T Cell Suppression in Transplantation Tolerance Through Linked Recognition[1]

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Allogeneic tissues transplanted to mice treated with CD4- and CD8-specific Abs are often accepted indefinitely due to the induction of immunologic tolerance. When transplantation tolerance was induced to grafts mismatched at multiple minor histocompatibility loci, Ag specificity was inferred because third party grafts, mismatched at the MHC, were rejected normally. However, some "third party" grafts were either accepted, or rejected more slowly. Tolerant mice possess $CD4^+$ cells, which suppress rejection by T cells reacting to the same grafts. Therefore, we hypothesized that tolerated third party grafts might share Ags with the original tolerizing graft, and that these Ags are a target for such suppression. To test this idea, we tolerized mice to a set of minor Ags (B10 minors) and challenged them with third party grafts that carried those minors, as well as an additional strong transplantation Ag, the class I MHC molecule, H-$2K^b$. This class I molecule acts as a good target for rejection in both naive mice and in mice tolerized to B10 minors. However, when this third party class I molecule is provided "linked" to those B10 minors on an F_1 graft, rejection was significantly impaired. The data suggest that suppression within tolerant animals operates locally (perhaps on the same APC) via linked recognition. In addition, our preliminary findings suggest that suppression via linked recognition can also lead to tolerance to the third party Ag. *The Journal of Immunology,* **1996, 156: 3602–3607.**

The specificity of immune responses has evolved to ensure adequate recognition of pathogens while still preserving self-tolerance. Although T cell activation requires the participation of diverse soluble mediators (1), and a variety of adhesion molecules (2), it is the TCR that determines the Ag specificity of the response (3). Triggering through this receptor can result in broadly "nonspecific" responses, but these are exceptional cases and occur as a result of activation by superantigens and other polyclonal stimulators (4–6). T cell tolerance is similarly Ag specific, and distinguishable from global immunosuppression (7, 8) by retention of third party responses (9–15). Recently, experimental protocols have been described that result in the induction of tolerance in the periphery of a mature immune system associated with the description of T cells that can suppress responses (Refs. 16–18, and Davies et al.[4]). The fine specificity of the peripheral response is remarkable in that responses to certain Ags, as well as the tolerizing Ags, can be impaired (19–23). In some cases, the authors concluded that the impaired "third party" response occurred (19), or was more substantial (23) if the tolerizing and third party Ags were presented by the same cell. However, dilution of Ag and loss of immunogenicity of one Ag when presented by the same APC as another Ag, could also have explained this data.

We have previously described a method that involves the induction of tolerance and suppression in the CBA/Ca mouse strain to the minor histocompatibility Ags of the B10.BR mouse strain. Here we investigate further the specificity of the tolerant state.

Materials and Methods

Mice

CBA/CaOla (H-2^k), B10.BROla (H-2^k), BALB.KOla (H-2^k), AKROla (H-2^k), C3H/HeOla (H-2^k) and BALB/cOla (H-2^d), B10.D2/nOla (H-2^d), and C57BL/10.ScSnOla (H-2^b) mice were provided by Harlan Olac, Bicester, U.K. CBK mice ($K^{b/b}$ and $K^{k/k}$, $A^{k/k}$, $E^{k/k}$, and $D^{k/k}$) were generated at the National Institute for Medical Research, Mill Hill, U.K. (24) by pronuclear microinjection of fertilized oocytes from CBA/CaOla inbred mice using a subclone containing the entire H-$2K^b$ structural gene and associated transcriptional control elements (a 10-kb *Eco*RI fragment from cosmid H8 (25)). Founders were bred to CBA/CaOla partners to maintain the transgene on an inbred CBA/Ca genetic background. As expected, H-$2K^b$ is expressed by all cell types in CBK mice. CBK, (CBK × B10.BROla)F_1 and (CBK × CBA/CaOla)F_1 were bred at the Pathology Department, Oxford University, Oxford, U.K. (CBA/CaOla × B10.BROla)F_1 and (CBA/CaOla × BALB/cOla)F_1 mice were bred and maintained at the Pathology Department, Cambridge University, Cambridge, U.K. All animals were treated in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Monoclonal Abs

