Antitumor Activity of a Thioether-Linked Immunotoxin: OVB3–PE

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A thioether-linked immunotoxin was made between *Pseudomonas* exotoxin and the monoclonal antibody OVB3. This conjugate, OVB3–PE, was cytotoxic for the human ovarium cancer cell line OVCAR-3 (ID of 2.5×10^{-12} M) and it was therefore tested for antitumor activity in a nude mouse model of ovarian cancer. This model employs the injection of a lethal number of OVCAR-3 cells into the peritoneal cavity of nude mice. When 0.2–1 μg of OVB3–PE was injected intraperitoneally on three successive days beginning 3–5 days after OVCAR-3 cell implantation, the survival of the tumor-bearing mice was increased 2–4-fold compared to that of untreated control mice. Median survival times for control mice ranged from 44 to 50 days while survival times of 150 days or greater were seen in mice treated with OVB3–PE. When OVB3–PE administration was delayed until 2–4 weeks after tumor cell implantation, OVB3–PE treatment also showed antitumor activity, but the duration of survival was less than with the early treatments. OVB3–PE was also cytotoxic for MCF-7 breast carcinoma cells, HT-29 colon carcinoma cells, and A431 epidermoid carcinoma cells.

INTRODUCTION

Ovarian cancer is an important cause of death in women. New approaches, such as immunotoxin therapy (*1*, *2*), are needed because ovarian cancer is frequently resistant to chemotherapy and because it often spreads to the peritoneal cavity before it is discovered so that surgery cannot remove all the tumor. In order to treat this disorder, we have developed a monoclonal antibody, OVB3, that has reacted with all adenocarcinomas of the ovary examined so far (25/25) as well as some adenocarcinomas of the breast and colon (*3*). This antibody was coupled previously to *Pseudomonas* exotoxin (PE)[1] by a disulfide bond (*3*, *4*) and the resulting conjugate, OVB3–PE, was shown to kill ovarian cancer cells in tissue culture and to prolong the life of immunodeficient mice with human ovarian cancer implants (*3*). Here we extend our original findings and describe a method to conjugate PE to OVB3 by a thioether bond. Thioether conjugates may have advantages in animals over disulfide conjugates since the carbon–sulfur bond is likely to be more stable in vivo.

In this study we have prepared a thioether conjugate of OVB3 and PE and evaluated its cell-killing activity in tissue culture and in a tumor model. In vitro it inhibited protein synthesis with an ID_{50} of $2–3 \times 10^{-12}$ M. The antitumor activity of the thioether conjugate was tested in the OVCAR-3 nude mouse model of human ovarian cancer. Mice implanted with 25×10^6 OVCAR3 cells die about 40 days after tumor implantation with massive ascites (*5*), but mice treated 3–5 days after implantation with OVB3–PE survive to 100 days or longer, with a few animals living for over 175 days. Furthermore, administration of OVB3–PE at day 26 after tumor implantation, when significant ascites and a large tumor load is present, causes the disappearance of ascites and prolongation of the life of the tumor-bearing animals.

[1] Abbreviations: PE = *Pseudomonas* exotoxin, MST = median survival time, ip = intraperitoneally, H and E = hematoxylin and eosin, SMCC = succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, SDS–PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Because OVB3–PE has been shown to be active against human ovarian cancer growing in mice, a phase I study using OVB3–PE has begun in women. The information contained in this paper was used to plan the initial protocol.

EXPERIMENTAL PROCEDURES

Reagents. Purified PE was purchased from Swiss Serum and Vaccine Institute, Berne, Switzerland. The OVB3 antibody was produced by in vitro culture at Damon Biotech. Purified antiTac antibody was a gift from T. Waldmann (NCI). Cross-linking and protein-modification reagents were obtained from Pierce Chemical Co.

