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Induction of tolerance in peripheral T cells with monoclonal antibodies*

Our goal has been to develop ways to tolerize the mature immune system to any defined antigen. In this report we show that peripheral (post-thymic) T cells of mice can become tolerant to a range of antigens (human and rat immunoglobulins, and bone marrow and skin grafts that differ at multiple minor transplantation antigens). In the case of human gamma globulin (HGG), this required that the antigen be given under the cover of a short course of non-depleting anti-CD4 antibody, while for tolerance to skin and marrow grafts anti-CD8 antibody was also required. Tolerance to HGG could be reinforced by repeated injections of HGG, but was lost in the absence of any further exposure to antigen. This reversal of tolerance with time was due to new T cells being exported from the thymus, as it was not observed in tolerized, adult thymectomized mice. In contrast, tolerance to marrow and skin grafts was permanent, presumably because the established grafts acted as a continuous source of antigen to reinforce the tolerant state. Tolerance could not be broken by the infusion of unprimed spleen cells and in one example (tolerance to Mls-1[a]) there was clear evidence that specific peripheral T cells were anergic. We propose that anergic cells may themselves participate in reinforcing the tolerant state by competing at sites of antigen presentation.

1 Introduction

The immune system is characterized by its capacity to respond to the outside world of "non-self" while remaining unresponsive to "self". This self tolerance operates through processes which inactivate lymphocytes either *centrally* in the thymus and BM, or *peripherally* for antigens that are more restricted in their expression in other tissues. In a therapeutic context it is essential to determine to what extent *peripheral* tolerance can be imposed on a mature immune system. If it were possible to re-establish tolerance in autoimmune diseases or to guarantee it to a transplanted allograft, then it might be possible to dispense with the conventional, long-term drug immunosuppression and its attendant risks and side effects.

We and others [1–9] have observed that mAb to CD4 can be potent immunosuppressive agents. Furthermore, this immunosuppression is associated with the creation of a tolerance-permissive environment such that specific unresponsiveness can be achieved to xenogeneic proteins and BM grafts mismatched for multiple minor transplantation antigens [10–13]. In the latter case anti-CD8 antibodies were also necessary, presumably to facilitate a symmetrical state of tolerance in $CD8^+$ cells [13].

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Abbreviations: ATx: Adult thymectomized **HGG:** Human gamma globulin

In this report we now extend these findings to demonstrate direct tolerance to skin allografts, and to answer the following questions relating to mechanisms of tolerance induction and maintenance.

(a) It has recently been shown that for tolerance to xenogeneic Ig depletion of $CD4^+$ T cells was not essential [14–16]. Can non-depleting anti-CD4 antibodies be used to permit *peripheral* tolerance to xenogeneic proteins and allogenic BM and skin grafts? We describe the use of non-depleting rat IgG_{2a} anti-CD4 and anti-CD8 antibodies for this purpose.

(b) Is the tolerant state induced with non-depleting antibodies truly *peripheral*, or does it require a functional thymus? We show that tolerance can be induced in mice that have been thymectomized as adults.

(c) What determines whether tolerance is maintained once it has been induced? It has been shown, and we here confirm, that euthymic mice will eventually lose tolerance to human gamma globulins (HGG) unless further *reinforcing* doses of "antigen" are given [16]. In contrast, thymectomized mice remain tolerant indefinitely. Given that antigen is required to maintain tolerance in euthymic mice, then one would expect BM and skin grafts to *reinforce* for themselves, once established. This is shown to be the case as tolerance is permanent, with no further need for an extrinsic source of antigen.

(d) What happens to antigen-specific T cells in the process of peripheral tolerance induction? In the case of tolerance to the superantigen Mls-1[a] we have previously shown, using a T cell *depleting* protocol, that the $V_\beta6^+$ T cells which recolonize the periphery are anergic to stimulation by antigen [13]. Now, using a *non-depleting* protocol, we show that tolerance to Mls-1[a] can be induced in the *periphery*, without a thymus, and that the tolerant T cells are anergic as they can no longer be triggered *in vitro*, neither by antigen nor by antibodies to the $V_\beta6^+$ antigen receptor.

(e) Can the tolerant state be overcome by infusion of normal lymphocytes? We have found that only primed T cells could break the tolerant state. We speculate that anergic cells in the tolerant recipient may be able to act as

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impotent competitors at sites of antigen presentation, which may in part explain the resistance of tolerant animals.

On the basis of these findings we suggest that peripheral T cell tolerance is feasible with relatively simple, low impact, antibody protocols, and that once induced, this tolerance should be robust and permanent when a constant source of antigen, such as a grafted organ, is present.

2 Materials and methods

2.1 Animals

Experimental mice were bred and kept in the conventional animal facility in the Department of Pathology, University of Cambridge, and were used in age- and sex-matched groups.

2.2 Isolation of mAb with transfected cells

The anti-CD4 mAb, clone YTS 177.9 was selected by its binding to a rat Tcell line NB2-6TG (kindly provided by Dr. J. Howard, Cambridge, GB) transfected with mouse CD4 [17]. Similarly, the anti-CD8 mAb, clone YTS 105.18 was selected by its binding to Ly-2-transfected mouse L cells ([18]; kindly provided by Dr. R. Zamoyska, London, GB). The isotype of both mAb was determined by their reaction with RG7/1.7, an mAb against rat IgG_{2a} [19].

2.3 Immunofluorescence staining of PBL

Mouse PBL were separated by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden). Cells at the interface were collected and washed in PBS with 1% BSA and 0.05% sodium azide. They were then stained with biotinylated mAb and followed by FITC-streptavidin (Amersham Int., Amersham, GB). The results were analyzed by FCM with a Cytofluorograph (model 50-H; Ortho, Westwood, MA) and data were processed by an Ortho 2150 computer.

