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Murine anti-mouse T cell monoclonal antibodies elicit anti-antibodies in mice: intra-species immunization model for estimating potential patient sensitization against humanized anti-T cell antibodies

Humanization of immunosuppressive anti-T cell monoclonal antibodies (mAb) raises the question as to how completely it helps to avoid formation of neutralizing anti-antibodies (anti-Ab) in patients. To get more information on intra-species sensitization against anti-T cell mAb, we produced two immunosuppressive mouse IgG2a anti-mouse Thy-1.2 mAb (MmT1 and MmT5) in AKR/J mice and measured the potential of MmT1 to elicit inhibitory anti-Ab in AKR/J ($H\text{-}2^k$), C57BL/6 ($H\text{-}2^b$), congenic B10.BR ($H\text{-}2^k$) and DBA/2 ($H\text{-}2^d$) mice. After one injection once weekly for 4 weeks of 5 μg MmT1 (200 μg/kg) in C57BL/6 mice, without the use of any adjuvants, high concentrations of anti-Ab directed against MmT1 (300 μg/ml) and MmT5 (100 μg/ml) were measured by enzyme-linked immunosorbent assay. Similar concentrations of anti-Ab were found in immunized DBA/2 and less in B10.BR mice. No syngeneic anti-Ab could be produced in AKR/J. From the C57BL/6 mice, we raised anti-$MmT1^+$, $MmT5^-$ idiotype (IDIO1) and anti-$MmT1^+$, $MmT5^+$ allotype (ALLO1) mAb. An *in vivo* test system was adapted to measure the inhibitory effects of circulating poly- or monoclonal anti-Ab. It revealed a reduction of *in vivo* depletion capacity not only of the sensitizing mAb (MmT1), but also of another anti-Thy-1.2 mAb (MmT5), with identical allotype but different idiotype.
From this we conclude that intra-species immunization following injection of anti-T cell mAb can produce high titer inhibitory anti-idiotype and anti-allotype antibodies. Implications for hyperchimeric or fully human anti-T cell mAb are discussed.

1 Introduction

Prolonged treatment of patients with rodent immunosuppressive anti-T cell monoclonal antibodies (mAb) is complicated by sensitization and appearance of anti-antibodies (anti-Ab), causing a loss of immunosuppressive effect [1–4]. Such polyclonal anti-Ab are directed against the variable or the constant part of the injected xenogeneic antibody. Experimental studies analyzed their inhibitory activity in mice [5, 6] and primates [7, 8]. In order to obviate the patient's antiglobulin response, chimeric mAb (constant region of human origin) have been produced. Unfortunately, chimeric antibodies induced anti-Ab, and their clinical relevance has engendered controversy [9, 10]. To improve antibody therapy, 'humanized', 'reshaped' anti-T cell antibodies have been constructed with the hypervariable regions of the rodent antibody introduced into normal human immunoglobulin genes [11].

Preclinical studies of non-human primate allograft recipients following a single injection of humanized anti-CD4 (OKT4A) T cell mAb showed induction of anti-idiotype antibodies [12].

No antiglobulin response was observed in the first preliminary communication on remission induction in two patients with non-Hodgkin lymphoma treated with the genetically reshaped human IgG1 mAb CAMPATH-1H [13]. Recently, Isaacs et al. [14] detected anti-Ab in patients retreated for rheumatoid arthritis with CAMPATH-1H. One patient even developed non-idiotypic antibodies. However, biological consequences of induced anti-Ab have not been described.

Systematic studies in humans clarifying the extent and *in vivo* relevance of immune response to species-adapted antibodies are questionable for ethical reasons.

Brüggemann et al. [15] have investigated the anti-Ab response in mice to xenogeneic, chimeric or mouse anti-NP mAb emulsified in Freund's adjuvants and found anti-Id antibodies even in Igh-matched mice and anti-allotype antibodies in Igh-mismatched mice. These results suggest that mAb administered in clinical therapy should be matched to the recipient's allotype. Since, however, the mAb used for immunization were specific for NP, possible therapeutical limitations posed by anti-Ab could not be assessed.

