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Immunological approach to inhibit formation of anti-antibodies to allo- and xenogeneic anti-T cell immunoglobulin

Inhibitory anti-antibodies induced in patients by xenogeneic or even by humanized anti-T cell antibodies remain an unresolved problem. Mice also produce anti-antibodies following injection of xeno- or allogeneic anti-T cell antibodies. Here we report a principle based on sequentially applied anti-T cell antibodies generated in different species, which results in suppressed anti-antibody formation and prolonged immunosuppression. Thus, a single priming injection in mice of mouse (MmT1 or MmT5 differing by idiotype only) or of rat (RmT1) anti-mouse Thy-1 monoclonal antibodies (mAb) or of rat anti-mouse L3T4 + Ly-2 (RmCD4 + CD8) mAb suppressed anti-antibody formation against subsequent booster injections of one of the above antibodies, provided that they differed in species origin from the priming antibody. Correspondingly, a sixfold and longer prolongation of 50% survival of fully mismatched skin grafts was observed. Less or no anti-antibody suppression and little prolongation of graft surival was obtained if the 'first' and the 'second' (and following) antibody injections were of the same species, differing by iso- or idiotype only. Finally, the suppressive principle did not manifest itself at all if the initial antibody injection included both the first and second antibody. These findings are discussed with reference to earlier studies on hapten/carrier effects as well as on immunosuppression attributed to 'non-depleting' rat anti-CD4/CD8 T cell antibodies.

1 Introduction

Much effort has been invested to overcome the antiglobulin response to anti-T cell monoclonal antibodies (mAb) [1–3]. These Ab are most strongly immunogenic when in a cell-binding form. For example, rat or mouse anti-mouse Thy-1.2 mAb elicit a strong anti-Ab response in Thy-1.2 mice but weakly in Thy-1.1 mice where they circulate in a fluid non-cell-coating form [4, 5]. Thus, injection of cell-coating anti-T cell Ig usually induces high levels of anti-Ab despite its potent immunosuppressive T cell-depleting activity. Even mice successfully tolerized to the constant regions of monoclonal rat Ig by classical deaggregation methods still developed anti-idiotype Ab after injections of an isotype-matched T cell-binding rat Ab [6]. Since mouse anti-mouse and humanized anti-T cell mAb have not eliminated the possibility of anti-Ab formation in mice or patients, we studied an approach based on a contrasting principle. Rather than improving the compatibility of anti-T cell Ig by other immunosuppressive modalities or by its adaptation to the species of the Ab recipient, we devised a method of sequential injections of Ab immunologically xenogeneic ot each other. This method was based upon the premise that the immune system of the Ab recipient would not respond to the priming and boosting immunogen in a conventional secondary response leading to anti-Ab production. Thus, a switch of animal species from which the first and the second anti-T cell Ab was generated was shown to prevent induction of anti-Ab formation and, consequently, to prolong skingraft survival in fully mismatched mice.

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2 Materials and methods

2.1 Animals

C57BL/6 (H2b) mice, originally obtained from Jackson Laboratory (Bar Harbour, ME), were raised and maintained in our breeding facilities. CBA (H2k) mice were bred from stock in our own laboraroty. For transplantation experiments 2–3-month-old female animals were used.

2.2 Antibodies

Table 1 lists the mAb used in this study along with their characteristics. The IgG fraction was prepared from culture supernatant by protein G chromatography, dialyzed into PBS; the protein concentration was estimated by measuring the absorbance at 280 nm. The binding activity of each purified mAb lot was checked by flow cytometry. The biotinylation was performed as described previously [7], using × 100 molar excess of biotinyl-ε-amino-caproic acid N-hydroxysuccinimide ester (Calbiochem, Bad Soden, Germany).

