Antilymphocytic Antibodies and Marrow Transplantation. XII. Suppression of Graft-Versus-Host Disease by T-Cell-Modulating and Depleting Antimouse CD3 Antibody Is Most Effective When Preinjected in the Marrow Recipient

By Josef Mysliwietz and Stefan Thierfelder

A hamster antimouse CD3 monoclonal antibody (MoAb) opened the way to experimental studies on the suppression of allograft rejection and cytokine-related morbidity after treatment with antibodies modulating the CD3/T-cell receptor complex (CD3/TCR). Because earlier attempts to suppress graft-versus-host disease (GVHD) in patients by in vitro treatment of donor marrow with anti-CD3 MoAb had remained inconclusive, we used a rat IgG2b antimouse CD3 MoAb (17A2) with fewer side effects to analyze suppression of GVHD in the mouse model. Detailed phenotyping of blood, spleen, and lymphnode T cells after the injection of 400 µg 17A2 in C57BL/6 mice showed 60% CD3 downmodulation and 50% T-cell depletion for spleen cells. Injection of these spleen cells, together with bone marrow cells, in fully mismatched preirradiated CBA mice delayed GVHD by only 6 days. Ex vivo treatment of donor cells with 17A2 was not effective. In contrast, conditioning of marrow recipients with a single injection of 17A2 delayed 50% GVHD mortality by 100 days and prevented GVHD altogether after prolonged treatment, with survivors showing complete chimerism and specific transplantation tolerance. This difference in antibody effect contrasts with earlier experiences with nonmodulating but more strongly T-cell-depleting MoAbs of the same isotype, which prevent GVHD no matter whether applied in vitro or injected into donor or recipient mice. Our data indicate that CD3/TCR reexpression in marrow recipients with no circulating 17A2 is the reason why ex vivo donor cell treatment with anti-CD3 MoAb is comparatively ineffective. Our data, which allow separate evaluation of cell-depleting and cell-modulating antibody activity, help to explain previous clinical failure to suppress GVHD and provide evidence in favor of conditioning the marrow recipient with anti-CD3 MoAb as a therapeutic alternative.

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MATERIALS AND METHODS

Animals. C57BL/6 mice originally obtained from the Jackson Laboratory (Bar Harbor, ME) were raised and maintained in our breeding facilities. CBA and (C57BL/6 × CBA)F1 mice were bred from stock in our own laboratory. Three-month-old female animals were used as bone marrow recipients.

Antibodies and flow cytometry. Flow cytometry was performed as described previously, using fluorescein (FITC)- or phycoerythrin (PE)-conjugated anti-L3T4 (Becton Dickinson, Heidelberg, Germany), anti-Lyt2 (Serva, Heidelberg, Germany), anti-B220 (Medac, Hamburg, Germany), anti-Thy-1.1 (Medac), MoAb, or polyclonal mouse F(ab')2-antirat IgG (Dianova, Hamburg, Germany). 17A2 (rat2b-antimouse-CD3) was donated by Dr R. MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland), anti-IL-2R (TIB 222; ATCC) and anti-NK-1.1 (HB-191) (Camon, Wiesbaden, Germany) were purified as described elsewhere.

In vivo modulation of CD3 and T-cell depletion after 17A2 treatment. C57BL/6 mice were injected intravenously (IV) with a single 400 (20 mg/kg), 100, or 25 μg dose of purified anti-CD3 (17A2) MoAb. Heparinized blood, spleen, and lymphnodes (axillary, inguinal, mesenteric) from three mice were pooled 1, 3, 7, and 10 days after injection, a cell suspension was prepared, and leukocytes were counted. One million leukocytes were double-labeled using saturating concentrations of anti-L3T4(PE)/anti-17A2(FITC), anti-Lyt2(PE)/anti-17A2(FITC), mouse F(ab')2-antirat IgG(FITC)/anti-Thy-1.2(PE), or anti-IL-2R(FITC)/anti-B220(PE) and, after standard NH4Cl lysis of erythrocytes, were processed on FACSscan (Becton Dickinson). The quantitative fluorescence measurements of CD3 expression were performed directly using FITC-labeled 17A2, or indirectly using purified 17A2 and mouse F(ab')2-antirat IgG (FITC), as described previously.