All hybridomas secreting CD4-specific (YTS 191.1.2, YTA 3.1.2 (26, 27, both rat IgG2b) and YTS 177.9.6.1 (28, rat IgG2a)) and CD8-specific (YTS 169.4.2.1, YTS 156.7.7 (26, 27, both rat IgG2b) and YTS 105.18.10 (28, rat IgG2a)) mAbs were made in this laboratory. All mAbs were grown either as ascites or in culture and purified by precipitation in saturated ammonium sulfate. Whether they were grown as ascites or in culture made no detectable difference to the outcome of the experiments.

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[4] J. D. Davies, G. Martin, J. Phillips, S. E. Marshall, S. P. Cobbold, and H. Waldmann. 1995. T cell regulation in adult transplantation tolerance. *Submitted for publication.*

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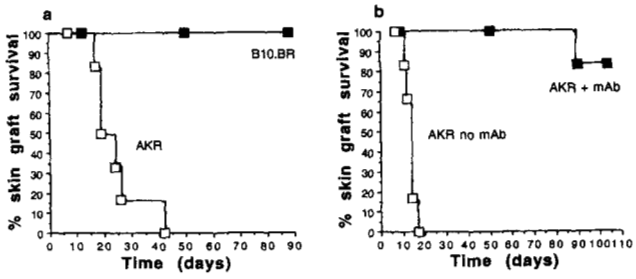

FIGURE 1. CD4- and CD8-specific mAbs induce specific tolerance to multiple minor histocompatibility Ags. *a*, CBA/Ca mice were made tolerant to B10.BR skin using the standard protocol. At least 2 mo after the first graft, specific tolerance to B10.BR skin was tested by regrafting with B10.BR (■, $n = 6$) and third party AKR (□, $n = 6$) skin. In all cases, the first B10.BR skin graft was not rejected. *b*, CBA/Ca mice were grafted with AKR skin with (■, $n = 6$) and without (□, $n = 6$) the standard tolerizing mAb protocol. The ability of CBA/Ca mice to be made tolerant to AKR skin graft proves the authenticity of AKR skin as a suitable graft to test for specific tolerance.

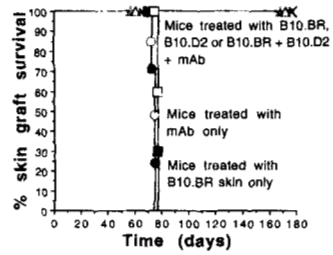

FIGURE 2. Tolerance is induced to graft minor Ags presented by both donor and host APC. (CBA/Ca × BALB/c)F_1 recipients were made tolerant to B10.BR ($n = 8$) B10.D2 ($n = 8$), or B10.BR plus B10.D2 ($n = 8$) skin using the mAb protocol indicated in *Materials and Methods*. On day 61, each mouse received a skin graft from both B10.BR (▲) and B10.D2 (△) donors. Since no grafts were rejected, the data have been pooled for clarity. Mice that had not received tolerizing skin or mAb therapy were grafted with BALB/K skin (X, $n = 6$). (CBA/Ca × BALB/c)F_1 mice that were grafted with B10.BR skin in the absence of tolerizing mAbs ($n = 7$) were regrafted on day 61 with both a B10.BR (●) and a B10.D2 (○) skin graft. (CBA/Ca × BALB/c)F_1 recipients that were treated with tolerizing mAbs in the absence of a graft ($n = 6$) were also grafted on day 61 with both a B10.BR (■) and a B10.D2 (□) skin graft.