2.3 Treatment protocols

C57BL/6 recipient mice were preinjected i.p. with a single 400-μg dose of mouse MmT1 or MmT5 mAb (differing by idiotype only) 3 days prior to transplantation of CBA skin grafts. MmT1 or xenogeneic, rat mAb RmT1 were then injected at 200 μg i.p., beginning at skin grafting and continued twice a week until rejection. In other groups of

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mice, reciprocal protocols were carried out, with rat mAb RmT1, 30H12 [8], RmT5 or RmCD4-2 + RmCD8-2 being preinjected and mouse mAb MmT1 or RmT1 or RmT5 injected twice a week until graft rejection. Tail blood samples for measurements of anti-Ab were collected 7–10 days after graft rejection.

For induction of classical tolerance to rat anti-RmCD4 and RmCD8 mAb, C57BL/6 mice were tolerized as described by Benjamin [6] by i.p. injection of 0.5 mg deaggregated (150 000 × g for 150 min) RmCD4-2 and RmCD8-2 (day 0) and afterwards immunized with 500 μg of the same mAb on day 9 (i.v.) and day 19 (i.p.) and bled on day 24 (Table 2A). In parallel experiment 400 μg MmT1 i.p. was preinjected on day − 3 (Table 2B).

Inhibition of anti-Ab response to allo- or xenogeneic anti-T cell mAb was studied in C57BL/6 mice preinjected i.p. on day − 3 with 400 μg of the "first Ab" and/or 500 μg i.p. of the "second Ab" on days 0, 14 and 21 or 0 and 9 (Table 2) and bled 7 days later.

2.4 Skin grafting

Segments of full-thickness tail skin, approximately 1 cm^2, were grafted under anesthesia onto a prepared vascularized bed on the recipient's lateral thoracic wall. The grafts were covered with a hydrophobic paraffin gauze dressing (Lohman, Neuwied, Germany) and a plaster bandage. The bandage was changed 14 days after grafting and then every other day for 1 week more. The graft status was monitored three times a week; rejection was defined as the complete loss of viable tissue. Differences in survival were analyzed using the Log-Rank method [9].

Table 1. Main features of the mAb used in this study

mAb	Ig isotype	Binding specificity	Ref.
RmT1	Rat IgG2b	Thy-1	[13]
RmT5	Rat IgG2a	Thy-1	[7]
30-H12	Rat IgG2b	Thy-1.2	[8]
RmCD4-2	Rat IgG2b	L3T4 (CD4)	[14]
RmCD8-2	Rat IgG2b	Ly-2 (CD8)	[14]
MmT1	Mouse IgG2a	Thy-1.2	[4]
MmT5	Mouse IgG2a	Thy-1.2	[4]
ALLO1	Mouse IgG1	Mouse IgG2a	[4]

2.5 Quantification of murine anti-Ab responses

Concentrations of mouse anti-Ig Ab were assessed by solid-phase ELISA. Microtiter plates were coated with mAb under study (MmT1, RmT1, RmT5, CD4 + CD8) or isotype-matched irrelevant controls by overnight incubation of 50 μl/well of a 10 μg/ml solution in 0.05 M carbonate buffer, pH 9.5. Before blocking and between each step in the assay, the plates were washed with PBS. After blocking with 1% (w/v) solution of milk powder in PBS for 1 h, serially diluted sera (beginning with 1/12) from treated animals or untreated controls or concentration standards (ALLO1, mouse anti-rat Ig) were added at 50 μl/well and incubated at room temperature for 1 h. For detection of polyclonal mouse anti-MmT1 or anti-MmT5 Ig, peroxidase-labeled goat anti-mouse Fc gamma IgG (Dianova, Hamburg, Germany), absorbed with MmT5 and MmT1 mAb coupled to tresyl-activated Sepharose 4B (Pharmacia, Freiburg, Germany) were used (the coupling procedure was performed as described by manufacturer). Peroxidase-labeled rat anti-mouse IgG (Dianova, Hamburg) were used for detection of mouse anti-RmT1, RmT5, -30-H12 or -CD4 + CD8 Ig. Bound indicator Ab were detected by the addition of o-phenylenediamine (OPD)-H_2O_2 substrate and absorbance was read at 405 nm. Concentrations of mouce anti-Ab wre calculated using Easy Software program (SLT Labinstruments, Creilsheim, Germany).