Construction of OVB3–PE by Thioether Linkage. To couple PE to OVB3 by a thioether bond, PE was first reacted with sulfo-SMPB (sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate). Typically PE at 2–3 mg/mL in borate buffer, pH 8.0, was reacted with a 3-fold molar excess of sulfo-SMPB. Immediately prior to use sulfo-SMPB was dissolved in dimethyl formamide (DMF) to a final concentration of 5–10 mg/mL (as appropriate). Usually 5 μL or less of the sulfo-SMPB solution was added per milliliter of PE solution. The reaction, which proceeded at 37 °C for 30 min, was quenched by the addition of excess glycine. PE modified in this manner was then resolved from low molecular weight reactants by HPLC gel-filtration chromatography (TSK-250, Bio-Rad, 21.5 × 600 mm). The running buffer for this column was 0.2 M $NaPO_4$, 1 mM EGTA, pH 7.0. With this chromatography system, both PE and chemically modified PE elute at approximately 140 mL. OVB3 was reacted for 1 h at 37 °C with a 3-fold molar excess of 2-iminothiolane in 0.2 M $NaPO_4$, 1 mM EGTA at pH 8.0. Reaction with 2-iminothiolane introduced approximately 0.9 mol of SH per mol of OVB3. This was determined with 5,5′-dithiobis(2-nitrobenzoic acid) (*4*). As with PE, excess glycine was used to quench the reaction. Chemically modified antibody was recovered by gel filtration on a TSK-250 (21.5 × 600 mm) column. The running buffer was the same as described above and the antibody eluted at 117 mL. Finally, PE, having a reactive maleimide group, was mixed

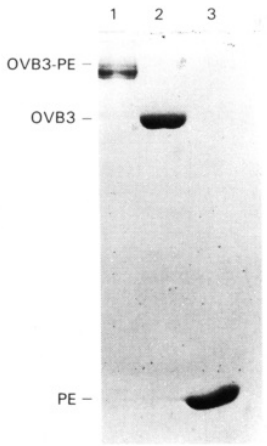

Figure 1. SDS–PAGE run under nonreducing conditions, using 10% acrylamide: lane 1, OVB3–PE (made by thioether linkage); lane 2, OVB3; and lane 3, PE.

with OVB3–SH. The two proteins were allowed to react at room temperature overnight and then further purified by gel filtration. The 1:1 conjugate which eluted at 105 mL was recovered and used in all subsequent experiments (see Figure 1 for SDS–page analysis of the final product). Protein concentration for the 1:1 conjugate was determined with the following conversion factor: absorbance of 1.3 at 280 nm was equivalent to 1.0 mg/mL of total protein.

AntiTac–PE was also made by thioether linkage and served as a control immunotoxin. AntiTac–PE (thioether) was made by using a similar protocol to the one used for the thioether-linked OVB3 immunotoxin. However, the one-to-one conjugate was purified by using a different separation strategy. The final reaction mixture was applied to a MonoQ (Pharmacia/LKB) column and eluted with a linear NaCl gradient. This separated unreacted antibody (which eluted at approximately 0.2 M NaCl) from a complex mixture that contained the immunotoxin and unreacted PE (this complex mixture eluted between 0.25 and 0.28 M NaCl). The one-to-one conjugate was then separated from high molecular weight material and unreacted PE on a HPLC sizing column. To ensure the conjugate was made correctly, it was tested on target HUT102 cells and had an ID_{50} value of 0.5 ng/mL.

Tissue Culture. OVCAR-3, MCF-7, A431, and HT-29 cells were maintained in DMEM, 10% FCS. HUT-102 cells were maintained in RPMI 1640, 10% FCS. For cytotoxicity studies, cells were plated at 1–2 × 10^5 cells/well in 24-well Costar tissue-culture plates (*6*). Adherent cells were seeded approximately 24 h before the addition of immunotoxin. HUT-102 cells were washed 3 or 4 times with tissue-culture medium and used the same day.

Cytotoxic Activity. Cytotoxic activity was determined by measuring inhibition of protein synthesis. Immunotoxins were added to cells in culture for 20 h. At the end of this period, [^{3}H]leucine at 10 μCi/mL was added for a further hour. Cells were washed with PBS, solubilized with 0.1 M NaOH, precipitated with excess TCA, and counted. Experiments were done with triplicate samples and usually repeated on 3 or 4 separate occasions.

