively, on the growth of A431 tumors in nude mice at various dose levels. Mice were injected with 3 × 10⁶ cells subcutaneously on day 0 and treatment with B3-PE or B3-PEArg57 was started on day 4 by administering 0.75 μg, 0.5 μg, and 0.25 μg every other day. At a dose level of 0.25 μg or 0.5 μg, the tumors disappeared almost completely after treatment but resumed their growth 3–4 weeks later. Animals treated with 0.75 μg showed no evidence of tumor after 3 months (P₂ < 0.0001).

To determine the anti-tumor activity of B3-LysPE40, groups of animals bearing A431 tumors were treated every other day i.p. with the immunonojugate at dose levels of 2.5, 10, and 20 μg. Treatment was initiated on day 4. As shown in Fig. 6, at the dose level of 20 μg, complete remission of the tumor was observed that lasted for at least 3 months (P₂ < 0.0001). With 10 μg and 2.5 μg, only partial remissions were observed.

**DISCUSSION**

In this study, we have shown that immunotoxins made with B3, a monoclonal antibody that reacts with many human carcinomas, kill cell lines reacting with the antibody and cause regression of a human epidermoid carcinoma. In addition, we have found that B3-PE causes regression of the MCF-7 breast carcinoma (unpublished data). B3 was coupled to several forms of PE. The toxicity in animals of immunotoxins made with native PE is mainly due to the binding of domain I of PE to liver cells (9). To minimize the toxic effect of PE due to its nonspecific binding, we made immunonojugates with two recombinant forms of PE, PEArg57 and LysPE40. PEArg57 is approximately 5-fold less toxic to murine L929 cells than native PE; it is also less toxic to mice than native PE. The anti-tumor activities of the immunonojugates...
B3-PE and B3-PEAr57 in vitro and in mice are very similar (Fig. 1). However, the LD$_{50}$ values of the two conjugates in mice showed only small differences. The single-dose LD$_{50}$ is 2.5 $\mu$g for B3-PE and 3.5 $\mu$g for B3-PEAr57. Although not significant, these findings suggest that this recombinant form of PE may have a small advantage over native PE for use in immunoconjugates due to its slightly lower toxicity. In contrast, LysPE40 appears to be a superior molecule for conjugation with monoclonal antibodies. B3-LysPE40 has no detectable cytotoxicity against cultured cells to five times. The single-dose LD$_{50}$ of the immunoconjugates due to antibody does not bind. Furthermore, 20 $\mu$g of B3-LysPE40 has a remarkable antitumor activity when injected three to five times. The single-dose LD$_{50}$ of the B3-LysPE40 is about 200 $\mu$g.

When administered i.v. in mice at a dose of 1 $\mu$g, B3-PE and B3Ar57 have half-lives of 20 hr; this is to be compared with B3-LysPE40, which has a half-life of only 4 hr. The absence of domain I of PE not only has an effect on the binding of PE to cells (9) but also shortens its half-life in mice. The biochemical basis of this difference in half-life remains to be determined.

All three conjugates produced complete regression of actively growing solid tumors using doses that were nontoxic to animals. When the tumors were allowed to grow to a larger size, four doses of the immunotoxin decreased the tumor size, the mass became hemorrhagic, and the overlying skin became necrotic probably due to infiltration of the tumor into the skin. Because of this skin necrosis leading to potential infection in these immunodeficient animals, further doses could not be administered to determine if these tumors could also be completely eliminated.

In summary, B3 is a monoclonal antibody that shows strong reactivity with many solid tumors, including mucinous and nonmucinous adenocarcinomas. Its limited reactivity with normal human tissues may make it useful for targeted therapy in humans. Because B3 reacts with monkey tissues in a manner similar to that of human tissues, it should be possible to determine if an immunotoxin composed of B3 and one or more forms of PE has properties that indicate it should be evaluated in patients with cancer.

We thank Ms. Betty Lovelace for technical assistance, and Dr. Waldemar Debinski for helping us with B3 iodination.