Antitumor Activity of an Immunotoxin in a Nude Mouse Model of Human Ovarian Cancer

David J. FitzGerald, Michael J. Bjorn, Robert J. Ferris, Jeff L. Winkelhake, Arthur E. Frankel,
Thomas C. Hamilton, Robert F. Ozols, Mark C. Willingham, and Ira Pastan

ABSTRACT

An immunotoxin composed of an antibody to the human transferrin receptor (454A12) and ricin A chain (RTA) was shown to inhibit the growth of NIH:OVCAR-3 tumors in a nude mouse model of human ovarian cancer. Inhibition of tumor growth by 454A12-RTA was related to the dose administered. The antitumor activity of the immunotoxin was blocked by coinjection of excess antibody with immunotoxin. An immunotoxin made using 454A12 and recombinant ricin A chain (rRTA) had an activity similar to that made with native RTA. The administration of 10 µg or greater of the immunotoxin 454A12-RTA/rRTA had significant antitumor activity. The injection of 30 µg of an irrelevant immunotoxin, MOPC21-RTA, or 30 to 500 µg of the 454A12 antibody had no antitumor activity.

INTRODUCTION

Ovarian cancer is one of the most frequent fatal gynecological malignancies occurring in women (1). Novel treatments appear necessary for this disease, since the effectiveness of combination chemotherapy is often limited by the development of multiple drug resistance (1). Ovarian cancer remains confined to the abdominal cavity virtually throughout its entire clinical course, frequently producing ascites and tumor deposits on multiple peritoneal surfaces. To study this disease and investigate new treatments, a mouse model has been developed which closely resembles the human disease. Human ovarian carcinoma (NIH:OVCAR-3) cells grow and develop as a lethal tumor within the peritoneal cavity of nude mice (2, 3). Ascites fluid is also produced. Because the tumor is confined to the peritoneal cavity, therapeutic materials can gain immediate access to tumor cells by i.p. injection.

Recently, we have shown that various immunotoxins are cytotoxic for NIH:OVCAR-3 cells when assayed in tissue culture (4, 5). One of these immunotoxins with high activity is made with an antibody to the human transferrin receptor (454A12).2 Antibodies to the transferrin receptor bind to most tumor cells and react with a limited number of normal tissues (6–10). TFRs are found on the cell surface of growing mammalian cells and mediate the uptake of iron by internalizing iron-transferrin complexes. When a monoclonal antibody to the TFR is bound to the receptor, that complex is efficiently internalized (11, 12).

Ricin is one of several protein toxins that inhibits protein synthesis in mammalian cells. To prevent nonspecific cell killing which is mediated by the B-chain of ricin, the A and B chains are separated, and only the A chain (RTA), which enzymatically inactivates ribosomes, is coupled to an appropriate antibody to create a specific immunotoxin. Trowbridge and Domingo first reported the construction of an immunotoxin composed of ricin A chain and an antibody to the human transferrin receptor (13). They showed that such an immunotoxin was active both in vitro and in vivo. The in vivo activity was measured as an antitumor effect. However, the injection of the monoclonal antibody alone also gave an antitumor effect.

In the current study, we have assessed the activity of an immunotoxin injected directly into the peritoneal cavity of tumor-bearing mice. We have chosen to use an antibody to the transferrin receptor to construct the immunotoxin, since it made a very active cell-killing agent in cell culture (4). The antibody was modified by conjugating it to the A chain of ricin. The A chain of ricin was prepared both by a conventional method, in which it was separated from ricin B chain, and by recombinant DNA technology.

MATERIALS AND METHODS

NIH:OVCAR-3 cells were grown to produce malignant ascites and solid tumor implants in the peritoneal cavity of B74 athymic nude mice. The tumor was maintained in carrier mice and transferred every 3 to 4 wk (2). 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 × 10⁶ cells i.p. In this model, untreated mice died routinely between 35 and 50 days (2).

