Inhibition of Growth of Human Tumor Xenografts in Athymic Mice Treated with Ricin Toxin A Chain-Monoclonal Antibody 791T/36 Conjugates

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ABSTRACT

Immunotoxin constructed by conjugating ricin A chain to monoclonal antibody 791T/36 specifically inhibits growth of human tumor xenografts which express the gp72 antigen recognized by the antibody component. Dose schedule tests showed that the major response was obtained during the first 5 days of treatment and further prolonged treatment did not improve therapy. Expression of gp72 antigen on tumor cells derived from xenografts in immunotoxin-treated mice was not markedly altered indicating that treatment did not lead to the expansion of tumor antigen deficient tumor cells. The experiments indicate that treatment for short duration with immunotoxin may be the most effective protocol.

INTRODUCTION

Monoclonal antibodies are now being used to target a variety of cytotoxic agents ranging from well-known chemotherapeutic agents like methotrexate to newer agents such as the ribosomal inhibitor protein RTA. These targeted drugs have been shown to have good cytotoxic indices when assayed in vitro on cell lines, and are now being considered as candidates for therapy of a wide variety of human tumors.

Monoclonal antibody 791T/36 recognizes a glycoprotein with a molecular weight of 72,000 present on the membrane of cells in a variety of human tumors including osteogenic sarcoma, and colon and ovarian carcinoma (4–6). The antibody is efficiently internalized after binding to cells; flow cytometry analysis of the internalization of 791T/36 antibody conjugates with human serum albumin labeled with tetramethyl rhodamine and retain antibody reactivity.

A large series of clinical immunoscintigraphy studies have demonstrated radiolabeled antibody 791T/36 localizes well in human tumors, and can be used as a diagnostic aid in colon (9, 10) and ovarian carcinoma (6, 11) and in osteogenic sarcoma (12). For this reason studies were carried out to determine if therapy of solid tumor xenografts would be effective and to determine optimal dose and time of therapy.

MATERIALS AND METHODS

Immunotoxins

Monoclonal Antibody 791T/36-RTA Conjugate. Monoclonal antibody 791T/36, produced in ascites form in BALB/c mice, was fractionated by Sepharose-protein A chromatography to yield IgG2b fraction for conjugate synthesis (8). Antibody RTA conjugates were prepared by coupling RTA to protein via a disulphide linkage using N-succinimidyl-3-(2-pyridyldithio)propionate as the bifunctional cross-linking agent. Two preparations were used in the therapy trials, the RTA antibody ratio determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and high-performance liquid chromatography being 2.9:1 for the first lot and 4.2:1 for the conjugate used in the majority of tests (Table 1). This latter preparation contained no free antibody or unconjugated RTA.

The antibody reactivity of conjugates was determined using a competitive inhibition flow cytometry assay (13, 14). This measures the ability of RTA conjugates to compete with fluorescein isothiocyanate-labeled 791T/36 antibody binding to tumor cells and compares this competitive ability with that of a standard lot of unconjugated antibody. By this criteria the RTA conjugates retained 82% of the binding activity of the standard antibody.

Melanoma Antibody-RTA Conjugate. Monoclonal antimelanoma antibody-ricin A chain conjugate (15) was used in control studies. This immunotoxin had an RTA antibody ratio of 1.5:1 and contained 5.7% free antibody.

Tumor Xenografts

Transplanted lines of osteogenic sarcoma 791T, which has approximately 1 × 10⁶ molecules/cell of the gp72 antigen (13, 14), and melanoma AAA232, which does not bind 791T/36 antibody, were established in athymic nude mice (Olac U. K. Ltd., Oxon, U. K.) by aseptic s.c. implantation of tumor tissue pieces. Mice were maintained on sterile bedding with sterilized food and water in isolators (Vickers Pathoflex Isolators, Hants, U. K.). For therapeutic experiments groups of six to 10 mice of the same sex and age were implanted s.c. with tumor fragments about 2 mm in diameter. Treatment was given by i.p. injection of RTA conjugate starting up to 11 days later. All injections were 0.5 ml of RTA conjugates at appropriate dilution in PBS. Control mice received the same volume of PBS alone. Tumor sizes were measured twice weekly with calipers in two horizontal planes. Mean tumor diameters in treated and control mice were compared for statistical significance using Student’s t test. After 20–58 days mice were killed and tumors were dissected and weighed. Statistical significance of the difference in tumor weight in treated and control groups was determined by the Wilcoxon nonparametric rank test.