Table 2. Reduced secondary anti-Ab response to mouse or rat mAb after booster injections with anti-T cell Ab[a)]

Exp.	First Ab (origin)	Immunization (days)	Second Ab (origin)	Immunization (days)	Anti-Ab[b)]
A			RmCD4+8 (rat)	0, 9, 19	84 ± 154.2
B	MmT1 (mouse)	− 3	RmCD4+8 (rat)	0, 9, 19	1.1 ± 1.4
C			MmT1 (mouse)	0, 14, 21	260 ± 140.6
D	RmT1 (rat)	− 3	MmT1 (mouse)	0, 14, 21	0.71 ± 0.96
E	RmCD4+8 (rat)	− 3	MmT1 (mouse)	0, 14, 21	0.92[c)]
F	RmT1 (rat)	− 3	RmT1 (rat)	0, 14, 21	254 ± 91.5
G	MmT1 (mouse)	− 3	RmT1 (rat)	0, 14, 21	2.45[d)]
H	**RmCD4+8+MmT1 (rat + mouse)**	**− 3**	**MmT1 (mouse)**	**0, 14, 21**	**251.5 ± 183.8**
I			RmT5 (rat IgG2a)	0, 9	215.8 ± 37.3
J	RmT1 (rat IgG2b)	− 3	RmT5 (rat IgG2a)	0, 9	10.8 ± 7.7

a) Groups of four to six C57BL/6 mice, 8–12 weeks of age were immunized i.p. using 400 μg of first and/or 500 μg of second Ab as indicated (origin and specificity of mAb is described in (Sect. 2.2 and Table 1). Tail blood was collected 6–10 days after the last injection and concentrations of anti-(second mAb) Ab were measured as described in Sect. 2.5.

b) μg/ml (mean ± SD) of anti-(second mAb) Ab.

c) In three of five mice no anti-Ab could be detected.

d) In three of four mice no anti-Ab could be detected.

2.6 Inhibition of binding of mAb

Serially diluted sera (beginning with 1/12) from mAb treated mice or untreated controls were incubated overnight with subsaturating concentrations of biotinylated mAb identical with that used in the treatment protocol and added to 5×10^5 thymocytes or lymph node cells. The binding of biotinylated mAb to the lymphocytes was revealed in microtiter plates using peroxidase-labeled avidin and OPD-H_2O_2 substrate. Serum titer causing a half maximal inhibition of binding of the biotinylated mAb was a measure of concentration of inhibiting anti-Ab.

3 Results

3.1 Inhibition of anti-antibody response to allo- or xenogeneic anti-T cell Ab

Despite a tolerizing injection of deaggregated rat RmCD4-2 + RmCD8-2 mAb (Sect. 2.3) a strong anti-Ab response (84 ± 154.2 μg/ml, Table 2A) was detected in C57BL/6 mice immunized with these Ab only. Interestingly, introduction to the above-described protocol of a single preinjection of mouse anti-Thy-1.2 mAb ((MmT1) on day −3 caused reduction of anti-RmCD4 + RmCD8 antibodies to 1.1 ± 1.4 μg/ml (Table 2B).

On the other hand, three injections of 500 μg/dose of allogeneic, otherwise immunosuppressive [10] mouse anti-Thy-1.2 mAb (MmT1) in C57BL/6 mice resulted in induction of 260 ± 140.6 μg/ml serum of anti-MmT1 Ab (Table 2C). In strong contrast, preinjection of 400 μg of xenogeneic rat anti-Thy-1 mAb (RmT1) inhibited almost completely (> 99%) anti-MmT1 Ab response which reached concentrations of only 0.71 ± 0.96 μg/ml (Table 2D). This inhibition was not due to binding inhibition between the preinjected and second Ab sharing the same antigen specificity (Thy-1), because comparable suppression was also observed after preinjection of rat anti-L3T4 (RmCD4-2) + anti-Ly-2 (RmCD8-2) mAb. In this case, in three of five mice no anti-MmT1 Ab could be detected (Table 2E).