Immunotoxins or control proteins (0.5 ml) were injected i.p. on Days 5, 8, and 11 unless otherwise indicated in the text. The amount of immunotoxin cited in the text is the quantity that was given in one injection. There were at least five mice per group.

To assess tumor invasion of mouse peritoneal tissue, mice were killed, and pieces of tissue from the peritoneal cavity were excised, sectioned, and stained with hematoxylin and eosin. On the first day of immunotoxin treatment, tissue pieces were excised from companion mice that had also been given injections of OVCAR-3 cells but received no immunotoxin.

Monoclonal antibody 454A12 (IgG1, κ) was initially selected as part of a screening for antibodies that bound human breast cancer tissue (6). Since it has been determined that 454A12 reacts with the human TFR, MOPC21 is a mouse myeloma antibody (IgG1) with no known reactivity.

Ricin was obtained from an acetic acid extract of castor beans (Ricinus communis L. var. communis) in a modification of the procedure of Olsnes and Pihl (14). The crude extract was chromatographed on an agarose affinity column and CM-sepharose ion exchange column. Following reduction with 2 mercaptoethanol, the RTA was chromatographed on DEAE- and CM-Sepharose and then on an agarose affinity column. RTA obtained from the Process Development Department of Cetus Corp.3 was also used as a component of immunotoxins in these studies. For conjugation, antibodies prepared as described previously (6) were derivatized using modifications of existing procedures (5), then coupled through reactive sulphydryl groups on RTA or rRTA to form disulfide-linked conjugates. Conjugates were purified using size exclusion and affinity chromatography.4 Purified conjugates contain less

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1 Present address: Department of Medicine, Duke University Medical Center, Durham, NC 27710.
2 The abbreviations used are: 454A12, monoclonal antibody to the human transferrin receptor; TFR, transferrin receptor; RTA, ricin, A chain; rRTA, recombinant ricin A chain; MOPC21, mouse myeloma antibody with no known reactivity; ID₅₀, 50% inhibitory dose.
3 M. Piatak, manuscript in preparation.
4 W. Bloch, manuscript in preparation.

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RESULTS

In Vitro Activity of 454A12 Immunotoxins. Immunotoxins made from monoclonal antibodies to the human TFR have been shown to be cytotoxic in vitro for various cell lines (4, 11, 13, 15, 16). An anti-TFR immunotoxin made with the antibody 454A12 (454A12-RTA) is a potent immunotoxin for NIH:OVCAR-3 cells; the ID$_{50}$ (24 h) for inhibition of protein synthesis for these cells in vitro was consistently between 5 and 10 ng/ml (0.05 nmol). A similar immunotoxin was made with the 454A12 antibody and rRTA. The cytotoxic activity of 454A12-RTA and 454A12-rRTA was carefully compared on OVCAR-3 cells and found to be quite similar. The ID$_{50}$ for 454A12-RTA was 0.048 nmol ± 0.010 and for 454A12-rRTA was 0.052 nmol ± 0.006 (SD). The in vitro specificity of the types of 454A12-RTA immunotoxins was confirmed by competition for activity using excess 454A12 antibody. No competition was seen when excess of an irrelevant antibody of the same isotype (MOPC21) was used.

Assessment of NIH:OVCAR-3 Invasion of Peritoneal Tissue. To assess the degree of tumor progression that corresponded to the first day of immunotoxin treatment, autopsies were performed on six mice 4 days after they had been given injections of 6 x 10$^7$ NIH:OVCAR-3 cells. Small clusters of tumor cells were seen attached to visceral organs. Histological sections were made and examined to determine the degree of tumor organization and depth of invasion into the peritoneal tissues. Most clusters of tumor cells were found attached to the surface of the liver, the wall of the gastrointestinal tract, and the peritoneal wall. Some invasion of lymphatics and lymph nodes was found; an example is shown in Fig. 2, C and D. In addition, large quantities of nonattached tumor cells were recovered by washing out the peritoneal cavity with saline (data not shown). Thus, it was determined that, at the time of immunotoxin injection, tumor cells had already implanted in host tissue.