2.4 Detection of murine antibody responses

Sera from experimental mice were titrated in flat-bottom flexible plates (Becton Dickinson, Mountain View, CA) coated with purified HGG or rat Ig and incubated for 30 min. The plates were washed with PBS/0.05% Tween 20 (Sigma, Poole, GB) and then incubated with biotinylated sheep anti-mouse IgG (Amersham) for 30 min, then washed as above. Streptavidin-horseradish-peroxidase (Amersham) was added for 15 min. After another three washes, the reaction was developed by 5 mg/ml o-phenylenediamine and 0.1% hydrogen peroxide. The absorbance at 490 nm was read and titers determined by comparison with a standard positive control.

2.5 Induction of tolerance to HGG and rat Ig

Tolerance induction to HGG was based on a previously described protocol [10]. Normal CBA/Ca mice were injected with anti-CD4 mAb i.v. on day −1, i.p. on day 0 and 1. One milligram of heat-aggregated HGG was given i.p. on day 0. The mice were rechallenged with 0.5 mg aggregated HGG on various days afterwards as described in the text. IgG responses to HGG were measured by ELISA.

To determine the responsiveness to rat Ig, normal CBA/Ca mice were injected on three consecutive days with rat IgG_{2a} or rat IgG_{2b} anti-CD4 mAb partially purified by ammonium sulfate precipitation of ascites fluid. Six weeks later, they were challenged with a mixture of a rat IgG_{2a} mAb to human CDw52 (YTH 34.5; [20]) and a rat IgG_{2b} mAb to human CD3 (YTH 12.5; [20]), first in CFA and then in IFA once a week for 3 weeks. The mice were then bled and their serum anti-rat Ig titers measured by ELISA using microtiter plates coated with HPLC-purified rat IgG_{2a} or rat IgG_{2b} mAb.

2.6 Preparation of BM cells for transplantation

BM cells were harvested from donor mice pretreated with anti-CD4 and anti-CD8 depleting antibodies to remove T cells. Live AKR/J (2×10^7) BM cells or 1×10^7 BM plus 1×10^7 spleen cells (B10.BR) were transferred into suitably prepared recipients to determine whether tolerance could be induced. Chimerism of AKR/J cells in CBA/Ca could be determined by measurement of donor Ig allotype or donor Th-1.1 allotype as described previously [13].

2.7 Skin grafting and adult thymectomy

The methods of skin grafting, assessment of graft survival and statistical analysis were as described before [13]. Mice were thymectomized at 4 weeks of age by the method of Monaco et al. [21] and used at least 4 weeks later. All were checked when killed and none were found incompletely thymectomized.

2.8 Adoptive transfer of spleen cells

Donor spleen cells were obtained from either naive CBA/Ca mice or CBA/Ca mice that had been presensitized to B10.BR spleen cells and skin grafts 4–8 weeks previously. These were transferred into tolerant CBA/Ca recipients that had been treated with mAb, grafted with B10.BR skin and had carried the grafts for more than 200 days. Each recipient mouse was injected i.v. with 5×10^7 spleen cells. As controls, adult thymectomized (ATx) mice were treated with rat IgG_{2b} anti-CD4 and anti-CD8 mAb to deplete $CD4^+$ and $CD8^+$ cells. Such mice are unable to reject allogeneic grafts [1] and were used here to demonstrate that the transferred cells could function.

2.9 MLC

Responder spleen cells were washed and resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated human AB serum. Stimulator spleen cells were treated with 25 μg/ml mitomycin C (Sigma) at 37 °C for 30 min and washed twice in

IMDM. Responder cells (3×10^5) and stimulators (3×10^5) were cultured in 96-well flat-bottom microtiter plates at 37 °C with 5% CO_2. On day 4, cells were pulsed with 10 μCi/ml [^{125}I]dUrd (Amersham) for 6 h. Incorporation was measured in a gamma counter. Geometric means of triplicates were calculated.

2.10 Stimulation of lymphocytes with mAb

The mitogenic anti-$V_\beta 6$ mAb, 46-6B5 was kindly given by Dr. H. Hengartner [22]. The mAb was coupled onto 96-well flat-bottom plates at 1 μg/ml at 37 °C overnight, then rinsed extensively with IMDM before use. The anti-CD3 mAb, 145-2C11, was a kind gift from Dr. J. Bluestone (University of Chicago, Chicago, IL; [23]), and was used at 1:100 dilution of serum-free tissue culture SN. Spleen cells (4.5×10^5) were added into each well, in IMDM and 10% heat-inactivated human AB serum. Proliferation after 4 days of culture was measured as above.