Surgery

Thymectomy was performed at 5 to 6 wk of age. A 1-cm^2 section of tail skin was grafted onto the flank of the thymectomized host not earlier than 2 wk post-thymectomy (28, 29). The anesthetic, 1 mg metomidate (Hypnodil; Janssen Biochimica, Berse, Belgium) with 0.4 μg fentanyl citrate (Sublimaze; Janssen Biochimica) was used for both procedures. Skin grafts were monitored for evidence of rejection daily over the first 3 wk post-grafting, 3 times a wk for the next 3 wk, and weekly until around 12 wk when the experiment was terminated. Grafts were scored as having been rejected when no viable graft could be detected. Of the grafts that were scored as having survived, close to 100% of the graft was viable. Statistical analysis of graft survival was by the Log-rank method (30).

Tolerance induction

Two different protocols were used for the induction of tolerance. The protocol used for the induction of tolerance in the CBA/Ca strain consisted of four injections of a mixture of 1 mg of YTS 177.9.6.1 mAb (specific for mouse CD4), and 1 mg of YTS 105.18.10 mAb (specific for mouse CD8, both nondepleting rat IgG2a) per injection. Each dose was given i.p. into each mouse over the first week after skin grafting (28). Thymectomy was performed before mAb treatment to distinguish peripherally induced tolerance from central tolerance (28). For the induction of tolerance in the (CBA/Ca × BALB/c)F_1 strain, euthymic recipients were immunosuppressed with a mAb mixture of 0.25 mg of each of YTS 191.1.2 (26), YTA 3.1.2 (27; both depleting rat IgG2b, CD4 specific), YTS 169.4.2.1 (26), and YTS 156.7.7, (27; both depleting rat IgG2b, CD8 specific) per injection, on days −3 (i.v.) and −1 (i.p.), followed by 0.5 mg of each of YTS 177.9.6.1 and YTS 105.18.10 (28) per injection on days 4, 6, 8, 11, 13, and 15 (i.p.). The presence of a thymus allows T cell reconstitution in these mice to a level of around 70% of the pretreatment level, in both the CD4 and CD8 subsets, by 45 days post-mAb treatment (21). In all cases, day 0 corresponds to the day of the first skin graft. Different tolerizing protocols were used because the experiments that included tolerizing the (CBA/Ca × BALB/c)F_1 were conducted at a time before we knew that the depleting mAbs were not needed for tolerance induction to minor histocompatibility Ags.

Results

CD4- and CD8-specific mAbs induce specific tolerance to multiple minor histocompatibility Ags

CBA/Ca mice can be made tolerant to a multiple minor histocompatibility Ag-mismatched skin graft (B10.BR) with nondepleting anti-CD4 and anti-CD8 mAbs (28). Tolerant mice do not reject a second B10.BR graft when given as long as 3 mo after the first, whereas mice given mAbs 3 mo previously but without a tolerizing graft reject B10.BR skin within 3 wk (J. Davies, data not shown). Here we show that such tolerant mice can reject third party minor Ag-mismatched grafts. A skin graft from the AKR mouse strain is rejected with a median survival time (MST)[5] of 19 days (Fig. 1*a*). The validity of AKR as a third party control is confirmed by showing that tolerance can be induced in CBA/Ca mice to AKR skin using the same mAb protocol as that used to tolerize these mice to B10.BR skin (Fig. 1*b*). The delay in rejection time of AKR skin by CBA/Ca mice tolerant to B10.BR skin (MST = 19 days, Fig. 1*a*) vs untreated CBA/Ca mice (MST = 14 days, Fig. 1*b*) will be discussed later.

