Combined Immunochemotherapy of Human Solid Tumors in Nude Mice

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ABSTRACT

In vivo immunochemotherapy of human solid tumors was studied in a nude mouse model. Large tumors (3 to 6 cm³) were induced by s.c. injection of the acute lymphoblastic leukemia T-cell line CEM. Transient tumor inhibition could be achieved by intratumoral injection of an intact ricin immunotoxin that specifically recognizes a determinant CD5 (T,p67) expressed on the cell surface. Injection of the in vitro active cyclophosphamide conjugate mafosfamid had little effect on the progression of tumor growth. A combination regimen of Immunotoxin and mafosfamid induced the most dramatic antitumor effect; a 72 to 100% reduction in tumor volume was observed within 3 to 4 days posttreatment. However, tumors relapsed within 5 to 13 days. Persistent, tumor regression was observed only when protocols using successive injections of combined immunotoxin/mafosfamid were used. All seven mice undergoing this treatment had a precipitous decrease in tumor size, and 86% survived greater than 30 days posttreatment. No residual tumor was detectable on Day 30 in five of seven mice. Regression was partly attributed to the selective activity of immunotoxin, since successive injections of an irrelevant control immunotoxin coupled to ricin in combination with mafosfamid did not reduce tumor size. Thus, we have demonstrated that a combination of anticancer chemotherapy and immunotoxin therapy yielded a greater antitumor effect than either therapy alone.

INTRODUCTION

IT, i.e., MoAb covalently bound to toxins such as ricin, diptheria toxin, abrin, gelonin, or pokeweed antiviral protein, represent a new class of highly selective and potent drugs in modern cancer therapy. Several preclinical studies have demonstrated the potential of cell type specific IT for the treatment of human cancer (1–15). Based on our previous experimental findings (3, 13, 14, 16–21), intact ricin IT are being utilized at the University of Minnesota (a) to selectively eliminate residual leukemic cells from stem cell grafts in autologous BMT for acute lymphoblastic leukemia and (b) to deplete donor T-cells from marrow grafts for GVHD prophylaxis in allogeneic BMT. Besides their current use as purgative reagents in BMT for GVHD prophylaxis in allogeneic BMT.

REFERENCES

1. This work was supported in part by National Cancer Institute Grants RO-1 31618 and RO-1 36725, American Cancer Society Grant IM-380, and the Minnesota Medical Foundation. CETCR Publication 29.

2. Recipient of New Investigator Award 1.R23CA-42111-01 from the NIH and Special Fellow of the Leukemia Society of America.

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5. The abbreviations used are: IT, immunotoxin(s); MoAb, monoclonal antibody(ies); PBS, phosphate-buffered saline; BMT, bone marrow transplantation; GVHD, graft-versus-host disease; LD50, 50% lethal dose.

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6 Clusters of differentiation were defined at the First and Second International Workshops on Human Leukocyte Differentiation Antigens.

7 Peukert, personal communication.
containing 500 mM lactose at a concentration of 5 μg/0.2 ml or 2.5 μg/0.2 ml. For combination therapy, IT and mafosfamid were mixed prior to injection. As controls, tumor-bearing mice were treated with T101-ricin alone, mafosfamid alone, M6 3D10-ricin plus mafosfamid, or were not treated. In all experiments, injection of intratumoral IT was preceded by an i.v. injection of 0.7 ml or 500 mM lactose in PBS. We have previously shown that i.v. lactose administration renders intact ricin IT cell type specific in this animal model (6). Various regimens of the reagents were administered to the tumors as detailed in “Results.” All tumor-bearing mice were observed up to 6 wk. Gross tumors were measured in 3 dimensions to achieve a more accurate evaluation of size. Tumor volume was estimated from measurements of 3 perpendicular axes.

RESULTS

Treatment of Large CEM Tumors with T101-Ricin IT Alone or Mafosfamid Alone. To evaluate the in vivo antitumor efficacy of T101-ricin IT alone against established CEM tumors, 2 nude mice bearing large CEM tumors (6.3 and 3.3 cm³, respectively) were treated with a single intratumoral injection of 5 μg T101-ricin IT plus i.v. lactose. Within 4 days following treatment, tumor mass was reduced by 47 and 64%, respectively (Table 1, Mice 1 and 2). However, regrowth occurred, and tumors reached a size of 6.2 and 5.7 cm³, respectively (Table 1; Fig. 1). Interestingly, we observed an arrest of tumor growth in Animals Mice bearing large CEM tumors were given injections intratumorally of 5 μg T101-ricin IT in 0.2 ml lactose (500 mM). This treatment was repeated 2 days later in mice 3 and 4. All mice received an injection i.v. of 0.7 ml 500 mM lactose prior to intratumoral IT.