Toxicity Testing

To assess the toxicity of 791T/36-RTA conjugates, groups of normal BALB/c mice or athymic mice were weighed individually and then injected i.p. with repeated daily injections of conjugate at appropriate dilutions in PBS. Mice were reweighed and examined daily.

Expression of gp72 Antigen of Tumor Xenograft-derived Target Cells

Tumor cell suspensions were prepared from tumor xenografts derived from control athymic mice and 791T/36-RTA treated mice. Tissue was finely minced and treated with 0.05% collagenase (Boehringer Mannheim, West Germany) to disaggregate tumor cells (5). Tumor cells were washed in Hanks’ balanced salt solution, incubated first with 791T/36 antibody (5 μg/2 × 10⁶ cells) and then with fluorescein isothiocyanate conjugated rabbit anti-mouse immunoglobulin (Dako, Bucks, U. K.). Flow cytometry analysis was carried out as described previously (5) on a FACS IV and the fluorescence intensity of cells was expressed as MLF/cell, calculated by multiplying the contents of each channel by...
TUMOR INHIBITION IN MICE TREATED WITH RTA IMMUNOTOXIN

Table 1 Treatment of human tumor xenografts with 791T/36-RTA immunotoxin

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Tumor</th>
<th>RTA preparation (RTA:antibody)</th>
<th>No. injections</th>
<th>Dose/injection (mg/kg)</th>
<th>Total dose (mg/kg)</th>
<th>Schedule</th>
<th>Tumor sizes measured at day:</th>
<th>Termination day T/C ratio</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>791T</td>
<td>791T/36-RTA (2.9:1)</td>
<td>20</td>
<td>5</td>
<td>100</td>
<td>Daily, Day 2-21</td>
<td>6.8, 11, 15, 18*</td>
<td>25.0 ± 0.6</td>
<td>3/10</td>
</tr>
<tr>
<td>2</td>
<td>791T</td>
<td>791T/36-RTA (4.2:1)</td>
<td>15</td>
<td>5</td>
<td>75</td>
<td>Daily, Day 3-17</td>
<td>10.6, 17, 23*</td>
<td>25.0 ± 0.14</td>
<td>0/10</td>
</tr>
<tr>
<td>3</td>
<td>791T</td>
<td>791T/36-RTA (4.2:1)</td>
<td>10</td>
<td>5</td>
<td>50</td>
<td>Daily, Day 3-12</td>
<td>10, 14, 17, 23*</td>
<td>25.0 ± 0.13</td>
<td>0/6</td>
</tr>
<tr>
<td>4</td>
<td>791T</td>
<td>791T/36-RTA (4.2:1)</td>
<td>5</td>
<td>5</td>
<td>25</td>
<td>Daily, Day 3-7</td>
<td>10, 14, 17, 23*</td>
<td>25.0 ± 0.25</td>
<td>0/6</td>
</tr>
<tr>
<td>5</td>
<td>AAA232</td>
<td>791T/36-RTA (4.2:1)</td>
<td>19</td>
<td>4</td>
<td>76</td>
<td>Daily, Day 3-21</td>
<td>11, 21, 25, 29, 32</td>
<td>58.0 ± 0.61</td>
<td>1/10</td>
</tr>
<tr>
<td>6</td>
<td>791T</td>
<td>INDI-RTA (1:5:1)</td>
<td>19</td>
<td>4</td>
<td>76</td>
<td>Daily, Day 3-21</td>
<td>10, 13, 17, 20</td>
<td>24.1 ± 0.35</td>
<td>0/6</td>
</tr>
<tr>
<td>7</td>
<td>791T</td>
<td>RTA</td>
<td>20</td>
<td>1.5</td>
<td>30</td>
<td>Daily, Day 3-22</td>
<td>8, 11, 13, 15, 18, 22</td>
<td>25.0 ± 0.87</td>
<td>0/10</td>
</tr>
<tr>
<td>8</td>
<td>791T</td>
<td>RTA</td>
<td>20</td>
<td>3</td>
<td>60</td>
<td>Daily, Day 3-22</td>
<td>8, 11, 13, 15, 18, 22</td>
<td>25.0 ± 0.70</td>
<td>1/10</td>
</tr>
<tr>
<td>9</td>
<td>791T</td>
<td>791T/36-RTA (4.2:1)</td>
<td>5</td>
<td>8</td>
<td>40</td>
<td>Daily, Day 11-15</td>
<td>14, 20*</td>
<td>20.0 ± 0.27</td>
<td>0/6</td>
</tr>
<tr>
<td>10</td>
<td>791T</td>
<td>791T/36-RTA (4.2:1)</td>
<td>1</td>
<td>40</td>
<td>40</td>
<td>Day 11</td>
<td>14, 20*</td>
<td>20.0 ± 0.32</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Significantly smaller tumor size (P < 0.05) compared with control mice (Students’ t test).
* T/G, ratio of tumor weights in treated and control mice. P < 0.05 (by Wilcoxon rank test).

