A new pharmacologic agent, anti-CD3(ab')2-ricin toxin A chain (RTA), was synthesized for the purpose of targeting T cells and as a means of treating established graft-versus-host disease (GVHD). The Fc region of anti-CD3 monoclonal antibody (MoAb) was removed to prevent its ability to activate T cells. The resulting F(ab')2 fragments were conjugated to deglycosylated RTA (dgRTA), a catalytic and potent phytoxin. The resulting immunotoxin (IT) was potent (greater than 95% inhibition) and selective in inhibiting T-cell mitogenesis in vitro. In vivo, the IT depleted 80% of T cells in mice receiving bone marrow (BM) transplants. Transplantation in an aggressive acute GVHD model using C57BL/6 donor cells and H-2 disparate B10.BR recipients resulted in an infiltration of CD3-expressing cells and a median survival time (MST) of 20 to 30 days. A 5-day course of anti-CD3F(ab')2-RTA (30 µg/d intraperitoneally) beginning 7 days after GVHD induction was beneficial in treating established GVHD in these mice, as evidenced by significantly prolonged survival (MST, greater than 80 days), superior mean weight values, and improved clinical appearance. Neither intact anti-CD3, unconjugated anti-CD3 (Fab')2 fragments, nor a mixture of anti-CD4 and anti-CD8 MoAbs (which are highly effective in prophylactic models) were as effective. F(ab')2 fragments made from anti-Lyt-1 (the murine homologue of human anti-CD5) linked to RTA were also not effective, despite the fact that both anti-CD3(ab')2RTA and anti-Lyt-1Fab')2RTA had similar half-lives of about 9 hours. The IT also increased MST in two aggressive models of GVHD across non-H-2 minor histocompatibility barriers, indicating that the usefulness of anti-CD3Fab')2-dgRTA is not limited to a single-strain combination. This agent should be further investigated as an alternative to current strategies for treating steroid refractory GVHD.

Graft-versus-host disease (GVHD) results when donor T lymphocytes react against the organs of the immunosuppressed recipient. It is a significant source of morbidity and mortality after bone marrow (BM) transplantation. Despite advances in preventing GVHD using in vivo chemotherapeutic prophylactic agents, ongoing GVHD is generally difficult to treat and represents a major clinical problem. Thus, better therapies for GVHD are needed.

Although numerous GVHD studies have addressed the topic of prophylaxis, few studies have been directed at studying ongoing GVHD. Thus, we devised mouse models with the express purpose of studying established GVHD. In the model, donor marrow, as a source of stem cells, is mixed with splenocytes, as a source of T cells, and transplanted into H-2 or non-H-2 disparate recipients, resulting in lethal GVHD directed against major (H-2) or minor (non-H-2) histocompatibility disparities. The role of T cells in GVHD in our H-2 disparate model has been established in previous studies.

One approach for eliminating GVHD-causing T cells has been to target T cells with monoclonal antibody (MoAb) linked to the potent catalytic phytoxin, ricin toxin A chain (RTA). RTA, the extractable A chain 30-kD component from the toxin ricin (from the plant Ricinis communis), was chosen for these studies because it requires only a single molecule in the cytosol for cell death. It is an enzyme with first order/single hit kinetics that inhibits the 28S RNA component of the 60S ribosome, thereby inhibiting protein synthesis. RTA was chemically deglycosylated before conjugation, as studies show that the removal of naturally occurring carbohydrates diminishes nonspecific reactivity without compromising activity.

The first in vivo studies with immunotoxin (IT) and GVHD therapy involved targeting of the CD5 differentiation antigen in humans and Lyt-1, the murine CD5 homologue. CD5 is a 65-kD glycoprotein that is broadly expressed on T cells. Transient responses at best were obtained in both models. A problem with this approach may be that CD5 functions as a T-cell costimulatory molecule recognizing the CD72 molecule on B cells. The MoAbs against CD5 or murine Lyt-1 trigger T-cell activation. Our previous studies show that ITs made with intact anti-CD3 MoAb that activate T cells have more nonspecific toxicity than ITs made with Fab')2. ITs that do not activate T cells. Therefore, in this study, we synthesized a nonactivating IT by removing the Fc region of anti-CD3 MoAb and then linked it to RTA.

Several facts indicated that anti-CD3 would be an excellent choice for targeting toxin to target cells. (1) The CD3 portion of the T-cell receptor (TCR) complex is expressed on all committed cytotoxic T cells and their precursors, and immunohistology studies reported herein show significant increases in CD3-expressing T cells in GVHD-target tissue. (2) Other studies showed that anti-human CD3-RTA effectively inhibited mitogen-activated T cells in vitro. (3) In clinical studies, anti-CD3-RTA plus the potentiator NH4Cl, when used to eliminate GVHD-causing T cells from donor

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Supported in part by US Public Health Service Grants No. RO1-CA31618, RO1-CA56725, P01-CA1237, P01-CA34945, and P01-AI35296 awarded by the National Cancer Institute and the National Institute of Allergy and Infectious Disease, Department of Health and Human Services. B.B. is a recipient of the Edward Mallinckrodt Jr Foundation Scholar Award.