Table 1 Treatment of CEM tumors with T101-ricin IT alone

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Initial tumor size (cm³)</th>
<th>Day of injection</th>
<th>Initial tumor reduction (%)</th>
<th>Final tumor size (cm³)</th>
<th>Survival (days)</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>6.3</td>
<td>0</td>
<td>47</td>
<td>6.2</td>
<td>23</td>
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<td>2</td>
<td>3.3</td>
<td>0</td>
<td>64</td>
<td>5.7</td>
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<tr>
<td>3</td>
<td>4.4</td>
<td>0</td>
<td>57</td>
<td>4.6</td>
<td>25</td>
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<td>4</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>9.7</td>
<td>15</td>
</tr>
</tbody>
</table>

* Percentage of initial tumor reduction = initial tumor size − tumor size posttreatment / initial tumor size × 100.

Fig. 1. Growth of CEM tumors following treatment with a combination of T101-ricin IT and mafosfamid: Protocol I. Two representative mice bearing 3- to 6-cm³ tumors were given injections intratumorally with 5 μg T101-ricin IT and 500 μg mafosfamid in 0.4 ml lactose (500 mM). When tumors regrew on Day 5 or 8, treatment was repeated with 2.5 μg T101-ricin IT and 500 μg mafosfamid (C, []). As a control, 2 CEM tumor-bearing mice were given intratumorally a single injection of 5 μg T101-ricin IT (C, []) or 500 μg mafosfamid (Δ, A). All mice treated with IT received an initial injection i.v. of 0.7 ml lactose (500 mM).

Table 2 Treatment of CEM tumors with mafosfamid alone

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Initial tumor size (cm³)</th>
<th>Day of injection</th>
<th>Initial tumor reduction (%)</th>
<th>Final tumor size (cm³)</th>
<th>Survival (days)</th>
</tr>
</thead>
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<tr>
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<td>5.7</td>
<td>0</td>
<td>0</td>
<td>30</td>
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<td>0, 2</td>
<td>0</td>
<td>36</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>0, 2</td>
<td>5</td>
<td>45</td>
<td>23</td>
</tr>
</tbody>
</table>

Fig. 2. Growth of CEM tumors following treatment with a combination of T101-ricin IT and mafosfamid: Protocol II. Two representative mice bearing tumors of 3 to 6 cm³ were given injections intratumorally with 5 μg T101-ricin IT and 500 μg mafosfamid (C, []). In 0.4 ml lactose (500 mM). On Day 2, a second injection was given with 2.5 μg T101-ricin IT and 500 μg mafosfamid. Two additional injections were administered 1 wk apart when tumor regrew. As controls, 2 tumor-bearing mice were given intratumorally 2 injections of 5 μg T101-ricin IT (C, []) or 500 μg mafosfamid (Δ, A) on Days 0 and 2. All mice treated with IT received an initial injection i.v. of 0.7 ml lactose (500 mM).
Treatment of Large CEM Tumors with T101-Ricin IT in Combination with Mafosfamid

Protocol I. To determine whether a combination of T101-ricin IT and mafosfamid would be more effective than either T101-ricin IT or mafosfamid alone, 2 tumor-bearing mice were given i.v. lactose followed by intratumoral injection of 5 \( \mu g \) T101-ricin IT combined with 500 \( \mu g \) mafosfamid (Fig. 1; Table 3, Mice 1 and 2). As shown in Fig. 1, the tumor mass was reduced to less than 0.3 cm\(^3\) within 3 to 4 days. However, we observed a relatively early recidivism on Days 5 to 8. After tumor progression was documented, we administered a second bolus injection consisting of 2.5 \( \mu g \) T101-ricin IT and 500 \( \mu g \) mafosfamid. Although tumor growth was completely inhibited for 6 to 8 days, tumors started to regrow on Days 15 and 16. By Day 30, tumors reached a size of 1.8 to 3.0 cm\(^3\) (Fig. 1). Interestingly, in Mouse 1 the tumor spontaneously regressed and became necrotic on Day 38. This mouse is surviving with no detectable tumor 40 days after initial treatment (Table 3, Mouse 1).

