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Antitumor effects of an immunotoxin made with *Pseudomonas* exotoxin in a nude mouse model of human ovarian cancer

(transferrin receptor/monoclonal antibody)

DAVID J. FITZGERALD, MARK C. WILLINGHAM, AND IRA PASTAN*

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, Bethesda, MD 20892

Contributed by Ira Pastan, May 19, 1986

ABSTRACT **An immunotoxin composed of *Pseudomonas* toxin coupled to an antibody to the human transferrin receptor was evaluated for its effect on ovarian cancer. In the tumor model employed, 60 million human ovarian cancer cells were injected into the peritoneal cavity of an immunodeficient nude mouse. By day 5, cancer cells were implanted and growing in small clusters throughout the peritoneal cavity. On days 5–8, 0.3–2 μg of immunotoxin was injected into the peritoneal cavity. Control mice died with malignant ascites at 34–58 days after the implantation of tumor cells, whereas immunotoxin-treated mice lived to 100 days or longer. Irrelevant immunotoxins or antibody alone had no antitumor activity. These findings suggest that intraperitoneal injection of immunotoxins may have a role in the treatment of ovarian cancer.**

Human ovarian cancer is a disease that remains confined to the peritoneal cavity throughout most of its course. In patients with ovarian cancer, relapse following an initial course of chemotherapy is usually associated with a poor prognosis. Acquired drug resistance often renders the disease refractory to retreatment with the same agent and cross-resistant to other agents (1). Some new strategies to overcome this resistance are directed toward restoring drug sensitivity to the tumor cells by the use of verapamil (2), which can increase drug accumulation, or buthionine sulfoximine, which decreases intracellular glutathione levels (3).

We have been developing an alternate approach to chemotherapy using immunotoxins (ITs) made from *Pseudomonas* exotoxin (PE) coupled to antibodies reactive with antigens present on the surface of ovarian cancer cells (4, 5). (For a review of ITs, see refs. 6 and 7.) We have previously assessed the activity of these ITs in cell culture (4, 5). We have now begun to utilize a nude mouse model of human ovarian cancer (8) to evaluate the efficacy of various ITs *in vivo*. In this model, human ovarian cancer cells are grown intraperitoneally (i.p.) as a malignant ascites in nude mice. After the tumor is established, the IT is injected into the peritoneal cavity. These experiments show that an IT composed of *Pseudomonas* toxin coupled to a monoclonal antibody to the human transferrin receptor (PE-anti-TFR) given in four doses of 0.3–2.0 μg significantly prolongs the life of tumor-bearing animals.

MATERIALS AND METHODS

Human NIH:OVCAR-3 cells were grown as a malignant ascites in the peritoneal cavity of B74 athymic nude mice (8). On day 1 of each experiment, cells were harvested from carrier mice, washed in normal saline, counted, and immediately injected into test animals. Each mouse received 6×10^7 cells i.p. ITs or control proteins (0.5 ml) were injected i.p. on days 5–8. In the text below the amount of IT cited was the quantity given in one injection. There were five mice per group in all experiments.

The anti-TFR antibody (9) was purified by DEAE chromatography from ascites fluid produced by injecting the HB21 hybridoma (ATCC) into pristane-treated BALB/c mice. Purified monoclonal antibody to the human interleukin 2 receptor (anti-Tac) and monoclonal antibody 103 were supplied by T. Waldmann and N. Richert, respectively, of the National Cancer Institute. Purified PE was purchased from Swiss Serum and Vaccine Institute (Berne, Switzerland). PE-anti-TFR, PE-103, and PE-anti-Tac were constructed by disulfide exchange as described elsewhere (5). A 1:1 PE-antibody conjugate was purified away from other products by using a high-performance liquid chromatography gel filtration column (TSK-250, 21.5 × 600 mm, Bio-Rad). When tested against OVCAR-3 cells in cell culture as described (4), the IT gave 50% inhibition of protein synthesis at a final concentration of 50 ng/ml.

Ascites fluid was examined by using phase-contrast microscopy with a Zeiss 1CM-405 inverted microscope and a 40× N.A. 0.85 long working distance oil-phase objective. The histologic appearance of small tumor implants was examined by fixing small portions of peritoneal wall removed at autopsy from tumor-injected mice at day 5, followed by routine histologic processing using paraffin embedding and hematoxylin/eosin staining.