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0006-4971/95/8611-0018$3.00/0

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graft rejection, resulting in an overall promotion of long-term BM engraftment. Anti-CD3 has also been conjugated to intact diphtheria toxin to the diphtheria-binding site mutant CRM9, resulting in selective and potent ITs.

In the current study, we show that anti-CD3 F(ab′)2-RTA has a deleterious effect on CD3-expressing cells in vivo, and in a model of aggressive ongoing GVHD, that anti-CD3 F(ab′)2-RTA is more effective than un conjugated anti-CD3 F(ab′)2 or anti-Lyt-1 F(ab′)2-RTA IT in the treatment of lethal GVHD induced by major or minor antigen disparate BM transplants.

**MATERIALS AND METHODS**

**Mice.** B10.BR/SgSnJ (H-2b, Ms 1b2a), C3H/HeJ (H-2a, Ms 1b2a), CB6F1 (H-2b, Ms 1a2a), B10.D2 (H-2d, Ms 1b2b), and DBA-2J (H-2d, Ms 1a2a) mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 (H-2d) mice were purchased from the Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD). Donors and recipients were females. Donors were 4 to 6 weeks old, and recipients were 8 to 10 weeks old at the time of BMT. The C57BL/6 and B10.BR donor/host strain combination are mismatched at the H-2 major histocompatibility complex (MHC) and at several minor antigen loci. The B10.BR and CBA donor/host strain combination are matched at H-2d, but not at the Ms minor antigen loci. The B10.D2 and DBA-2 donor/host strain combination are matched at H-2d, but not at the Ms minor antigen loci.

**Reagents.** Anti-CD3 is a hamster IgG MoAb produced from hybridomas 145-2C11 and was generously provided by Dr Jeffrey Bluestone (University of Chicago, Chicago, IL). It is reactive against the CD3 epsilon component of the murine TCR and was purified by ammonium sulfate precipitation followed by ion exchange chromatography on DE52 (Whatman, Hillswro, OR). F(ab′)2 fragments were prepared as previously described by incubating antibody at 37°C for 90 minutes with a 1/100 ratio of pepsin (Sigma Chemical Co, St Louis, MO) to antibody in 0.1 mol/L citrate buffer, pH 3.9. The reaction was halted with the addition of 3.0 mol/L tris-HCl (pH 8.6), and the mixture was dialyzed against borate-buffered saline, pH 8.5. The F(ab′)2 fragments were purified on a protein A-Sepharose column. IT was produced by linking the purified fragments to a selective 3-log depletion of CD3-expressing cells and largely prevented lethal GVHD without the need for posttransplant immunosuppression. Although it is more difficult to control ongoing GVHD in vivo, these in vitro findings were encouraging.

Anti-CD3-RTA was more rapidly and completely internalized as compared with anti-CD5–RTA and anti-CD2–RTA. In a different study in which internalization was measured with radionucleotide-labeled antibodies, anti-CD3–RTA was internalized faster than anti-CD5–RTA, anti-CD4–RTA, or anti-CD8–RTA. In mice, studies have confirmed that anti-CD3–RTA or anti-CD3 F(ab′)2–RTA can be administered in vivo and eliminate the CD3+ cells responsible for BM graft rejection, resulting in an overall promotion of long-term BM engraftment. Anti-CD3 has also been conjugated to intact diphtheria toxin or to the diphtheria-binding site mutant CRM9, resulting in selective and potent ITs.

In the current study, we show that anti-CD3 F(ab′)2-RTA has a deleterious effect on CD3-expressing cells in vivo, and in a model of aggressive ongoing GVHD, that anti-CD3 F(ab′)2-RTA is more effective than un conjugated anti-CD3 F(ab′)2 or anti-Lyt-1 F(ab′)2-RTA IT in the treatment of lethal GVHD induced by major or minor antigen disparate BM transplants.

**Flow cytometry.** MoAbs were directly labeled with bio- tin, fluorescein isothiocyanate (FITC), or phycocerythrin (PE) as previously described. Single-cell splenocyte preparations were suspended in buffer (phosphate-buffered saline [PBS] + 5% colostrum-free bovine serum + 0.015% sodium azide). Pelleted cells were incubated for 15 minutes at 4°C with 0.4 μg of an anti-Fe receptor MoAb (clone 2.4G2, provided by Dr Jay Unkless, Rockefeller University, New York, NY) to prevent Fc binding. Optimal concentrations of directly conjugated (biotin-, PE-, or FITC-labeled) MoAb were added to a total volume of 100 to 130 μL, and the mixture was incubated for 1 hour at 4°C. For the biotin-labeled MoAb, fluorescence was indirectly measured by adding streptavidin-labeled Red 613 (GIBCO, Grand Island, NY) for an additional 1 hour at 4°C. After final washing, cells were fixed in 1% paraformaldehyde. The following labeled MoAbs and reagents, obtained from Phar- Mingen (San Diego, CA) unless otherwise indicated, were used: anti-CD3 (clone 145-2C11, hamster IgG), anti-CD8 (clone 53-6-72, rat IgG2a, provided by Dr Ledbetter), anti-CD4 (clone GK1.5, rat IgG2b, provided by Dr Gloria Koo, Rahway, NJ), and an irrelevant rat IgG2b anti-human antibody (clone 3A1e). All samples were analyzed on a FACSscan (Becton Dickinson, Palo Alto, CA) using consort-30 software. A minimum of 20,000 events were examined. Background subtraction using a directly conjugated irrelevant antibody control was performed for each sample.