Since we observed a second recidivism in both mice receiving Protocol 1, we increased the IT dose in the second bolus injection to improve the therapeutic efficacy. Three tumor-bearing mice were initially treated with intratumoral injection of 5 \( \mu g \) T101-ricin IT and 500 \( \mu g \) mafosfamid (Table 3, Mice 3 to 5). This treatment was repeated when we observed recidivism on Days 5 to 13, following initial reduction of tumor size by 72 to 96\% (Table 3). Tumor size was 0.13 to 1.8 cm\(^3\) when tumor progression was documented. Although in 2 of 3 mice tumor mass was virtually eliminated after second injection, all 3 mice in this group displayed symptoms of ricin poisoning (weight loss, skin hemorrhage, respiratory distress, loss of balance) (6). These mice died on Days 15 to 23 (Table 3, Mice 3 to 5).

Protocol II. Our first combination protocol described above resulted in initial reduction of tumor mass by 90 to 100\% in 4 of 5 mice (Table 3). However, early recidivism was observed in all mice, and the outcome of the second bolus injection was not satisfactory. Based on these findings, we modified our protocol in an attempt to reduce the incidence of early recidivism. Seven tumor-bearing mice received a local treatment of 5 \( \mu g \) T101-ricin IT combined with 500 \( \mu g \) mafosfamid after i.v. lactose administration, as in Protocol I. This resulted in 100\% reduction of tumor size in 5 of 7 treated mice within 2 days (Table 3, Mice 6 to 12). To eradicate residual CEM cells that escaped initial treatment, a second intratumoral bolus injection of 2.5 \( \mu g \) T101-ricin IT and 500 \( \mu g \) mafosfamid was administered on Day 2. Fig. 2 shows the course of therapeutic response in 2 representative nude mice. Despite this consolidation therapy, recidivism occurred within 5 to 7 days after second injection. Regrowing tumors were treated with 2 to 3 more injections 1 wk apart. This serial treatment protocol successively reduced the remaining tumor burden, and in 100\% of mice a persistent, complete regression was obtained (Table 3). Eighty-six \% of tumor-bearing nude mice undergoing this protocol survived over 30 days posttreatment. No residual tumor was detectable in 5 of 7 mice, but a calcified necrotic mass (Fig. 3A) or a fibrotic scar (Fig. 3B) was observed at the previous site of tumor. In the remaining 2 mice, the tumor mass was reduced to less than 10\% of its initial size (Table 3).

Specificity of T101-Ricin/Mafosfamid Treatment

To determine whether or not the efficacy of T101-ricin/mafosfamid treatment was dependent on specific binding of T101-ricin to CD5 on the surface of the CEM cell line, tumor-bearing mice underwent subsequent treatment with an irrelevant control immunotoxin, M6 3D10-ricin, combined with mafosfamid. Three mice (Nos. 13 to 15) bearing tumors of 5.2 to 6 cm\(^3\) were treated with our more aggressive Protocol II in a manner identical to Mouse 7 (Table 3). Despite 4 successive injections of control immunotoxin/mafosfamid, there was no initial reduction of tumor size in any of the mice tested. Tumor size increased to 30.4 to 34.4 cm\(^3\) over 28 days of observation. By contrast, Protocol II utilizing T101-ricin/mafosfamid resulted in initial tumor reductions of 60 to 100\%.

Tumor Destruction: Morphology

After treatment with 5 \( \mu g \) T101-ricin IT alone, the upper part of the tumor mass became necrotic within 2 days. On Days 3 to 7 a small defect was observed on the top of the tumor (Fig. 4B). This open wound healed, and tumor continued to grow in its periphery.

After treatment with 500 \( \mu g \) mafosfamid alone, tumor con-
The tissue defect rapidly healed and was replaced by a white flat fibrotic scar (Fig. 4E). Tumor recidivism occurred adjacent to the site of tumor necrosis (Fig. 4F). Tumor recidivism occurred adjacent to the site of tumor necrosis (Fig. 4F).

**DISCUSSION**

Immunotoxins have been used in experimental and clinical settings for the ex vivo treatment of donor bone marrow for purposes of GVHD prophylaxis (reviewed in Refs. 14 and 16). A major challenge in the field of IT research is to develop these highly potent and selective anticancer reagents for in vivo use. Studies using in vivo IT for treatment of residual disease in mice have shown promise (18, 29, 31, 32). In a recent study, we have investigated the potential of intact ricin IT in treatment of human solid tumors (6). Utilizing a novel in vivo nude mouse model of human neoplasia, we have shown that established CEM solid tumors of small size (0.3 to 0.5 cm² or 0.06 to 0.15 cm³) can be effectively treated with T101-ricin IT. Our findings using non-target cell lines and irrelevant IT have clearly shown that the specificity of intratumoral IT therapy (a) depends on systemic administration of lacrose to IT treatment and (b) is not absolute.