RESULTS

To evaluate IT activity, 60 million ovarian cancer cells were injected on day 1 and test materials were administered i.p. on days 5–8. The delay before initiation of treatment was to allow the establishment of the tumor. To assess the progress of tumor development at the time of treatment, a group of mice was killed on day 5. The peritoneal cavity on day 5 contained a large number of nonattached tumor cells that could be washed out with saline (Fig. 1*A*). Histological sections revealed cell clusters attached to the surface of the liver, the serosal surface of the gastrointestinal tract, and the mesentery (Fig. 1*B*). Some of these clusters were organized and surrounded by a connective tissue layer and some invasion of lymphatics and lymph nodes was found. Thus, at the time of treatment the mouse model closely resembled human ovarian cancer, having numerous solid tumors and malignant ascities. Untreated animals died at 35–50 days with massive ascites and multiple tumors within the peritoneal

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Abbreviations: PE, *Pseudomonas* exotoxin; anti-TFR, monoclonal antibody to the transferrin receptor; PE-anti-TFR, PE coupled to anti-TFR; IT, immunotoxin; anti-Tac, monoclonal antibody to the human interleukin 2 receptor.

*To whom reprint requests should be addressed at: Laboratory of Molecular Biology, National Cancer Institute, 9000 Rockville Pike, Building 37, Room 4B20, Bethesda, MD 20892.