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Our aim was to set up an animal model that more precisely reflects the clinical situation, in as much as it allows us to measure the reversal of the immunosuppressive potential of anti-T cell mAb induced by anti-antibodies.

Taking advantage of the Thy-1 di-allelic system, which allowed us to study intra-species sensitization potential of immunosuppressive mouse anti-Thy-1.2 mAb, we could detect anti-Ab, which reduced the *in vivo* depletion capacity not only of the sensitizing mAb (MmT1), but also of another anti-Thy-1.2 mAb (MmT5), which shares the former's allotype, but not its idiotype.

Thus, even anti-Ab against allotypic variants can neutralize the therapeutic effect.

2 Materials and methods

2.1 Animals

C57BL/6, congenic B10.BR, CBA/J, DBA/2 and AKR/J mice, originally from the Jackson Laboratory (Bar Harbor, ME), were raised and maintained in our breeding facilities.

2.2 Sensitization of mice against mouse anti-mouse T cell mAb

MmT1 and MmT5, both mouse anti-mouse Thy-1.2 mAb, were derived from a fusion of spleen cells of AKR/J mice (Thy-1.1) immunized with thymocytes of AKR/Cum (Thy-1.2) with the myeloma cell line P3 × 63-Ag 8.653, according to the general procedure described by Köhler and Milstein [16]. MmT1 and MmT5 are of the mouse IgG2a subclass and are immunosuppressive *in vivo*. A single injection of these antibodies in recipient mice (*e.g.* C57BL/6) prevents GVH by fully mismatched Thy-1.2 donors. Injection of MmT1 in mice may induce two types of anti-antibodies. One is directed against the unique hypervariable region, the other against allotypic determinants on the immunoglobulin.

The sensitization potential of MmT1 (Igh-1^d allotype) was measured by injecting 5 μg (200 μg/kg) (which is also an applicable dose in human treatment) i.p. in C57BL/6 mice (Igh-1^b allotype), congenic B10.BR (Igh-1^b), DBA/2 (Igh-1^c) or in AKR/J (Igh-1^d) mice once weekly for 4 weeks. C57BL/6 and AKR/J mice were also treated with 50 μg and 250 μg doses. Tail vein blood was taken 10 days after the last injection, and the anti-antibody response was measured by solid-phase ELISA: polystyrene microtiter plates were coated with either 10 μg/ml MmT1 or MmT5 in carbonate-bicarbonate buffer (50 mM, pH 9.5). After blocking unspecific binding with PBS containing 1 % FCS, serial dilutions of sera preincubated either in C57BL/6 or AKR/J serum were added. Antibodies bound to MmT1 or MmT5 were detected by using biotinylated goat anti-mouse IgG subclass-specific antibodies (Amersham, Braunschweig, FRG) absorbed to mouse IgG2a, and horseradish peroxidase (HRP) avidin. Anti-Ab concentrations were calculated by comparing the titration curves of the mouse sera with those of the standard mAb (10 μg/ml ALLO1).

2.3 Production of mouse mAb with anti-idiotype or anti-allotype specificity for MmT1

One C57BL/6 mouse from the sensitization experiment was boosted 3 weeks after the last sensitizing injection with another 5 μg MmT1. Three days later, spleen cells were fused with the myeloma cell line P3 × 63-Ag 8.653. Hybridoma supernatants were tested by the MmT1 ELISA method described above. mAb that showed positive reactions were further analyzed by MmT5 ELISA. Binding on MmT1 but not on MmT5 should indicate anti-Id specificity; binding on both MmT1 and MmT5 suggested anti-allotype specificity. For confirmation of the predicted specificity, culture supernatants were preincubated with sera of mouse strains differing in IgG2a allotype (C57BL/6, CBA/J and AKR/J), or with purified MmT1 or MmT5, and added to MmT1-coated microtiter plates. Preincubation of the culture supernatants with calf serum served as 100 % control. Detection of bound antibodies was identical to MmT1 ELISA.