Given the current limitations of our model system, we have extended our previous studies and evaluated the efficacy of T101-ricin IT alone, in combination with mafosfamid, a synthetic cyclophosphamide derivative, against large CEM tumors (3 to 6 cm³ containing 40 to 60 x 10⁶ cells). Mafosfamid in combination with T101-ricin IT was selected for the following reasons. (a) IT have been shown to be cell type specific and extremely potent (1-15, 28, 29, 31-34) and have been used safely to deplete leukemia cells from human autologous grafts prior to reinfusion and lymphohematopoietic recovery of the treated patient (35). (c) These 2 approaches kill cells by different mechanisms. IT inhibit cellular protein synthesis by inactivation of the 60S ribosomal subunit (36), whereas mafosfamid prevents cells from dividing by DNA alkylation (26). (d) Most importantly, in a recent study, we have demonstrated that a combination of T101-ricin IT and mafosfamid was superior to either T101-ricin IT or mafosfamid alone in inhibiting target T-leukemia cells in vitro (19). Combined treatment with 1 µg/ml T101-ricin IT and 50 µg/ml mafosfamid eliminated all but...
IN VIVO IMMUNOCHEMOTHERAPY

Fig. 4. BALB/c-nu/nu mice bearing CEM tumors following IT, mafosfamid, or combined IT and mafosfamid treatments. A, mouse bearing a CEM tumor of 4.8 cm³ before treatment. B, mouse bearing a CEM tumor that has been treated intratumorally with 5 µg T101-ricin IT when it measured 6.3 cm³. Three days later, note the defect on the top of tumor. The size of tumor decreased to 3.3 cm³. C, mouse in which a CEM tumor of 4 cm³ has been injected intratumorally with 500 µg mafosfamid. At 3 days posttreatment, the tumor almost doubled in size to 7.7 cm³. D, mouse in which CEM tumor of 5.5 cm³ has been treated intratumorally with 5 µg T101-ricin IT combined with 500 µg mafosfamid. At 3 days posttreatment, a crater-like tissue defect was present at the previous site of tumor. E, mouse at 8 days following treatment described in Fig. 1D. The defect healed, but tumor relapsed adjacent to scar tissue.

Table 4. Toxicity of s.c. T101-ricin IT/mafosfamid to healthy nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Signs of toxicity</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mafosfamid (500 µg)</td>
<td>None</td>
<td>100, 38, 40</td>
</tr>
<tr>
<td>T101-ricin IT (5 µg)</td>
<td>Wt loss, hemorrhages, respiratory distress, loss of balance</td>
<td>0, 4, 5</td>
</tr>
<tr>
<td>T101-ricin IT (2.5 µg)</td>
<td>Wt loss, local tissue damage, scar formation (in 1 of 2 mice)</td>
<td>100, 40+, 40+</td>
</tr>
<tr>
<td>Mafosfamid (500 µg) + T101-ricin IT (5 µg)</td>
<td>Limited local tissue damage, scar formation</td>
<td>100, 40+, 40+, 40+</td>
</tr>
</tbody>
</table>

Higher concentrations of mafosfamid did not result in increased tumor cell elimination (26, 33).

Based on these in vitro findings, we used 500 µg mafosfamid. We calculated that this concentration should yield a supraoptimal intratumoral drug concentration, since 500 µg in 3- to 6-ml tumor volume equal a concentration of 83 to 166 µg/ml. We used 5 µg T101-ricin IT, which should yield an effective IT dose, since 5 µg in 3 to 6 ml equal 0.8 to 1.7 µg/ml. We assumed that this combination protocol would eliminate all CEM cells in the established xenograft.