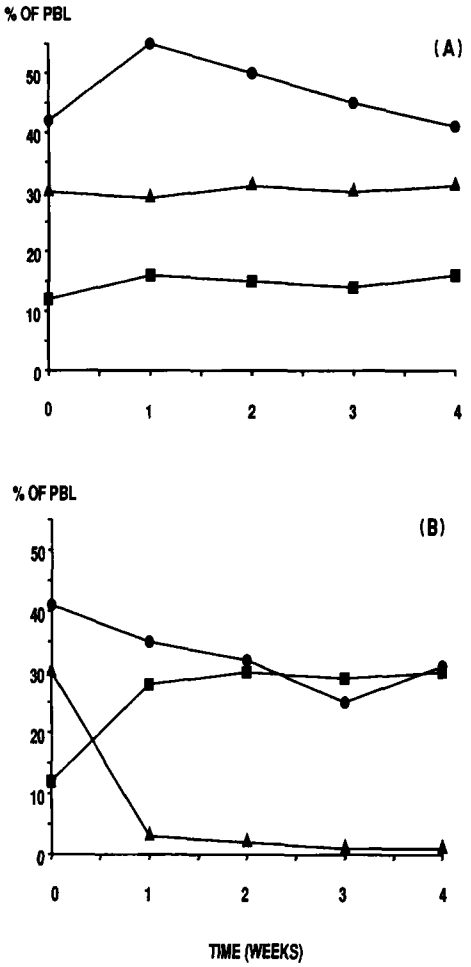

Figure 1. A rat IgG_{2a} CD4 mAb does not deplete $CD4^+$ cells. ATx CBA/Ca mice ($n = 3$) were given a single injection of (A) YTS 177.9 (rat IgG_{2a} anti-CD4 epitope a) or (B) YTS 191.1 (rat IgG_{2b} anti-CD4 epitope a) at a dose of 2 mg/mouse and bled weekly. PBL were stained with biotinylated mAb against CD4 (YTA 3.1 epitope b, ▲), CD8 (YTS 156.7 epitope b, ■) and Thy-1 (YBM 29.2, ●), followed by FITC-streptavidin. The results were analyzed by FCM. Similar results were obtained after repeated mAb injections for up to 3 weeks (data not shown).

3 Results

3.1 The rat IgG_{2a} anti-CD4 mAb does not deplete T cells *in vivo* but rather modulates antigen expression

Two milligrams of the rat IgG_{2a} anti-CD4 mAb YTS 177.9 was injected into ATx mice, which allowed us to distinguish antibody-mediated target cell depletion, temporary antigenic modulation or lymphocyte redistribution. Control ATx mice received 2 mg of the depleting rat IgG_{2b} antibody YTS 191.1. PBL were analyzed by FCM, using biotin-labeled anti-CD4 and anti-CD8 antibodies against different epitopes to those injected, before and at various times after treatment. As can be seen in Fig. 1b, injection of a rat IgG_{2b} anti-CD4 mAb resulted in depletion of about 90% $CD4^+$ cells from the periphery, associated with a decreased percentage of total T cells and an increase in $CD8^+$ cells. In contrast, the rat IgG_{2a} mAb treatment did not significantly change the cellular profile (Fig. 1a). However, the fluorescence intensity of $CD4^+$ cells in these mice was substantially diminished (not shown). As the injected antibodies do not block the detecting anti-CD4 reagent we interpret this as antigenic modulation. A single injection resulted in antigenic modulation of PBL $CD4^+$ cells for up to 3 weeks, after which the fluorescence intensity returned to normal levels.

When this non-depleting anti-CD4 mAb was used together with YTS 105.18, the rat IgG_{2a} CD8 mAb, for 3 weeks of therapy it emerged that the combination produced minimal reduction in T cell numbers, but significant modulation of CD4 antigen expression (Fig. 2). The status of the $CD8^+$ cells remaining after therapy is not clear because the staining antibodies (to epitopes a and b) are both partially blocked by the injected YTS 105.18 (epitope c). However, the total number of rat IgG_{2a}-coated cells was greater than $CD4^+$ cells alone and there was also a significant Thy-$1^+CD4^-CD8^-$ population in treated naimsl which, when taken together, suggest that $CD8^+$ cells had also not been depleted.

3.2 Tolerance to HGG and rat IgG

Previous work using low doses of rat IgG_{2b} anti-CD4 mAb pairs or anti-CD4 $F(ab')_2$ mAb fragments has suggested that mAb-facilitated tolerance to certain protein antigens may not need depletion of $CD4^+$ T cells [14–16]. In the present study, we investigted if the non-depleting anti-CD4 mAb could also do the same. Mice were given YTS 177.9 (rat IgG_{2a}) or YTS 191.1 (rat IgG_{2b}) in a 3-day course and one injection of HGG (0.5 mg/mouse) on the second day (day 0). When rechallenged with HGG 4 weeks later, both sets of anti-CD4 mAb-treated mice had become tolerant to HGG (Fig. 3). This tolerance was specific to HGG as control mice, which received YTS 177.9 but no HGG, responded normally.

The same anti-CD4 mAb regimen also induced tolerance to other Ig of the same isotype as is the case for depleting anti-CD4 mAb [12]. Data in Fig. 4 show that 1 mg of YTS 177.9 rendered mice completely tolerant to rat IgG_{2a}. The tolerance so induced was specific as these mice were still able to respond to rat IgG_{2b} Ig. Likewise, mice that were tolerant to rat IgG_{2b} by treatment of YTS 191.1 were

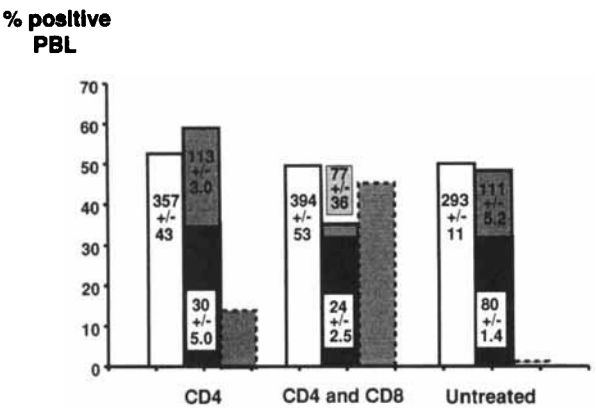

Figure 2. The effect of injection of rat IgG_{2a} anti-CD4 and anti-CD8 mAb on the peripheral blood T cells. Normal CBA/Ca mice ($n = 4$) were given injections of the rat IgG_{2a} anti-CD4 mAb (YTS 177.9 epitope a) alone or together with a rat IgG_{2a} anti-CD8 mAb (YTS 105.18 epitope c) for 3 weeks. Animals received three antibody injections per week to a total antibody dose of 7 mg. One week after the last mAb injection, mice were bled and PBL stained with biotinylated anti-Thy-1 (open bars), anti-CD4 epitope b (solid bars), anti-CD8 eptiopes a and b (shaded bars) followed by FITC-streptavidin, or FITC-anti-rat Ig to detect *in vivo* bound mAb (broken bars). The results were analyzed by FCM with linear amplifiers and mean fluorescence values are indicated.