Assay for Anti-RTA Antibody

Sera was collected from athymic mice bearing 791T xenografts and from immunocompetent BALB/c mice treated with RTA-791T/36 immunotoxin. Serum was assayed for anti-RTA antibody using a solid phase radioimmunoassay. Briefly, wells of Terasaki microtest plates (Becton Dickinson, Oxnard, CA) were coated with RTA (20 μg/well) and washed with a washing buffer [PBS containing 0.1% bovine serum albumin and 0.1% rabbit serum (16)]. Mouse serum (10 μl/well), was added to the wells, and incubated for 1 h at room temperature. After washing four times in washing buffer, bound mouse immunoglobulin was detected with 125I labeled affinity purified F(ab')2 fragment of rabbit anti-mouse immunoglobulin reactive with mouse IgG and IgM (16).

RESULTS

Toxicity of 791T/36-RTA. Five daily injections of 20 or 30 mg 791T/36-RTA killed all mice (Table 2, test 1). Five daily injections of 10 mg/kg killed 20% of mice in one test (test 2) and produced 26% loss of body weight in another test (test 1). Doses of 5 mg/kg were well tolerated for up to 5 days with no mortality and acceptable loss of body weight (tests 3 and 4). When dosing at this level was extended beyond 5 days, there was greater toxicity as shown by loss in weight and increased mortality (test 4). These results show that for repeated injection of the 791T/36-RTA conjugate, 5–10 mg/kg/day is the upper acceptable level.

Influence of 791T/36-RTA on Growth of Osteosarcoma 791T Xenografts. Athymic mice implanted s.c. with osteosarcoma 791T tissue were treated by i.p. injection of 791T/36-RTA immunotoxin starting 2–11 days after tumor implantation (Table 1). Treatment significantly retarded tumor growth in all of these experiments as determined by tumor size determination at three time points and/or tumor weights at the termination of the test. In the first experiment (Table 1, experiment 1) mice received 20 daily injections each of 5 mg immunotoxin/kg. This produced a marked inhibition of tumor growth (Fig. 1) and the
Tumor inhibition in mice treated with RTA immunotoxin

The 791T xenografts are well established 11 days after tumor implantation. The influence of 791T/36-RTA immunotoxin treatment starting at this time was tested (Table 1, experiments 9 and 10): mean tumor diameter was 0.6 cm at initiation of treatment. Treatment for 5 days (8 mg/kg/day; total dose, 40 mg/kg) effectively inhibited tumor growth, this being reflected in the growth curves (Fig. 3) and in the reduction in tumor weight at termination of the test (T/C, 0.27; P < 0.05). A single bolus treatment (40 mg/kg) initiated 11 days after tumor implantation was equally effective (Fig. 3) with a final T/C ratio of 0.32 (Table 1). There was no mortality in either of the two groups of mice.

Expression of the gp72 Antigen on Tumor Xenograft-derived Target Cells. To determine if the cells in xenografts from immunotoxin-treated animals had altered levels of antigen expression, mice were implanted with tumor fragments and tumors were allowed to develop untreated for 11 days (Table 3, experiment 1). Mice were then treated for 5 days with 8 mg/kg/day of immunotoxin and 4 days later the xenografts were removed and collagenase treated to produce single cell suspensions. The antigen density was assessed by reacting the cells with 791T/36 antibody and then with fluorescein-labeled anti-

Table 3 Antigen expression on tumor cells derived from sarcoma 791T xenografts in 791T/36-RTA immunotoxin treated mice

<table>
<thead>
<tr>
<th>Exp. Days</th>
<th>Dose (mg/kg/day)</th>
<th>Total dose (mg/kg)</th>
<th>791T/36</th>
<th>Normal mouse IgG&lt;sub&gt;0&lt;/sub&gt;</th>
<th>Average (MLF/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11-15 (5)</td>
<td>8</td>
<td>771</td>
<td>28</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3-17 (15)</td>
<td>4</td>
<td>921</td>
<td>54</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*With respect to day of tumor xenografting.

