

Thymus Requirement and Antigen Dependency in the “Infectious” Tolerance Pathway in Transplant Recipients¹

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We have shown that features of infectious tolerance, as originally described in thymectomized mice, may be applied to euthymic rat recipients of heart transplants. We now report on studies aimed at exposing mechanisms underlying the infectious tolerance pathway, with emphasis on the role of thymus and alloantigen. Pretransplant thymectomy diminished the efficacy of CD4-targeted therapy, with donor-specific tolerance induced in ~50% of recipients. Thymus was required for generation of regulatory T cells under the cover of CD4 mAb therapy and for the ability of these cells to confer infectious tolerance. However, thymus was not mandatory to maintain an infectious-permissive environment in cohorts of adoptively transferred recipients. Intra-graft expression of IL-2, IL-4, and IL-10 genes was diminished in euthymic and thymectomized tolerant hosts. However, grafts in the latter group showed significant IFN- γ gene expression, suggesting a less efficient down-regulation of Th1-like cells in the absence of regulatory cells. Indeed, exogenous challenge with rIL-2 or freshly alloactivated spleen cells recreated rejection in thymectomized, but not euthymic, hosts, suggesting that a state of cytokine-responsive anergy contributes to the “noninfectious” form of tolerance in thymectomized rats. The infection-tolerant state did not result from “graft adaptation,” and regulatory T cells restricted for the original alloantigen were exposed to its continuous stimulation. The effective memory for suppression was dependent upon persistent donor-specific alloantigen stimulation; it disappeared within 3 weeks after its removal. Hence, both central and peripheral immune mechanisms, orchestrated by the tolerizing alloantigen, contribute to the infectious tolerance pathway in CD4 mAb-treated rat transplant recipients. *The Journal of Immunology*, 1998, 160: 5765–5772.

Donor-specific tolerance, defined as the indefinite survival of a well-functioning organ allograft in the immunocompetent host in the absence of immunosuppression, remains the elusive goal in clinical organ transplantation. The possible mechanisms of tolerance include clonal deletion of alloreactive cells, clonal anergy, cell-mediated suppression (1), and what has been recently termed “infectious” tolerance (2). Once generated, infectious tolerance becomes self-sustaining, imposing itself by adoptively transferred lymphocytes over multiple cell generations to new cohorts of test recipients (2–5). Thus, infectious tolerance represents a natural host-regulatory mechanism, which is allowed to amplify and becomes dominant under cover of the initial therapy. The dissection of this mechanism should add to our understanding of why long-term unresponsiveness may be maintained without concomitant immunosuppression in transplant recipients.

The infectious tolerance, as originally described by Dr. H. Waldmann’s group, was induced and perpetuated in adult thymec-

tomized (ATX)³ mouse recipients of nonvascularized skin allografts treated with a mixture of nonlytic CD4 and CD8 mAbs (2–4). We have recently shown, however, that cardinal features of such a classic peripheral form of tolerance may be also applied to euthymic Ag-primed rat recipients of vascularized transplants (6). Hence, 1) treatment of sensitized rats with RIB-5/2, a CD4-non-depleting mAb, abrogated accelerated (<36 h) rejection and produced indefinite cardiac allograft survival; 2) tolerant cells in mAb-treated hosts could disable naive or even alloimmune cells, so that they failed to trigger rejection; and 3) donor-specific and organ-nonspecific tolerance could be transferred by spleen cells alone, and with no concomitant therapy, into new cohorts of cardiac or renal test allograft recipients. Furthermore, we have documented that regulatory CD4⁺ Th2-like IL-4-producing cells are instrumental for the maintenance of the infectious tolerance pathway (7). Given the phenomena associated with this clinically relevant rat transplant model, we have now performed studies aimed at exposing mechanisms underlying the induction and maintenance of infectious tolerance, with particular emphasis on the role of the thymus and alloantigen. We provide evidence that the induction of infectious tolerance in rat recipients is thymus dependent, consistent with the role of the thymus in generating regulatory T suppressor cells (8). Once established, these immunoregulatory T cells remain critically dependent upon the continuous presence of a tolerizing donor-specific alloantigen and may now operate in ATX test recipients. Hence, both central and peripheral immune mechanisms for induction and maintenance to CD4 mAb-induced infectious tolerance must exist.

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Received for publication December 1, 1997. Accepted for publication February 11, 1998.

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¹ This study was supported by National Institutes of Health Grant AI23847 and by North Atlantic Treaty Organization Grant CRG.951353.

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³ Abbreviations used in this paper: ATX, adult thymectomy/thymectomized; BN, Brown Norway; LBNF₁, (LEW \times BN)F₁; WF, Wistar-Furth; MST, mean survival time; PE, phycoerythrin; AU, arbitrary unit.

