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ORIGINAL ARTICLE

# Suppression extends to major histocompatibility antigens linked to tolerizing minor histocompatibility antigens, but not the other way round

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## Summary

'Active suppression', a mechanism of transplantation tolerance, can spread to newly introduced minor antigens once these antigens are linked to tolerizing antigens. We explored whether this suppression can extend to major histocompatibility (MHC) antigens and whether this phenomenon can be demonstrated once tolerance is induced to a MHC antigen. Mice were tolerized using donor bone marrow plus CD4 and CD8 monoclonal antibodies. The following strain combinations were used: AKR (H-2k) into CBA (H-2k), a multiple minor difference and B6 (H-2b) into B6$^{bm12}$ (H-2b), a MHC class II difference. Tolerance was tested by a donorskingraft. CBA mice tolerant to AKR received a second skin carrying either AKR antigens plus additional multiple minor antigens [F1(AKRxBalb.K)] or carrying additional minors and a MHC class I antigen (B10.AKM-H2M). B6$^{bm12}$ (H-2b) tolerant to B6 (H-2b) were grafted with skin from a Balb.B donor (Balb minors linked to the tolerizing class II antigen) or from a B10.A(3R) strain (a MHC class I antigen linked to the tolerizing class II antigen). CBA mice tolerant to AKR accepted F1(AKRxBalb.K) skin, whereas F1(CBAxBalb.K) were rejected. Rejection of B10.AKM/H2M skin by tolerant mice was delayed as compared with nontolerant mice. Tolerant and nontolerant B6$^{bm12}$ mice rejected Balb.B skin and B10.A(3R) skin within the same time. Thus, in this model, suppression was linked to minors. Alloreactivity against minors and majors could be suppressed. Suppression linked to a class II antigen could not be demonstrated.

## Introduction

In the last three decades, 1 year renal allograft survival in most centers improved from 70% to more than 90%. Long-term allograft survival failed to improve as dramatically and 10 years renal allograft survival has remained about 60% [1]. Most grafts are lost because of chronic rejection. Moreover, immunosuppressive regimens are a major cause of morbidity. In theory, both problems can be overcome by induction of transplantation tolerance. Therefore, exploration of the mechanisms of tolerance and ways to induce and maintain a tolerant state are important topics in transplantation research. In murine models, transplantation tolerance can be induced against skin [2], heart [3], pancreas islet cells [4], liver [5] and bone marrow [6] with the use of various immunosuppressive drugs sometimes combined with myeloablation, i.e. radiation or cytotoxic drugs [7]. Mechanisms involved in the process of tolerance induction range from deletion of the alloreactive clones [8,9] to active suppression [10]. At first side, clonal deletion might yield the most robust form of tolerance [11]. However, complete deletion is difficult to achieve without the help of toxic myeloablative strategies [7,12,13]. Active suppression implies an ongoing balance between suppression and alloreactivity [14], but has shown to be

very robust [15]. Active suppression has interesting features; it can be transferred to the immunesystem of the recipient [6,10]. Furthermore, it can protect against alloreactivity to third party antigens, when these antigens are presented together with the tolerizing antigens on the same cell [6,16]. This latter phenomenon is called linked suppression. The necessity of the presence of the second party disappears with time and the recipient becomes tolerant to the third party itself. Up to now, linked suppression was described in models in which tolerance was induced across a multiple minor histocompatibility barrier, with third party minor antigens linked to the tolerizing minors. In addition, there was some evidence that linked suppression could extend to major antigens.

In this study we aimed to explore this so-called linked suppression. First, will it prevent alloreactivity against major histocompatibility antigens linked to the minors? Secondly, does it occur after tolerance induction across a major histocompatibility difference? The latter might have some clinical potency: after tolerizing to one MHC difference, tolerance might, one by one, extend to other MHC differences.