In Vivo Activity of 454A12 Immunotoxins. To evaluate the efficacy of immunotoxin treatment in vivo, human ovarian cancer cells (NIH:OVCAR-3) were grown in the peritoneal cavity of nude mice to produce a lethal tumor with ascites. Four to 5 days after the inoculation of tumor cells, 0.5 ml of immunotoxin or control protein material were injected into the peritoneal cavity. Immunotoxin treatment was always delayed for 4 to 5 days after transplantation to allow the injected cells to establish small tumors in the peritoneal cavity. In one experiment, the first injection of immunotoxin was delayed until Day 11 (Table 1).

Mice treated with a control solution containing only albumin died at a median time of 34 to 50 days after the inoculation of 6 x 10$^7$ NIH:OVCAR-3 cells (Table 1; Fig. 3). The administration of immunotoxin resulted in a dose-dependent prolongation of the life of test animals (Fig. 3). In this experiment the smallest dose of 454A12-RTA (3 µg) prolonged the life of the mice by an average of 20 days. Ten µg of immunotoxin extended the life span by 45 days, and the two larger doses by 70 to 80 days (see Table 1). The administration of antibody alone (either 500, 100, or 30 µg), an immunotoxin made with an irrelevant antibody, MOPC21-RTA (30 µg), or rRTA (30 µg) did not significantly extend survival. In fact, mice treated with either an irrelevant immunotoxin or an immunotoxin that had no in vivo antitumor activity (data not shown) did not live longer than the control mice given injections of albumin but died approximately 10 days earlier when given dose levels of 30 µg or greater.

In one experiment, immunotoxin administration was delayed and begun on Day 11. A significant antitumor response was also seen even with this delayed treatment (Table 1, Experiment 5).

Experiments 1 and 2 (Table 1) were carried out using immunotoxins made from RTA which had been prepared from extracts of castor beans by conventional chromatographic techniques. Experiments 3 to 5 were carried out with immunotoxins made from rRTA. As noted above, both immunotoxins were equally potent when assayed for their ability to inhibit protein synthesis in vitro. In vivo, the native and recombinant ricin A immunotoxins showed roughly similar activities.

Blocking of Antitumor Activity by Competing Antibody. To determine the ability of unconjugated 454A12 to block the antitumor effect of 454A12-RTA, 500 µg of 454A12 and 25 µg of 454A12-RTA were injected together. The presence of excess native 454A12 blocked the antitumor activity of this immunotoxin (Table 2). This blocking activity was specific, since 500 µg of MOPC21 did not alter the antitumor activity of 454A12-RTA.

Evaluation of Tumor Burden after Immunotoxin Treatment. To assess the tumor status in animals from groups treated with either 30 or 100 µg of 454A12-RTA, 16 mice that had survived to 70 days or longer were sacrificed and autopsied (13 of the 16 are noted in Table 1). Of these, 4 appeared completely tumor free. Eight had solid tumor masses of various sizes, ranging in diameter from a few mm to 2 cm or greater. Six of the 8 had extraperitoneal tumors which appeared to be along the “needle track” created when the cells were injected into the peritoneal

![Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of immunotoxins. Samples were applied to a 7.5% nonreducing polyacrylamide gel. Loadings were: A, 454A12 (5 µg); B, 454A12-RTA (15 µg); and C, 454A12-rRTA (15 µg). Gels were stained with Coomassie Brilliant Blue.](image-url)
cavity. The remaining 4 mice had overt ascites. In contrast to the above result, all animals that died before Day 50 had ascites.

DISCUSSION

We have shown a dose-dependent inhibition of tumor growth by the administration of an immunotoxin directed against the human transferrin receptor. The positive response, which was assessed by the increased life span of tumor-bearing animals, was obtained in a unique in vivo model system that closely resembles human ovarian cancer. An important feature of the result stems from the fact that the first injection of immunotoxin was given at a time when the tumor was already established within the peritoneal cavity. Of the animals receiving a dose of immunotoxin in the therapeutic range (10 to 100 μg per injection), at least 4 appeared to be tumor free beyond Day 70. We expect that additional injections of immunotoxin would have a further therapeutic effect, but such experiments have not yet been performed.