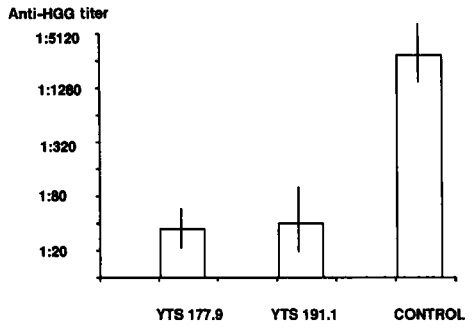

Figure 3. Tolerance to HGG induced by a rat IgG_{2a} anti-CD4 mAb. Normal CBA/Ca mice ($n = 6$ per group) were given 1 mg/mouse YTS 177.9 (rat IgG_{2a} anti-CD4) or YTS 191.1 (rat IgG_{2b} anti-CD4) on days −1, 0 and 1. One milligram heat-aggregated HGG was injected on day 0. The mice were rechallenged with 0.5 mg HGG on days 28 and 35. Control mice received 1 mg of YTS 177.9 as others, but immunized with HGG only on days 28 and 35. Serum anti-HGG titers were measured on day 45 by an ELISA assay.

responsive to rat IgG_{2a} (Fig. 4). This clearly demonstrates that the tolerance is highly specific in a reciprocal manner to the two different rat isotypes.

The fact that rat IgG_{2a} anti-CD4 mAb produced tolerance to rat IgG_{2a} proved useful for the studies described below where we injected rat IgG_{2a} anti-CD4 and anti-CD8 mAb for up to 3 weeks to induce tolerance to allografts without any problems from anti-globulin responses.

3.3 Peripheral tolerance to HGG

It has previously been shown that tolerance to HGG induced by depleting anti-CD4 antibody is lost in normal

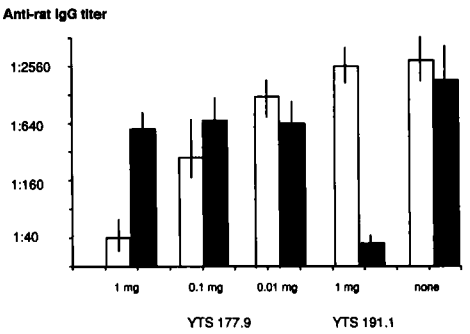

Figure 4. Injection of a rat IgG_{2a} anti-CD4 mAb can induce specific tolerance to rat IgG_{2a}. Normal CBA/Ca mice ($n = 6$ per group) were given three injections of YTS 177.9 or YTS 191.1 at the indicated doses. Six weeks later, they were rechallenged with rat IgG_{2a} and rat IgG_{2b} (rat anti-human mAb) in adjuvant. Anti-rat IgG_{2a} (open bar) and anti-rat IgG_{2b} titers (solid bar) were measured from 4-week bleeds with an ELISA assay using plates coated with HPLC-purified rat IgG_{2a} or rat IgG_{2b} mAb, respectively.

mice unless maintained by further challenge with antigen [16]. It was therefore of interest to investigate the role of the thymus in the induction and maintenance of tolerance in mice where the anti-CD4 antibody does not deplete T cells. Normal and ATx mice were given three injections of non-depleting rat IgG_{2a} anti-CD4 mAb and immunized with HGG. When rechallenged with HGG 6 weeks after mAb therapy, it was found that both groups of mice were tolerant to HGG (Fig. 5). This demonstrates that the thymus is *not necessary* for the induction of tolerance and, therefore, the tolerance so induced must be *peripheral*. However, while thymectomized mice would never again respond to HGG, euthymic mice deprived of further HGG (for more than 200 days) eventually recovered their responsiveness to HGG. We infer that the loss of tolerance to HGG in euthymic mice was due to *new T cells* exported from the thymus which restore responsiveness in the absence of antigen. It should be noted that control euthymic and ATx mice were treated identically except that they received no HGG during antibody treatment, so the failure of ATx mice to respond cannot have been due to any nonspecific immunosuppression.

3.4 Tolerance and anergy in mice grafted with multiple minor antigen-mismatched BM

A combination of rat IgG_{2a} anti-CD4 and rat IgG_{2b} anti-CD8 permitted chimerism and tolerance by AKR/J BM (Mls-1^a; Fig. 6a). As tolerance could be achieved without depletion of $CD4^+$ cells we had the opportunity to follow the fate of $CD4^+V_\beta6^+$ CBA/Ca T cells in the periphery of the recipient. Four weeks after mAb and BM infusion the spleen cells of these animals were unable to respond by proliferation to Mls-1^a *in vitro* and were also poorly reactive to solid-phase stimulation with a mitogenic anti-$V_\beta6$ mAb (Table 1). Two-color FCM analysis showed normal numbers of $CD4^+V_\beta6^+$ cells in the periphery (these were shown to be of recipient Thy-1.2 allotype). Clearly the $CD4^+V_\beta6^+$ T cells were anergic to stimulation. As the anti-CD4 mAb does not deplete T cells these were assumed to be peripheral T cells that had been anergized.