Materials and Methods

Animals and grafting techniques

Inbred male adult (10–12-wk-old) rats weighing 200 to 250 g were used (Harlan Sprague Dawley, Indianapolis, IN). Lewis (LEW, RT1^b) served as recipients of cardiac allografts from (LEW × BN)_F₁ (LBNF₁) hybrids. Brown Norman (BN) (RT1^a) rats were used as skin donors. Wistar-Furth (WF, RT1^a) rats were employed as third-party donors for specificity experiments. Full-thickness skin was sutured bilaterally to appropriate defects in the chest of prospective rat recipients. Hearts were transplanted to the abdominal great vessels by standard microvascular techniques. Their function was monitored daily by palpation, and rejection was defined as the day of cessation of heart beat.

The model of infectious tolerance

LEW rats were sensitized with BN skin grafts (day -7), followed 1 wk later by transplantation of LBNF₁ hearts (day 0). These cardiac allografts are rejected in an accelerated manner in <36 h (6, 7, 9). However, intermittent treatment with RIB-5/2 (5 mg/rat i.v. at days -7, -4, -1, 0, +3, +4, +7, +10, +14, and +21), a mouse antirat CD4-nondepleting mAb (10), results in permanent cardiac allograft acceptance, with features characteristic for the infectious tolerance pathway (6, 7). Skin grafts are rejected despite RIB-5/2 therapy (6). Rats maintaining their transplants for >100 days were used in the present study.

Host immune manipulations

ATX was performed following partial median sternotomy in otherwise untreated rats at 10–12 wk of age. These were then sensitized with skin grafts 4–8 wk later, followed 1 wk later by transplantation of donor-strain hearts. Some animals underwent ATX at >100 days posttransplant. To test for tolerance induction, long-term allograft recipients were challenged with a secondary donor-specific (LBNF₁) or third-party (WF) heart transplant. In an adoptive transfer assay, 100 × 10⁶ erythrocyte-free spleen cells were administered i.v. into sublethally gamma-irradiated (450 rads) syngeneic rats (one donor spleen/two test recipients). These were then challenged 24 h later, unless specified otherwise, with donor-specific test cardiac allografts. In an attempt to break tolerance, long-term engrafted hosts were injected i.v. with 1) spleen cells (100 × 10⁶ cells/rat once a week for 3 wk) from sensitized rats undergoing accelerated cardiac allograft rejection, and 2) human rIL-2 (20,000 U (~1.4 μg)/day for 5 days; Collaborative Research, Bedford, MA). Finally, long-term LBNF₁ cardiac allografts "parked" in tolerant LEW recipients for >100 days were retransplanted into normal LEW rats. The "graft-free" hosts were then challenged with a fresh LBNF₁ heart at different times after removal of the original transplant.

RT-PCR analysis

For evaluation of intragraft cytokine gene expression, competitive template RT-PCR was performed (11). Total RNA was prepared from the grafts and reverse transcribed into cDNA. A cDNA equivalent of 5 ng total RNA was amplified in a 25-μl reaction volume containing 250 μM of each deoxynucleotide triphosphate, 10 μM of the primer pair, 2.5 μl 10-fold PCR buffer, and 0.5 U *Taq* DNA polymerase (Perkin-Elmer/Cetus, Emeryville, CA). As control DNA fragment, a synthetic gene containing nonhomologous DNA with binding sites for 5' and 3' cytokine-specific primers was constructed. The gene was cloned into pBluescript II SK⁺ (Stratagene, La Jolla, CA). The sense and antisense primer sequences derived from rat CD3, CD25, IL-2, IFN-γ, IL-4, IL-10, and β-actin cDNA have been described (11). For quantification, cDNA samples were adjusted to equal input concentrations based on their β-actin cDNA content. For this purpose, 10-fold serial dilutions of the control fragment (500–5 pg) were mixed with identical volumes of cDNA prepared from the tissue RNAs; these were then coamplified by using β-actin-specific primers. After electrophoresis of the PCR products, the cDNA samples were adjusted to equal β-actin cDNA content by ethidium bromide fluorescence. The adjusted cDNA samples and specific primers were then subjected to another coamplification with 10-fold dilutions of the control fragment using primers specific for each cytokine. The level of cytokine gene expression was expressed in AU (arbitrary unit)/μl cDNA. One AU was defined as the lowest concentration of the control fragment that yielded a detectable amplification product at the conditions used.

Evaluation of host alloantibody responses

Donor-specific IgM and IgG alloantibody responses were determined in recipient serum, as described (6). Briefly, cervical BN lymph node target cells (1.5 × 10⁵) were incubated with serially diluted (1:4 → 1:64) heat-

inactivated serum samples for 30 min at 4°C. To stain for IgM and IgG, the washed cells were then reacted with a mixture of FITC-conjugated goat Ab specific for the Fc portion of rat IgG and phycoerythrin (PE)-conjugated goat Ab specific for the μ chain of rat IgM (Jackson ImmunoResearch, West Grove, PA). After staining, the cells were washed, fixed in 1% neutral buffered formalin, and analyzed on an EPICS C cell sorter (Coulter, Hi-aleah, FL). The use of paired secondary Abs allowed simultaneous assessment of IgG (FITC channel) and IgM (PE channel). Their levels were expressed as mode channel fluorescence.