## Materials and methods

### Mice

The CBA, AKR, Balb.K, Balb.B and B10.A(3R) mice were purchased by Harlan Olac (Bicester, Great Britain) and maintained at the animal facility of the Laboratories of Experimental Medicine of the Academic Medical Centre (Amsterdam, The Netherlands). F1(Balb.KxAKR) mice and F1(Balb.KxCBA) mice were bred in the same animal facility. B10.AKM/H2M, B6, and B6$^{bm12}$ were obtained of The Jackson Laboratory (Bar Harbor, ME, USA). Table 1 lists the various mice strains used in the experiments and their genetic background. All animals were treated in accordance to the rules of the local animal ethical committee and according to the principles of laboratory animal care. The research protocol was approved by the local animal ethical committee.

**Table 1.** Mouse strains and their histocompatibility typing.

| Mouse strain | Majors | Minors |
|---|---|---|
| CBA | H-2K$^k$I$^k$D$^k$L$^k$ | CBA |
| AKR | H-2K$^k$I$^k$D$^k$L$^k$ | AKR |
| Balb.K | H-2K$^k$I$^k$D$^k$L$^k$ | Balb |
| B10.AKM/H2M | H-2K$^k$I$^k$D$^q$L$^q$ | Black (shares minors with AKR) |
| B6 | H-2K$^b$I$^b$D$^b$L$^b$ | Black |
| B6$^{bm1}$ | H-2K$^{bm1}$I$^b$D$^b$L$^b$ | Black |
| B6$^{bm12}$ | H-2K$^b$I$^{bm12}$D$^b$L$^b$ | Black |
| Balb.B | H-2K$^b$I$^b$D$^b$L$^b$ | Balb |
| B10.BR | H-2K$^k$I$^k$D$^k$L$^k$ | Black |
| B10.A(3R) | H-2K$^b$I$^{b/k}$D$^d$L$^d$ | Black |

### Surgery

Skin grafting was performed according to a modified procedure of Medawar [17]. Full thickness donor tail skin, approximately 0.7 × 1.0 cm was transplanted onto the lateral thoracic wall of recipients. A second skin graft was transplanted on the opposite side. Skin grafts were monitored at least three times a week after removal of the bandages at day 8 after transplantation. Rejection was complete when no viable graft tissue was seen on the thoracic wall.

### Monoclonal antibodies

Rat hybridomas producing monoclonal antibodies against mouse CD4 and CD8 were a kind gift of Professor H Waldmann, Dunn School of Pathology, Oxford, U.K. Antibodies were produced, purified and dialyzed into phosphate-buffered saline (PBS) in our laboratory.

### T cell depletion

T cell depletion of the donor before harvesting bone marrow cells was achieved with a single intraperitoneal injection of 0.5 mg YTS 191.1 plus 0.5 mg YTS 3.1. (rat IgG2b anti mouse CD4 monoclonal antibodies) and 0.5 mg YTS 156.7 plus 0.5 mg YTS 169.4 [18].

### Preparation of bone marrow cells

Mice were T cell depleted, using the above mentioned monoclonal antibodies. Then 3–5 days after T cell depletion, mice were culled and bone marrow cells were obtained by flushing the femoral bones with ice cold Iscore's modified Dulbecco's medium (IMDM)/2% fetal calf serum (FCS). Cells were washed, counted and, right before injection, resuspended in 200 µl 0.9% NaCl.

### Tolerizing protocol

The various strains used and their H2 typing are listed Table 1. Tolerance to AKR minors was achieved by the intravenous administration of 1X10$^5$ T cell depleted AKR bone marrow cells on day 1 in CBA recipients. In addition mice received three intraperitoneal injections on day 1,3 and 5 after bone marrow infusion consisting of 0.5 mg nondepleting CD4 rat IgG2a monoclonal antibodies (177.9) plus two synergistic and depleting CD8 rat IgG2b monoclonal antibodies (Hybridoma YTS 156.7 and YTS 169.4, 0.25 mg of each). After 10 weeks tolerance

**Table 2.** Origin and H-2 typing of second skin graft after tolerance induction across a minor histocompatiblity barrier.

| Recipient strain | Tolerance to AKR | Second skin graft | Antigens linked to tolerizing antigens |
|---|---|---|---|
| CBA | Yes | F1(AKRxBalb.K) | Balb minors |
| CBA | Yes | F1(CBAxBalb.K) | None |
| CBA | No | F1(AKRxBalb.K) | |
| CBA | Yes | B10.AKM/H2M | MHC class shared and new minors |
| CBA | No | B10.AKM/H2M | |