**Radiiodination.** The labeling of IT with iodine-125 (125I) was performed using lodo-beads according to the manufacturer's specifications (Pierce, Rockford, IL) and an adaptation of the method first described by Markwell. A 20% loss of protein content during the labeling procedure was assumed, based on previous studies, and all concentrations were adjusted using this value. Over 82% of the counts of labeled antibody were precipitable with trichloroacetic acid.

**Blood clearance of 125I-labeled IT.** Blood clearance was determined as previously reported. Between 0.17 hours and 72 hours after injection intravenously (IV) of 20 μCi radiolabeled IT, five C3H/HeJ mice in each group were anesthetized and bled by retroorbital venipuncture at nine different time points. Whole blood (25 μL) was aliquoted from each animal and counted in a liquid scintillation counter. Triplicate samples of the original injection standard were

**Flow cytometry analysis.** MoAbs were directly labeled with bio- tin, fluorescein isothiocyanate (FITC), or phycocerythrin (PE) as previously described. Single-cell splenocyte preparations were suspended in buffer (phosphate-buffered saline [PBS] + 5% colostrum-free bovine serum + 0.015% sodium azide). Pelleted cells were incubated for 15 minutes at 4°C with 0.4 μg of an anti-Fe receptor MoAb (clone 2.4G2, provided by Dr Jay Unkless, Rockefeller University, New York, NY) to prevent Fc binding. Optimal concentrations of directly conjugated (biotin-, PE-, or FITC-labeled) MoAb were added to a total volume of 100 to 130 μL, and the mixture was incubated for 1 hour at 4°C. For the biotin-labeled MoAb, fluorescence was indirectly measured by adding streptavidin-labeled Red 613 (GIBCO, Grand Island, NY) for an additional 1 hour at 4°C. After final washing, cells were fixed in 1% paraformaldehyde. The following labeled MoAbs and reagents, obtained from Phar- Mingen (San Diego, CA) unless otherwise indicated, were used: anti-CD3 (clone 145-2C11, hamster IgG), anti-CD8 (clone 53-6-72, rat IgG2a, provided by Dr Ledbetter), anti-CD4 (clone GK1.5, rat IgG2b, provided by Dr Gloria Koo, Rahway, NJ), and an irrelevant rat IgG2b anti-human antibody (clone 3A1e). All samples were analyzed on a FACSscan (Becton Dickinson, Palo Alto, CA) using consort-30 software. A minimum of 20,000 events were examined. Background subtraction using a directly conjugated irrelevant antibody control was performed for each sample.

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Mouse splenocytes were incubated with IT for four hours at 37°C. For blocking studies, cells were treated with 300 μg/mL unconjugated anti-CD3F(ab')2 for 30 minutes before addition of IT. After treatment, cells were washed, and PHA was added. After 3 days, cells were pulsed with tritiated thymidine and harvested. Data are presented as cpm × 10^-3 ± one SD unit.

Table 1. Specific Activity of Anti-CD3F(ab')2-RTA Against PHA-Activated Murine T Cells

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>CD3F(ab')2-RTA</th>
<th>CD3F(ab')2-RTA + 300 μg/mL CD3F(ab')2</th>
<th>Hamster F(ab')2-RTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53.6 ± 14.8</td>
<td>64.1 ± 10.1</td>
<td>43.2 ± 8.9</td>
</tr>
<tr>
<td>0.1</td>
<td>50.4 ± 14.9</td>
<td>65.4 ± 3.6</td>
<td>58.8 ± 5.2</td>
</tr>
<tr>
<td>1</td>
<td>21.1 ± 3.2</td>
<td>59.5 ± 2.2</td>
<td>59.6 ± 3.5</td>
</tr>
<tr>
<td>10</td>
<td>2.7 ± 0.4</td>
<td>42.8 ± 18.6</td>
<td>48.0 ± 18.6</td>
</tr>
</tbody>
</table>