2.4 Inhibition of MmT1 binding to T cells by anti-idiotype or anti-allotype mAb

Serial twofold dilutions (100 μg/ml) of ALLO1 or IDIO1 were incubated overnight with subsaturating amounts of FITC-labeled MmT1. C57BL/6 thymus cells (5×10^5) were added for 1 h and then analyzed by FACScan. FCSC Microbeads Standards (Becton Dickinson, Heidelberg, FRG) were used as fluorescence standards for calculating MmT1 molecules bound to T cells.

2.5 FACS analysis of T cell depletion with MmT1 or MmT5 in C57BL/6 mice presensitized to MmT1

To study the effect of either anti-allotype and anti-Id antibodies or anti-allotype antibodies alone on the depletion capacity of anti-T cell mAb, we sensitized mice against MmT1 and depleted with either MmT1 or MmT5 (same allotype, different idiotype). Therefore, C57BL/6 mice (5 per group) were injected with 5 μg MmT1 i.p. once a week for 4 weeks. One week later, they received a final dose of either 150 μg MmT1 or 150 μg MmT5 i.p., which was earlier determined to suboptimally deplete T cells in spleen and lymph nodes; the same mAb doses were injected in unsensitized control mice. Three days later, these mice were killed and spleen and lymph nodes removed. Single cell suspensions of spleen and lymph node cells were prepared by passing them through nylon meshes. Subsequently, 2×10^5 cells were phenotyped with different antibody combinations (CD4-PE, CD3-FITC; CD8-PE, CD3-FITC; rat anti-mouse-FITC) and analyzed by FACScan. Statistical analyses were performed by using the Mann-Whitney Test.

2.6 FACS analysis of T cell depletion with MmT1 in C57BL/6 mice pretreated with anti-MmT1 mAb

In contrast to the above sensitization experiment, mice received either 1 mg of an mAb against the idiotype (IDIO1) or 1 mg of an mAb against the allotype (ALLO1)

of MmT1. One day later, they were challenged with the suboptimal depleting dose of MmT1.

3 Results

3.1 Sensitization against mouse anti-mouse T cell mAb MmT1

Injection of 5 μg MmT1 (mouse anti-mouse Thy-1.2) once a week for 4 weeks in C57BL/6, congenic B10.BR or DBA/2 mice raised anti-Ab, as demonstrated with MmT1-coated microtiter plates. Raising the dose to 50 μg or to 250 μg did not change the anti-Ab titer in C57BL/6 mice. However, it was not possible to generate anti-Ab in AKR/J mice under the same conditions. Incubation of the sera with AKR/J serum adsorbed anti-allotype antibodies. The anti-Id antibodies, which were not bound to AKR/J serum, were measured on MmT1-coated microtiter plates. AKR/J-preadsorbed sera were negative with MmT5. For the purpose of standardization, aliquots of 10 μg/ml ALLO1 were titrated along with the sera, allowing an estimation of the anti-MmT1 concentrations. Fig. 1 shows anti-Ab concentration in sensitized C57BL/6, B10. BR, DBA/2 or AKR/J mice.

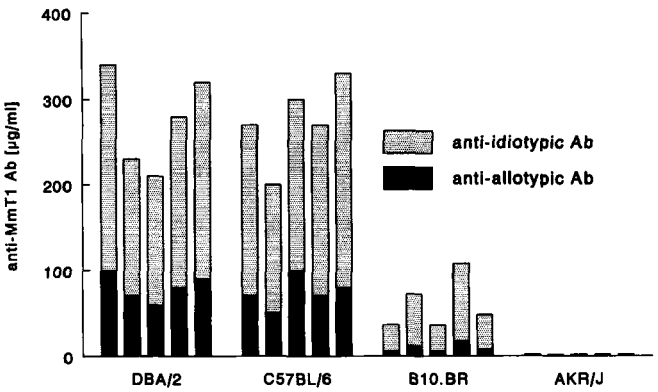

Figure 1. Anti-Ab concentrations in mice after four sensitizing injections, once weekly, of 5 μg MmT1 (mouse anti-mouse Thy-1.2). Anti-Ab concentrations in C57BL/6, congenic B10.BR, DBA/2 or AKR/J mice were determined on MmT1-coated microtiter plates either after absorption with AKR/J serum (detection of anti-Id antibodies) or with C57BL/6 serum (detection of anti-Id and anti-allotype antibodies). Serum concentrations were related to an ALLO1 (anti-MmT1-allotype mAb) titration curve.