Tolerance can be induced to donor minor Ags presented in the context of both donor and host MHC

It was possible that tolerance had been induced only to Ags expressed within the graft itself. Alternatively, some Ags might have been processed and presented on host APCs. To address this, we tolerized (CBA/Ca × BALB/c)F_1 mice to grafts bearing B10 minor Ags associated with H-2^d or H-2^k. We could then investigate whether animals were tolerant to donor Ags alone, or also to donor minor Ags associated with host MHC. (CBA/Ca × BALB/c)F_1 mice were made tolerant to B10.BR, B10.D2, and B10.BR plus B10.D2 skin. On day 61 all mice were regrafted with both B10.BR and B10.D2 skin grafts, in the same graft bed. Irrespective of whether the recipient was made tolerant to B10.BR or B10.D2, all grafts survived for longer than 176 days (Fig. 2). The mice that were given a tolerizing graft with no mAb therapy rejected their second grafts with a MST of 14 days. The extended survival of B10.D2 skin on B10.BR-tolerized mice, and of B10.BR on B10.D2 tolerized mice, is not due to residual effects of the mAb since mice that were treated with the mAb protocol without a tolerizing graft rejected both B10.D2 and B10.BR grafts with a MST of 16 days. As a positive control for endogenous "processing," (CBA/Ca × BALB/c)F_1 mice were grafted with BALB/K skin in the absence of a tolerizing graft and mAb treatment. All BALB/K grafts survived for longer than 176 days. This was not surprising since recipient BALB minor Ags are presented by the host in the context of both H-2^k and H-2^d. Mice in all groups were grafted with skin from C57BL/10 mice on day 116. In all cases, the C57BL/10 grafts were rejected with a MST of 14 days. We conclude from this data that donor B10 minor Ags are reprocessed and

[5] Abbreviation used in this paper: MST, median survival time.

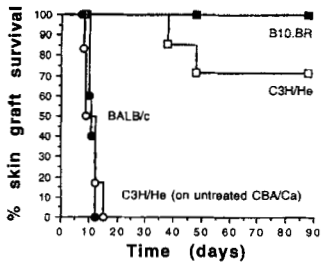

FIGURE 3. Tolerant mice do not reject skin grafts from all third party mouse strains. CBA/Ca mice were made tolerant to B10.BR skin grafts using the standard mAb protocol. At least 2 mo after grafting, all mice received a second B10.BR graft (■) with a third party graft from C3H/He (□, $n = 7$), or BALB/c (●, $n = 5$) mouse strains. In all cases, the first B10.BR skin graft was not rejected. In a separate experiment, untreated CBA/Ca mice were grafted with C3H/He skin (○).

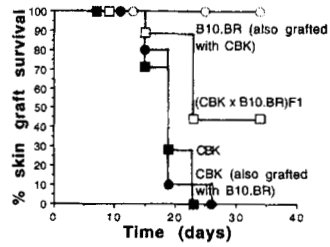

FIGURE 4. Some third party grafts are not rejected if third party and tolerizing Ags are presented by the same APC. CBA/Ca mice were made tolerant to B10.BR skin using the standard mAb protocol. At least 2 mo after the first graft, the mice were split into three groups and grafted with skin from the following mouse strains: CBK (■, $n = 7$), (CBK × B10.BR)F_1 (□, $n = 9$), or CBK (●, $n = 10$) plus B10.BR (○, $n = 10$). The first two groups were also grafted with CBA/Ca skin. In all cases the CBA/Ca skin survived for >34 days and all first B10.BR grafts were in good condition by the end of the experiment. In cases in which CBK and B10.BR grafts were placed simultaneously on the tolerant CBA/Ca, the survival of both grafts is shown.

presented by the host APC in the context of both H-2^k and H-2^d during the period of tolerance induction.

Some multiple minor Ag-mismatched third party skin grafts are not rejected by mAb-induced tolerant mice