Statistical analysis

The graft survival curves were analyzed by the Kaplan-Meier test, and statistical significance in graft survival between experimental groups was determined by log rank χ^2 test. Values were expressed as the mean ± SEM, with differences considered statistically significant if $p < 0.05$.

Results

ATX prevents the development of infectious tolerance in CD4 mAb-treated rat recipients

As infectious tolerance was originally induced and perpetuated in thymectomized mice (2–5), we first asked whether ATX may affect the infectious tolerance pathway in our rat transplantation model. First, we confirmed our earlier findings (12) by demonstrating that thymic extirpation at 4–8 wk before the skin allografting did not influence host sensitization, as cardiac allografts were rejected uniformly in <36 h, similarly to euthymic sensitized rat recipients (Fig. 1A). However, as shown in Figure 1A, ATX in naive animals 4–8 wk before skin graft-induced sensitization profoundly influenced the tolerogenic effects of CD4-targeted therapy (mean survival time (MST) = 53.2 days, $p < 0.0001$, as compared with euthymic controls). Moreover, unlike in euthymic rats, only about 50% of ATX and RIB-5/2 mAb-treated animals maintained well functioning cardiac allografts for >100 days; the remainder rejected their transplants within 17 days (Fig. 1A). The permanent graft acceptance rates were similar in groups of rat recipients, which received heart transplant at 5 wk (60%), 7 wk (50%), or 9 wk (50%) following thymectomy. Long-term ATX and CD4 mAb-treated hosts developed donor-specific tolerance, which could be manifested by acute rejection (7–8 days) of third-party (WF) and acceptance (MST = >70 days) of donor-matched (LBNF₁) secondary heart grafts ($n = 2$ rats/group). Thus, although pretransplant thymectomy diminished the overall efficacy of CD4-targeted therapy, donor-specific tolerance may still have been induced in about 50% of CD4 mAb-conditioned ATX hosts.

The next question we asked was whether ATX affects the cornerstone of the infectious tolerance pathway, i.e., the induction of permanent graft survival in new cohorts of "test-tube" animals after infusion of spleen cells from original tolerant hosts. Interestingly, ATX abrogated the ability of spleen cells (100 × 10⁶) from CD4 mAb-treated long-term (>100 days) allograft recipients to prolong test cardiac allograft survival after adoptive transfer (Fig. 1D). These new euthymic, lightly gamma-irradiated but otherwise untreated test animals rejected their transplants within 7 days (MST = 6.3 days), similarly to control rats (Fig. 1B), which were either infused with the same number of nontolerant recipient-type spleen cells (MST = 6.8 days) or did not receive any cells at all (MST = 10.2 days). Hence, pretransplant thymectomy prevented the development of the infectious tolerance pathway in engrafted rat recipients.

ATX prevents induction but not maintenance of infectious tolerance-mediating regulatory T cells

Because the regulatory T cells develop gradually in CD4 mAb-treated hosts and become fully operational at >100 days posttransplant, we hypothesized that thymic extirpation at such a late phase should not affect the allograft outcome. Indeed, cardiac allografts