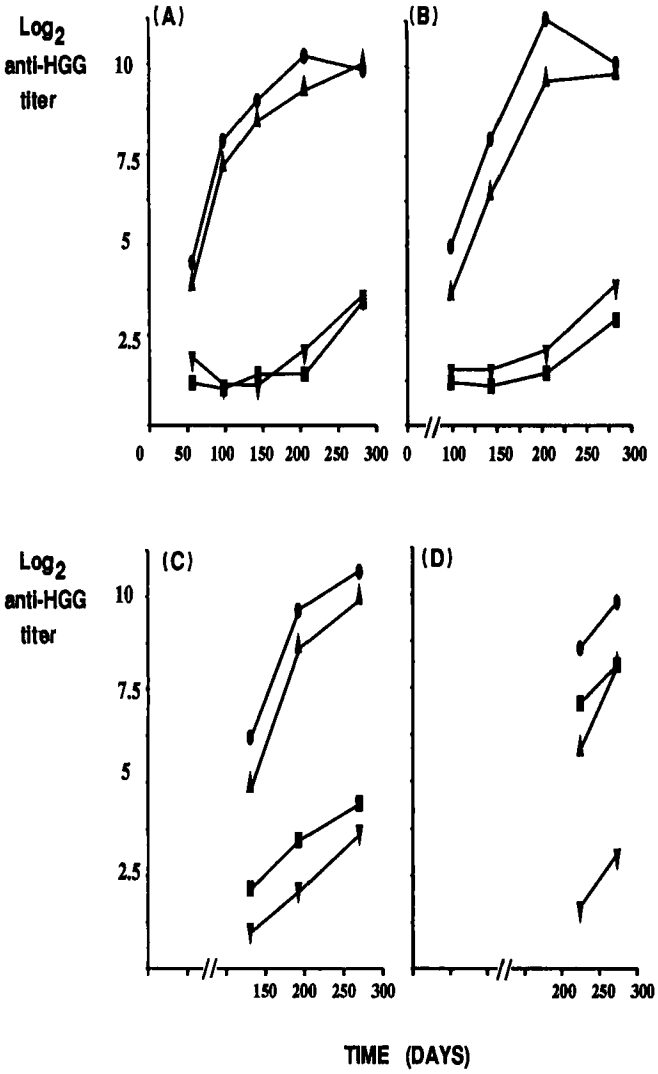

Figure 5. Tolerance to HGG in normal and thymectomized mice. Tolerance to HGG was induced in normal (■) or ATx mice (▼) with rat IgG_{2a} anti-CD4 mAb and HGG as in the legend to Fig. 3 (3 mg of mAb in three doses, with HGG given on day 0). Six to eight mice were randomly selected from the groups at various times after tolerance induction and rechallenged with two doses of 0.5 mg HGG i.p. and bled 7 days later. Control mice were normal (●) or ATx (▲) mice which had received anti-CD4 mAb only with no HGG on day 0. These mice were also challenged with HGG on the same days that the mAb + HGG-treated mice were rechallenged. (A) Mice first rechallenged on days 40, 49 and bled on day 56, with further challenge followed by bleeds on days 98, 143 and 213; (B) mice first rechallenged on days 82, 91 and bled on day 98, with further challenges followed by bleeds on days 143 and 213; (C) mice first rechallenged on days 127, 136 and bled on day 143 with a further challenge and bleed on day 213; (D) mice first rechallenged on days 213, 224 and bled on day 233. All the mice were boosted on days 266 and 276, and bled on day 283.

To confirm that the thymus was not essential for tolerance of *peripheral* Tcells AKR/J BM was again used, this time to tolerize ATx mice to Mls-1^a. Again the spleen T cells became anergic to Mls-1^a and $V_\beta 6$ stimulation (Table 2). Neither the injection of BM nor antibody alone could reproduce this. Clearly the combination of antibodies and BM was required to bring about long-term peripheral Tcell anergy of Mls-1^a-specific T cells.

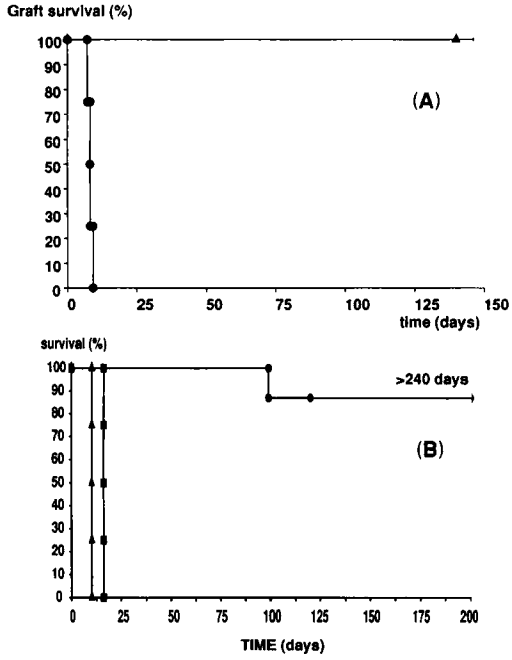

Figure 6. (a) Tolerance to AKR/J BM and skin grafts. Normal CBA/Ca recipient mice were injected for 3 weeks with a combination of rat IgG_{2a} anti-CD4 and rat IgG_{2b} anti-CD8 mAb. AKR/J BM cells (2×10^7) were infused i.v. 2 days after the beginning of mAb treatment. Four weeks after the end of antibody treatment, they were grafted with AKR/J skin (▲). Control mice were antibody treated only (●). (b) Tolerance to B10.BR BM and skin grafts. Recipient CBA mice ($n = 8$) were given 1–3 weeks of injections of a combination of rat IgG_{2a} anti-CD4 and anti-CD8 mAb, following an infusion of 10^7 T-depleted spleen cells and 10^7 BM cells (●) from B10.BR mice. Control mice ($n = 4$) were given either BM without mAb (▲) or anti-CD4 and anti-CD8 antibodies alone (■). All groups were grafted 30 days later with B10.BR skin. Chimerism of donor allotype Ig measured at day 60 and day 260 after BM injection was $2.3 \pm 1.9\%$ and $0.9 \pm 0.7\%$, respectively. None of the mice in the BM only or antibody alone groups showed any detectable chimerism ($< 0.2\%$). Skin grafts in the tolerant group were held indefinitely (> 240 days: one graft was scored as a rejection on day 96, as shown, but later recovered with full hair growth) while control groups rejected with median survival time (MST) = 10 days (BM only group) and 16 days (antibody only group). Second B10.BR skin grafts given to the tolerant (BM plus antibody) group 90 days after the first skin were all accepted (MST > 150 days), while the control groups rejected such second grafts within 7 days (not shown).