Serum concentration of 17A2 (anti-CD3) MoAb and anti-17A2 antibodies after in vivo treatment. Concentration of 17A2 (rat IgG2b) was determined in enzyme-linked immunosorbent assay (ELISA), using mouse-antirat IgG (Dianova) as the capture and the same peroxidase-labeled antibodies as the indicator. Sera from treated mice or purified 17A2 as the concentration standard were titrated twofold and the concentration of rat IgG in serum was calculated. To determine anti-17A2 antibodies, ELISA microtiter plates (Greiner, Nütingen, Germany) were coated with purified 17A2. Sera from 17A2-treated mice or affinity-purified polyclonal mouse-antirat IgG (Dianova) as the concentration standard were titrated twofold and indicated with peroxidase-labeled rat-antimouse IgG (Dianova). The concentration of anti-17A2 antibodies was calculated from mouse-antirat IgG standard curves.

Bone marrow transplantation and X-radiation were performed as described in detail previously. Briefly, in vivo treatment was as follows: groups of six irradiated (8.5 Gy) CBA (H-2d) or (C57BL/6 × CBA)F1 recipient mice were injected IV with a given concentration of purified anti-CD3 (17A2) MoAb. A mixture of 5 × 10^7 spleen and 2 × 10^7 bone marrow cells from C57BL/6 mice was incubated in 1 mL minimal essential medium (MEM; Biochrom, Berlin, Germany) with 5× saturating concentration of purified 17A2 MoAb for 30 minutes at room temperature. Afterwards 150 μL of a mixture of 2 × 10^7 bone marrow and 5 × 10^7 spleen cells per mouse was injected into lethally irradiated CBA or (C57BL/6 × CBA)F1 recipient mice. Statistical evaluation of survival curves was performed using the logrank method.

Test for chimerism and specific transplantation tolerance. Heparinized blood was collected from the tails of mice who received cells from C57BL/6 mice was incubated in 1 mL minimal essential medium (MEM; Biochrom, Berlin, Germany) with 5× saturating concentration of purified 17A2 MoAb for 30 minutes at room temperature. Afterwards 150 μL of a mixture of 2 × 10^7 bone marrow and 5 × 10^7 spleen cells per mouse was injected into lethally irradiated CBA or (C57BL/6 × CBA)F1 recipient mice. Statistical evaluation of survival curves was performed using the logrank method.

Fig 1. T-cell depletion and CD3 antigen modulation in lymph node, spleen, and blood of C57BL/6 mice 1, 3, 7, and 10 days after a single IV injection of 400 μg of 17A2 (anti-CD3 MoAb). Double-color FACS analysis and quantitative fluorescence measurements were performed as described in Materials and Methods. (*) CD3 antigen expressed as number of directly (FITC) labeled 17A2 molecules bound per cell × 10^4. (**) CD3 antigen downmodulation: diminution of fluorescence intensity of T cells (mean of CD4* and CD8*) from 17A2-injected mice expressed as a percentage of untreated control.
bone marrow transplants and the cells were double-labeled with anti-H2k FITC and biotinylated anti-H2b MoAb. After probing with avidin-PE and FACScan processing, live lymphoid cells were evaluated and compared with cells of untreated control mice. Bone marrow-reconstituted mice were tested for specific tolerance 50 days after transplantation. They were grafted with marrow donor-type (C57BL/6) and third-party (Balb/c) tail skin grafts, both placed on the lateral thoracic wall. Dressings were removed on day 12 and grafts were inspected every day for signs of rejection.

RESULTS

Suppression of GVHD by anti-CD3 MoAb (17A2) was tested after antibody treatment of marrow donors or recipients or of donor cells in vitro. Changes in T-cell depletion and modulation after treatment of C57BL/6 donor mice were measured by detailed immunophenotyping.

T-cell depletion with anti-CD3 MoAb. Single injections of 400 μg 17A2 in C57BL/6 mice produced a reduction of T cells, which were measured on day 1, 3, 7, and 10 postinjection in blood, spleen, and lymphnode cells (Fig 1). Cell depletion was most pronounced in blood (80%) on day 1 and approximately 50% T-cell reduction was measured in spleen and lymphnode cell suspension pooled from axillary, inguinal, and mesenteric lymphnodes. Splenic T cells had reached a 50% reduction by day 3, at which time peripheral blood lymphocytes (PBL) T-cell counts had already increased, although CD3 was still modulated (see below). T-cell counts on days 7 and 10 had stabilized at about half the normal values. Virtually the same pattern was observed after a single injection of 100 μg 17A2. Markedly diminished T-cell depletion and modulation were noted in blood, lymph nodes, and spleen after the injection of 25 μg 17A2 (data not shown).