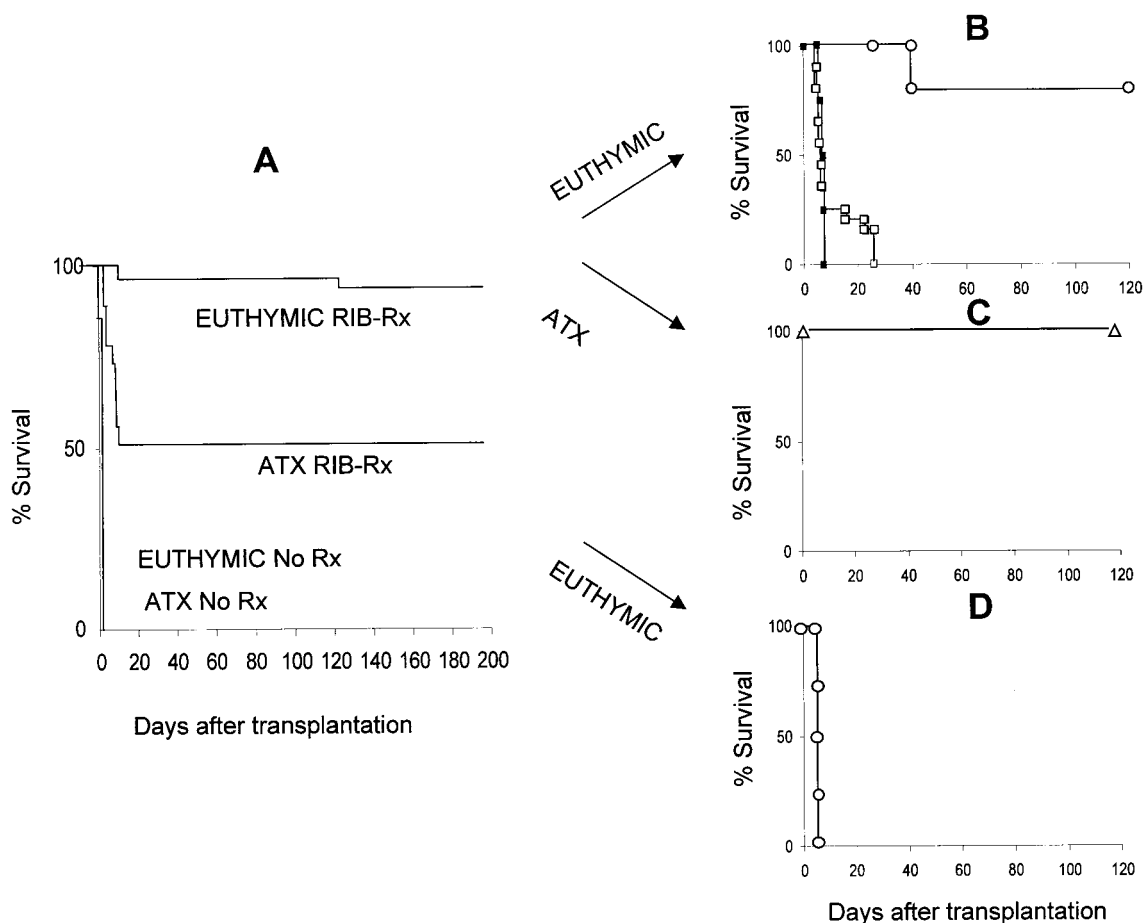


FIGURE 1. The influence of ATX in presensitized rat recipients (at 10–12 wk of age) upon the development of infectious tolerance to cardiac allografts. MST and SEM values are shown. *A*, Cardiac allograft survival in CD4 (RIB-5/2) mAb-treated (5 mg/rat 10 times, day $-7 \rightarrow$ day $+21$) vs untreated rats. ATX + RIB-5/2 mAb treatment, MST = 53.2 ± 12.3 days ($n = 13$)*; euthymic + RIB-5/2 mAb treatment: >100 days ($n = 30$); ATX, no RIB-5/2 mAb treatment: 0.8 ± 0.3 days ($n = 4$); euthymic, no RIB-5/2 mAb treatment: 0.4 ± 0.2 days ($n = 10$) (*the ATX + RIB-5/2 mAb group exhibited a bimodal graft survival pattern: six rats maintained cardiac allografts for >100 days and the remainder rejected their transplants in 13.0 ± 4.0 days ($n = 7$); the cumulative graft survival in this recipient group is significantly shorter as compared with that of euthymic controls ($p < 0.0001$)). *B*, Cardiac allograft survival in test euthymic rats after adoptive transfer of spleen cells (100×10^6) from the following: \circ , RIB-5/2 mAb-treated euthymic tolerant (>100 days) hosts: 115.8 ± 13.9 days ($n = 7$)*; \blacksquare , untreated naive rats: 6.8 ± 0.3 days ($n = 4$); and \square , no cell transfer: 10.3 ± 2.1 days ($n = 12$) (*statistical comparisons: \circ vs \blacksquare , $p < 0.01$; \circ vs \square (0%), $p < 0.0001$; \blacksquare vs \square , NS). *C*, Cardiac allograft survival in test ATX rats after adoptive transfer of spleen cells (100×10^6) from: \triangle , RIB-5/2 mAb-treated euthymic tolerant hosts >100 days ($n = 4$). *D*, Cardiac allograft survival in test euthymic rats after adoptive transfer of spleen cells (100×10^6) from: \diamond , RIB-5/2 mAb-treated ATX tolerant (>100 days) hosts: 6.3 ± 0.3 days; $n = 4$ *) (*statistical comparison: *C* vs *D*, $p < 0.0001$).

continued to function for >50 days in a group of RIB-5/2 mAb-treated recipients, despite ATX performed at 100–120 days posttransplant ($n = 4$; data not shown). Moreover, such a late thymectomy did not affect the ability of spleen cells to tolerize new sets of test recipients. We next asked whether these regulatory T cells, which require thymus for their induction, may then function in a thymus-free environment of test recipients. As shown in Figure 1C, transfer of spleen cells from RIB-5/2 mAb-treated tolerant euthymic hosts prompted acceptance of cardiac allografts in new cohorts of ATX “test-tube” rats. Moreover, we found that spleen cells from secondary ATX long-term hosts retained their suppressive effects upon sequential transfer to tertiary ATX test animals (MST = >50 days; $n = 5$; data not shown). These results imply that the thymus is critical for generation of regulatory T suppressor cells under cover of peritransplant CD4 mAb therapy and for the ability of cells to confer tolerance in an infectious manner. However, the presence of the thymus is not mandatory to

maintain an infectious-permissive environment in new generations of adoptively transferred test recipients.