3.2 Production of mouse mAb with anti-idiotype or anti-allotype specificity for MmT1

Supernatants of hybridomas resulting from a fusion of P3 × 63-Ag 8.653 with spleen cells of hyperimmune C57BL/6 mice were screened for specific antibody production by ELISA using MmT1-coated plates, as described above. mAb from six positive wells were further analyzed. All were of mouse IgG1 isotype. Three of them showed a positive reaction only with MmT1, suggesting anti-idiotype specificity. This could be confirmed by preincubation either with sera of mouse strains differing in IgG2a allotype (AKR: Igh^d-; C57BL/6: Igh^b-; CBA/J: Igh^j-allotype) or with MmT1 or MmT5, followed by detection of non-absorbed mAb on MmT1-coated microtiter plates. The binding to coated MmT1 was only inhibited by fluid MmT1.

The anti-allotype mAb bound to IgG of AKR/J serum, to MmT1 and to MmT5, but there was no detectable binding to IgG of C57BL/6 or CBA/J sera. The amount of non-adsorbed mAb, as detected on MmT1-coated microtiter plates, was as high as in the calf serum control (Fig. 2).

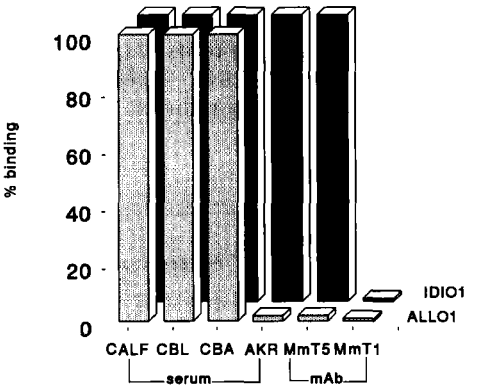

Figure 2. Specificity of anti-MmT1 mAb. Anti-MmT1 mAb ALLO1 or IDIO1 were preadsorbed with either purified MmT1 or MmT5 or normal C57BL/6 (IgG2a $Igh\text{-}1^b$ allotype), CBA/J ($Igh\text{-}1^j$ allotype) or AKR/J ($Igh\text{-}1^d$ allotype) serum. Unbound IDIO1 or ALLO1 were tested on MmT1-coated microtiter plates, following addition of biotinylated goat anti-mouse IgG1 and HRP-avidin. 100% binding: IDIO1 and ALLO1 binding on MmT1-coated microtiter plates after preincubation with calf serum.

3.3 Inhibition of MmT1 binding to T cells by anti-idiotype or anti-allotype mAb

Preincubation of FITC-labeled MmT1 with IDIO1 inhibits binding of MmT1 to T cells over 6 log2 titers, whereas ALLO1 inhibits only marginally (Fig. 3). This inhibition may be due to formation of larger MmT1/ALLO1 complexes, which were washed away during the test procedure.