**Table 3.** Origin and H-2 typing of second skin graft after tolerance induction across a MHC class II histoincompatiblility barrier.

| Recipient strain | Tolerance to B6 | Second skin graft | Antigens linked to tolerizing antigens |
|---|---|---|---|
| $B6^{bm12}$ | Yes | Balb.B | Balb minors |
| $B6^{bm12}$ | No | Balb.B | |
| $B6^{bm12}$ | Yes | B10.A(3R) | MHC class I plus class II |
| $B6^{bm12}$ | No | B10.A(3R) | |

was tested by grafting an AKR skin or a third party skin (Balb.K). Control mice received antibodies only.

Tolerance induction across a H-2 class class II difference was performed by the intravenous infusion of $1X10^5$ T cell depleted B6 bone marrow cells on day 1 into $B6^{bm12}$ recipients plus six intraperitoneal injections of the above mentioned cocktail on day 1,3,5,7,9 and 10 after bone marrow infusion. Tolerance was tested by grafting B6 skin after 10 weeks. Control $B6^{bm12}$ recipients received antibodies only.

### Linked tolerance

Four to 6 weeks after the first skin graft, a second skin graft was placed on the contralateral chest wall. Tables 2 and 3 demonstrate the types of skin used after tolerance induction across a minor histocompatibility barrier and across minor and major histocompatibility barrier respectively.

### Statistical analysis

Median survival time (MST) was calculated as the time that 50% of the grafts had rejected. Statistical analysis was performed using a log rank test.

## Results

### Tolerance induction to multiple minors

*CBA mice can be made tolerant to AKR*

Tolerance to multiple minor antigenic differences was proven by transplantation of the H-2k AKR skin into the H-2k CBA/Ca recipient mouse. These two strains differ at the level of multiple minor antigens. The recipients were pretreated with monoclonal antibodies and AKR bone-marrow. Six weeks later the recipients accepted AKR skin indefinitely, whereas controls – treated with antibodies only – all rejected AKR skin within 100 days ($P < 0.003$). Third party skin, with again a multiple minor difference to the CBA/Ca strain (Balb.K) were rejected by the control group and the tolerized group within 30 days.

*CBA recipients tolerant to multiple minors (AKR) accept skin carrying tolerizing minors plus new minors*

Next, experiments were performed to study whether these CBA mice, made tolerant to AKR, would also be tolerant to co-expressed new minor antigens. Therefore we transplanted CBA recipients, tolerized to AKR, with donor skin derived from a F1(AKRXBalb.K) mouse. These grafts were accepted indefinitively, despite the new antigens. To rule out dilution of antigens as a possible cause of acceptance of the F1(AKRXBalb.K) donor skin, control experiments were performed with F1(CBAXBalb.K) donorskin grafted on CBA/Ca mice tolerant to AKR. The latter grafts were rejected on day 10 ($n = 3$), 12 ($n = 3$), 49 and 65 after transplantation. The median survival time was 12 days for F1(CBAXBalb.K) donorskin versus undefined for F1(AKRXBalb.K) donorskin ($P < 0.0007$) (Fig. 1).

*CBA recipients tolerant to multiple minors (AKR) demonstrate delayed graft rejection of a B10.AKM-H2M skin graft*

It has been shown that the B10 background shares minors with AKR [16]. As compared the CBA/Ca strain, there is a difference at the level of class I antigens and at the level of multiple minors. This strain was readily available in our lab, so we choose it as a second skin donor, to investigate whether CBA/Ca mice tolerant to AKR and – via the shared antigens to the Black minors –, would

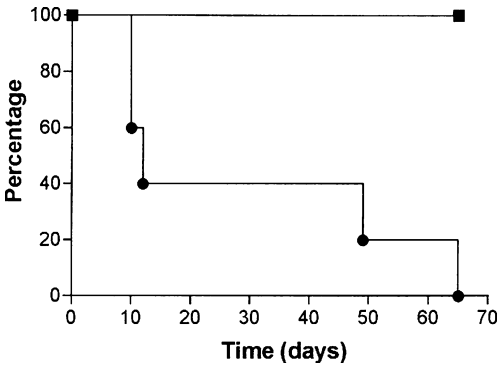


**Figure 1** Linked tolerance to a new set of minor antigens. CBA recipients tolerant to AKR accepted F1(AKRxBalb.K) skin grafts (■) indefinitely, whereas F1(CBAxBalb.K) skin grafts (●) were rejected ($n = 8$ in each group, $P < 0.0007$).