Animal Experiments. B75 female mice 6–9 weeks old were used to grow OVCAR-3 ascites tumors. Usually, 25 × 10^6 washed cells were injected into recipient mice on day 1. Untreated, these mice died from their tumor burden 40–50 days later (*5*, *7*). To prevent undue suffering, mice beginning to show distress from their tumor burden were killed prematurely. Tumor-bearing mice were treated by

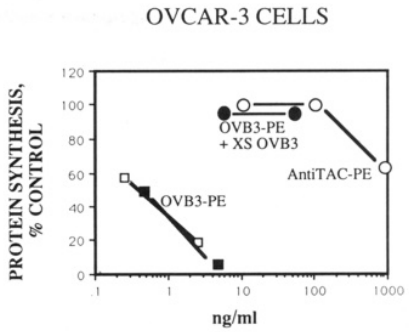

Figure 2. Inhibition of protein synthesis by OVB3–PE. Various concentrations of OVB3–PE (two individual experiments are shown) or antiTac–PE shown as total protein in ng/mL were added to OVCAR-3 cells for approximately 20 h. Following this, [^{3}H]leucine was added to individual wells to a final concentration of 10 μCi/mL for 1 h. Inhibition of protein synthesis was determined by measuring the radioactivity in TCA-precipitable material in immunotoxin-treated wells compared to that of untreated wells. Parallel experiments were conducted where excess OVB3 (75 μg/mL) was added to cells immediately prior to the addition of the OVB3–PE.

Table I. Cytotoxic Activity of OVB3–PE for Various Adenocarcinomas

cell line	OVB3–PE ID_{50},[a] ng/mL	cell line	OVB3–PE ID_{50},[a] ng/mL
OVCAR-3	0.5	A431	0.4
MCF-7	0.3	HT-29	0.5

[a] ID_{50}s were assessed after an overnight incubation with immunotoxin.

ip administration of immunotoxins. The early-treatment protocols (experiments 1–5) involved giving injections of OVB3–PE beginning on days 3–5 and various numbers of individual injections were administered. For the late-treatment protocol (experiment 6), immunotoxin treatments were initiated 19–33 days after the injection of the tumor cells. In the case of late treatments, five injections were given approximately every other day. All immunotoxin preparations were formulated in normal saline/human serum albumin (10 mg/mL), filter sterilized (0.22 μM, GV-Millipore), and injected ip in a volume of 0.5 mL.

RESULTS

OVB3–PE (Thioether): Evaluation of Cytotoxic Activity. To assess the cytotoxic potential of a thioether-linked immunotoxin, a one-to-one conjugate was made between the monoclonal antibody OVB3 and *Pseudomonas* exotoxin (PE). OVB3–PE was purified by gel-filtration chromatography and evaluated for its cytotoxic activity by adding various concentrations of the immunotoxin to OVCAR-3 cells. After a 20-h incubation, inhibition of protein synthesis was determined. As shown in Figure 2 and Table I, the ID_{50} was 0.5 ng/mL (2.5 × 10^{-12} M). When the control immunotoxin, antiTac–PE, which does not bind OVCAR-3 cells was added, an ID_{50} of greater than 1000 ng/mL was noted. Also excess OVB3 competed at least 100-fold for the cytotoxic activity of OVB3–PE. OVB3–PE was also assessed for cytotoxic activity against other cancer cell lines and for its antitumor activity against OVCAR-3 tumors growing in nude mice (see below).

Antitumor Activity Mediated by Early Treatment with OVB3–PE. The thioether conjugate of OVB3–PE was tested for antitumor activity in the same nude mouse model of human ovarian cancer previously used to test the