Although it was necessary for us to deplete $CD8^+$ cells to get chimerism in this strain combination, this was not the case in another (B10.BR BM plus spleen cells into CBA/Ca) where we found that the pair of rat IgG_{2a} anti-CD4 and anti-CD8 mAb were sufficient to establish chimerism and tolerance (Fig. 6b). It should be noted that, in a previous publication [13], we were not able to achieve tolerance in this strain combination when we gave BM without spleen cells and used a different frequency of antibody treatment.

3.5 Tolerance to skin grafts mismatched for multiple minor transplantation antigens without depletion of $CD4^+$ T cells

We wished to determine whether a longer course of mAb treatment would permit tolerance to be induced to skin

Table 1. Tolerance of Mls-1^b mice to Mls-1^a antigen is associated with anergy of $V_\beta6^+$ cells[a)]

Mouse no.	Responses to (cpm) AKR/J	BALB/c	Anti-$V_\beta6$	Anti-CD3	Negative	$V_\beta6^+CD4^+$ cells (%)
Exp. 1						
1	772	11117	583	12530	596	10.2
2	439	8964	1173	9879	460	12.1
3	961	6498	1050	7719	703	8.7
4	1203	12866	1163	13496	1082	7.5
5	859	10784	764	11230	977	7.8
Normal mice						
1	20756	9633	10440	11980	868	7.0
2	27529	17253	10530	13291	642	10.2
Marrow only						
1	38264	10042	13950	19087	815	8.1
2	25832	8679	7267	13563	976	9.0
Exp. 2						
1	1078	13414	1158	14632	1050	5.6
2	879	9412	926	13211	573	4.0
mAb only						
1	15494	8987	4677	15469	751	4.6
2	18751	13133	8546	18211	955	5.1
Normal mice						
1	21162	13451	7882	14835	1120	3.9
2	12775	12551	5833	20112	997	5.2

a) Normal CBA/Ca mice were treated with rat IgG_{2a} anti-CD4 (YTS 177.9) and rat IgG_{2b} anti-CD8 (YTS 156.7) for 3 weeks with a total of 10 mg mAb/mouse. AKR/J BM cells (2×10^7) were injected. Four weeks after stopping antibody treatment, spleen cells from these mice were tested in MLC and fluorescence stained for two-color FCM. AKR/J spleen cells used as negative control contained $< 1\%$ $CD4^+V_\beta6^+$ cells. Figures given for cpm are geometric means of triplicates.

Table 2. Tolerance and anergy to Mls-1^a in ATx Mls-1^b mice[a)]

Mouse	Responses to (cpm)[b)] AKR/J	BALB/c	Anti-$V_\beta6$	Negative	$V_\beta6^+CD4^+$ cells[c)] (%)
1	1348	8081	696	885	2.8
2	1145	10231	812	521	2.9
3	998	7311	976	833	3.7
4	1039	6508	793	978	3.3
Control[d)]	12463	10283	7374	957	3.8
Mice	15578	8864	5824	1021	2.9

a) ATx CBA/Ca mice were injected with equal amounts of rat IgG_{2a} anti-CD4 mAb (YTS 177.9) and rat IgG_{2b} anti-CD8 mAb (YTS 156.7) for 2 weeks (total 7 mg mAb/mouse). AKR BM cells (2×10^7) were infused 2 days after the beginning of mAb treatment. Eight weeks later, their spleen cells were stimulated *in vitro* as described in Sect. 2.9.

b) Numbers given are cpm (geometric means of triplicate samples) of each individual mouse.

c) Spleen $V_\beta6^+CD4^+$ cells were stained with mAb 44-22-1 [22] detected by FITC-MARG2A (Serotec, Oxford, GB) and biotin-YTS 191.1 detected by PE-streptavidin (Serotec). Samples were analyzed by two-color FCM using logarithmic amplifiers. Labeled AKR/J spleen cells as negative controls contained $< 1.0\%$ $V_\beta6^+CD4^+$ cells.

d) Control mice were ATx CBA injected with mAb only.

allografts. CBA/Ca mice were transplanted with B10.BR skin and injected with rat IgG_{2a} anti-CD4 and anti-CD8 mAb for 2 weeks (7 mg mAb/mouse in total). At the end of mAb treatment, fluorescence staining revealed no significant depletion of $CD4^+$ T cells and residual antibody could not be detected after day 42 (data not shown). All the mAb-treated mice accepted their skin grafts for > 90 days. A second donor-type skin, together with a third-party (BALB/c) graft was transplanted at that time. Both the first and second B10.BR grafts continued to survive for > 200 days, confirming tolerance, while the BALB/c skin was rejected without delay (Fig. 7a), thereby proving the mice were immunocompetent. As $CD4^+$ T cells are the major subpopulation determining rejection in this model, it seems likely that tolerance had been induced in mature peripheral $CD4^+$ T cells. Since $CD8^+$ cells can reject B10.BR skin in their own right, albeit slowly, complete tolerance was not observed when the rat IgG_{2a} mAb to CD4 was used without the anti-CD8 mAb ([13] and Fig. 7b). It should be emphasized that long-term tolerance to skin and indeed BM does not require any further extraneous reinforcing doses of antigen. CBA/Ca mice tolerant to B10.BR skin or BM which were left for 120 and 370 days, respectively, before testing with a second skin graft were still completely tolerant ([13] and data not shown).