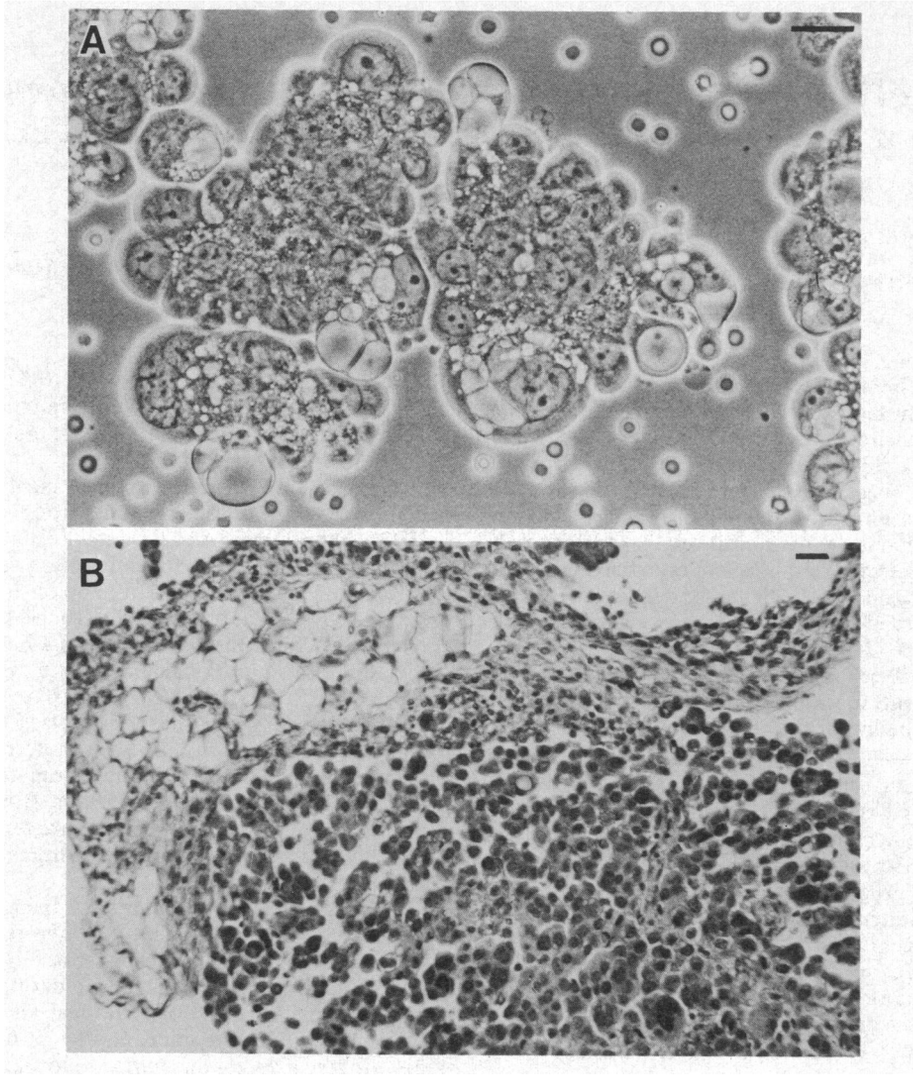

FIG. 1. Appearance of OVCAR-3 cells. (*A*) Ascites fluid removed from tumor-bearing mice contained large numbers of nonattached OVCAR-3 cells. (Bar = 20 μm.) (*B*) Tumor appearance of OVCAR-3 implants on day 5 after injection of 6×10^7 OVCAR-3 cells. Small cell clusters were cut out of peritoneal wall, sectioned, and stained with hematoxylin/eosin. (Bar = 20 μm.)

cavity (8). The upper panel of Fig. 2 shows a mouse sacrificed on day 47. Massive ascites and large intraabdominal tumors are evident.

The administration of injections of PE-anti-TFR on days 5–8 increased the survival of tumor-bearing mice compared with controls. The injection of 1 or 2 μg increased the median survival time to $\approx$100 days, whereas control mice died on or about day 50 (Fig. 3). The lower panel of Fig. 2 shows a mouse treated with 2 μg of PE-anti-TFR that appeared tumor-free on day 99 (see below). Four injections of 0.3 μg gave enhanced survival, with a median survival time of 78 days. Thus, there was evidence for a dose-dependent response (Fig. 3).

We have performed five individual experiments with PE-anti-TFR and several control experiments with antibody alone and irrelevant ITs to show that the increased survival of the mice reflected a specific effect of PE-anti-TFR on NIH:OVCAR-3 cells. The injection of antibody alone (100 μg) did not increase survival (Table 1, experiment 6). In other experiments (not shown) mice given lower doses of antibody alone also did not survive longer than controls. Furthermore, the anti-TFR antibody had no growth inhibitory activity in tissue culture (data not shown) and did not compete for transferrin binding.

To demonstrate that increased survival was due to the IT specifically directed against the TFR receptor, an IT directed against the human interleukin 2 receptor (10) was administered and found to be inactive (Table 1, experiment 5). To show that activity did not reside in the IgG1 subtype of the antibody, an IT was made with an irrelevant IgG1 antibody designated 103 (11). This antibody recognizes a cytoplasmic protein of 36 kDa. Treatment with this IT, at 2 μg per injection, did not inhibit tumor growth (data not shown). The mice that received a coinjection of PE-anti-TFR (2 μg per injection) with excess anti-TFR (100 μg per injection) showed no increase in survival (Table 1, experiment 6). Although excess anti-TFR blocked PE-anti-TFR activity, excess (100 μg) of the 103 antibody did not (data not shown).

A total of 25 mice was given either 2 or 1 μg of PE-anti-TFR and, of these, 3 were tumor-free at the conclusion of the experiment. One mouse was sacrificed on day 111 (Table 1, experiment 1), 1 mouse from experiment 4 remained alive with no sign of tumor on day 170, and another from the same group was sacrificed on day 99 (Fig. 2, lower panel) with no sign of any tumor. The IT-treated mice that died on or about day 100 had i.p. solid tumor masses, intraperitoneal ascites, and extraperitoneal solid tumors. Control mice that died between days 35 and 55 had i.p. solid tumors and ascites but rarely had any extraperitoneal tumors. No effort was made to give more frequent injections of IT to achieve longer survivals. It is not possible to give more than 2 μg of a PE-containing