Table 2. Depletionary Effect of Anti-CD3F(ab')2-RTA on Mice Undergoing Syngeneic BMT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Positive</th>
<th>Absolute Cell Count (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>36</td>
<td>0.11</td>
</tr>
<tr>
<td>400 μg anti-CD3F(ab')2</td>
<td>27</td>
<td>0.05</td>
</tr>
<tr>
<td>150 μg anti-CD3F(ab')2-RTA</td>
<td>9</td>
<td>0.02</td>
</tr>
<tr>
<td>anti-Thy1.2 + C' (in vitro)</td>
<td>25</td>
<td>0.18</td>
</tr>
</tbody>
</table>

C57BL/6 mice were given 3 Gy TBI on day -1 and 1 day later were given a syngeneic BMT with 15 × 10^6 C57BL/6 BM cells and 15 × 10^6 splenocytes. Different groups (n = 3 per group) were given IP injections of PBS, 400 μg anti-CD3F(ab')2 once on day 0, or 150 μg anti-CD3F(ab')2-RTA once on day 0. One group was given BM mixed with T-cell-depleted splenocytes. Splenocytes were harvested on day 2 and examined for CD3+ cells by flow cytometry.

RESULTS

IT composition. Purity of the IT was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described. Both anti-CD3F(ab')2-RTA and anti-Lyt-1F(ab')2-RTA were heterogeneous, consisting of one, two, or three toxin molecules linked to F(ab')2, but the 1:1 species generally predominated (data not shown). Less than 13% unconjugated F(ab')2 was present after purification, but no free toxin was detected.

Inhibitory effect of anti-CD3F(ab')2-RTA on mouse splenocytes in vitro. To determine whether anti-CD3F(ab')2-RTA selectively inhibited mouse T cells, C57BL/6 splenocytes were preincubated with anti-CD3F(ab')2-RTA for 4 hours at concentrations of 0, 0.1, 1, and 10 μg/mL (Table 1). Inhibition of PHA-induced activation was dose-dependent, and at 10 μg/mL, counts per minute (cpm) were reduced 95% (2.7 × 10^5 as compared with 53.6 × 10^5 in the untreated control) without the benefit of IT potentiators. Two control groups showed specificity: (1) The addition of 300 μg/mL unconjugated anti-CD3F(ab')2 blocked the inhibitory effect of anti-CD3F(ab')2-RTA. (2) An irrelevant control polyclonal hamster antibody conjugated to dgrTA did not inhibit PHA activation.

Depletionary effects of anti-CD3F(ab')2-RTA. To determine whether anti-CD3F(ab')2-RTA had a depletionary effect, C57BL/6 mice were irradiated and then given syngeneic BMTs to avoid complications of the sometimes immunosuppressive nature of GVHD occurring in allogeneic transplants. We studied the ability of anti-CD3F(ab')2-RTA to deplete CD3+ cells (Table 2). Irradiation was given on day -1. Syngeneic BM and a single dose of anti-CD3F(ab')2-RTA were given on day 0. On day 2, mice were killed and splenocytes studied by flow cytometry. Mice given 400 μg unconjugated anti-CD3F(ab')2 had 0.05 × 10^6 CD3+ cells in the spleen, while mice given less than half that dosage of anti-CD3F(ab')2-RTA had 2.5-fold fewer CD3+ T cells. Compared with PBS-treated controls, mice given anti-CD3F(ab')2-RTA had about fivefold fewer CD3+ cells (a reduction of 82%). These findings show the depletionary effect of anti-CD3F(ab')2-RTA as compared with anti-CD3F(ab')2 in the BMT setting. Anti-CD3F(ab')2-RTA treatment resulted in the same number of NK1.1+ cells as in the PBS control group, further indicating that anti-CD3F(ab')2-RTA selectively depleted CD3-expressing cells. A significant reduction in these CD3+ T cells (42%) was still observed in anti-CD3F(ab')2-RTA-treated mice at even 14 days postinjection of IT. A depletionary effect of anti-CD3F(ab')2-RTA was also observed in normal mice, where a significant inhibition of both CD4-expressing and CD8-expressing cells was observed (data not shown).