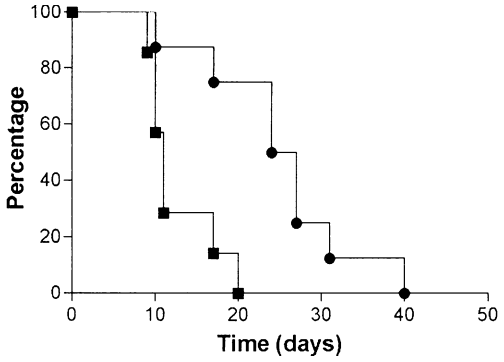


**Figure 2** Linked suppression spreads to MHC antigens. CBA recipients tolerant to AKR (●) showed delayed rejection of B10.AKM skin grafts as compared with nontolerant CBA (■) ($n = 8$ in each group, $P < 0.0039$).

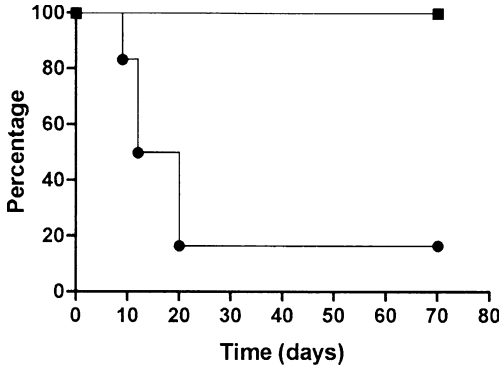


**Figure 3** $B6^{bm12}$ mice can be made tolerant to B6. $B6^{bm12}$ mice treated with monoclonal antibodies plus B6 bone marrow (■) accepted B6 skin permanently, in contrast to $B6^{bm12}$ mice treated with antibodies only (●) ($n = 8$ in each group, $P < 0.02$).

demonstrate tolerance to linked H2 class I antigens. Recipients did not show full tolerance to the B10.AKM-H2M strain, but graft survival was significantly increased as compared with controls: grafts were rejected at day 10, 17, 24 ($n = 2$), 27 ($n = 2$), 31 and >31 after transplantation by the CBA/Ca recipients tolerant to AKR versus day 9, 10 ($n = 2$), 16 ($n = 2$), 17 ($n = 2$) and 20 days after transplantation by the nontolerant CBA/Ca. Fig. 2 shows a median survival time of B10.AKM-H2M skin grafts of 26 days, when grafted on CBA mice tolerant to AKR, versus 16 days when grafted on CBA mice treated with antibodies only ($P < 0.0039$).

### Tolerance induction across a MHC class II difference

*$B6^{bm12}$ mice can be tolerized to B6 with a regimen of monoclonal antibodies and B6 derived T cell depleted bone marrow cells*

To explore the possibility of tolerizing across a MHC class II difference, we treated $B6^{bm12}$ mice with monoclonal antibodies against B and T cells plus T cell depleted bone marrow cells derived from a B6 strain. These strains differ only at the level of a MHC class II locus. Tolerance was tested by grafting B6 skin 8 weeks after the last antibody injection. B6 skin was permanently accepted by the $B6^{bm12}$ mice, whereas controls treated with antibodies only rejected B6 donorskin at day 9, 10 ($n = 3$), 21 ($n = 3$) and >42 after transplantation. Median survival time of B6 skin in control recipients was 16 days ($P < 0.02$) (Fig. 3) ($N = 6$).