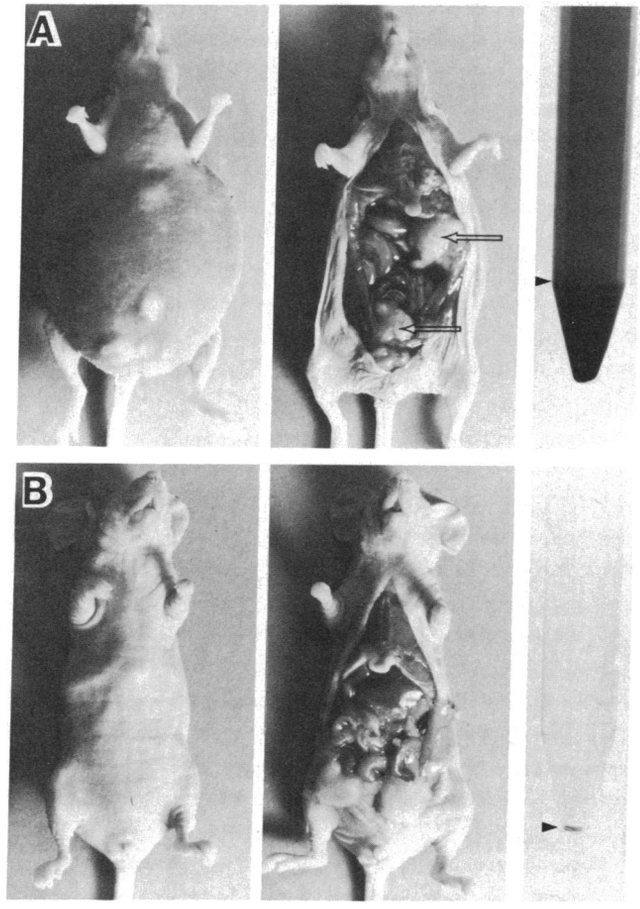

FIG. 2. Mouse model of ovarian cancer, with and without the administration of an active IT. (*A*) Typical mouse showing a large amount of ascites, floating tumor cells, and several solid tumor masses. On day 47, this mouse was killed and nonattached cells were recovered by removing ascites fluid and centrifuging at 1000 × *g*. The cell pellet appears at the right with about 2 ml of packed cells. (*B*) Typical mouse that was treated with active IT. The mouse appears free of tumor on day 99, and no tumor cells can be washed out of the peritoneal cavity.

IT per day to mice because of their extreme sensitivity to the toxin.

Table 1. Survival of nude mice bearing NIH:OVCAR-3 tumors following treatment with PE-anti-TFR or control proteins

Experiment	Antibody	Toxin	Dose, μg	Median survival, days
1	—	—	—	34
	Anti-TFR	PE	2.0	110
2	—	—	—	45
	Anti-TFR	PE	2.0	90
3	—	—	—	58
	Anti-TFR	PE	2.0	91
4	—	—	—	52
	Anti-TFR	PE	0.3	78
	Anti-TFR	PE	1.0	99
	Anti-TFR	PE	2.0	99
5	—	—	—	38
	Anti-Tac	PE	2.0	38
6	—	—	—	53
	Anti-TFR	PE	2.0	>75
	Anti-TFR	—	100	55
	Anti-TFR + anti-TFR	PE	2.0 100	39

Data are expressed as the median day of survival. Routinely, the mice in each group died within 10 days of the median day of survival.

Small numbers of cancer cells are often deposited outside the peritoneal cavity along the needle track at the time of injection of the cancer cells and these eventually grow into extraperitoneal tumors. These extraperitoneal tumors were not affected by doses of IT that were active in the peritoneal cavity. The injection of IT into the peritoneal cavity provides a high concentration of IT within and in the vicinity of the peritoneal cavity and even results in killing of tumors implanted in the peritoneal wall. Apparently the concentration outside the peritoneal cavity is not high enough to kill cells lodged outside the cavity.

DISCUSSION

This paper provides evidence that PE-ITs can achieve a significant antitumor effect in an animal. Other investigators have shown antitumor effects in various animal model systems using ITs made with ricin A chain (12, 13), diphtheria toxin A chain (14, 15), pokeweed antiviral protein (16), or saporin (17). In some of these studies, antibody alone was just