With the inverse protocol, preinjection of 400 μg MmT1 prevented induction of anti-RmT1 Ab in three of four mice (Table 2G), while injecting 500 μg/dose of RmT1 alone, resulted in high concentrations (254 ± 91.5 μg/ml) of anti-RmT1 Ab (Table 2F).

This suppressive protocol, based on the sequential injection of anti-T cell mAb originating from different species failed, however, if the initial injection included both the first and second Ab. In this case the concentrations of anti-Ab against the "booster" antibody were comparable to those where the preinjection was omitted (251.5 ± 183.8 μg/ml *vs.* 260 ± 140.6 μg/ml, Table 2H *vs.* C).

Isotype differences between RmT1 (rat IgG2b) followed by RmT5 (rat IgG2a) also caused reduced anti-Ab response against the second mAb (215.8 ± 37.3 μg/ml *vs.* 10.7 ± 7.7 μg/ml; Table 2I,J) although anti-Ab levels were over ten times higher than that achieved with Ab derived from different species.

3.2 Skin graft survival and anti-Ig responses after sequential treatment with antibodies from different species

The *in vivo* relevance of our suppression of anti-Ab was studied in mice grafted with minor and MHC antigens mismatched skin grafts. C57BL/6 recipients were injected i.p. with a single 400 μg dose of rat anti-Thy-1 mAb (RmT1) 3 days prior to transplantation of allogeneic CBA skin grafts; 200 μg of mouse anti-Thy-1.2 mAb (MmT1) were injected on the day of skin grafting and continued twice weekly until rejection. For serological studies heparinized tail blood samples were collected from individual mice 7–10 days after graft rejection. As shown in Fig. 1A (4), 50% graft survival of up to 70 days occurred in mice treated with the RmT1/MmT1 protocol compared to only 22 days in mice treated with MmT1 only [$p = 0.021$; Fig. 1A-(2)]. The anti-Ab analysis in the former group revealed no anti-MmT1 response in four of five mice; in one mouse

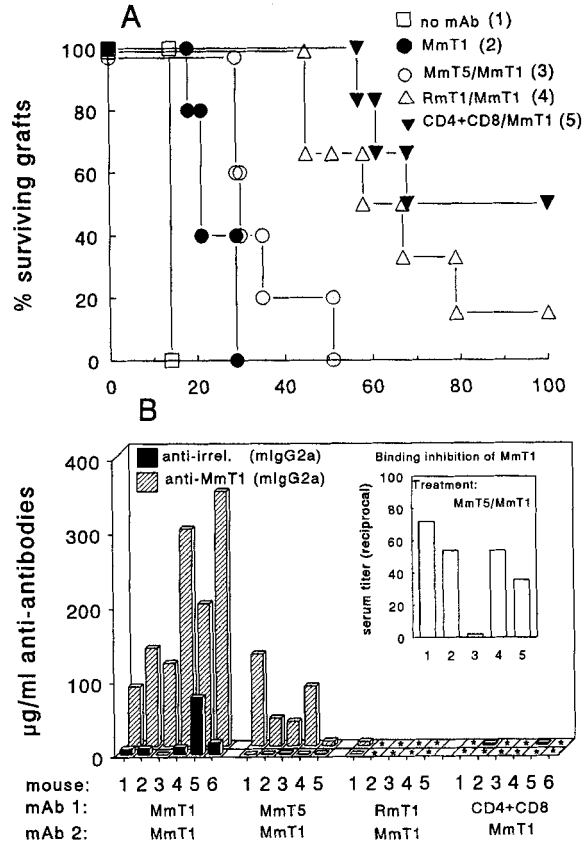

Figure 1. Prolonged survival of minor and MHC incompatible skin grafts and reduced anti-mouse Ig response in mice treated with anti-T cell mAb. (A) Groups of six C57BL/6 mice were grafted with CBA tail skin and injected with indicated mAb as described in Sect. 2.3. Log-rank analysis of the survival (in days) curves gave a following *p* values of statistical significance: 1 *vs.* 2 = 0.022, 2 *vs.* 4 = 0.021, 2 *vs.* 3 = 0.041, 3 *vs.* A4 = 0.035, 4 *vs.* 5 = not significant. (B) Heparinized tail blood samples were collected 7–10 days after graft rejection (see A) and concentrations of anti-Ab were quantified as described in Sect. 2.5. Insert: Polyclonal anti-MmT1 Ab in mice pretreated with MmT5/MmT1 inhibit the binding of MmT1 to lymph node cells from C57BL/6 mice. Representative data from two to three independent experiments are shown. *: No anti-Ab could be detected.