Effect of anti-CD3F(ab')2-RTA on ongoing GVHD across the H-2 barrier. To determine whether anti-CD3F(ab')2-RTA could inhibit established GVHD across the H-2 barrier, B10.BR mice were given 8 Gy total body irradiation (TBI) and then transplanted with 25 × 10^6 C57BL/6 BM cells and 25 × 10^6 splenocytes. In situ studies showed that at day 9 after BMT, there is a dramatic infiltration of CD3-expressing lymphocytes observed in the colon of these mice (data not shown). No such cells were observed in controls given BM grafts without GVHD-causing splenocytes. Treatment with anti-CD3F(ab')2-RTA was initiated a week after GVHD induction to permit a graft-versus-host reaction before treatment. Mice were given daily IP injections of 30 μg IT beginning on day 7 and continuing through day 11. Figure 1A shows that anti-CD3F(ab')2-RTA-treated mice had a median survival time (MST) of greater than 80 days. At 3 months posttransplant, 22% of the animals were still surviving. In contrast, control PBS-treated animals had an MST.
of only 20 days, and none survived beyond 45 days post-BMT. Treatment with 50 μg anti-CD3F(ab')2 × 5 days on days 7 through 11 (250 μg total) did not result in significant protection against GVHD. Treatment with 400 μg of intact CD3 on day 7 resulted in a characteristic high percentage of early mortality (MST, 8 days) due to the activation of T cells and toxic side effects of anti-CD3 treatment, as previously reported. Transient and nonsignificant protection from GVHD was noted when a combination of 400 μg anti-CD4 and anti-CD8 MoAbs was administered on day 7. As a positive control, T cells were eliminated from the donor grafts before BMT using anti-Thy1.2 plus complement in one group of animals. Eighty-eight percent of these mice were survivors on day 90, indicating that elimination of all donor T cells can prevent GVHD mortality in this model. We also studied the effects of an irrelevant control hamster F(ab')2-RTA in a separate experiment using the exact dose and schedule for GVHD therapy as with anti-CD3F(ab')2-RTA (data not shown). No extension of MST was observed. Collectively, these findings indicate that anti-CD3F(ab')2-RTA is potent and specific in its ability to inhibit an ongoing GVHD response across a full MHC disparity.

To select the day of GVHD treatment initiation, we identified the onset of GVHD in the mice shown in Fig 1A by monitoring weight loss over time. Our data in Fig 1B show that the first detectable GVHD occurred on days 7-11. However, after an initial decline, weights of mice treated with anti-CD3F(ab')2-RTA stabilized and remained around 18 g for the duration of the experiment. More precipitous drops in weight were noted in mice with higher GVHD mortality. These groups included mice treated with control PBS, anti-CD3F(ab')2 alone, or a combination of anti-CD4 plus anti-CD8 MoAbs. Only the group of mice in which T cells were eliminated from the donor graft before BMT with anti-Thy1.2 plus complement underwent a steady increase in weight over the 76-day interval. These findings indicated that although anti-CD3F(ab')2-RTA inhibited GVHD, it was still present in these treated mice.
Table 3. Effect of Various Dosages of Anti-CD3F(ab')2-RTA on Established GVHD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (µg/d)</th>
<th>Schedule</th>
<th>MST (d)</th>
<th>% Actuarial Survival Rate at Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>—</td>
<td>d 7-11</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>10</td>
<td>d 7-11</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>30</td>
<td>d 7-11</td>
<td>42*</td>
<td>12*</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>50</td>
<td>d 7-11</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>75</td>
<td>d 7-11</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>400 + 25</td>
<td>d 7-11</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>400</td>
<td>d 7 only</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Thyl.2 + C'</td>
<td>—</td>
<td>in vitro</td>
<td>&gt;78*</td>
<td>100*</td>
</tr>
</tbody>
</table>

B10.BR recipients were given lethal TB1 (n = 8 per group) on day -1, and on day 0 were transplanted with C57BL/6 BM and spleen cells (25 × 10⁶ of each). On day 7, mice were given a 5-day course of various concentrations of anti-CD3F(ab')2-RTA or unconjugated anti-CD3F(ab')2 fragments, or a mixture of unconjugated anti-CD3F(ab')2 fragments plus unconjugated RTA. One group was given a single injection of 400 µg anti-CD3. Another was given donor BM and spleen cells that were treated with anti-Thyl.2 + C' before BMT. Survival was monitored.

* P < .05 compared with PBS control.

Tissues from mice in the same experiment that were treated with unconjugated anti-CD3F(ab')2, anti-CD4 plus anti-CD8, or intact anti-CD3 and that were moribund between day 19 and day 39 were collected immediately before death (n = 1 or 2). Tissues were embedded in paraffin, blocked, sectioned, and stained with hematoxylin and eosin for histopathologic examination. All mice showed evidence of colonic GVHD including infiltrates and tissue necrosis. Two mice treated with anti-CD3F(ab')2-RTA that survived were killed on day 90 post-BMT. These mice showed positive signs of GVHD in the colon, liver, and lung. Infiltrates and tissue necrosis were the most prominent features.

Effect of varying dosages of anti-CD3F(ab')2-RTA on ongoing GVHD across the H-2 barrier. In a different experiment, B10.BR recipients given C57BL/6 BM and spleen were treated with increasing concentrations of anti-CD3F(ab')2-RTA (Table 3). Mice given control PBS had an MST of 26 days, similar to the previous experiment, and died of GVHD. MST was increased to 36 days in mice given a 5-day (days 7 through 11) course of 10 µg per injection anti-CD3F(ab')2-RTA and to 42 days in mice given a course of 30 µg per injection anti-CD3F(ab')2-RTA. However, MST decreased when the dosage was increased to 50 or 75 µg per injection. A group of control mice given a 5-day course of 400 µg unconjugated anti-CD3F(ab')2 plus 25 µg unconjugated RTA showed no protective effect from GVHD onset. A group of mice given anti-CD3 had an MST of only 9 days. Positive controls in which T cells were eliminated before transplant with anti-Thyl.1.2 plus complement in vitro had an MST of greater than 76 days. Using these data, we selected the dose of 30 µg per injection anti-CD3F(ab')2-RTA for subsequent studies. Our findings on the effectiveness of the IT were reproducible, and we established a working dose and schedule.

