Antilymphocytic Antibodies and Marrow Transplantation. XIV.
Antibody-Induced Suppression of Graft-Versus-Host Disease in C3-Decomplemented Mice Differentiates Two T-Cell–Depletion Pathways

By Stefan Thierfelder, Josef Mysliwietz, Gertrud Hoffmann-Fezer, and Udo Kummer

Remarkable differences in the suppression of graft-versus-host disease (GVHD) have been found for anti-Thy-1 antibodies to relate to (1) antigen density and antibody coating on the target cells, (2) antibody isotype, and (3) uptake of complement component C3 by cobra venom factor (CVF) differentiates two T-cell elimination pathways in mice: four rat IgG2c anti-Thy-1 mononuclear antibodies (MoAbs) with low uptake of mouse C1q lost most of their T-cell–depleting and consequently GVHD-preventing effect in C3-depleted H2 IA incompatible semiallogeneic (C57BL/6xCBA)F1 mice. In contrast, eight rat IgG2b, mouse IgG2a, and 2b anti-Thy-1 MoAbs with high affinity for C1q still remained strongly T-cell–depleting and prevented GVHD even in fully mismatched CBA mice depleted of C3. In conjunction with our observation that anti-Thy-1 MoAbs also suppress GVHD in C5-deficient AKR mice, we conclude that complete complement activation until T-cell lysis is not required for our antibodies to be effective in vivo. Activation, but only until deposition of C3b on target cells for opsonisation via C3b receptors, is necessary with the less immunosuppressive anti-Thy-1 IgG2c isotype with low affinity for C1q. Mouse C1q uptake and C3/C4 deposition on target cells were measured with labeled antibodies and localized in T-cell areas. Interestingly, not even activation until C3b is necessary with the most immunosuppressive C1q–high-affine isotypes. As far as the latter is concerned, we discuss whether elimination of antibody-coated cells via Fc receptors is enhanced by binding to C1q receptors and/or by intercalating C1q expressed on macrophages.

MATERIALS AND METHODS

Animals. All strains of inbred mice reported in this article, originally obtained from The Jackson Laboratory (Bar Harbor, ME), were raised and maintained in our breeding facilities. Both male and female mice between 2 and 3 months of age were used in these studies.

Complement-dependent lympholysis. Lymphocytotoxicity was evaluated with the complement-dependent dye exclusion test. Freshly drawn serum from male C57BL/6 mice was incubated undiluted with a suspension of mouse lymph node cells and antibody for 1 hour.

Antibodies. Table 1 lists the MoAbs used in this study along with their characteristics. RmT6, RmT7, RmT8 rat IgG2c anti-Thy-1 was produced in our laboratory (J. Haunschild, personal communication, April 1990) as described for RmT2.I MoAb reacting with mouse C1 and Clq was generated according to a principle published previously, where rats were immunized with antibody-coated cells intercalating mouse Clq. After native agarose gel electrophoresis it was found to bind to mouse Clq and to recognize the same band as a polyclonal POX rabbit antihuman Clq (Dakopatts, Hamburg, Germany), which cross-reacts strongly with mouse Clq (E. Ego, M. Wasiliu, and E. Lederer, personal communication, April 1990). MoAbs to mouse C4 and C3 deposited on antibody-coated cells were generated in rats following the same principle of immunization with, however, a different screening system as detailed elsewhere. A class switch family of anti-Thy-1.1 MoAbs producing cell clones were kindly provided by

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**Table 1. Main Features of the MoAbs Used in This Study**

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<th>MoAb</th>
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<th>Ig Isotype</th>
<th>Binding Specificity</th>
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<td>Hamster × mouse</td>
<td>IgG</td>
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*Myeloma × spleen cell donor.
†This study.
‡Cedarlane Lab, Hornby, Ontario, Canada.

I.D. Bernstein University (Scattle, WA). Binding of rat anti-Thy-1 MoAb to Thy-1-positive cells was fully inhibited by mouse anti-Thy-1.1 or Thy-1.2 MoAb listed in Table 1.

**X-radiation.** Prospective marrow recipients received a lethal dose of total body X-radiation of 8.5 Gy at a dose rate of 1.04 Gy/min.

**Labeling of RmC1q MoAb with biotin.** Two milligrams of biotin N-hydroxysuccinimide ester were diluted in 250 μL dimethyl sulphoxide and mixed with a 10-fold volume of purified rat antiserum C1q (RmC1q7H8) MoAb at a concentration of 1.45 mg/mL in 0.1 mol/L NaHCO₃ (pH 8.2). After 1.5 hours of incubation the biotin-conjugated protein was dialyzed overnight against phosphate-buffered saline (PBS) with 0.1% sodium azide, and stored at 4°C.