only 5.6 μg/ml were detected (Fig. 1B RmT1/MmT1). In contrast, mice treated with MmT1 alone showed 192.0 ± 95.5 μg/ml anti-MmT1 Ab. These anti-Ab were mainly anti-idiotypic with concentrations of 20.0 ± 26.14 μg/ml measured against isotype-matched, irrelevant control mAb (Fig. 1B MmT1/MmT1).

Suppression of anti-Ab and graft rejection were not linked to Thy-1 specificity. Comparable results were observed in mice preinjected with rat anti-L3T4 and anti-Ly-2 mAb [RmCD4-2 + RmCD8-2; Fig. 1A (5)]. In this case, no anti-MmT1 Ab could be detected in four of six mice, while in two mice only about 2 μg/ml anti-Ab were detected (Fig. 1B CD4 + CD8/MmT1).

Similarly improved survival was observed with the inverse protocol, *i.e.* preinjection of mouse anti-Thy-1.2 (MmT1) followed by rat anti-Thy-1 mAb (RmT1). In this case prolongation of 50 % graft survival to 70 days [Fig. 2A (6)] occurred with the MmT1/RmT1 protocol compared to 25 days using RmT1 alone [Fig. 2A (2)]. The anti-Ab analysis in this former group showed no anti-Ig response to RmT1 in three of five mice; in two mice, minute concentrations of 0.04 μg/ml anti-Ab could be detected (Fig. 2B MmT1/RmT1). By contrast, mice without preinjection of MmT1 showed anti-RmT1 antibody levels of 388.2 ± 146.6 μg/ml (Fig. 2B RmT1/RmT1).

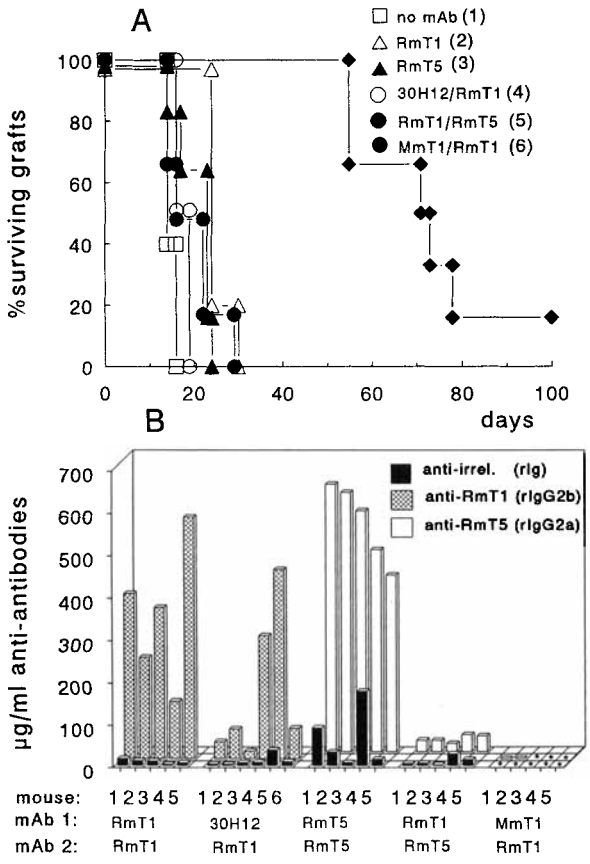

Figure 2. Prolonged survival of minor and MHC antigens incompatible skin grafts and reduced anti-rat Ig response in mice treated with anti-T cell antibodies. (A) and (B) As described in Fig. 1; *p* values of statistical significance: 1 *vs.* 2 = 0.009, 2 *vs.* 6 = 0.024, 2 *vs.* 4 = 0.005, 3 *vs.* 5 = not significant.