Effect of anti-CD3F(ab')2-RTA in protecting against established GVHD across non-H-2, minor histocompatibility barriers in two different mouse models. To determine whether anti-CD3F(ab')2-RTA could be used to treat GVHD generated across non-H-2 minor histocompatibility antigen disparities, CBA mice were given 8 Gy TBI and then 20 × 10⁶ T cell-depleted, H-2-match B10.BR BM cells and 50 × 10⁶ splenocytes (twice the splenocyte number used in the C57B/6 into B10.BR model). Table 4 shows that anti-CD3F(ab')2-RTA treatment significantly extended the MST from 18 days in PBS controls to 46 days in IT-treated mice. Daily weight measurements showed that anti-CD3F(ab')2-RTA enhanced the anti-GVHD effect compared with untreated controls, but that GVHD-related weight loss still occurred later (data not shown). Whereas GVHD generation in the mismatched C57BL/6 into B10.BR model is CD4+ T cell-dependent, GVHD in this minor model is CD8+ T cell-dependent, indicating that this agent was active against both types of T cells.

In a separate experiment in a different model in which minor antigens are mismatched, DBA/2 recipients given 8 Gy TBI and 20 × 10⁶ T cell-depleted B10.D2 BM cells and 50 × 10⁶ splenocytes developed GVHD. The MST was 21 days in a group of mice given control PBS injections. Anti-CD3F(ab')2-RTA significantly extended the MST to 34 days in this minor mismatched model. Weight loss data indicated that the mice that died did undergo GVHD-related weight decline (data not shown). In both of these models, in vitro depletion of T cells from donor grafts before BMT resulted in an MST of greater than 83 days, with 100% of the animals surviving at day 60. Collectively, these findings show that anti-CD3F(ab')2-RTA is not only effective against lethal GVHD induced across the major histocompatibility barrier, but is also effective against GVHD in aggressive models in which minor antigens, and not major antigen differences, serve as targets for lethal GVHD. The data also

Table 4. In Vivo Effect of Anti-CD3F(ab')2-RTA Injections on Established GVHD Induced Across the Minor Histocompatibility Barrier in Two Different Strain Combinations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (µg/d)</th>
<th>Schedule</th>
<th>MST (d)</th>
<th>% Survival at Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.BR into CBA</td>
<td>—</td>
<td>d 7-11</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>—</td>
<td>d 7-11</td>
<td>46*</td>
<td>20*</td>
</tr>
<tr>
<td>Anti-Thyl.1.2 + C'</td>
<td>—</td>
<td>in vitro</td>
<td>&gt;85*</td>
<td>100*</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>30</td>
<td>d 7-11</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>30</td>
<td>d 7-11</td>
<td>34*</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Thyl.1.2 + C'</td>
<td>—</td>
<td>in vitro</td>
<td>&gt;85*</td>
<td>100*</td>
</tr>
</tbody>
</table>

BMts were performed with two strain combinations. First, CBA recipients (n = 10 per group) were given 8 Gy lethal TBI on day -1, and on day 0 were transplanted with 20 × 10⁶ B10.BR BM cells and 50 × 10⁶ spleen cells. On day 7, mice were given a 5-day course of anti-CD3F(ab')2-RTA or control PBS. Another group was given donor BM and spleen cells that were treated with anti-Thyl.1.2 + C' before BMT. Survival was monitored. A second experiment was performed identically, except B10.D2 BM and splenocytes were transplanted into irradiated DBA/2 recipients (n = 10 per group).

* P < .05 compared with PBS control.
Table 5. Effect of Anti-Lyt-1-F(ab')2-RTA on Established GVHD in the Mismatched C57BL/6-Into-B10.BR Model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μg/d)</th>
<th>Schedule</th>
<th>MST (d)</th>
<th>% Actualual Survival at Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Lyt-1-F(ab')2-RTA</td>
<td>30</td>
<td>7-11</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Anti-human CD5-RTA</td>
<td>30</td>
<td>7-11</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3F(ab')2-RTA</td>
<td>30</td>
<td>7-11</td>
<td>77*</td>
<td>62*</td>
</tr>
</tbody>
</table>

B10.BR recipients (n = 8 per group) were given lethal TBI on day -1, and on day 0 were transplanted with C57BL/6 BM and spleen cells (25 x 10⁶ of each). On day 7, mice were given a 5-day course of PBS, anti-Lyt-1-F(ab')2-RTA, anti-CD3F(ab')2-RTA, or a control anti-human CD5-RTA. Survival was monitored.