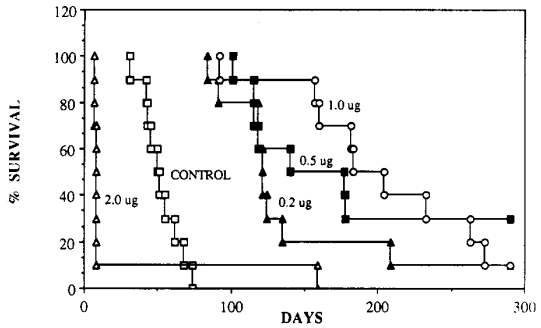

Figure 3. Inhibition of tumor growth by OVB3–PE. OVCAR-3 (25×10^6) cells were injected into the peritoneal cavity of B75 athymic nude mice on day 1. On days 3, 4, and 5, various amounts of OVB3–PE (ranging from 0.2 to 2.0 μg) were injected ip in 0.5 mL of sterile normal saline, 10 mg/mL of human serum albumin. The survival of each group of mice was then followed. There were 10 mice in each group.

antitumor activity of the OVB3–PE disulfide conjugate (*3*) and various other immunotoxins (*7*, *8*). In this model, 25×10^6 OVCAR-3 cells were injected intraperitoneally into athymic nude mice on day 1, and immunotoxin treatment was begun on day 3 or later. The immunotoxin was also given by the ip route. The mice were then monitored for immunotoxin-mediated antitumor effects. The OVCAR-3 tumor was lethal and untreated mice usually died between days 40 and 50. At death these mice had one or two large tumor masses, studding of the abdominal cavity with small tumor implants, and 5–7 mL of ascites fluid containing approximately 500×10^6 unattached tumor cells.

When OVB3–PE was injected on days 3, 4, and 5 after the injection of the tumor cells, there was a large increase in the duration of survival compared to that of the untreated animals. The duration of increased survival was generally related to the amount of immunotoxin given (Figure 3 and Table II, experiment 1). In this experiment, the median survival time (MST) was increased from 50 days in control mice to 121 days for mice receiving three injections of 0.2 μg, to 158 days for mice receiving 0.5 μg, and to 193 days for mice receiving 1.0 μg of immunotoxin. The injection of three doses of 1.0 μg of OVB3–PE was the maximum tolerated daily dose. Injection of 2.0 μg per day for 3 days was lethal for at least 50% of the mice.

Two experiments (nos. 2 and 3 of Table II) were carried out where OVB3–PE was first administered on day five and injections were given on days 5, 6, and 7. In both cases a significant antitumor effect was seen (Table II). Additional experiments were then carried out to determine (1) whether a greater number of immunotoxin injections would increase median survival and (2) whether beginning OVB3–PE treatment 2 days after tumor inoculation would be more effective than beginning 5 days after inoculation. The results of experiment 4 indicated that seven injections were no more effective than three when 0.2 μg of immunotoxin were given but were marginally better when 0.5 μg were administered. Experiment 5 indicated that at early times after tumor inoculation there was little or no differences in survival when treatment was initiated 2 days after inoculation or 5 days after inoculation.

Antitumor Activity Mediated by Late Treatment with OVB3–PE. To determine if it were possible to achieve antitumor effects even when the OVCAR-3 tumors had reached a large size and significant ascites was present, the first injection of OVB3–PE was delayed by 2–4 weeks after the injection of tumor cells. In one experiment (Table III, experiment 6), five daily injections of 1 μg of OVB3–PE were administered to tumor-bearing mice beginning on day 19 or 26. As a control for early treatment, OVB3–PE (1 μg) was also administered starting on day 5. The results indicated that the administration of OVB3–PE beginning on day 19 or 26 resulted in both antitumor activity and increased survival compared to control mice receiving only diluent (Figure 4 and Table III). The increase in survival time for mice beginning treatment on day 19 or 26 was approximately 26 days. In contrast, the increase in survival for mice beginning treatment on day 5 was greater than 100 days.