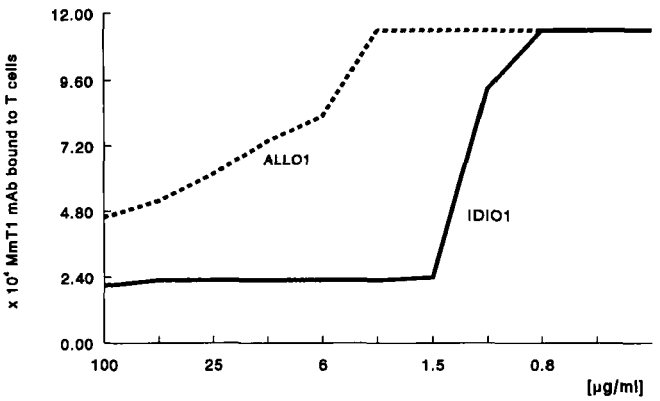

Figure 3. Inhibition of FITC-labeled MmT1 by anti-MmT1 mAb. Serial twolfold dilutions of IDIO1 or ALLO1 mAb (100 μg/ml) were incubated overnight with subsaturating amounts of FITC-labeled MmT1.5 × 10^5 C57BL/6 thymocytes were added for 1 h and then analyzed by FACS. Bound MmT1 molecules were calculated using FCSC Microbeads Standards.

3.4 FACS analysis of T cell depletion with MmT1 or MmT5 in C57BL/6 mice presensitized to MmT1

To investigate the biological effects of anti-Ab formation in mice which were treated once weekly for 4 weeks with 5 μg MmT1, the sensitized mice were challenged with a suboptimal depleting dose (150 μg) of either MmT1 or MmT5. Three days later, lymph nodes and spleen were analyzed by FACS with either anti-CD3 and anti-CD4 or anti-CD3 and anti-CD8, allowing the determination of the amount of remaining T cells. Cell-bound MmT1 was not detectable. As shown in Fig. 4a, the depletion capacity of MmT1 is significantly reduced by immunizing it to itself ($p < 0.05$). However, the depletion capacity of MmT5 – same allotype, but different idiotype – is also greatly reduced ($p < 0.05$, Fig. 4b).

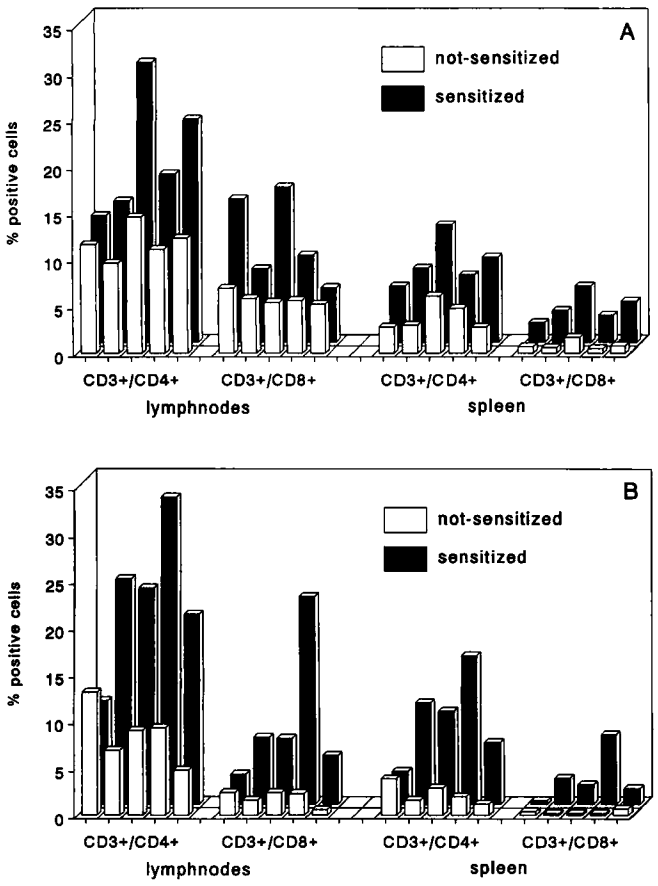

Figure 4. T cell depletion with MmT1 or MmT5 in C57BL/6 mice sensitized to MmT1. C57BL/6 mice, 5 per group, were injected with 5 μg MmT1 over a period of 4 weeks once weekly and then challenged with a suboptimal depleting dose of MmT1 (A) or MmT5 (B). Three days later, spleen and lymph nodes were removed and analyzed by FACS using anti-CD3-FITC/anti-CD4-PE or anti-CD3-FITC/anti-CD8-PE. $CD4^+$ or $CD8^+$ cells are expressed as percentage of cells in the preparation.