CBA/Ca mice made tolerant to B10.BR skin using CD4- and CD8-specific Abs reject only 30% of skin grafts from the C3H/He (multiple minor histocompatibility Ag mismatch) mouse strain, but they reject all skin grafts from the MHC-mismatched BALB/c strain (Fig. 3). This is not due to an inability of untreated CBA/Ca mice to reject C3H/He skin grafts, since they are rejected with a MST of 9 days. To explain this, we hypothesized that tolerance to Ags that are shared by the tolerant and third party grafts might allow tolerance to be spread to other Ags presented on the third party APC (spreading or infectious tolerance). In the case described in Figure 3, the shared Ags would be those minor Ags that are potentially shared by the B10.BR and C3H/He strains. However, there is no reason to assume that infectious tolerance could not apply to any Ag. We tested this by comparing the survival times of secondary grafts from the tolerizing strain, B10.BR; a third party strain, CBK, with a defined Ag mismatch between it and the CBA/Ca and B10.BR (strain known to be rejected by CBA/Ca mice that are tolerant to B10.BR skin); and an F_1 between these two donor strains, i.e., a strain that will present both tolerizing Ags and third party Ags on the same APC. The reason for using a MHC-mismatched graft rather than a minor histocompatibility Ag-mismatched graft is that minor Ag-congenic strains are not readily available for the CBA/Ca strain, and therefore defining shared and nonshared Ags in the strain combination used was not possible.

Some grafts that are known to present Ags that are not shared with Ags from the tolerizing graft are not rejected by tolerant mice if the unshared Ags are presented by the same APC as the shared Ags

B10.BR-tolerant CBA/Ca mice were grafted with CBK plus CBA/Ca, (CBK × B10.BR)F_1 plus CBA/Ca, or CBK plus B10.BR skin placed simultaneously in the same graft bed. The mice receiving CBK and (CBK × B10.BR)F_1 grafts were simultaneously grafted with CBA/Ca skin to control for any effects of a second graft (Fig. 4). Over 40% of the (CBK × B10.BR)F_1 grafts showed no signs of rejection over the time monitored (34 days). The rejection rate of the F_1 grafts is significantly different from that of CBK skin grafted simultaneously with CBA/Ca ($p < 0.01$) or simultaneously with B10.BR ($p < 0.006$). Although around 50% of the F_1 grafts were rejected within 23 days, the rejection rate of the F_1 grafts compared with that of the B10.BR grafts was not statistically significant ($p = 0.17$).

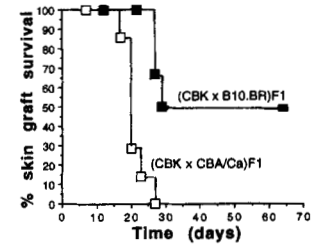

FIGURE 5. The lack of rejection of some third party skin grafts is not due to a simple dilution of Ag in the F_1. CBA/Ca mice made tolerant to B10.BR skin on day −60 were regrafted with skin from either a (CBK × B10.BR)F_1 (■, $n = 6$) or (CBK × CBA/Ca)F_1 (□, $n = 7$) mouse. All first B10.BR grafts were in good condition at the end of the experiment.

Lack of rejection of F_1 grafts is not due to a dilution of Ag

The lack of rejection of some (CBK × B10.BR)F_1 skin grafts was not due to a dilution of the H-2K^b Ag on the F_1 since all grafts from (CBK × CBA/Ca)F_1 mice were rejected within 27 days, whereas in this experiment, 50% of the (CBK × B10.BR)F_1 grafts survived for longer than 64 days ($p < 0.003$; Fig. 5).

Lack of rejection of F_1 skin grafts is not due to a lack of immunogenicity of (CBK × B10.BR)F_1 Ags in a CBA/Ca recipient that is tolerant to B10.BR Ags

(CBA/Ca × B10.BR)F_1 mice were transplanted with skin from (CBK × B10.BR)F_1, (CBK × CBA/Ca)F_1, and CBK donors, together with a CBA/Ca graft placed simultaneously in the same graft bed. Another group of F_1 mice was grafted with both CBK and B10.BR skin. The (CBK × B10.BR)F_1 grafts were rejected at a rate indistinguishable from all other grafts that expressed CBK Ags (Fig. 6). Five of six B10.BR grafts were not rejected by the (CBA/Ca × B10.BR)F_1 recipients. We conclude that the presentation of CBK Ags within a (CBK × B10.BR)F_1 graft does not diminish the immunogenicity of the CBK Ags as presented to a CBA/Ca mouse that is tolerant to B10.BR Ags. We found that H-2K^b grafts were rejected somewhat faster in the F_1 than in the mAb-tolerized CBA/Ca mice. This might represent an effect of