To follow tumor growth, each mouse was weighed and scored for abdominal girth on a weekly basis. An increase in body weight began two weeks after injection of the tumor cells. By day 26, most of the mice had gained 10 g and had an abdominal girth score of 3–4+ (scale of 0–4+). The increase in body weight was due to the build-up of ascites fluid within the abdominal cavity. The body weight of the untreated mice continued to increase until death (Figure 4A). In the group of mice that received OVB3–PE (1 μg/injection × 5 between days 26 and 32) there was a rapid decrease in body weight (Figure 4B), a reduction in abdominal girth, and a 26-day prolongation of survival (Figure 4C). Similar survival data to that shown in Figure

Table II. Median Survival Times (MST) of Tumor-Bearing Mice Treated with OVB3–PE

group	OVB3–PE, μg/injection	MST, days	no. of injections	day of injection
		Experiment 1 (n = 10)		
1	0	50	3	3, 4, 5
2	0.2	121	3	3, 4, 5
3	0.5	158	3	3, 4, 5
4	1.0	193	3	3, 4, 5
5	2.0	8[a]	3	3, 4, 5
		Experiment 2 (n = 10)		
1	0	44	3	5, 6, 7
2	0.2	87	3	5, 6, 7
3	0.5	87	3	5, 6, 7
4	2.0	42[a]	3	5, 6, 7
		Experiment 3 (n = 10)		
1	0	48	3	5, 6, 7
2	0.05	56.5	3	5, 6, 7
3	0.1	70.0	3	5, 6, 7
4	0.2	64.5	3	5, 6, 7
5	0.5	69.5	3	5, 6, 7
6	1.0	91.5[b]	3	5, 6, 7
		Experiment 4 (n = 7)		
1	0	53	3	5, 6, 7
2	0.2	81	3	5, 6, 7
3	0.5	83	3	5, 6, 7
4	2.0	10[a]	3	5, 6, 7
5	0.2	83	7	5, 6, 7, 9, 10, 11, 12
6	0.5	118	7	5, 6, 7, 9, 10, 11, 12
7	2.0	10[a]	7	5, 6, 7, 9, 10, 11, 12
		Experiment 5 (n = 10)		
1	0	46	5	2, 3, 5, 7, 10
2	1.0	90[c]	5	2, 3, 5, 7, 10
3	1.0	90	5	5, 7, 10, 12, 14

[a] At least 50% of mice in this group died from dose-related toxicity. [b] Three mice alive past day 160. [c] Four mice alive past day 140.

Table III. Survival of Tumor-Bearing Mice Receiving Early and Late Treatments with OVB3–PE

experiment 6 (n = 10); group	OVB3–PE, μg/injection	MST, days	no. of injections	day of injection
1	0	37	5	5, 6, 7, 10, 11
2	1.0	156	5	5, 6, 7, 10, 11
3	1.0	63	5	19, 20, 21, 24, 25
4	1.0	63	5	26, 27, 28, 31, 32