* P < .01 compared with PBS control.

show that anti-CD3F(ab')2-RTA is active in models in which CD8+ T cells play a major role.

Effect of anti-Lyt-1-F(ab')2-RTA on ongoing GVHD induced across the H-2 MHC barrier. As anti-Lyt-1-F(ab')2-RTA is the murine equivalent of anti-human CD5-RTA currently used in clinical trials, we directly compared anti-Lyt-1F(ab')2-RTA and anti-CD3F(ab')2-RTA for their efficiencies in treating ongoing GVHD in the same experiment. Table 5 shows that anti-Lyt-1-F(ab')2-RTA in a 5-day course did not result in a significant anti-GVHD effect in B10.BR recipients of C57BL/6 BM/spleen. In contrast, a 5-day course of anti-CD3F(ab')2-RTA significantly increased MST to day 77, as compared with day 21 in controls. Anti-human CD5-RTA, a noncrossover control IT, did not inhibit GVHD.

To determine whether the failure of anti-Lyt-1-F(ab')2-RTA as opposed to anti-CD3F(ab')2-RTA to protect against GVHD was related to IT potency or perhaps to a difference in the clearance of the two proteins in vivo, we performed additional studies. Pharmacokinetic studies showed that the serum half-lives of anti-Lyt-1-F(ab')2-RTA and anti-CD3F(ab')2-RTA were similar (8.9 and 9.0 hours, respectively) in normal B10.BR mice. Both ITs accumulated in a similar manner in the spleen and thymus. In contrast, the abilities of these two ITs to inhibit T-cell proliferation in PHA assays were different, because the 50% inhibition concentration (IC₅₀) of anti-Lyt-1-F(ab')2-RTA was 10.2 μg/mL and the IC₅₀ of anti-CD3F(ab')2-RTA was 2.1 μg/mL, when compared in the same assay without the addition of any potentiators. Collectively, these findings suggest that the failure of anti-Lyt-1-F(ab')2-RTA to inhibit GVHD is more likely related to an issue of potency of the IT, rather than clearance and localization. A second anti-Lyt-1F(ab')2-RTA was prepared using another MoAb, SK69.6.38 generously provided by Dr Edward Boyse (Memorial Sloan-Kettering Cancer Center, New York, NY) and findings were identical.

**DISCUSSION**

Ongoing GVHD is acknowledged as a significant clinical problem and few murine studies have been directed at studying ongoing GVHD. The major finding of this study was that anti-CD3F(ab')2-RTA extended MST in major and minor antigen-mismatched BMT recipients with established GVHD longer than any other IT thus far reported. MST was also extended in two additional models of lethal GVHD where donors and recipients were disparate at minor antigen loci only. The effects of anti-CD3F(ab')2-RTA were specific, because flow cytometry studies on mice given syngeneic transplants [to study the effects of anti-CD3F(ab')2-RTA in the absence of the immunosuppressive effects of allogeneic transplants] revealed that anti-CD3F(ab')2-RTA was selectively depletionary in its effect on CD3-expressing T cells, but not on cells expressing the unrelated NK1.1 marker. An irrelevant IT control, hamster CD3F(ab')2-RTA, did not extend MST.

Previous studies using mice indicated that effective IT could be synthesized using anti-CD3 MoAb to target T cells, but only if we inhibited the ability of the MoAb to trigger T-cell activation through the removal of the Fc region.15 ITs made with activating, intact anti-CD3 caused a high mortality rate and a precipitous release of inflammatory cytokines. In fact, therapy with anti-CD3 alone has shown potential for preventing organ graft rejection, but has been limited by toxic side effects due to in vivo activation of T cells induced by ligation of the TCR and the Fc region of anti-CD3. Anti-CD3 ITs made with nonactivating anti-CD3F(ab')2 did not cause cytokine release, were less toxic to nontarget organ systems, deleted CD3-expressing T cells, and prevented BM rejection in vivo. Evaluation of anti-CD3F(ab')2-RTA and anti-CD3-RTA in the same in vitro PHA experiment showed that anti-CD3F(ab')2-RTA is more potent.13 Previous studies have demonstrated that ITs made with F(ab')2 are more potent than ITs made with intact antibody.50,51 However, this is not always the case.41-43 Collectively, these studies indicate that certain F(ab')2-ITs such as anti-CD3F(ab')2-RTA may have advantages.

We believed that it was important to address the activation issue in these studies. CD5 interacts with costimulatory molecule CD72 on antigen-presenting B cells.12 Anti-CD5 MoAb triggers activation of CD5-expressing human T cells.13 Likewise anti-Lyt-1 triggers murine T cells to proliferate.12 Thus, we prepared nonactivating anti-Lyt-1F(ab')2-RTA and compared it with anti-CD3F(ab')2-RTA in the same experiment. The same RTA was used to prepare both reagents to ensure consistency of the toxic moiety of the ITs. Although the clearance and biodistribution of the two agents were similar, anti-CD3F(ab')2-RTA was more effective in treating established GVHD. One factor that correlated with the superior in vivo activity of anti-CD3F(ab')2-RTA was its superior fivefold in vitro potency measured by PHA assay. We concluded that preparation of anti-Lyt-1F(ab')2-RTA provided no advantage.