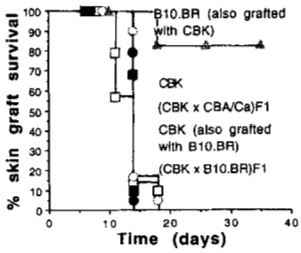

FIGURE 6. (CBA/Ca × B10.BR)F_1 mice reject third party grafts whose graft Ags are presented by the same APC as the B10.BR graft Ags. (CBA/Ca × B10.BR)F_1 mice were grafted with skin from CBK (■, $n = 5$), (CBK × B10.BR)F_1 (□, $n = 7$), (CBK × CBA/Ca)F_1 (●, $n = 6$), and CBK (○, $n = 6$) plus B10.BR (▲, $n = 6$) mice. Groups 1, 2, and 3 were also grafted with CBA/Ca skin. In all cases, the CBA/Ca grafts survived for >35 days. When CBK and B10.BR were grafted simultaneously, the survival of both sets of grafts is shown.

genetic background on immune responsiveness or signify some spillover effect of tolerance generated by mAbs. The rejection of one of the B10.BR grafts was unexpected. These mice were also grafted with a CBK skin graft at the same time and in the same graft bed. It is possible that, on a rare occasion, inflammation caused by the rapid rejection of one graft can have an effect on the survival of a syngeneic graft that has been placed in the same site.

Suppression of the CBK response can result in tolerance to H-2K^b

Although the co-presentation of B10.BR and CBK Ags extended the survival of H-2K^{b+} grafts, the data does not distinguish between continuous suppression of the nontolerant response and suppression that might lead to tolerance of the CBK-specific response. To address this issue, we used as hosts mice that had previously been mAb tolerized to B10.BR skin (first graft) and regrafted with skin from (CBK × B10.BR)F_1 alone, B10.BR alone (experiment 1), (CBK × B10.BR)F_1 plus CBA/Ca, and B10.BR plus CBK (experiment 2). All groups were then tested for tolerance to H-2K^b by regrafting with CBK skin alone (third graft). At the time of grafting with the third graft, five of six B10.BR grafts and three of six (CBK × B10.BR)F_1 grafts in the first experiment, and all B10.BR and CBA/Ca grafts and three of six (CBK × B10.BR)F_1 grafts in the second experiment (Table I) were in good condition. The mice that maintained their CBK grafts for the longest tended to be those that maintained their (CBK × B10.BR)F_1 second grafts. Therefore, the presence of CBK Ags grafted at the same time as the second B10.BR skin graft did not induce the prolongation of the third CBK skin graft unless presented as an F_1 graft. After day 7 post-third graft in experiment 1, all grafts were monitored by an investigator from another group. Using pooled data from experiments 1 and 2, the p value on the survival of the third graft, CBK, for the groups that received (CBK × B10.BR)F_1 (with and without CBA/Ca) vs B10.BR (with and without CBK), as their second grafts is $p <= 0.012$.

Interestingly, in the cases in which the CBK third grafts were eventually rejected, the second (CBK × B10.BR)F_1 grafts were also rejected. In the two cases in which the H-2K^b graft was not rejected, neither were the F_1 grafts. The presence of the CBA/Ca skin grafted at the same time as the second (CBK × B10.BR)F_1 graft, and CBK skin grafted at the same time as the B10.BR grafts, made no difference to the survival of the third grafts. All mice that rejected their F_1 second grafts before grafting with the third graft also rejected their third CBK grafts in a time not unlike that of the other two groups.