4 I. Spitler, unpublished findings. "With respect to day of tumor xenografting.
mouse immunoglobulin. Tumor cells from untreated mice had an MLF of 915–1073, this being comparable to the level of which express 79IT/36 antibody defined gp72 antigen. Xeno cytotoxicity can be reproduced in vivo; 79IT/36-RTA immunoantibody (8). The present studies establish that this in vitro clonal antibody 79IT/36 are specifically cytotoxic in vitro for similar to those used for the therapy experiments (Table 1).

36-RTA ranging from 25 to 120 mg/kg; sera reacting very RTA Conjugate. Immunocompetent mice generated a marked reduction in binding of 79IT/36 antibody with tumor-derived target cells (MLF, 606–921) compared with controls (MLF, 526–803).

Anti-RTA Antibody Response in Mice Receiving 79IT/36-RTA Conjugate. Immunocompetent mice generated a marked anti-RTA antibody response when treated with doses of 79IT/36-RTA ranging from 25 to 120 mg/kg; sera reacting very strongly with RTA at the maximum dilution (1/10,000) tested (Table 4). Only marginal antibody responses were detected in sera from athymic mice treated with 79IT/36-RTA (Table 4). These included mice treated with immunotoxins on protocols similar to those used for the therapy experiments (Table 1).

**DISCUSSION**

Immunotoxins constructed by linking ricin A chain to monoclonal antibody 79IT/36 are specifically cytotoxic in vitro for tumor cells which express the gp72 antigen recognized by this antibody (8). The present studies establish that this in vitro cytotoxicity can be reproduced in vivo; 79IT/36-RTA immunotoxin specifically inhibiting growth of tumor xenografts which express 79IT/36 antibody defined gp72 antigen. Xenografts of a melanoma which does not react with 79IT/36-RTA to an anti-melanoma antibody (15) did not inhibit tumor growth. The greater difficulty in obtaining complete regression in hu

<table>
<thead>
<tr>
<th>Serum donor</th>
<th>Treatment 79IT/36-RTA (mg/kg)</th>
<th>10³</th>
<th>10⁴</th>
<th>10⁵</th>
<th>10⁶</th>
<th>10⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocompetent (BALB/c)</td>
<td>25</td>
<td>9,861 ± 630</td>
<td>7,819 ± 902</td>
<td>9,725 ± 1,203</td>
<td>4,588 ± 402</td>
<td>NT ²</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>NT</td>
<td>32,223 ± 4,668</td>
<td>30,084 ± 1,541</td>
<td>27,725 ± 5,675</td>
<td>27,621 ± 361</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>NT</td>
<td>40,878 ± 3,010</td>
<td>35,282 ± 2,381</td>
<td>32,436 ± 1,095</td>
<td>23,966 ± 1,314</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>NT</td>
<td>1,367 ± 201</td>
<td>835 ± 319</td>
<td>531 ± 270</td>
<td>296 ± 283</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>NT</td>
<td>0 ± 572</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>NT</td>
<td>1,104 ± 210</td>
<td>538 ± 451</td>
<td>274 ± 106</td>
<td>104 ± 68</td>
</tr>
</tbody>
</table>

³ Three to six mice/group.

² Mouse serum added to microtest plates containing RTA. After washing bound mouse immunoglobulin determined by reaction with [¹²⁵I]rabbit anti-mouse immunoglobulin (Fab')₂ (16).

² NT, not tested.

If tumor penetration is the rate-limiting step in immunotoxin

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5 V. S. Byers, manuscript submitted.

6 V. S. Byers, unpublished findings.
therapy, then it may only be feasible to treat small tumor deposits such as micrometastases known to be present in colorectal cancer and osteogenic sarcoma patients following surgical debulking of the primary tumor. It should be possible, however, to improve tumor penetration using immunotoxins constructed with F(ab')2 and Fab fragments. This approach is strongly supported by the finding of increased tumor localization of F(ab')2 fragments of anti-CEA antibodies in colorectal cancer patients (23) and xenografts (24). The construction of immunotoxins with antibody fragments is now being investigated.

REFERENCES


Inhibition of Growth of Human Tumor Xenografts in Athymic Mice Treated with Ricin Toxin A Chain-Monoclonal Antibody 791T/36 Conjugates


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