Fifty days after a single injection of 400 μg of 17A2, CD4+ cells had reached normal values; CD8+ cells recovered slowly, reaching some 65% to 75% of untreated controls (data not shown).

T-cell modulation after anti-CD3 MoAb. Figure 1 shows expression of CD3 on CD4+ and CD8+ T cells. Reduction of CD3 expression on T cells was measured by staining 17A2 after incubation with FITC-labeled mouse F(ab')2-antirat Ig secondary antibody and/or by using FITC-labeled 17A2 directly. Fluorescence was related to FCSC Microbead Standards. The use of sensitive double staining with mouse (Fab')2-antirat IgG(FITC) and mouse anti-Thy-1(PE) was crucial for detection of in vivo 17A2-coated cells, showing strongly diminished CD3 expression. The sensitivity threshold using other reagents, such as complete goat-IgG antirat Ig or even IgG mouse-antirat Ig, was not sufficient. On day 3 postinjection of 400 μg, the day when a mixture of spleen cells and bone marrow cells was used for...
transplantation, all splenic CD4+ and CD8+ cells were saturated with circulating 17A2; CD3 downmodulation can be assumed because the concentration of bound 17A2 was saturated with circulating 17A2; CD3 downmodulation can be assumed because the concentration of bound 17A2 was saturated with circulating 17A2; CD3 downmodulation can be assumed because the concentration of bound 17A2 was saturated with circulating 17A2; CD3 downmodulation can be assumed because the concentration of bound 17A2 was saturated with circulating 17A2. The diminished antigen density was still seen, even after 17A2 had disappeared from serum on days 7 and 10 after treatment (Figs 1 and 2). Complete modulation must have occurred on PBL because approximately 40% to 50% of PBL on day 3 post injection of 100 or 400 µg 17A2 did not stain with directly labeled 17A2 nor with FITC-labeled antirat Ig secondary antibodies. They had lost their CD3 antigen completely, at least below the threshold of sensitivity of the FACS. During in vivo treatment with 17A2, we did not find increased expression of IL-2R on T cells as reported for 145-2C11.14

Effect of anti-CD3 MoAb on GVHD. Transplantation of \(5 \times 10^7\) spleen cells together with \(2 \times 10^7\) bone marrow cells to irradiated, minor and major MHC class I and II-mismatched CBA mice induced acute mortality, which was only slightly delayed if donor or donor cells were pretreated with anti-CD3 MoAb (Fig 3). In contrast, the injection of 400 or 200 µg antibody in marrow recipients 20 hours after irradiation and 4 hours before transplantation suppressed GVHD mortality by about 50% on day 100 posttransplantation and 20% to 40% on day 200. In one experiment, permanent survival of 80% of chimeras was observed after single injection of 200 µg 17A2 (Fig 3, experiment 2). Prolonging antibody treatment by further injections of 200 µg on days 3, 6, and 9 after transplantation led to long-term survivors with 90% to 97% chimerism on days 50 and 100. Compared with untreated controls, GVHD suppression in 17A2-treated recipients was statistically significant (\(P < .05\)) for all above described treatments. The differences in survival resulting from dose of 17A2 or prolonging of treatment were not statistically significant (Fig 3). Chimeras showed long-lasting changes in blood T-cell frequency (Table 1). Even 50 days after the last injection of 17A2, the frequency of CD4+ cells was reduced to about 50% and CD8+ cells were reduced to 10% of untreated controls. On day 200 posttransplantation, recovery of blood CD8+ cells improved to 50% of untreated controls; percentage of splenic CD4+ cells had reached control values and splenic CD8+ cells were reduced by 65%. C57BL/6-into-CBA chimeras (chimerism was proved on days 30, 50, and 100 posttransplantation and regularly found to be greater than 90%), grafted 50 days after bone marrow transplantation with donor C57BL/6 or third party (Balb/c) skin, accepted donor skin and promptly rejected third-party skin. Besides changes in the frequency of CD4+ and CD8+ lymphocytes, we regularly observed an increase in donor NK 1.1 cells (Table 1); however, this was not predictive for GVHD in our mice. The formation of antiantibodies against 17A2 was negligible in these experiments (Table 1).