**Binding of biotinated RmC1q to thymocytes coated with antibodies and C1q.** Fifty microliters of a saturating dilution of anti-Thy-1 MoAb was incubated for 30 minutes with 5 × 10⁴ C57BL/6 or AKR/J thymocytes in microliter plates at 4°C for enzyme-linked immunosorbent assay (ELISA) as described elsewhere. The plates were washed twice in PBS, incubated for 30 minutes with 50 μL of reciprocal dilutions of partly purified mouse C1q, and then again washed twice in PBS before 50 μL of diluted biotinated RmC1q was added, followed by detection with POX avidin (Vector, Burlington, CA) and 1,2-phenylene-diamine (OPD) as chromogen. Partly purified mouse C1q was obtained by fractionated polyethylene glycol (PEG) precipitation as described before.

**Immunofluorescence of cell suspensions.** Direct staining was performed with the following fluorescins or phycoerythrin (PE)-conjugated MoAbs: GK 1.5 (rat IgG2b anti-L3T4, PE); 53.6.7. (rat IgG2a anti-Lyt-2, fluorescein isothiocyanate [FITC]); 53.7.3 (rat IgG2a anti-Ly-1, FITC). All MoAbs above were received from Becton Dickinson (Heidelberg, Germany); polyclonal F(ab)₂ anti-mouse μ antibodies were received from Jackson Immuno Research (Hamburg, Germany); MoAbs RmT1, †RmC1q, RmC4, RmC3, †and 145-2C11 (hamster-anti-CD3), ‡a gift from Dr J. Bluestone, The Ben May Institute, Chicago, IL, were conjugated with FITC as described previously. For quantitative determination of mouse C1, C4b, and C3b deposition, 0.5 × 10⁶ of C57BL/6 lymphnode cells in fresh undiluted syngeneic serum was incubated with serial dilutions of purified RmT1 or RmT2 MoAb in fresh undiluted C57BL/6 serum for 30 minutes at 37°C. After washing the cells were double-labeled with saturating concentrations of RmC1q, RmC4, or RmC3 MoAb (FITC) and F(ab)₂-goat-antimouse μ antibody (PE) for 15 minutes. The samples were processed on fluorescence-activated cell analyzer (FACScan; Becton Dickinson) without final wash; propidium iodide was added to monitor the dead cells. Quantitative measurements were performed as described elsewhere.

**Immunohistochemistry.** Five-micrometer thick, acetone-fixed (10 minutes) cryostat sections of spleens and lymph nodes of C57BL/6 mice were incubated in the first step with RmT1 together with either CVF-treated C3-depleted serum or, for positive controls, with untreated fresh serum or, for negative controls, with heat-inactivated serum of C57BL/6 mice. The second incubation step was either with biotin-labeled rat antiserum C3 (RmC3) or RmC4 MoAb. Peroxidase-labeled avidin was used in the third incubation. Each incubation lasted 60 minutes and was stopped by washing in PBS. Peroxidase activity was shown with amino-ethyl-carbazole. The sections were counterstained with hematoxilin.

**BMT.** Groups of irradiated (C57BL/6xCBA)F1 or CBA (H-2b) mice were injected with a mixture of 5 × 10⁷ spleen and 2 × 10⁶ BM cells of C57BL/6 (H-2b) mice 4 hours later as described before.

**Complement depletion with CVF.** Mice were injected with 10 U of CVF (Cordis Lab, Miami, FL) from Naja haje for C3 depletion.

**RESULTS**

**C3 depletion by CVF.** Deposition of C3b and C4b on mouse T cells coated with anti-Thy-1 MoAb (RmT1) was tested following incubation with serum of mice injected with 10 U of CVF. C4b deposition was found in RmT1-coated T-cell zones, ie, perilineral lymphatic sheaths of spleen white pulp (Fig 1) and paracortex of lymphnodes of both CVF-treated and control mice. In contrast, C3b was present in T-cell areas only in those spleens incubated with fresh serum and lacking in spleen sections incubated with RmT1 plus CVF-treated C3-depleted serum of C57BL/6 mice. Virtually no deposition of C3b on antibody-coated cells was measured with sera collected from mice during the first 2 days following injection of CVF (Fig 2). A second injection of CVF on day 2 prolonged C3 depletion for another 2 days but did not change the isotype-related differences in immunosuppression described below.