3.5 FACS analysis of T cell depletion with MmT1 in C57BL/6 mice pretreated with anti-MmT1 mAb

In this experiment, 1 mg of defined anti-MmT1 mAb (ALLO1 or IDIO1) was injected in mice 1 day before they received the suboptimal depleting dose of MmT1. IDIO1 and ALLO1 reduced the depletion capacity of MmT1 significantly ($p < 0.05$). However, there is a significant difference between the IDIO1 and the ALLO1 group ($p < 0.05$). When equal doses are injected, anti-allotype mAb reverse the immunosuppressive effect of MmT1 less than the anti-Id mAb do (Fig. 5).

As expected, IDIO1 did not inhibit the T cell depletion capacity of MmT5, which shares the same allotype with MmT1, but not the same idiotype (data not shown).

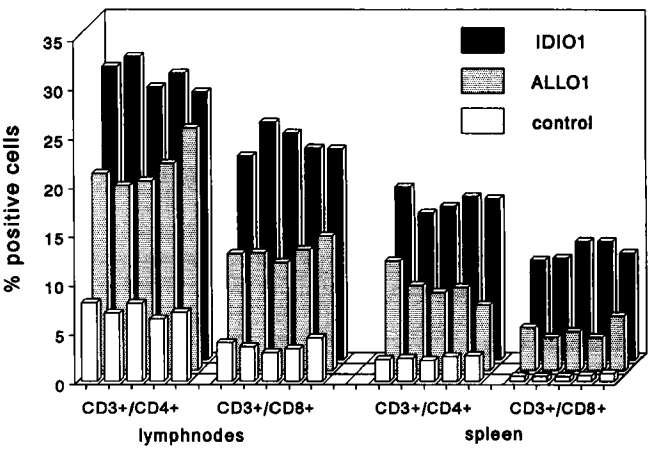

Figure 5. T cell depletion with MmT1 in C57BL/6 mice after injection of anti-MmT1 mAb (IDIO1 or ALLO1). C57BL/6 mice, 5 per group, were injected with 1 mg IDIO1 or ALLO1. One day later, they were challenged with a suboptimal depleting dose of MmT1 (150 μg). Three days later, spleen and lymph nodes were removed and analyzed by FACS using anti-CD3-FITC/anti-CD4-PE or anti-CD3-FITC/anti-CD8-PE. $CD4^+$ or $CD8^+$ cells are expressed as percentage of cells in the preparation.

4 Discussion

Formation of anti-Ab against anti-T cell mAb of the same species has not been reported so far, but it could be expected, considering the concept of idiotypy that was brought up when individual human myeloma proteins were shown to possess unique antigenic determinants that were not found in other myeloma proteins or in normal serum Ig [17].

From basic studies on idiotypes (for review see [18]), one has to question "whether tolerance can ever be attained to the unique variable region (*i.e.*, idiotype) of a given therapeutic monoclonal antibody" (Benjamin et al. [5]). However, not only anti-Id Ab, but also anti-allotype Ab may be induced after injection of an mAb of the same species, as on most IgG subclasses different allotypes have been identified. Although these are determined by minor structural differences when compared to the differences between xenogeneic isotypes, they may also contribute to a loss of effectiveness of an injected anti-T cell mAb [19, 20].

In the present study, four sensitizing injections (in C57BL/6 mice Igh-1^b, congenic B10.BR or DBA/2 Igh-1^c) of a clinically relevant dose of 5 μg MmT1 mouse anti-mouse Thy-1.2 (Igh-1^d; 200 μg/kg) produced anti-Ab with concentrations up to 300 μg/ml (anti-Id and anti-allotype Ab), without the use of Freund's or other adjuvants. After

preadsorption of the anti-allotype specificities with AKR serum, the remaining anti-Id Ab were measured in concentrations up to 200 μg/ml. This shows that about one third of the induced anti-Ab are anti-allotype Ab. Increase of the sensitizing 5 μg dose of MmT1 to 50 μg or 250 μg did not change the anti-Ab titer in C57BL/6 mice. Using allogeneic Igh-mismatched mice, we tried to mimic the clinical situation in which humanized mAb are applied regardless of the Igh allotype of the patient. Furthermore, the induction of anti-Id antibodies in our mouse model is informative in that inhibitory anti-Id antibodies must be expected, even with human anti-T cell alloantigen mAb, whose production may become possible from human combinatorial libraries [21].