We repeated the GVHD experiments with C57BL/6 donors in semiallogeneic (CBL/6 × CBA)F1 recipient mice. This donor-recipient incompatibility is notoriously easy to overcome. Even the injection of normal rabbit Ig causes some delay of GVHD. Again, conditioning of recipient mice with 17A2 was most effective. Pretreatment of donors or donor cells ex vivo showed a clearly delayed, but 100% mortality (Table 2). Taking advantage of the sensitivity of this mouse model, we titrated the spleen cells from donors pretreated with an MoAb of irrelevant specificity in the following experiments.

### Table 1. Blood Cell Phenotype, Antibody, and Antiantibody Concentration in Serum After Prolonged Treatment of Bone Marrow Transplanted Mice With Anti-CD3 (17A2) MoAb

<table>
<thead>
<tr>
<th>Day post last injection</th>
<th>CD3+ CD4+</th>
<th>CD3- CD4+</th>
<th>CD3+ CD8+</th>
<th>CD3- CD8+</th>
<th>H2b+</th>
<th>NK1.1⁺</th>
<th>Serum Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.0</td>
<td>3.9</td>
<td>0.0</td>
<td>9.6</td>
<td>97.7</td>
<td>17.9</td>
<td>6.5</td>
</tr>
<tr>
<td>8</td>
<td>0.7</td>
<td>0.5</td>
<td>5.1</td>
<td>2.5</td>
<td>ND</td>
<td>16.9</td>
<td>0.7</td>
</tr>
<tr>
<td>15</td>
<td>0.9</td>
<td>0.1</td>
<td>1.4</td>
<td>0.4</td>
<td>ND</td>
<td>14.0</td>
<td>0.0</td>
</tr>
<tr>
<td>50</td>
<td>5.3</td>
<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>97.9</td>
<td>21.8</td>
<td>ND</td>
</tr>
<tr>
<td>200</td>
<td>7.7</td>
<td>0.0</td>
<td>6.3</td>
<td>0.0</td>
<td>98.7</td>
<td>12.9</td>
<td>ND</td>
</tr>
<tr>
<td>Untreated</td>
<td>14.1</td>
<td>11.9</td>
<td>11.9</td>
<td>6.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

CBA recipients were injected with 400 µg 17A2 IV 4 hours before transplantation of bone marrow/spleen cells from C57BL/6 mice and additionally with 200 µg IV on days 3, 6, and 9 after transplantation.

Abbreviation: ND, not determined.

*Heparinized tail blood from four to six animals was pooled, double-stained, and processed in FACScan as described in Materials and Methods.

Table 2. Effect of Anti-CD3 MoAb on Suppression of GVHD in the C57BL/6-to-(C57BL/6 × CBA)F1 Combination

<table>
<thead>
<tr>
<th>Anti-CD3 Treatment</th>
<th>Survival (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor cells†</td>
<td>Day 30</td>
</tr>
<tr>
<td>400 µg</td>
<td>66</td>
</tr>
<tr>
<td>100 µg</td>
<td>100</td>
</tr>
<tr>
<td>Recipients‡</td>
<td>400 µg</td>
</tr>
<tr>
<td>200 µg</td>
<td>83</td>
</tr>
<tr>
<td>100 µg</td>
<td>100</td>
</tr>
<tr>
<td>25 µg</td>
<td>83</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0</td>
</tr>
</tbody>
</table>

*Groups of 6 (C57BL/6 × CBA)F1 mice were irradiated with 9 Gy 24 hours before transplantation of \(5 \times 10^7\) spleen cells and \(2 \times 10^7\) bone marrow cells of C57BL/6 donors.

†Bone marrow and spleen cells from C57BL/6 donors were incubated in vitro with 17A2 and transplanted.

‡Donors were injected IV with 400 µg 17A2; bone marrow/spleen cells were prepared 3 days later and transplanted.

§17A2 injected IV at indicated dose 4 hours before transplantation.
ANTI-CD3 SUPPRESSES GVHD

semiallogeneic GVHD model. The cell-dose-dependent delay of GVHD was correlated to a delay of GVHD after the transplantation of 5 x 10^7 spleen cells from donors who had been pretreated with 17A2 (Fig 4). Because 17A2 induced 50% T-cell depletion in the spleen (see above), we expected a delay of GVHD mortality corresponding to the delay after the transplantation of 2.5 x 10^7 spleen cells of our control mice. However, anti-CD3 MoAb delayed GVHD significantly more (P = .009, Fig 4). These results support the conclusion that immunosuppression by 17A2 cannot be explained by its T-cell depletion alone. The CD3/TCR modulation must at least have played a role.