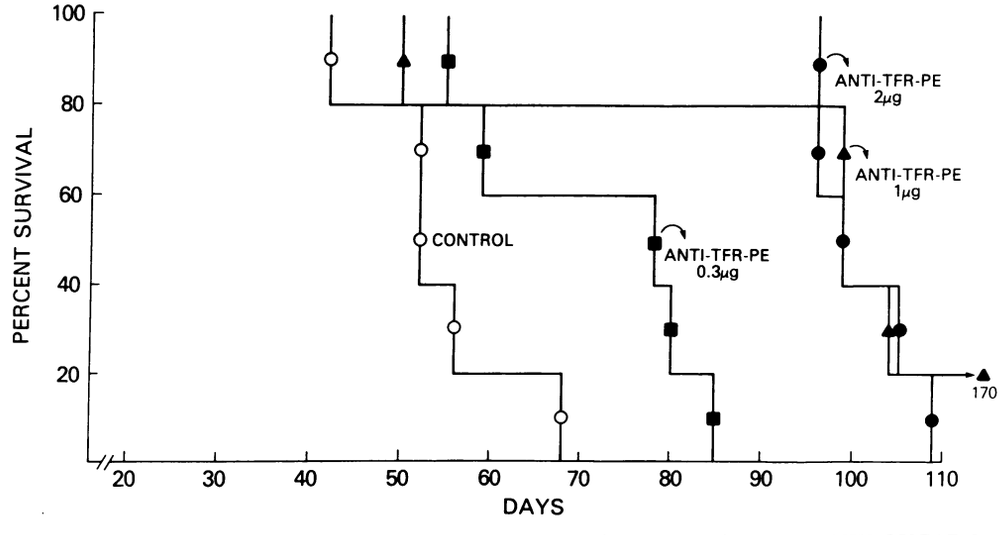

FIG. 3. Activity of PE-anti-TFR in a nude mouse (B74, athymic nude) model of human ovarian cancer. NIH:OVCAR-3 cells (6×10^7) were injected on day 1 and test materials were administered i.p. on days 5–8. There were five mice per group. This graph depicts the survival data listed as experiment 4 in Table 1.

as effective or almost as effective as the IT (13, 14), suggesting that toxin-independent mechanisms were operating. In the current study, the antibody alone had no effect on tumor growth. In addition, when excess antitransferrin antibody was given with IT, it blocked IT action, establishing that the IT was binding to a specific and saturable binding site.

One potential advantage of ITs employing PE is their high activity. Generally, ITs made with whole toxins are much more active than A chain toxins (5, 18–20). This is probably because a portion of the molecule is retained that enables the toxin to penetrate through cell membranes. In other studies, we have found that an IT made using ricin A chain and a different monoclonal antibody to the human TFR (454A12) was active in this ovarian cancer model but required higher doses of IT (30–100 μg per injection) to achieve a similar therapeutic result (unpublished results).

The current study utilized an antibody to the human TFR to carry the toxin to cancer cells. Though much of the efficacy of this tumor-specific antibody-toxin conjugate in a mouse resides in the species-specific nature of the antibody, this system still demonstrates that properly selected tumor-specific ITs can be expected to be therapeutically useful. It remains to be determined whether or not TFR can serve as a target antigen for treatment of human ovarian cancer. TFRs have been detected in a number of normal tissues, including skin, epithelia of the gastrointestinal tract and bladder, kidney, endocrine pancreas, hepatocytes, Kupffer cells, testis, pituitary, and brain capillary endothelium (21, 22), and in many different kinds of cancer tissue, including ovarian tumors (23, 24). However, we have identified a number of monoclonal antibodies that react with ovarian cancer cells and a more limited number of normal tissues than the antibody used in this study (ref. 5; unpublished data). In preliminary studies, some of these are active in the nude mouse model (unpublished data).

This study shows an antitumor activity of an IT made with PE in an ovarian cancer model. It demonstrates a positive result in a model system employing large numbers of tumor cells and the model can be used to evaluate the *in vivo* activity of ITs made from other antibodies reactive with ovarian cancer tissue. Ovarian cancer is often accompanied by ascites containing many cancer cells. The ascites is due to tumor cell invasion and obstruction of regional lymphatics. The injection of ITs directly in the peritoneal cavity ensures that tumor cells in the ascites and those growing superficially are exposed to a high initial concentration of drug. Furthermore, the IT, like other proteins, leaves the peritoneal cavity by the same lymphatics as cancer cells. These considerations, together with the fact that the blockage of lymphatics often seen in patients with ovarian cancer will slow the diffusion of IT to the blood, may help provide a therapeutic activity against human ovarian cancers.

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