The ease with which we raised anti-Ab against the variable antibody-binding region and the constant part of our anti-T cell mAb is interesting, but may come as no surprise. Rat anti-T cell mAb had shown an increase of xenogeneic immunogenicity when bound on T cells and injected in mice [5]. When we injected rat anti-mouse Thy-1.2 mAb in Thy-1.1. mice to let it circulate in a fluid, non-cell-coating form, the anti-Ab response was much lower than when we injected it in Thy-1.2 mice [6]. This may explain our failure to produce anti-Ab in AKR/J (Thy-1.1) mice, the strain in which MmT1 (Thy-1.2) was generated. Unresponsiveness is not related to the MHC type, since we induced anti-Id Ab in B10.BR mice, which share the MHC molecule H-2^k with AKR/J.

In vivo consequences of the polyclonal anti-MmT1 immune response were studied using T cell depletion as the test value. Injection of a suboptimal dose of MmT1 or MmT5 into MmT1-presensitized mice distinguishes the biological relevance of the allotypic or idiotypic parts of the immune response. We could demonstrate that anti-MmT1 antibodies distinctly reduced the *in vivo* depletory capacity not only of MmT1, but also of the other anti-Thy-1.2 mAb (MmT5) with identical allotype but different idiotype.

To study further the anti-Id or the anti-allotype immune response of an anti-T cell mAb separately *in vivo*, we generated mAb specific for the idio- or allotype of MmT1. As expected, anti-MmT1-IDIO1 mAb considerably inhibited T cell depletion by MmT1 *in vivo*. Also, anti-MmT1-ALLO1 reduced T cell depletion by MmT1 significantly, but to a lesser degree.

Comparing the *in vitro* binding inhibition of MmT1 by anti-ALLO1 or anti-IDIO1, anti-ALLO1 inhibits only marginally and at high concentrations. In this case, formation of antigen-antibody complexes with increased clearance rather than direct blocking of MmT1-binding to T cells must have inhibited MmT1.

With our *in vivo* test system, in which we measured not only the amount of induced anti-Ab, but also their consequences on target cell elimination, we extend the findings of Brüggemann et al. [15]. The authors had found a strong anti-Ab response against both V and C domains after injection of mouse anti-NP (Igh^b) mAb into allogeneic (Igh^a) mice. No anti-Ab were observed following injection of this mAb into syngeneic mice even after adjuvants had been used. However, injection of mAb in a Igh-matched strain induced detectable amounts of anti-Ab.

Recent findings of Isaac et al. [14] showed induction of anti-Ab in patients retreated for rheumatoid arthritis with humanized CAMPATH-1H mAb, but their clinical relevance could not be investigated. The present study demonstrates the potential of intra-species immune response against an anti-T cell mAb. The fact that not only anti-Id Ab but also anti-allotype Ab reduce the *in vivo* efficiency of species-adapted mAb suggests that Igh-matching for clinical purposes may not be completely academic. Avoiding anti-allotype antibodies would thus allow the change to a new T cell mAb in case of circulating anti-Id antibodies to the first antibody. Further clinical experience will tell us about the significance of intraspecies anti-Ab in prolonged antibody treatment or retreatment. Even continuous treatment of C57BL/6 with MmT1 every second or third day could not prevent the appearance of anti-Id antibodies (data not shown). On the other hand sensitization against humanized anti-T cell mAb in patients whose immune response is suppressed, either by the underlying disease or by additional immunosuppressive therapy, may well turn out to be less important than in an experimental animal with a normo-reactive immune system.

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