3.3 Skin graft survival and anti-Ig response after sequential treatment with mAb differing in idiotype and/or isotype

Compared with the 60- to over 100-day 50 % graft survival using sequential species-mismatched mAb treatment, clearly poorer (up to 30-day) 50 % graft survival was observed with MmT5 followed by MmT1 [Fig. 1A (3)]. Both mouse antibodies share the Thy-1.2 specificity, isotype (IgG2a) and Igh type but differ by idiotype. Unlike the xenogeneic antibody combination [RmT1/MmT1, Fig. 1A (4) and 1B], the anti-MmT1 response in mice injected with isotype-matched pairs was only moderately reduced and reached 88.5 ± 51.4 μg/ml about 10 days after graft rejection (Fig. 1B MmT5/MmT1). Surprisinlgy, these anti-Ab were not directed against the shared iso-determinants (IgG2a) but recognized predominantly the idiotype of the second mAb (MmT1) as proved by the almost negative binding of the irrelevant IgG2a mAb (Fig. 1B MmT5/MmT1). These anti-Ab showed also considerable neutralizing capacity. As shown in Fig. 1B (insert), serum titer causing half-maximal inhibition of binding of MmT1 to C57BL/6 lymph node cells ranged between 1 in 27 to 1 in 72.

Similarly, sequential treatment with rat anti-Thy-1 mAb matched by isotype (IgG2b) but differing in idiotype, did not significantly improve graft survival in the 30H12/RmT1 combination [Fig. 2A (4)]. Anti-Ab analysis revealed 156.1 ± 172.5 μg/ml of anti-RmT1 Ab 7 days after graft rejection (Fig. 2B 30H12/RmT1).

Finally, sequential injection of rat mAb differing both in idiotype and isotype also did not prolong graft survival [Fig. 2A (5)]. Preinjetion of C57BL/6 mice with RmT1 (rat IgG2b-anti-Thy-1) followed by RmT5 (rat IgG2a anti-Thy-1, different idiotype) injected twice weekly until CBA skin raft rejection caused an intermediate suppression of anti-RmT5 response (30.2 ± 8.4 μg/ml; Fig. 2B RmT1/RmT5) as compared to 541.0 ± 91.7 μg/ml (Fig. 2B RmT5/RmT5) detected in mice without the preinjection step. Apparently, this inhibition was not sufficient for improvement of skin graft survival as showed in Fig. 2A(5).

4 Discussion

The study of anti-Ab production in mice injected with allogeneic, partially of fully T cell-depleting or modulating mAb provides a model for understanding antiglobulin responses in human 'species-adapted' antibody therapy. We have studied the immunological consequences *in vitro* and *in vivo*, of injecting allogeneic, T cell-depleting mAb, in a mouse skin graft model where graft rejection is reproducibly observed within 4 weeks in both the CBA-to-C57BL/6 and C57BL/6-to-CBA combination ([14] and Kremmer et al., manuscript submitted). Our data showing only 10-day improved graft survival after changing from a course of mouse anti-mouse-Thy-1.2 mAb MmT1 to MmT5 differing in idiotype only was unexpected and may prove disappointing from a clinical perspective. Correspondingly, this injection protocol was not successful for avoiding anti-Ab production [Fig. 1A (3) and 1B MmT5/MmT1]. In contrast, the sequential injection of anti-T cell antibodies from different species resulted in the suppression of