In Protocol I, 5 mice bearing large CEM tumors of 3 to 6 cm³ were given i.v. injections of lactose followed by intratumoral application of 5 µg T101-ricin IT mixed with 500 µg mafosfamid. Although the tumor mass was effectively reduced to 0 to 28% of its initial size within 3 to 4 days, we observed an early relapse in all animals. The outcome of a second treatment was not satisfactory. Because of the high incidence of tumor relapse, a more aggressive treatment was used that consisted of 5 µg T101-ricin IT and 500 µg mafosfamid injected intratumorally followed by a second treatment with 2.5 µg T101-ricin IT and 500 µg mafosfamid 2 days later. The second
injection was given in hope of eliminating residual tumor cells that escaped initial treatment and led to an early recidivism. Recidivism still occurred within 1 wk after the second injection. Four to 5 serial injections (Protocol II) of T101-ricin IT and mafosfamid were necessary for persistent, complete regression in 5 of 7 treated mice, and tumor mass in the 2 remaining mice measured only 0.1 to 0.3 cm³ (3 to 6% of tumor initial size). Eighty-six% of treated mice survived over 30 days. Treatment was specific since an irrelevant immunotoxin (directed against guinea pig idiotype) combined with mafosfamid did not prevent tumor growth when administered according to Protocol II. A group of tumor-bearing mice given T101-ricin alone achieved results of a transient arrest of tumor growth. The combined treatment was clearly more effective than T101-ricin IT alone. Surprisingly, mafosfamid by itself was not effective against CEM cells in vivo. This lack of the predicted antitumor efficacy of mafosfamid might be due to (a) the faster clearance of mafosfamid in vivo or (b) a low intratumoral concentration if the drug was not distributed well.

The reason why IT in combination with mafosfamid gave greater tumor kill than the individual agents is not yet known. Ricin A-chain is a potent inhibitor of protein synthesis by inactivating the 60S ribosomal subunit. Even a single molecule in the cytosol can destroy a cell (36). Thus, when a sufficient number of surface receptors have bound T101-ricin/lactose, a single A-chain molecule may find its way into the cytosol compartment and kill the cell. In contrast, mafosfamid is a DNA alkylation agent (26). It is possible that cells resistant to ricin might be destroyed by drug treatment, and vice versa, drug-resistant mutants might prove susceptible to immunotoxin treatment. Other explanations are possible: (a) mafosfamid might behave similarly to lysosomotropic agents, such as NH₄Cl or carboxylic ionophores (1, 6), that promote entry of ricin A chain into the cytosol; (b) the cellular level of aldehyde dehydrogenase, an enzyme responsible for the degradation of the active cyclophosphamide metabolite (37), may decrease due to IT-mediated inhibition of protein synthesis; or (c) IT in combination with mafosfamid treatment may trigger a type of rejection response or localized necrosis. Despite i.v. administration of lactose, s.c. injection of 5 μg T101-ricin IT was lethal to healthy nude mice not bearing tumors. Also, 3 mice receiving a second intratumoral bolus injection of 5 μg T101-ricin IT (Protocol I) displayed symptoms of ricin poisoning. Thus, for the remaining studies, we reduced the IT concentration of subsequent injections to 2.5 μg T101-ricin IT/mouse. Our findings, as well as the findings of others (38), show that ricin toxicity severely limits the in vivo potential of intact ricin IT for treatment of systemic or malignant disease. Such problems may be solved by (a) using ricin with the galactose binding site chemically altered to make “better” IT (39); or (b) using hemi-toxins that do not have receptors on human cells, such as pokeweed anti-viral protein (PAP) (12). Surprisingly, the lethal dose of IT (5 μg) in combination with 500 μg of mafosfamid was not fatal to non-tumor-bearing mice. Also, less toxicity was observed when mice received multiple intratumoral injections of immunotoxin/mafosfamid. The reason for these results is yet unknown. Possibly, mafosfamid might affect biodistribution or enhance the rate of immunotoxin degradation in normal animals.

Despite limitations, Protocol II, by utilizing immunochemotherapy, was able to control tumor progression. Cytoreduction was at least in the order of 90%, since injection of 5 × 10⁶ tumor cells will initiate tumors in this model. Final tumor size was less than 10% of the initial volume in all mice. No remaining tumor was detectable in 5 to 7 treated mice. Our findings in this in vivo nude mouse model support our previous in vitro studies (19). Considering the degree of heterogeneity in primary neoplasms, the combined use of reagents that interfere at different stages of the cellular machinery is likely to improve the efficacy of treatment. Given the limitations of the nude mouse model, further investigation in a syngeneic murine tumor model is necessary. Nevertheless, this combination strategy has obvious clinical potential in the treatment of common human solid tumors, such as lymphoma, melanoma, osteogenic sarcoma, and ovarian, breast, lung, colon, and brain cancer, against which several different monoclonal antibodies are now available (40–47).

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