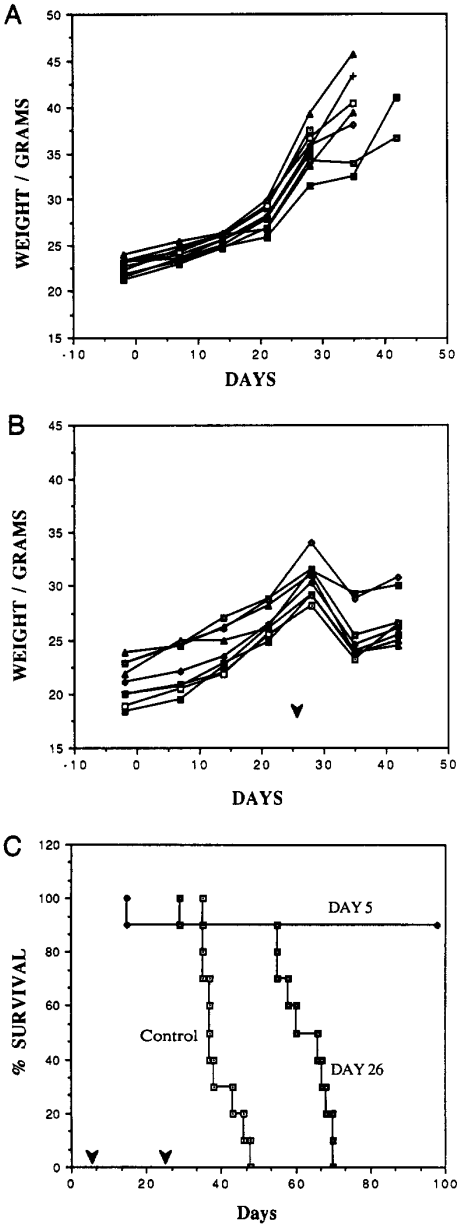

Figure 4. Antitumor effect of OVB3–PE on mice bearing large tumor burden with ascites. OVCAR-3 (25×10^6) cells were injected into the peritoneal cavity on day 1. Beginning on day 26, five individual injections (see Table III for exact days) of OVB3–PE were given to mice with prominent distention from ascites accumulation. As a control for early treatment, one group of mice was treated beginning on day 5. A shows the progressive increase in weight of control mice that were not treated with immunotoxin (data for individual mice are shown). B shows the weight of mice treated with OVB3–PE beginning on day 26. The arrow indicates the day when immunotoxin treatment was initiated. C shows the survival curves for mice treated with OVB3–PE (1 μg/injection) beginning on day 26 (■) or day 5 (◆) or untreated (⊡). The arrows indicate the days when immunotoxin treatment was initiated. Additional details are provided in Table III (experiment 6).

4C were obtained for the mice that were treated with OVB3–PE between days 19 and 25 (Table III).

An additional experiment was performed to determine the fate of large tumors treated with cytoreductive doses of OVB3–PE. In this experiment, the tumors grew slower than in the previous experiment so treatment was delayed until day 33. To estimate the tumor burden at the time when OVB3–PE was injected, three mice were sacrificed on day 33. By aspirating the peritoneal cavity an average of 4×10^8 OVCAR-3 tumor cells per mouse was recovered from ascites fluid. In addition, there was a single solid tumor mass in each mouse (approximately 1.0×1.0 cm in size). A comparison that was made with the tumor burden present on days 0–5 showed there were roughly 50–100 times more tumor cells present on day 33 than on days 0–5. Five immunotoxin injections were given beginning on day 33. To assess the effect of this treatment, two mice from this treatment group were killed and examined for evidence of viable tumor 4 days after the final injection of immunotoxin. Gross anatomical examination revealed that the immunotoxin treatment eliminated the ascites fluid and unattached tumor cells. However, the solid tumor mass remained evident in both mice and the tumor had similar dimensions to the pretreatment solid tumor. This posttreatment tumor mass was examined microscopically following fixation and H and E staining. The tumor cells appeared viable.

Cell Killing of OVB3–PE on Breast and Colon Cancer Cells. The reactivity of OVB3 for various human tumor samples was reported previously and has been extended here (*3*). With use of immunocytochemistry on frozen sections of cancer specimens, OVB3 has been found to react with 25/25 ovarian carcinomas and approximately 25% of breast cancers and colon tumors. Because of this reactivity with tumors other than ovarian, we investigated the cytotoxicity of OVB3–PE on cell lines derived from breast and colon tumors. OVB3–PE was found to have an ID_{50} of less than 1 ng/mL for both a breast cancer cell line, MCF-7, and a colon cancer cell line, HT-29 (Table I). Thus OVB3 has potential for use in the treatment of other adenocarcinomas besides ovarian cancers. OVB3–PE was also tested on epidermoid carcinomas and found to have potent cell-killing activity for A431 cells (Table I) and to a lesser extent, KB cells (data not shown). Recently, OVB3–PE was shown to have antitumor activity against HT-29 tumors (*9*).

DISCUSSION

We show here that it is possible to make a very potent immunotoxin by conjugating native PE to OVB3 by a thioether linkage.

OVB3 was originally selected as an ovarian-specific monoclonal antibody. We have examined many tumors for reactivity with OVB3 and found that OVB3 has preferential reactivity for ovarian cancer (25/25), but it also reacts with a significant percentage of adenocarcinomas of the breast and colon. As shown in Table I, OVB3 is also active against a breast (MCF-7), a colon (HT-29), and an epidermoid carcinoma (A431) cell line. While OVB3–PE inhibited these cell lines with an ID_{50} of 0.5 ng/mL, a 200-fold higher concentration was needed to kill HUT-102 cells (data not shown). Thus, the cytotoxicity of OVB3–PE was relatively specific for cells bearing the OVB3 antigen.