ATX allows the development of clonal anergy in CD4 mAb-treated rats

Our recent data demonstrating the persistence of donor reactive T cells in long-term tolerant hosts rule out clonal deletion as a possible mechanism of tolerance maintenance in ATX hosts (13). Moreover, as thymectomy prevented the development of regulatory T cells, we then asked whether clonal anergy may have contributed to the tolerance maintenance in ATX recipients. To address this issue, we have analyzed intragraft Th1/Th2-type cytokine gene programs in transplant recipients by employing competitive template RT-PCR. As shown in Figure 2, unlike in untreated hosts undergoing accelerated graft rejection, intragraft IL-2, IL-4, and IL-10 gene expression was diminished in both euthymic and ATX + CD4 mAb-treated long-term (>100 days)

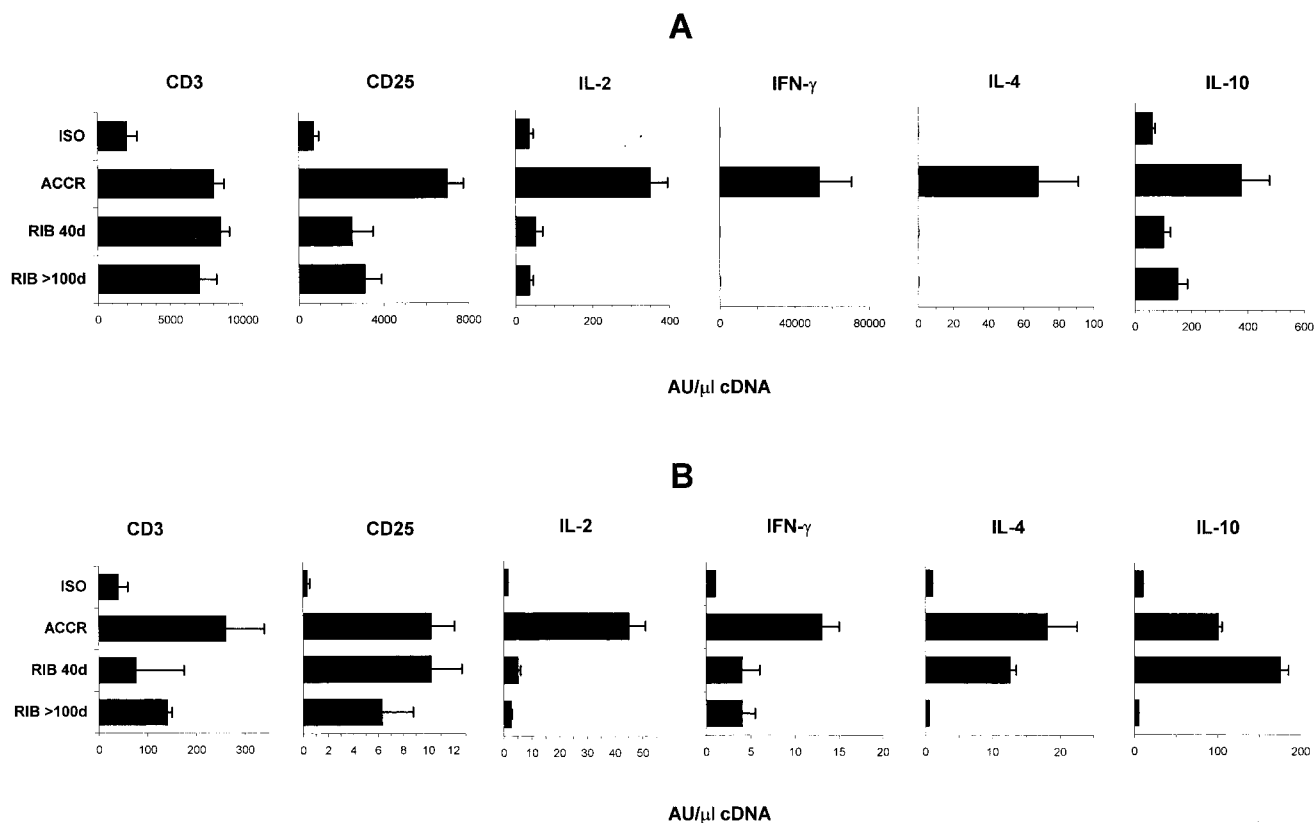


FIGURE 2. Competitive template RT-PCR analysis of CD3, CD25, IL-2, IFN- γ , IL-4, and IL-10 gene expression in cardiac allografts harvested at days 40 and 100+ from euthymic (A) and ATX (B) rat recipients treated with RIB-5/2 mAb (5 mg/rat 10 times between days -7 and +21). ACCR (accelerated graft rejection), control allografts from untreated hosts undergoing <36 h rejection; ISO, >100-day control cardiac isografts. The mRNA levels are expressed in AU/ μ l cDNA; 1 AU is defined as the lowest concentration of the control fragment that yields a detectable amplification product under the conditions used. Each value represents the mean \pm SD of two or three analyses of four to six tissue samples/group. Group A and B experiments were performed on two different occasions; the same isograft and accelerated graft rejection mRNA samples served as controls for both experimental series.