99–100% of anti-Ab levels (Table 2) and significantly prolonged graft survival. Thus, a switch of antibody species after the first injection of a 'priming' antibody which, by itself, did not affect graft survival caused prolonged immunosuppression during the course of the subsequent second Ab injections. The suppressive effect of this approach did not depend on the ordered specificity of the first or second Ab since these Ab were interchangeable in their sequence for producing the observed immunosuppression. A failure to prolong survival was, however, observed with two anti-T cell mAb from the same species differing in isotype and/or idiotype. Thus, skin graft survival of mice treated with a first injection of rat IgG2b anti-Thy-1 (RmT1) followed by injections of rat IgG2a anti-Thy-1 (RmT5) was not better than in the control groups treated with RmT1 and 40H12 (differing in idiotype only) or with RmT1 alone [Fig. 2A (2, 4, 5)]. This observation may be explained by insufficient suppression of anti-Ab formation which may then reach levels high enough to inhibit T cell binding of the rat IgG2a Ab in our mouse model (Table 2I,J). These findings contrast with much longer skin graft-survival times after sequential high-dose treatment with rat IgG2b/IgG2a anti-mouse CD4 + CD8 mAb reported by Cobbold et al. [11, 12]. Here the authors did not attribute the effect of their Ab treatment to species differences between first and second Ab but to a 'non-depleting' and modulating quality of the IgG2a Ab used. In other experiments involving isotype-specific tolerance to rat Ig, the same investigators did not utilize a combined Ab treatment protocol but used instead their Ab isotypes singly. Thus, they injected rat IgG2a (or IgG2b) anti-CD4 + CD8 mAb and rechallenged with rat IgG2a (or correspondingly, IgG2b) mAb (with human specificity) in adjuvant 6 weeks later. In contrast, the present approach was always most suppressive when switching Ab species. Furthermore, it was not dependent on non-T cell-depleting Ab specificities and was highly suppressive when using Ab combinations involving mouse IgG2a or rat IgG2b isotypes. These Ab have previously been shown to prevent mortality from secondary disease in fully mismatched mice [13, 14]. Furthermore, our findings were also valid when using anti-Thy-1 mAb (RmT1/MmT1 and MmT1/RmT1) without including anti-CD4 + CD8 mAb in the Ab treatment (Figs. 1 and 2). In addition to the necessary species switch of Ab, the time lapse between first and second Ab injection was found to be crucial. Thus, the suppressive principle failed if first and second Ab were combined in the priming injection. Intutively, one might have expected that increasing the immunosuppressive dose by combining mAb would have increased rather than decreased suppression of anti-Ab production.

It is tempting to compare the present results with classical experiments on epitope-specific suppression [15, 16], *i.e.* the remarkably specific suppression of anti-hapten Ab in carrier/hapten-carrier-immunized mice with which our data share the phenomenon of almost complete inhibition of a specific Ab response. However, in this hapten-carrier model Ab against hapten are suppressed, whereas those against the carrier protein are not. In contrast, if one assumes the Fc portion and idiotype of our boosting anti-T cell Ig protein to function as carrier and hapten epitope, respectively, we find in our mice an inhibited Ab response to both target specificities. One might also make an analogy between our findings and previous experiments involving helper T cell-dependent carrier effects which showed reduced secondary Ig responses to haptens complexed with certain xenogeneic protein carriers [17–19]. However, the interpretation of our results must be more complex since the effects of Ab-induced depletion of T cells would most certainly be superimposed with any type of carrier-mediated suppressor activity. B cells like cytotoxic T cells (CTL) require 'help' from helper T cells (T_H) to differentiate into effector cells. This means that antigen receptor occupancy in the absence of lymphokines produced by T_H cells results in unresponsiveness. Thus, one might expect that the initial T cells depletion caused by the injection of the first antibody prevented the second 'mismatched' Ab from inducing its own helper cell- and cytokine-mediated anti-Ab response. The study shows that anti-Ab can already appear during a first course of heavy Ab treatment. While it documents their consequences leading to curtailed graft survival or prolongation of immunosuppression if anti-Ab are avoided, the type of ultimate rejection of skin grafts in mice with low concentrations of anti-Ab is still under investigation.

In summary, the present study delineates a versatile approach for preventing the body's immune system from making Ab to species-adapted as well as to non-chimeric xenogeneic anti-T cell mAb. As such, this approach may prove useful for prolonging immunosuppression in cases requiring chronic Ab therapy.

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