*$B6^{bm12}$ mice tolerant to a MHC class II difference reject skin grafts carrying the tolerizing class II difference plus a new set of minors in the same time as nontolerant $B6^{bm12}$ mice*

The next experiments where performed to test the quality of the tolerance created in the $B6^{bm12}$ mice across the above described class II mismatch. Was it possible to create suppression of rejection to a new set of minors (Balb) linked to the tolerizing MHC class II antigens? Balb.B donorskin grafts, grafted on $B6^{bm12}$ recipients tolerant to B6, were rejected at day 11, 14 ($n = 4$) and day 18 after transplantation, whereas Balb.B donorskin grafts were rejected at day 11 ($n = 2$), 14, 18 and 24 ($n = 2$) by control $B6^{bm12}$ recipients. Median survival time of the second skingraft in the tolerant group was 16 days versus 14 days in the nontolerant recipients (not significant, NS) (Fig. 4).

As we could not demonstrate suppression of rejection of *minor* antigens linked to a tolerizing MHC class II antigen, we were not surprised that mice, tolerant to the above mentioned class II antigen, showed no suppression of rejection of *major* transplantation antigens linked to the tolerizing class II antigen.

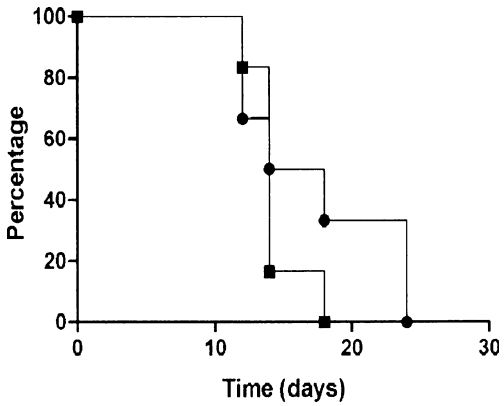


**Figure 4** $B6^{bm12}$ tolerant to B6 mice show no linked suppression to linked minor antigens. $B6^{bm12}$ mice tolerant to B6 (■) rejected Balb.B skin in the same time as nontolerant $B6^{bm12}$ mice (●) ($n = 6$ in each group, NS).

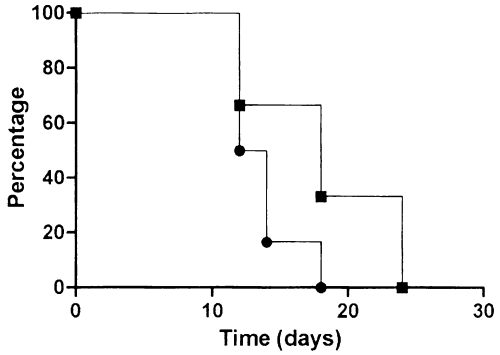


**Figure 5** B6$^{bm12}$ mice tolerant to B6 demonstrate no linked suppression to MHC antigens. B6$^{bm12}$ mice tolerant to B6 (■) rejected B10.A(3R) skin in the same time as nontolerant B6$^{bm12}$ mice(●) ($n = 6$ in each group, NS).

B6$^{bm12}$ mice tolerant to a MHC class II difference rejected second skin grafts from a B10.A(3R) strain at day 12 ($n = 2$), 18 ($n = 2$), and 24 ($n = 2$) after transplantation (mean graft survival time 18 days) whereas nontolerant B6$^{bm12}$ mice rejected these grafts at day 12 ($n = 3$), 14 ($n = 2$) and 18 (median graft survival time 13 days, NS) (Fig. 5).