DISCUSSION

The present observations concern an interrelationship between antibody action and type of conditioning for suppression of GVHD. Extending experimental studies on antimouse CD3 MoAb to the suppression of GVHD, we show in the mouse GVHD model that this modulating antibody is relatively ineffective in ex vivo T-cell treatment, but successful when used for conditioning bone marrow recipients. This difference in immunosuppressive effect contrasts with that of primarily T-cell-depleting MoAbs, which are equally effective in both types of treatment.17,19 Whereas purging with anti-T-cell antibodies depends on their Fc-parts,20 an important anti-CD3 antibody effector function extends to its variable T-cell-binding part that causes downmodulation of the CD3/TCR complex. F(ab')2 fragments of anti-CD3 MoAb have thus been shown to conserve much of its immunosuppressive activity by prolonging survival of skin allografts.15

In the present study, pretreating of C57BL/6 donor mice with 17A2 resulted in dose-dependent T-cell elimination and downmodulation of the CD3/TCR receptor complex, which were most pronounced in blood and spleen cells. We transplanted spleen and bone marrow cells of mice injected with up to 400 μg 17A2 3 days earlier. At this time, splenic T cells had decreased to about 50%. They also showed a reduction in CD3 expression of about 60%, as measured by binding 17A2 (Fig 1). The spleen T cells, despite their reduced number and TCR downmodulation, hardly delayed acute GVHD mortality at all (Fig 3). In vitro tests for T-cell function, in which spleen cells from mice injected with anti-CD3 MoAb 145-2C11 and tested for generation of cytotoxic T-lymphocyte–mediated lysis (CTL) of complete MHC-disparate target cells, showed no CTL activity for at least 5 weeks posttreatment.13 Clearly, in vivo conditions for GVHD-inducing T cells must be quite different. Other factors, eg, preirradiation of marrow recipients, cytokines, or upregulation of target antigens, may have contributed to in vivo T-cell stimulation and early recovery of graft-versus-host response. On the other hand, successful marrow-recipient conditioning—not only in the notoriously ‘easier' parent-to-F1 system, but also in fully mismatched CBA mice—proved the potential anti-GVHD effectiveness of 17A2. It underscores the importance of circulating anti-CD3 MoAb for maintaining inhibition and dysfunction of donor cell TCR in the early stages after transplantation. It is conceivable that T-cell–depleting antibodies like anti-Thy-1 MoAb (and we found the same for antimouse CD4/CD8 antibody pairs [data not shown]) are equally effective in donor, donor-cell, or recipient treatment as long
as they cause sufficient T-cell elimination.\textsuperscript{17-21} The 50% T-cell-depleitory effect of 17A2 is clearly insufficient to explain its suppression of GVHD in fully mismatched mice. A rat IgG2c antiserum Thy-1 MoAb, causing 92% T-cell suppression, hardly delayed GVHD in fully mismatched mice, in contrast to the IgG2b isotype with 98% depletion and prevention of GVHD.\textsuperscript{21} Therefore, we conclude that TCR modulation in addition to T-cell depletion must be operative in suppressing GVHD with anti-CD3 MoAb. Our observation that prolonged recipient treatment with 17A2 can lead to specific transplantation tolerance is more difficult to understand. Whether transferred donor T cells underwent programmed cell death (apoptosis)\textsuperscript{31} or were gradually substituted by tolerant T cells deriving from the transplanted stem cells and differentiated in the marrow recipient’s thymus was not further investigated. Even subclinical GVHD cannot be dismissed completely, considering the reduced frequency of CD4\textsuperscript{+} and CD8\textsuperscript{+} cells that we had observed in some chimeras beyond day 100 posttransplantation.

OKT3 is presently used for treatment of GVHD symptoms after bone marrow transplantation. It may not be completely academic to discuss conditioning marrow recipients with anti-CD3 MoAb for GVHD prophylaxis. Bone marrow engraftment, a clinical problem when T-cell-depleting MoAbs are used, has been shown to be promoted by anti-CD3 treatment.\textsuperscript{32} Mitogenic side effects of anti-CD3 MoAb can be mitigated or avoided by isotype-matching,\textsuperscript{33} steroids,\textsuperscript{34} pentoxifylline,\textsuperscript{35} by antibodies to tumor necrosis factor,\textsuperscript{36,37} or by anti-CD3 F(ab\textsuperscript{2})\textsubscript{2} fragments.\textsuperscript{38}

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