Table I. The effects of exogenous immunomodulation upon cardiac allograft survival in ATX + CD4 mAb-treated rats^a

Experimental Group	Treatment Regimen	Allograft Survival (days)	MST \pm SEM
ATX	rIL-2	6, 7, 8	7.0 \pm 0.6 ^b
Euthymic	rIL-2	>100 (\times 4)	>100 \pm 0
ATX	Specific sensitized cells	22, 24, 28	24.7 \pm 1.8 ^c
Euthymic	Specific sensitized cells	>100 (\times 7)	>100 \pm 0

^a LEW rats were sensitized with BN skin grafts (day -7), followed 1 week later by transplantation of LBNF₁ hearts. Treatment with RIB-5/2, a CD4 nondepleting mAb (5 mg/rat 10 \times , day -7 \rightarrow +21) induced tolerance to cardiac allografts in ca. 50% of ATX + CD4 mAb-treated rats. At >100 days postcardiac engraftment, tolerant hosts were challenged with 1) specifically sensitized spleen cells (100 \times 10⁶ cells/rat/week for 3 weeks), or 2) human rIL-2 (20,000 U[\sim 1.4 μ g]/day for 5 days). Statistical analysis: p < 0.0001 when b and c is compared.

recipients; grafts from ATX tolerant rats showed significant IFN- γ gene expression. In an attempt to break tolerance in our model, long-term allograft recipients were then challenged with exogenous rIL-2 or with freshly alloactivated spleen cells. Indeed, as shown in Table I, this treatment recreated graft rejection in ATX but not in euthymic hosts. The challenge with rIL-2 was more effective than that utilizing alloimmune cells (MST = 7.0 days and 24.7 days, respectively; p < 0.0001). These results imply that unlike in euthymic hosts, a state of cytokine-responsive anergy con-

tributes to the maintenance of cardiac allografts in ATX + CD4 mAb-treated rats.

ATX does not influence CD4 mAb-mediated effects upon host alloantibody network

We have previously shown that accelerated rejection in sensitized rats is accompanied by a strong systemic anti-donor IgM response, which peaks around the time of allograft loss and then switches to IgG alloantibody response at 7–10 days (6). The tolerogenic RIB-5/2 mAb regimen depresses the level and duration of IgM and prevents the switch from IgM to IgG (6, 7). We have now asked how pretransplant ATX, which diminishes the overall efficacy of CD4-targeted therapy and prevents the development of regulatory T cells, will influence host circulating alloantibody responses. As shown in Figure 3, both IgM and IgG alloantibody levels were virtually abolished throughout in ATX recipients regardless of the functional status of the allograft itself. Thus, although depression of humoral alloreactivity is important for the acquisition of infectious tolerance pathway in euthymic rats, diminished systemic IgM/IgG levels do not correlate with the allograft outcome in ATX hosts.

Regulatory T cells in the infectious tolerance pathway are alloantigen dependent

Finally, we wished to determine whether persistence of donor alloantigen is required for regulatory T cells to maintain infectious tolerance