## Discussion

These results show, as has been demonstrated before, that transplantation tolerance against minors can be achieved with the use of monoclonal antibodies and bone marrow [10,19,20]. Then, when the tolerizing antigens are presented to the immune system of the recipient in combination with a new set of minor antigens, tolerance spreads to these new antigens. To explore whether suppression linked to minor antigens can extend to a major antigen, we choose B10.AKM-H2M mice as second skin donors. This strain was readily available in our lab and previously, it has been shown that the B10 background shares antigens with the AKR background. In addition, although B10.AKM-H2M is an inbred strain, there still might be some minor antigens expressed derived from the AKR founder, encoded in regions flanking the H2 gene. Tolerance was not achieved but the alloreactive response seems to be suppressed as skin graft rejection is delayed. This is in concordance with the result of Davies *et al.* [16]. She demonstrated linked suppression to (CBKxB10.BR)F1 skin grafts in CBA made tolerant to B10.BR. Her tolerizing protocol consisted of the same monoclonal antibodies as we used but as tolerizing agents she used skin instead of bonemarrow. In this experiment the linked antigen was a single MHC class I antigen. Two of seven mice became truly tolerant, showing no rejection of a third skin graft derived from a CBK strain. Chen *et al.* also showed linked suppression extending to class I and II antigens in the less stringent murine heart transplantation model. First, he injected fully immunocompetent CBA/Ca mice with spleencells of CBA/Ca made tolerant to Balb/c. Then he transplanted the Balb/c tolerant CBA/Ca with a heart graft derived from a F1(Balb/cXC57/Bl10) The latter grafts were accepted by 50% of the recipients, whereas control C57/Bl10 grafts were rejected in 8 days [21]. In our experiments none of the CBA tolerant to AKR accepted permanently skin grafts of B10-AKM-H2H. Apparently, regulatory mechanisms of the immune response were present and decreased the alloreactive response against the linked antigens but lacked the power to induce full tolerance to an allo MHC class I molecule and new minor antigens.

Tolerance could be achieved across a MHC class II difference using the same cocktail of monoclonal antibodies although we had to use a higher dose. This is what we expected, first because mice with a black background are less susceptible to tolerizing protocols as compared with other strains [22,23] and second because tolerance had to be established across a H-2 barrier. When tolerant recipients were confronted with their tolerizing antigens in combination either with new multiple minor antigens alone or new MHC antigens, linked tolerance could not be induced nor was there any evidence of linked suppression. Apparently, regulatory mechanism seemed to be of less importance in tolerance across this MHC class II barrier. Several explanations can be given. First, we had to use a higher dose of monoclonal antibodies in the B6 into the B6$^{bm12}$ combination in order to achieve tolerance. High doses of the anti-CD4 antibodies might induce some degree of -polyclonal- deletion within the CD4 T cell population and thus deletion of potential regulator clones. Another possibility is that re-exposure of recipient CD4 cells to donor class II on donor dendritic cells and activated B cells also facilitates clonal deletion of recipient CD4 cells [24]. It has been demonstrated that within this cell population regulatory mechanisms develop [6,10,21]. That induction of tolerance to skin in murine models across a H-2 barrier requires some degree of deletion, has been made plausible by Turka *et al.* [25]. He showed that clonal deletion and death of the bulk of alloreactive T cells was an important mechanism in achieving tolerance to murine skin grafts across full MHC barriers.

Another explanation is the interstrain variability in the mechanism by which tolerance is established and that the balance clonal deletion/active regulation in the B10 strain is shifted towards deletion. Previously, we tolerized B10.BR mice to CBA. These mice showed linked suppression by accepting F1(CBAxAKR) skin, indicating that mice with a B10 background are capable of linked suppression (data not shown). Furthermore, there is some

evidence that even after tolerizing to a class II antigen in B6 mice regulatory mechanism develop as linked tolerance developed against a single minor antigen (male Y antigen linked to the tolerizing class II) [26]. Finally, the fact that we did not see any regulation might be because of the antigenic strength of our test graft: New minor antigens (Balb skin) were presented to the recipients in the context of class I and II molecules. Depletion of effector CD8 cells with depleting monoclonal antibodies might have led to acceptance of Balb skin, by uncovering weaker regulating mechanisms within the CD4 population.

The above mentioned tolerizing protocol could not induce tolerance in the MHC class I difference strain combination. $B6^{bm1}$ donor skin grafted on B6 recipients 10–12 weeks after bone marrow infusion plus antibody treatment was all rejected within 14 days and within the same time as the control group.

We conclude that in this particular strain combinations regulation lacks the power to establish tolerance across a MHC barrier. After inducing tolerance to a MHC class II antigen regulatory mechanisms fail to suppress the alloresponse to multiple minors.

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