Then why was CD3 more effective than CD5 as a target molecule? There are several possible explanations. (1) In addition to the previously discussed studies in which anti-CD3-RTA was more completely and rapidly internalized than anti-Lyt-1-RTA, other subcellular compartmentalization studies in which anti-CD5-RTA was radiolabeled and then directly measured in organelle fractions isolated from Percol gradients show that anti-CD5-RTA IT is rapidly
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transferred to the lysosome, where unwanted degradation takes place.44 (2) Anti-CD5 IT permits the expansion of GVHD-causing T cells, which do not express CD5. Studies show that a large proportion of T lymphocytes lack CD5 expression after BMT.53,46 When human EM cells from GVHD patients were treated with an anti-CD5 IT and then cultured in vitro for 16 days, CD5-negative T cells mostly expressing CD3 were detected. CD5-CD3+ cells that escape IT treatment retain an allogeneic cytotoxic repertoire capable of GVHD reaction.47

These results with anti-CD3F(ab')2–RTA are the best that have been attained in several published studies dealing with the treatment of ongoing GVHD in the same mismatched mouse model of allogeneic BMT.10,36,48 Anti–Ly–1 antibody linked to RTA10 or labeled with radionuclide49 was only partially protective. Attempts to treat established GVHD using the potent anti-T cell drug rapamycin,48 a fungal macroline, also produced transient protection.

All of these murine studies and the clinical studies resulted in significant but transient anti-GVHD effects. The lack of complete inhibition of GVHD during the effector phase rather than the afferent phase indicates that it is far more difficult to inhibit GVHD once an alloresponse is initiated in vivo. This could be attributed to the difficulty of effectively delivering therapy to a relatively small GVHD-causing population of cells in a complex biologic environment. Alternatively, a network of cells may be involved in GVHD once the process has begun, thus making specific targeting difficult. Protection was more limited when mice were transplanted with BM with minor antigenic disparities because minor histocompatibility antigen differences sometimes can be even more potent at eliciting GVHD responses than fully mismatched MHC differences, because different mechanisms of GVHD may be operative in the two different systems34 or because we gave higher numbers of donor T cells in the minor model. This is a topic of future investigation.

The difficulty in inhibiting ongoing GVHD is evidenced by our results with anti-CD3F(ab')2 fragments alone. In past studies, we showed that unconjugated anti-CD3F(ab')2 fragments can be used prophylactically to prevent GVHD. In these current studies, anti-CD3F(ab')2 fragments were much less effective when compared with anti-CD3F(ab')2–RTA in treating established GVHD. Fragments given on day 7 post-BMT did not inhibit established GVHD in this model. This could be explained by the flow cytometry studies, which showed that anti-CD3F(ab')2 fragments caused the modulation of CD3 from the cell surface of the T cells and only partial T-cell depletion. In contrast, the anti-CD3F(ab')2–RTA, which was much more effective against ongoing GVHD in the present study, caused the deletion of CD3+ T cells. The internalization of anti-CD3F(ab')2–RTA eliminated the GVHD-causing cells. Whereas modulation may be an effective means of GVHD prophylaxis, it is better to delete T cells to treat GVHD once the response has been initiated.

Although we have achieved a high degree of protection against ongoing GVHD with IT, we have not cured the mice, as evidenced by late onset of the disease. To further extend MST with anti-CD3F(ab')2–RTA, it may be necessary to combine it with other ITs or approaches such as drugs.49 Some reasons that anti-CD3F(ab')2–RTA is not fully protective are as follows. (1) Inadequate dosing and schedule may have resulted in incomplete elimination. This has been a serious limitation to the clinical use of IT, as clinical studies to date have mostly been limited by vascular leak syndrome and hepatotoxicity. However, we hope that a more thorough understanding of IT toxicity and agents that might diminish their toxicity will be of future help. (2) Immunohistology studies show that other donor cells, such as macrophages and natural killer cells, may play some role in established GVHD in this model (unpublished data, January 1995). Perhaps even greater anti-GVHD effects might be achieved by targeting these cells as well. (3) Antibody responses may develop against the antibody or toxin component of IT. Although several immunoglobulin responses have been noted in clinical trials,30 it is likely that optimal doses of anti-T cell IT will not only target GVHD-causing cells, but also might inhibit the development of immunoglobulin responses, as these responses are also T cell-dependent.

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Therapy for ongoing graft-versus-host disease induced across the major or minor histocompatibility barrier in mice with anti-CD3F(ab’)2-ricin toxin A chain immunotoxin

DA Vallera, PA Taylor, A Panoskaltsis-Mortari and BR Blazar