Regardless of the treatment, essentially all the mice that died between Days 25 and 50 had a large tumor burden composed of abundant ascites fluid and solid tumor implants. However, only a quarter of those animals with tumor that survived past Day 70 had ascites. The remaining three quarters were either tumor free or had solid tumors. Since most of the mice with solid tumors also had large needle track tumors, it is possible that the peritoneal cavity was seeded from s.c. tumors outside the peritoneal cavity.

The rationale for using anti-TFR immunotoxins as anticancer reagents in humans is based on studies that indicate that the TFR is only expressed at readily detectable levels on a limited number of normal human tissues. However, it is almost universally present on tissue derived from tumors. Further, by injecting the immunotoxin into the peritoneal cavity, it should...
Animals were monitored, and the duration of survival noted. This is Experiment 1, in which nude mice were treated with 0, 3, 10, 30, or 100 fig of the immunotoxin 454A12-RTA. The doses received per individual injection are indicated in the text. Inhibition of tumor growth was determined by increased survival time of the treated animals.

Table 1 Inhibition of tumor growth by 454A12-RTA and 454A12-rRTA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Antibody</th>
<th>Toxin</th>
<th>Dose (µg)</th>
<th>Median day of survival</th>
<th>Range</th>
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<tbody>
<tr>
<td>1 (10)*</td>
<td>454A12</td>
<td>0</td>
<td>100</td>
<td>49</td>
<td>29–58</td>
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<tr>
<td></td>
<td>454A12</td>
<td>RTA</td>
<td>100</td>
<td>87</td>
<td>76–96</td>
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<tr>
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<td>RTA</td>
<td>3</td>
<td>34</td>
<td>23–42</td>
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<tr>
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<td>454A12</td>
<td>RTA</td>
<td>10</td>
<td>77</td>
<td>63–91</td>
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<tr>
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<td>30</td>
<td>&gt;111*</td>
<td>&gt;110–111</td>
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<tr>
<td></td>
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<td>RTA</td>
<td>100</td>
<td>114*</td>
<td>&gt;110–111</td>
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<tr>
<td>3 (5)</td>
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<td>30</td>
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<tr>
<td></td>
<td>454A12</td>
<td>rRTA</td>
<td>100</td>
<td>&gt;74*</td>
<td>&gt;73–&gt;74</td>
</tr>
<tr>
<td>4 (5)</td>
<td>454A12</td>
<td></td>
<td>30</td>
<td>20</td>
<td>20–26</td>
</tr>
<tr>
<td></td>
<td>MOPC21</td>
<td>RTA</td>
<td>30</td>
<td>20</td>
<td>20–26</td>
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<tr>
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<td>rRTA</td>
<td>30</td>
<td>44</td>
<td>41–75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>92*</td>
<td>56–93</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers in parentheses, animals per group.

Table 2 Specific blocking of the antitumor effect of 454A12-RTA with excess unconjugated antibody

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Av. survival days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>41</td>
</tr>
<tr>
<td>454A12-RTA (25 µg)</td>
<td>85</td>
</tr>
<tr>
<td>454A12-RTA (25 µg) + MOPC21 (500 µg)</td>
<td>93</td>
</tr>
<tr>
<td>454A12-RTA (25 µg) + 454A12 (500 µg)</td>
<td>26</td>
</tr>
</tbody>
</table>

Interact with tumor cells while still at a high concentration and be considerably diluted when it enters sensitive cells in the bone marrow.

Previous studies by other investigators have shown antitumor effects with either RTA immunotoxins or immunotoxins made with native ricin plus added lactose (17, 18). Here we demonstrate antitumor activity using an RTA-immunotoxin where the RTA was prepared using either "conventional" chromatographic techniques or "recombinant" techniques. As such, 454A12-RTA represents a novel type of immunotoxin.

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