Discussion

The immunologically mature adult can be made specifically tolerant to Ag using a number of different protocols. These include Ag ingestion (9), i.v. injection (10), and mAb treatment at the time of immunization (11–14). We have worked extensively on protocols that involve treatment of the host with nondepleting CD4- and CD8-specific mAbs at the time of grafting with multiple minor histocompatibility Ag-mismatched skin grafts. Tolerance induced in this way is life-long, independent of a thymus (28), and associated with the development of regulatory CD4$^+$ T cells that can prevent naive T cells from rejecting a graft. Once suppressed, naive cells themselves become tolerant to the graft. We have referred to this phenomenon as "infectious tolerance" (18).

Mice made tolerant to a particular Ag have been shown to respond to some but not all third party Ags (19, 21). In both cases tolerance is Ag specific; therefore, the tolerizing and third party Ag combination seem to be critical to the survival of the third party graft. These data, combined with the knowledge that the induction of tolerance results in suppression of responses to the tolerizing Ag, have led us to hypothesize that the response to the third party Ag is suppressed. We constructed experiments to assess the nature of Ag presentation on suppression induced to multiple minor Ags with CD4- and CD8-specific mAbs. Since minor Ags were shown by us, and others (31), to be presented for tolerance by both donor and host APC, we chose an MHC donor Ag as the third party Ag to which suppression was directed. Co-presentation of tolerizing Ag with third party Ag was shown to be necessary for lack of rejection of the third party graft. This lack of rejection was not due to a dilution of third party Ag, nor was it due to a loss of immunogenicity of third party Ag when presented on the same cell as the tolerizing Ag. The only remaining explanation is that mAb-induced tolerance resulted in suppression of the response to third party Ags when presented on the same cell as the tolerizing Ag.

Tolerance induced to myelin basic protein by oral administration also induces suppression to other Ags if tolerizing Ag is present (22). The mechanism is thought to involve the secretion of IL-4, IL-10 (32), and TGF-β (32, 33) by the suppressive cells. In an in vitro system, Lombardi et al. have shown that anergic T cell clones can suppress proliferation of nonanergic T cells (23). They argue that suppression is caused by competition for both locally produced IL-2 and the surface of the APC. Recently, using the same strain and mAb combinations as we describe here, we have shown that IL-4 is involved in the transfer of transplantation suppression.[4] The cytokine IL-4 is known to deviate a naive response toward a Th2 phenotype (34–36) and away from a Th1 (37, 38) inflammatory phenotype (39). This strongly suggests that, in our system, the tolerant cell population contains cells of the Th2 phenotype, and that IL-4 secreted by tolerant Th2 T cells in the vicinity of nontolerant T cells, at the time of Ag presentation, will cause the latter to respond in a Th2 manner. Since the Ag-specific T cell secretes IL-4 only in the direction of the APC that presents Ag to it (40), we also suggest that IL-4 appears to induce specific suppression by acting within a limited area that encompasses only those tolerant and nontolerant cells that are bound to the same APC. This model predicts that suppression might lead to tolerance. When tested we found that suppressed recipients did show some tolerance to the third party Ag when rechallenged with third party Ag (CBK skin) alone. Interestingly, the mice that slowly rejected their CBK skin grafts also rejected the grafts to which they were suppressed. The explanation for this is not clear but it might relate to the balance of tolerant and nontolerant cells in individual mice. Whether the mechanism of linked suppression is the same as that

Table I. *Suppression of the response of mAb tolerant mice to (CBK × B10.BR)F$_1$ skin grafts leads to tolerance in those mice to CBK graft antigens*[a,b]