**Isotype-related consequences of CVF treatment for T-cell elimination.** Table 2 summarizes T-cell counts in lymphnode-cell-depleted mice after injection of saturating amounts (1 mg) of IgG2b (RmT1) or IgG2c (RmT2) anti-Thy-1 MoAb. Three days after injection of RmT1, a 97% reduction of cells carrying the pan T-cell marker Lyt-1 and a 90% reduction T cells in C3-depleted mice were counted. Reduction of T cells was much less with RmT2: 79% in normal and 46% in C3-depleted mice.

**Isotype-related differences in immunosuppression in C3-depleted mice.** Mortality from GVHD was prevented in semiallogeneic radiation chimaeras conditioned with RmT1 or RmT2 injected after irradiation and before transfusion of 2 × 10⁷ BM together with 5 × 10⁷ spleen cells of C57BL/6 mice. RmT2, in contrast to RmT1, failed to prevent GVHD if the mice were in addition depleted of C3 by CVF that was...
Fig 1. Immunohistochemical staining of spleen sections for the presence of labeled MoAb to C4b and C3b deposited on T cells during C' activation (from fresh or CVF-treated mouse serum) by anti-Thy-1 MoAb (RmT1). Spleen periarteriolar lymphatic zone shows labeled anti-C4b (A) or anti-C3b (B) on RmT1-coated cells incubated with fresh serum and anti-C4b (C) or absence of anti-C3b (D) after incubation with CVF-treated C3-depleted serum of C57BL/6 mice.

injected after irradiation (Fig 3). A comparable reversal of prevention of GVHD with 100% mortality between days 20 to 68 was noted when further rat IgG2c (RmT6, RmT7, RmT8) anti-Thy-1 MoAbs were injected together with CVF. Prevention of GVHD following injection of RmT1 or further anti-Thy-1 MoAb of rat IgG2b (BD 30-H12), mouse IgG2a (MmT1, MmT4), or IgG2b (5a-8) isotype remained unaffected by C3 depletion even in homozygous fully H-2, IA mismatched CBA mice. When tested approximately 100 days later these mice were full (95% and better) chimaeras tolerating marrow donor skin grafts. In further experiments, RmT1 or RmT2 antibodies were injected in prospective C57BL/6 donors depleted of C3 by CVF. Three days later their spleen and BM cells were transferred to irradiated (C57BL/6xCBA)F1 mice. Again, failure to survive from GVHD was noted for recipients of cells from donors treated with RmT2 but not RmT1.

Uptake of C1q by T cells coated with various anti-Thy-1 MoAb isotypes. In contrast to four rat IgG2c anti-Thy-1 MoAbs, a plateauing retention of C1q on thymocytes saturated with rat IgG2b or mouse IgG2a and IgG2b anti-Thy-1 MoAbs was found after incubation with dilutions of partly purified mouse C1q (Fig 4). Similar differences were obtained for C57BL/6 lymphocytes. Comparison of uptake of C1q by thymocytes coated with mouse IgG3 anti-Thy-1.1 MoAb or its IgG2a, IgG2b, and IgG1 switch variants showed low uptake of C1q for IgG3 similar to the rat IgG2c isotype and marginal C1q binding of IgG1 (Fig 5).

Deposition of C1, C4, and C3 on antibody-coated T cells. C1, C4b, and C3b were bound on C57BL/6 lymph node cells during incubation with rat IgG2b (RmT1) or IgG2c (RmT2) in the presence of undiluted fresh serum from C57BL/6 mice. Their amounts were measured with FITC-labeled anti-C1, anti-C4b, and anti-C3b MoAbs in FACS. No difference between RmT1 and RmT2 was found for C3b. C1 was about four times more on cells incubated with RmT1 as compared with RmT2; C4b was about one third (Fig 6).

DISCUSSION

Although suppression of GVHD is usually approached by incubation of antibodies with donor cells and comple-
Fig 2. Depletion of C3 in (C57BL/6xCBA)F1 (○) or CBA (●) mice injected with 10 U of CVF. C3 levels were assayed in ELISA using labeled anti-C3 MoAb in the presence of thymocytes that had been preincubated with RmT1 and serum of mice collected after injection of CVF.