Immunotoxin therapy has been shown to be effective in treating intraperitoneal tumor models of human cancer (*7–10*). Here we show in some instances that OVB3–PE can increase survival of nude mice bearing OVCAR-3 tumors by greater than 100 days. The injection of irrelevant immunotoxins such as antiTac–PE has no antitumor activity in this model (*7*). For OVB3–PE, 1 μg per injection gave the most pronounced antitumor effects. The median survival times of 193 days in experiment 1 and 156 days in experiment 6 represent some of the longest

survival times reported to date for an immunotoxin-mediated antitumor effect. We have also shown that OVB3–PE prolongs the life of tumor-bearing mice when given 19–33 days after OVCAR-3 implantation at a time when ascites had developed and large tumor implants were present.

In every experiment where OVB3–PE was administered at a dose of 0.5 or 1.0 μg/injection, there was a significant increase in the survival of mice bearing OVCAR-3 tumors. However, from experiment to experiment, we have noted some variability in the median survival times of the various treatment groups. For example, the MST for the 0.5 μg per injection dose ranged from 158 days in experiment 1 to 69 days in experiment 3 and for 1.0 μg from 193 days in experiment 1 to 91 days in experiment 3. We have noted intermediate MSTs in other experiments. Various lots of immunotoxins were checked for changes in cell-killing activity upon storage. No systemic loss of activity was noted. We do not have an explanation for the variability in the mouse experiments except to suggest that animal-to-animal variation may be greater than the small variations seen in tissue-culture systems.

The administration of OVB3–PE 2–4 weeks after initiating tumor growth was carried out to test for antitumor activity against a large established tumor. OVB3–PE was effective when injections were begun either 19, 26, or 33 days after the injection of tumor cells. Antitumor activity was characterized by increased survival time, decreased body weight, diminished abdominal girth, and a reduced number of tumor cells recovered immediately posttreatment. The antitumor effect was most pronounced against ascites tumor cells. In two mice that were autopsied 5 days posttreatment, ascites tumor cells were not evident. However, in those same mice, the solid tumor was of similar size to tumors examined immediately prior to treatment. It would appear that the large solid tumor was poorly permeable to the immunotoxin.

Bjorn et al. had reported that it was possible to produce active immunotoxins when PE was thioether-linked to various monoclonal antibodies (*11*). In this report, we have described a novel method to make thioether-linked PE immunotoxins which is different from that of Bjorn et al. By first reacting PE with sulfo-SMPB, and OVB3 with 2-iminothiolane we have made a very potent immunotoxin with an ID_{50} of 2.5×10^{-12} M. In more recent experiments we have used SMCC in place of sulfo-SMPB and shown no difference in conjugate potency (data not shown). In this report, only data using native PE coupled to OVB3 is presented. We have made OVB3 conjugates with PE40, a recombinant form of PE lacking the toxin's binding domain, but these showed little or no cytotoxicity for cells and were not pursued further (data not shown).

The dosing schedule used in these experiments has been designed with certain clinical parameters in mind. Patients receiving PE conjugated to antibodies have been found to develop neutralizing antibodies to PE 10–12 days after the initial immunotoxin injection. Because of this, our injection protocol did not span more than 2 weeks. Thus, we achieved antitumor responses with an injection schedule that could be directly applied to patient treatment. Likewise, after the initial evidence that thioether conjugates mediated an antitumor response when the immunotoxin was given 3–5 days after tumor implantation, we decided to treat more advanced tumors. While it is difficult to relate the staging of human disease to mouse tumor load, it was clear that we could achieve a significant antitumor effect when the tumor burden had increased by a minimum of 50-fold. OVB3–PE gave an antitumor effect in the dose range of 5–50 μg/kg in mice when administered at early times after tumor implantation. When administered after 3 weeks of tumor growth, 50 μg/kg also gave a significant antitumor response. At 100 μg/kg OVB3–PE was often lethal. Mice died 24–72 h after injection of severe liver toxicity.

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