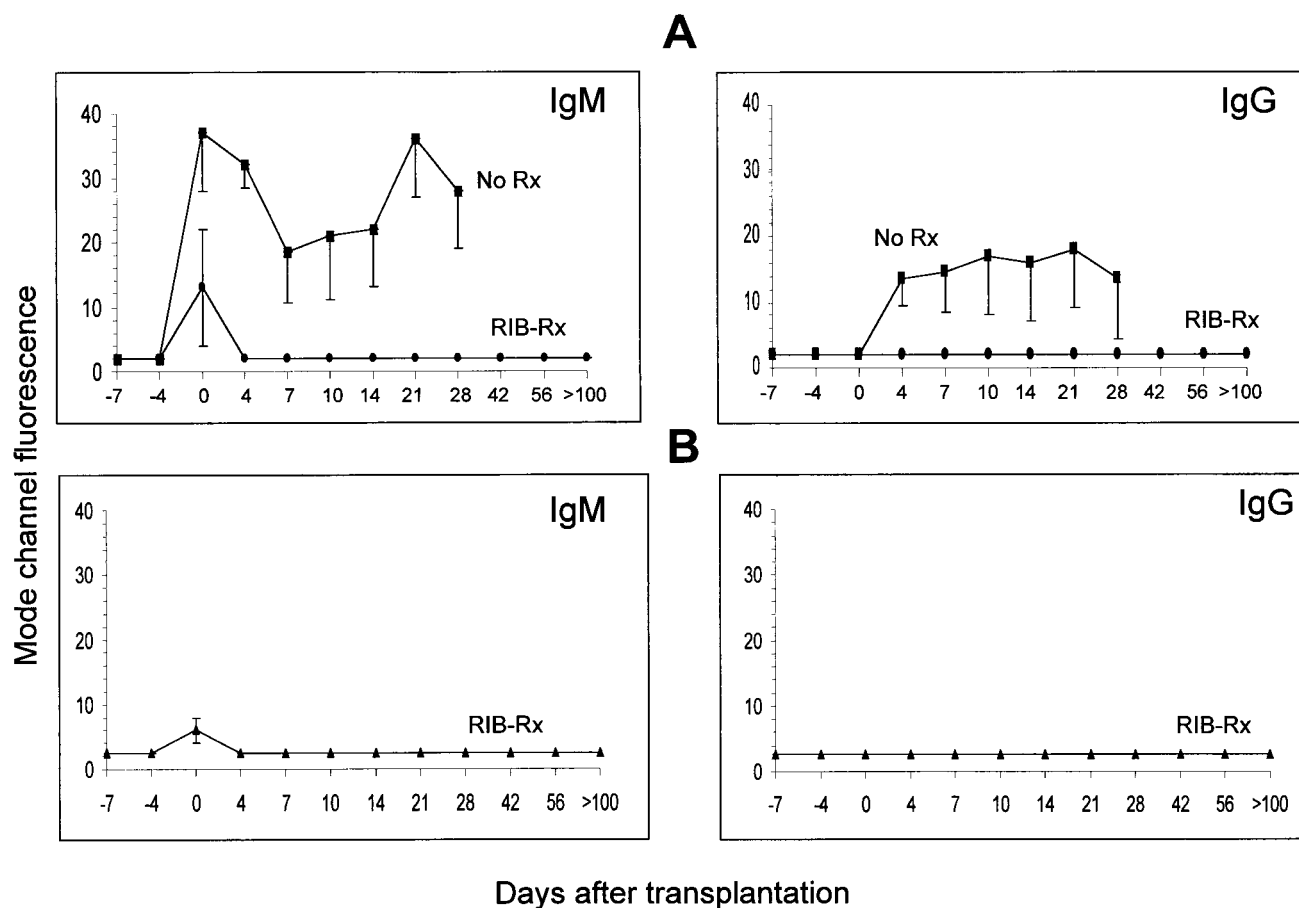


FIGURE 3. Flow cytometry analysis of circulating IgM and IgG alloantibody responses in euthymic (*A*) and ATX (*B*) engrafted rats, which were either untreated (No Rx) or underwent CD4-targeted therapy (RIB-Rx). Lymph node target cells from naive BN rats were incubated with experimental serum samples and stained with PE-conjugated anti-rat IgM (*upper panel*) or FITC-conjugated anti-rat IgG (*lower panel*). Each point represents the average mode channel staining \pm SEM at 1:4 serum dilution ($n = 3-7$ for each data point). Data on IgM/IgG alloantibody responses in euthymic untreated/RIB-5/2 mAb-treated rat recipients are derived from Refs. 6 and 7.

pathway. First, we evaluated the immunogenicity of long-term cardiac allografts in RIB-5/2 mAb-treated hosts, because the replacement of donor APCs by recipient APCs over time may have resulted in the loss of functional allostimulatory capacity *in vivo*. However, normal LEW secondary recipients rejected acutely retransplanted LBNF₁ hearts, despite such hearts having been “parked” in LEW-tolerant hosts for >100 days (MST \pm SD = 8.5 ± 0.5 days; $n = 5$; data not shown). We then performed a series of experiments in which primary heart grafts were removed at >100 days from CD4 mAb-treated recipients, and secondary hearts were placed at 1, 7, 14–17, and 30–90 days thereafter (Fig. 4A). By that time, there are no detectable RIB-5/2 mAb levels in the circulation (6). As shown in Figure 4A, such graft-free recipients accepted secondary donor-type transplants after a delay of up to 17 days, after which the operational tolerance decayed with time. Indeed, only two of four grafts survived >100 days in 30-day graft-free hosts ($p < 0.05$), compared with a 90-day graft-free environment, in which all hearts were rejected promptly within 2 wk ($p < 0.01$). Moreover, spleen cells from <17-day, but not from 90-day, graft-free hosts conferred tolerance into new cohorts of test recipients (MST = >50 days; $n = 3$ rats/group; data not shown). To confirm that the maintenance of infectious tolerance is Ag dependent, we used another experimental system in which spleen cells from tolerant rats were adoptively transferred into syngeneic secondary hosts, which were then challenged with donor-specific test cardiac allo-

grafts. As shown in Figure 4B, tolerant cells rapidly lost their suppressive capacity in the absence of donor alloantigen, and by 3 days they were unable to prevent rejection of test cardiac allografts. This contrasted with a stable tolerance achieved when tolerant cells were infused on the day of or 1 day before transplantation (100% and >80% graft acceptance, respectively). Hence, the effective memory for suppression in the infectious tolerance pathway depends upon the continuous donor-specific alloantigen stimulation.