ment, we had previously found that, except for the IgM isotype, which was not included in this study, 1 mg of anti-Thy-1 MoAb, whether used in vitro or injected in recipients before transplantation or in donors, produced comparable results.11 In vivo T-cell depletion by these antibodies could thus be related to its immunosuppressive consequences by probing the cells of the antibody-treated donor in the GVHD model. We used MoAbs that reacted with Thy-1.2 cells. Their isotypes include the most immunosuppressive examples found so far. Pan T Lyt-1 (CD5), Lyt-2 (CD8), or L3T4 (CD4) antigens are less densely expressed on T cells. Injections of corresponding MoAbs do not prevent GVHD in fully mismatched mice. Even the concentration of the Thy-1.1 alloantigen on T lymphocytes is too low for optimal antibody coating, complement activation, and suppression of GVHD.22 Regarding the depletory effect of CVF, we found an almost complete lack of C3b deposition, as a consequence of C3 depletion, on antibody-coated cells in the presence of serum samples collected from mice until 48 hours after injection of CVF (Figs 1 and 2). This finding is concordant with the markedly diminished C3 levels (>1%) shown in immune adherence activity that were reported for sera of guinea pigs and mice treated with CVF.22

The main focus of our investigation was to differentiate between the C'-fixing rat IgG2c anti-Thy-1 isotype and C'-fixing rat IgG2b, mouse IgG2a and IgG2b isotypes regarding the suppression of GVHD. Only the former lost most of its T-cell–reducing and immunosuppressive effect in C3-depleted mice, whereas the latter isotypes showing high uptake of C1q remained little affected by CVF. The high intrinsic affinity for human Clq of the rat IgG2b

Table 2. T-Cell Elimination in Lymph Nodes of C3-Depleted C57BL/6 Mice Three Days After Intravenous Injection of RmT1 (lgG2b) or RmT2 (lgG2c) Rat anti-Thy-1 MoAb

<table>
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<th>Treatment</th>
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<th>Lyt-1+/μ</th>
<th>μ-</th>
<th>Rat Ig*</th>
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<td>24.0</td>
<td>59.0</td>
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<tr>
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<td>8.3</td>
<td>32.0</td>
<td>62.0</td>
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Abbreviation: ND, not done.

*Percentage of positive lymphoid cells from pooled lymph nodes of four normal or treated mice was determined with FITC- or PE-labeled MoAbs in FACS.

†Ten units of CVF per mouse was injected 6 hours before RmT1.

Fig 3. Survival of (C57BL/6xCBA)F1 mice depleted of C3 by injection of CVF after irradiation followed by 1 mg of anti-Thy-1 MoAb 4 hours before transplantation of BM and spleen cells of C57BL/6 mice.

Fig 4. Uptake of C1q by C57BL/6 (Thy-1.2) thymocytes precoated with rat anti-Thy-1 MoAbs of various isotypes. Cells were saturated with MoAb and incubated with dilutions of C1q partly purified from mouse serum. C1q uptake was measured in ELISA with biotinylated rat antirat C1q MoAb. Rat IgG2b: (■) RmT1, (△) BD30-H12. Rat IgG2c: (○) RmT2, (□) RmT6, (●) RmT7, (△) RmT8. Mouse IgG2a IgG2b: (□) RmT1, (△) RmT4.
isotype was discovered by Hughes-Jones et al using synergistic MoAbs specific for the rat class I major histocompatibility antigen RT1A, which is also expressed on rat red cells. Synergistic, i.e., noncompetitive, binding of these antibodies to distinct epitopes on the same cell membrane antigen produced pairs of closely positioned MoAbs that intercalated Clq on the red cells. We had noted that Thy-1.2 is more densely expressed on T cells, allowing, for example, an average concentration of 59,000 RmT1 molecules per lymphnode cell, enough for normal, nonsynergistic rat IgG2b MoAb to intercalate Clq with high affinity. Furthermore, we demonstrated that a correlation exists between uptake of human Clq by cell-bound anti-Thy-1 MoAb or polyclonal rabbit or rat ATG and their immunosuppressive effect on GVHD and/or skin graft rejection. C'-dependent cell lysis is a classic but not a reliable indicator for in vivo antibody activity. Our anti-Thy-1 rat IgG2b and 2c MoAb isotypes are fully lytic, in the presence of mouse complement, for their target T cells despite their different uptake of Clq and immunosuppressive potency. This finding underlines the fact that intrinsic affinity for Clq and, consequently, uptake of Clq, rather than cell lysis, is a better indicator of in vivo potency of the tested anti-Thy-1 MoAb regarding cell depletion and immunosuppression. Using polyclonal rabbit anti-human Clq crossreacting strongly with mouse Clq or a monoclonal antimouse Clq antibody that we generated in rats, we have shown in the present study that the isotype-related differences found for uptake of human Clq also hold true in principle for mouse Clq, although we find an even lower uptake of human compared with mouse Clq for the rat IgG2c isotype (data not shown).