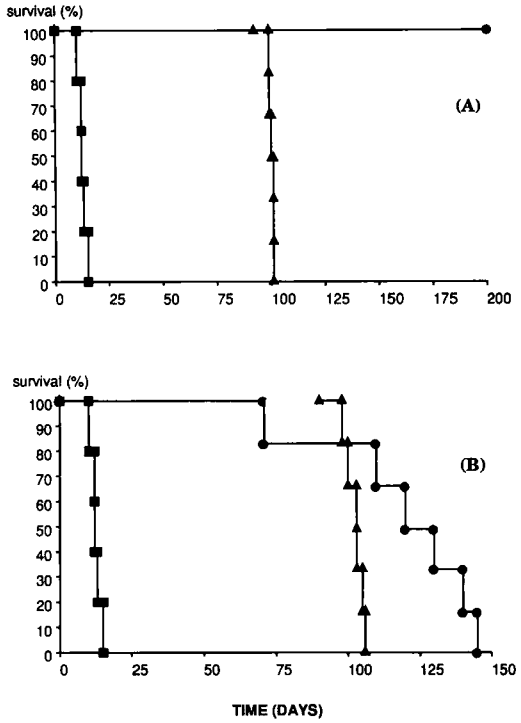

Figure 7. Tolerance to skin grafts alone with non-depleting anti-CD4 and anti-CD8 mAb. Normal CBA/Ca mice ($n = 6$) were injected with YTS 177.9 together with a rat IgG_{2a} anti-CD8 mAb YTS 105.18 (A) every other day for 2 weeks. Another group of mice were given YTS 177.9 alone (B). Three days after the first mAb treatment (day 0), they were grafted with B10.BR skin. On day 90, animals that carried B10.BR skin were regrafted with B10.BR and BALB/c skin. (●) mAb-treated mice, first and second grafts; (■) untreated mice, MST = 13 days; (▲) BALB/c grafts, MST = 10 days.

3.6 The demonstration of peripheral tolerance to skin allografts in ATx mice

To confirm that we were looking at bona fide peripheral tolerance induced by the skin grafts, we repeated the experiment in ATx mice. As the rat IgG_{2a} mAb do not kill T cells, we expected that immune functions in these mice should return to normal soon after the cessation of antibody therapy. Certainly by day 60 they could reject allogeneic skin grafts at the normal rate, confirming immunocompetence. As was found in euthymic mice, tolerance was induced to the test skin and second B10.BR grafts were accepted indefinitely (Fig. 8).

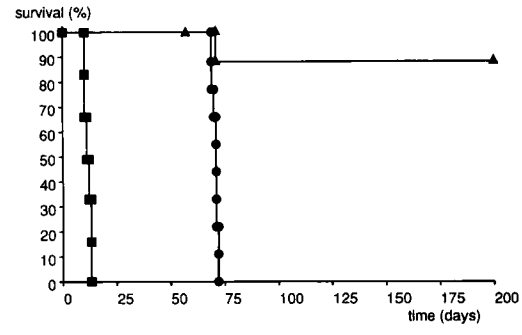

Figure 8. Tolerance to skin grafts can be established in ATx mice. ATx mice ($n = 9$) were injected with rat IgG_{2b} anti-CD4 and rat IgG_{2a} anti-CD8 mAb every other day for 2 weeks. B10.BR skin was grafted 3 days after the beginning of mAb treatment. On day 60, mice were regrafted with B10.BR and BALB/c skin. BALB/c grafts (●) were rejected by tolerant ATx mice carrying B10.BR skin grafts with MST = 12 days (▲). Control ATx mice were also grafted with B10.BR skin (■) MST = 12 days.

3.7 The "peripheral" tolerant state is refractory to infusion of normal lymphocytes

In the above skin allograft tolerance model, we asked whether the adoptive transfer of normal lymphocytes could terminate the tolerant state. Spleen cells (50×10^6) transfused from normal donors could not restore the ability to reject the graft (Table 3). A perennial problem with this type of experiment is to show that the infused cells survive and function. In part this was controlled for by transferring the same number of spleen cells into T-depleted ATx mice. In this case the transferred cells were able to mediate skin graft rejection. Only "primed cells" transferred into tolerant mice could break the tolerant state (Table 3). Similar results were found in the mice tolerant to HGG, where tolerance could not be broken by adoptive transfer of normal cells unless recipient $CD4^+$ T cells had been depleted first (Wise, M., in preparation). Whatever the difficulties of controlling such experiments may be, we must conclude that tolerant animals resist the ability of virgin T cells to express their normal function.

Table 3. Transfer of normal lymphocytes does not break tolerance in normal mice[a)]

Animals	Cells injected[b)]	Graft survival after cell transfer
Tolerant CBA	B10.BR spleen cells	>100 days × 4
	Naive CBA spleen cells	>100 days × 4
	Sensitized CBA spleen cells[c)]	15, 17, 17, 21
T-depleted[d)] ATx CBA	Naive CBA spleen cell	12, 14, 14, 15
	Sensitized CBA spleen cells	8, 8, 9, 10
	None	>100 days × 4

a) CBA/Ca mice were rendered tolerant to B10.BR skin by grafting on day 3 with injections of rat IgG_{2a} anti-CD4 and anti-CD8 mAb for 3 weeks (total of 7 mg).
b) Mice carrying B10.BR skin for >200 days were selected and injected i.v. with 5×10^7 spleen cells.
c) Sensitized spleen cells were from CBA mice which had been injected with B10.BR spleen cells and had rejected B10.BR skin graft.
d) ATx CBA mice were depleted of Tcells by injection of anti-CD4 and anti-CD8 mAb at least 4 weeks before they were grafted with B10.BR skin.