Post-Second Graft		Post-Third Graft		CBK (third graft) Post-Third Graft	
Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
(CBK × B10.BR)F$_1$ (second graft)					
27[c]	23[d]	—	—	13	18
27	23	—	—	13	18
29	23	—	—	13	18
>104(++)[2]	23	27	—	24	23
>104(++)	>34(++)	>38(+)	20	27	20
>104(++)	>34(++)	>38(++)	30	>38(++)	34
—	>34(++)	—	>48(++)	—	>51(++)
B10.BR (second graft)					
10	>34(++)[e]	—	>48(++)	10	14
>104(++)	>34(++)	>38(++)	>48(++)	17	14
>104(++)	>34(++)	>38(++)	>48(++)	17	14
>104(++)	>34(++)	>38(++)	>48(++)	17	18
>104(++)	>34(++)	>38(++)	>48(++)	17	18
>104(++)	>34(++)	>38(++)	>48(++)	17	18
—	>34(++)	—	>48(++)	—	18
—	>34(++)	—	>48(++)	—	18

[a] CBA/Ca mice that had been made tolerant to B10.BR skin (on day −7 and day 0 for Expt. 1, and day 0 for Expt. 2), using the mAb protocol, were regrafted 61 (Expt. 1) and 128 (Expt. 2) days later with (CBK × B10.BR)F$_1$ (n = 6) or B10.BR (n = 6) (Expt. 1) and with (CBK × B10.BR)F$_1$ plus CBA/Ca (n = 7) or B10.BR plus CBK (n = 8) (Expt. 2). All mice were grafted once again (day 128 for Expt. 1 and day 162 for Expt. 2), but this time with skin from CBK mice only. In both experiments, all first B10.BR and CBA/Ca grafts survived for the duration of the experiment. The survival times of the second (CBK × B10.BR)F$_1$ and B10.BR grafts and the third CBK grafts are shown.

[b] All numbers indicate the day on which 100% of the graft was rejected. A score of ++ indicates that the graft is fully viable macroscopically and that it has full hair growth. A single + indicates a fully viable graft but less than full hair growth.

[c] This data is also shown in Figure 5 (■) and has been duplicated here so that the fate of grafts on individual mice can be followed.

[d] This data is also shown in Figure 4 (□) and has been duplicated here so that the fate of grafts on individual mice can be followed.

[e] This data is also shown in Figure 4 (○) and has been duplicated here so that the fate of grafts on indivivual mice can be followed.

for the transfer of suppression[4] is not known; however, this question is currently under investigation.

Around 50% of the grafts that carried both tolerizing and third party graft Ags were rejected within a time similar to that of controls. This might indicate that a threshold level of suppression is required to achieve long-term graft acceptance, and that the response to the (CBK × B10.BR)F$_1$ graft is balanced at a point very close to that threshold. The slightly prolonged survival of AKR skin on B10.BR tolerant vs untreated mice might also be due to suppression generated to the third party Ags by tolerant cells that were specific for shared Ags. In this case the balance of shared and nonshared Ags was such that the suppression (Th2 response) induced was not strong enough to overpower the rejection (Th1) response.

The phenomenon of linked recognition is well known to immunologists in defining a need for lymphocyte cell-cell proximity. For example, hapten-specific B cells interact with a Th cell that is specific for Ag presented by that B cell (the carrier; Refs. 41–44) in what has been termed "intramolecular help." In addition, Th cells help CD8 T cells through intermolecular help where two Ags are presented on the same immunizing cell (45, 46).

CD4$^+$ cells have clearly been shown to be necessary for suppression in the basic model. However, the way in which they might control the CD8 response to minor Ags and the MHC class I molecule needs to be resolved. One possibility is that suppression of CD4 responses leads to a lack of help for a CD8 response. This could indirectly lead to tolerance (9). Another possibility is that CD4 suppression acts to down-regulate the local APC and so interferes with CD8$^+$ T cell priming. Finally, CD4$^+$ suppressor cells might directly suppress CD8$^+$ T cells.

The requirement that cells of two different specificities must recognize specific Ag on the same cell will limit the number and types of such responses that are suppressed. An obvious advantage to having a peripheral mechanism of suppression physiologically is that already tolerant T cells would have the capacity to suppress T cell responses to peripheral self-Ags that are not expressed in the thymus. The fact that new Ag must be presented on the same APC as tolerizing Ag would reduce the chance of unwanted suppression of advantageous immune responses.

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