Studying the clearance of anti-RT1A' antibody-coated rat red cells, Yousaf et al measured a prolongation in rats treated with CVF. It was explained by the CVF-induced lack of C3 that could no longer be deposited on the antibody-coated cells and contribute to their binding to corresponding receptors on phagocytes. We interpret our results with CVF and rat IgG2c anti-Thy-1 MoAb as due to a similar weakening of the classical Fe and C3-receptor-dependent effector mechanism.

It remains to be explained why the Clq-affine anti-Thy-1 MoAbs were so little affected by C3 depletion that they eliminated T cells and prevented GVHD not only in C3-depleted semiallogeneic F1 mice but also in homologous H2 IA region two haploype incompatible chimaeras. In the latter, considerably more difficult combination the Clq-low-affine rat IgG2c anti-Thy-1 MoAb failed to delay GVHD even when C3 depletion by CVF had been omitted.

We did not find clear differences in the amount of deposition of C3b on target T cells and only small differences for C4b. The question, therefore, remains whether the isotype-related difference in uptake of Clq is the sole explanation for in vivo differences in antibody effector mechanisms. Opsonization by macrophages, complement-mediated lysis, and ADCC have been discussed as important effector mechanisms in cell suppression of certain antibody isotypes. As such, ADCC does not require C' activation and would not explain the correlation between Clq uptake and immunosuppression. Classical ADCC with K cells as effectors is not ruled out by the present findings. However, there is recent evidence for a mechanis-
tic role of Clq in ADCC of IgG-coated cells, too. Murine resident peritoneal macrophages with low levels of endogenous Clq synthesis were reconstituted in their ADCC of antibody-coated red cells by exogenous Clq. In contrast, reconstitution with serum Clq did not increase ADCC above the level that activated, ‘inflammatory’ macrophages induce all on their own. Macrophages synthesize and secrete Clq by themselves, at least when they are activated. Whether they use it directly as a link with intercalation of the globular heads of Clq in preformed immune complexes and the fibrillar stems to be bound to Clq-specific receptors on macrophages is still open. Leu et al, when discussing their findings, emphasized a pivotal role of Clq in the regulation of receptor-mediated phagocytosis and ADCC activation.

One might wonder about the advantage of exploiting the earliest subcomponent of the complement cascade as a powerful cell-depleting effector mechanism. In the race between cell depletion and cell renewal, antibodies that deplete as promptly as possible will be most successful. Integrating Clq may be safer than using only later complement components, which may, eg, be deficient. For instance GVHD was also completely prevented by injection of Clq-high-affine anti-Thy-1 MoAbs in prospective AKR/J recipient mice challenged with C57BL/6 spleen and BM cells. AKR mice are genetically fully deficient of C5. Even depletion of C3 and the later components of the C'-cascade regarding anti-immune complexes and the fibrillar stems to be bound to Clq-specific receptors on macrophages is still open. Leu et al, when discussing their findings, emphasized a pivotal role of Clq in the regulation of receptor-mediated phagocytosis and ADCC activation.

The question remains as to whether mouse IgG3 anti-Thy-1 MoAbs in mice correspond to the rat IgG2c isotype. Their heavy chain constant region genes have a high (87%) degree of homology. As for rat IgG2c, we found low uptake of mouse Clq on IgG3 MoAb-coated Thy-1.1 thymocytes. Unfortunately Thy-1.1 density on postthymic T cells is too low to cause strong T-cell depletion and prevention of GVHD comparable with what is possible with anti-Thy-1.2 MoAb. Testing whether CVF reduces the C3b-dependent effector mechanism of IgG3 anti-Thy-1 MoAb in the same way as we found for rat IgG2c anti-Thy-1 MoAb must, therefore, be adjourned until generation of an anti-Thy-1.2 mouse IgG3 isotype. However, it is of interest that in another, lymphoma cell-depleting mouse model, Denkers et al found this C'-fixing antibody less effective in vivo than its C'-fixing IgG2a and 2b switch variants, which would be concordant with the differential uptake of Clq that we found for the same antibodies.

In summary, differentiation between a C3-9–independent effector mechanism of antibody isotypes with high intrinsic affinity for Clq and a less potent C3-dependent effector mechanism of antibody isotype(s) with low affinity for Clq led us to focus our attention on the earliest subcomponent of the complement cascade regarding antibody-induced cell depletion and immunosuppression.

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without the need of their prior purification. I. Antibodies to mouse Clq. Hybridoma 8:615, 1989
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S Thierfelder, J Mysliwietz, G Hoffmann-Fezer and U Kummer