4 Discussion

Since the first demonstrations that anti-CD4 antibodies can create a tolerance-permissive environment *in vivo*, there have been numerous attempts to establish antibody-

mediated tolerance in the transplantation of allografts [13, 24–27]. Long-term survival of islet and fetal heart grafts has been possible in rodents, as well as tolerance of skin grafts in conjunction with cyclosporin A. In the latter tolerance depended upon an intact thymus [27]. In general all the above models have used anti-CD4 antibodies that depleted their target cells *in vivo*. Non-depleting anti-CD4 mAb have also been shown to be immunosuppressive [28, 29]. In this report we have focused on the question of whether *non-depleting* anti-CD4 antibodies (combined where necessary with either depleting or non-depleting anti-CD8 mAb) can be used to induce tolerance to a range of antigens, in particular to HGG and skin and BM allografts.

One of the outstanding questions has been whether tolerance can be induced in the mature T cells of the *peripheral* immune system without any influence of the thymus. Since most previous studies with allografts have depended on depletion of a large proportion of peripheral Tcells, it was not clear whether tolerance in those studies had been induced in *immature* Tcells newly generated from the thymus. Our data with thymectomized mice show unequivocally that mature *peripheral* Tcells are tolerizable under antibody cover. In these studies they have been tolerized to minor transplantation antigens present in, or released for reprocessing by, the allograft. The duration of *peripheral tolerance* would seem to depend upon the persistence of the tolerogen (antigen) to continually inactive Tcells as they are exported from the thymus [30]. Mice tolerant to HGG remained so indefinitely in the absence of a thymus, whereas euthymic mice came out of the tolerant state by day 213. In a previous report we showed, and here confirm that euthymic mice regularly reminded of antigen could have their tolerant state reinforced indefinitely [16]. In the case of skin and BM grafts we presume that the presence of grafts continuously provided tolerogen to maintain the tolerant state.

In the transplantation models we have used we know that both $CD4^+$ and $CD8^+$ cells participate in the rejection process [3]. Therefore, to achieve transplantation tolerance with mAb, it is important to consider how to control both the $CD4^+$ and $CD8^+$ T cell subsets. It is apparent that non-depleting rat IgG_{2a} or $F(ab')_2$ anti-CD8 mAb [31] can facilitate tolerance in $CD8^+$ Tcell in the way that anti-CD4 antibodies do so for $CD4^+$ T cells. However, there are circumstances where simply depriving $CD8^+$ Tcells of the help from $CD4^+$ cells was sufficient to attain tolerance [31]. In such cases the antigenic differences between donor and recipient were weak (Qa-1 or H-Y). In our examples there were many minor antigens differing between host and graft so that perhaps "help" may have come from within the $CD8^+$ population itself [32]. Treatment with anti-CD8 mAb may then have interfered with this $CD8^+$ help as well as the $CD8^+$ effectors. The net outcome though was operational tolerance of $CD8^+$ cells as they could no longer participate in rejection of first or second grafts.

We were able, in CBA/Ca mice tolerized to AKR/J BM to show the persistence of anergic peripheral Tcells expressing their $V_\beta 6^+$ receptors. We have previously described a similar situation in mice conditioned with *depleting* antibodies. As then, anergy was not possible without antibody treatment. In contrast, Rammensee et al. have since reported Tcell anergy induced simply by injection of Mls-1^a spleen cells into Mls-1^b mice [33]. As Mls-1^a-expressing cells would eventually disappear it is perhaps not surprising that anergy was short lived in the experiments of Rammensee et al., but in our model, hemopoietic chimerism was established to maintain a continued source of Mls-1^a.

There are now many reports of tolerance by anergy in peripheral Tcells [33–35]. It is compelling to conclude that anergy is one natural mechanism to maintain peripheral tolerance throughout life. In the examples shown here the anergic state seemed absolute. However, in a previous report we identified some tolerant mice where $V_\beta 6^+$ Tcells were unresponsive to Mls-1^a but could still be stimulated by anti-$V_\beta 6$ mAb [36]. This finding raises the question whether anergy is quantitative rather than absolute. In other words, is it possible that Tcells have ways of raising their triggering thresholds?

We have in the past tried but failed to find cells with suppressor qualities by adoptive transfer into irradiated hosts in the HGG tolerance model [16]. We have seen that the transfer of normal cells into tolerant mice in any of the three models (HGG; Wise, M., in preparation; BM plus skin, [36]; and skin alone, Table 3) does not break the tolerant state. This cannot be simply a question of "space" in terms of the immune system physically excluding transferred cells because primed cells were capable of rejecting in the same environment. One explanation would be that tolerant, perhaps anergic, Tcells themselves pre-empt any response of naive cells at sites of antigen presentation, competing with the transferred normal cells and preventing the development of a response-permissive microenvironment.

In this report we have shown that peripheral virgin Tcells can be tolerized to antigens that we can classify as processable (HGG and minor non-MHC antigens). In the accompanying article we proceed to demonstrate ethat CD4 and CD8 antibodies can also be used to allow primed cells to be tolerized by "minors" and indeed to allow operational tolerance to MHC-disparate grafts [37]. It is clear that at no stage of their development are T cells excempt from tolerogenesis if the right circumstances are contrived.

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Note added in proof: We have recently obtained tolerance to B10-BR skin using anti-CD4 mAb alone, but starting 1 week before skin grafting.

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