Discussion

The principal findings of this study on the mechanisms underlying the induction and maintenance of infectious tolerance in rat allograft recipients are as follows: 1) thymus is critical for the induction of regulatory T cells under the cover of CD4-targeted therapy and for the ability of these cells to confer infectious tolerance from primary engrafted hosts; 2) thymus is not required to maintain the infectious-permissive environment in new cohorts of test recipients infused with regulatory T cells; 3) clonal anergy, accompanied by diminished expression of Th1- and Th2-type cytokines, contributes to the “noninfectious” form of tolerance in ATX hosts, which are devoid of operational regulatory T cells; and 4) maintenance of the infectious tolerance pathway depends on the persistence of donor-type alloantigen.

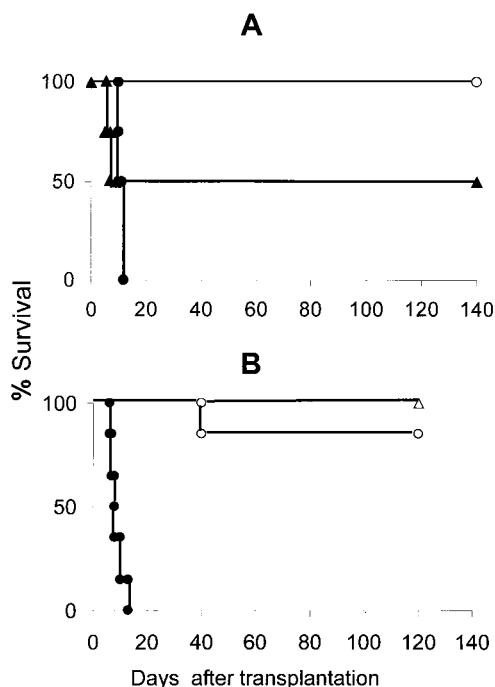


FIGURE 4. The role of donor-specific alloantigen in the infectious tolerance pathway. MST and SEM values are shown. *A*, The survival of secondary (LBNF₁) cardiac allografts after removal of the original transplants in CD4 (RIB-5/2) mAb-treated tolerant hosts (>100 days posttransplant). ○, 1- to 17-day graft-free recipients, >100 days posttransplant ($n = 9$) (*); ▲, 30-day graft-free recipients, 76.0 ± 30.5 ($n = 4$). ●, 90-day graft-free recipients, 11.5 ± 0.3 ($n = 4$) (*statistical comparisons: △ vs ●, $p < 0.05$; ○ vs ●, $p < 0.01$; ▲ vs ●, NS). *B*, The survival of cardiac allografts (transplanted at day 0) in test rat recipients, subjected to adoptive transfer of spleen cells (100×10^6) from CD4 (RIB-5/2) mAb-treated tolerant hosts (>100 days posttransplant): △, at day 0 (the day of allografting), >100 days ($n = 5$) (*); ○, 1 day before allografting, 115.8 ± 13.9 ($n = 7$); ●, >3 days before allografting, 8.7 ± 1.0 days ($n = 6$) (*statistical comparisons: △ vs ●, $p < 0.001$; ○ vs ●, $p < 0.001$; △ vs ○, NS).

Thymus is required for the acquisition of central tolerance to self-Ags, in which autoreactive T cells are deleted or anergized by exposure to the self-Ags presented by either bone marrow-derived or thymic stromal cells (14). Similar intrathymic mechanisms are important for inducing central tolerance to alloantigens, as evidenced by permanent allograft acceptance or tolerance following intrathymic injection of donor cells or soluble Ag (15, 16). In our own studies, administration of donor-type Ag into the recipient thymus abrogated accelerated rejection and significantly prolonged cardiac allograft survival in sensitized rats (17, 18). However, the role of the thymus in the acquisition of peripheral tolerance remains unclear. It has been shown that pretransplant ATX neither abolished donor-specific blood transfusion-mediated tolerogenic effects in fully MHC-incompatible rat renal allograft recipients (19) nor affected the infectious tolerance pathway to minor histocompatibility-nonvascularized skin grafts in mice conditioned with CD4 + CD8 mAbs (2–4). In contrast, thymus was essential for rapid and stable tolerance to MHC class I-mismatched renal allografts in miniature swine (20) and for the maintenance of tolerance to skin grafts in mixed allogeneic bone marrow mouse chimeras (21). Similarly, pretransplant ATX abrogated CTLA4Ig-induced tolerance after the blockade of CD28-B7 T cell costimulation in rat renal allograft recipients (22).

Our present data document the requirement for the thymus in the induction of infectious tolerance pathway in the accelerated rejection model. Hence, unlike in euthymic rats, only about 50% of ATX animals maintained long term functioning cardiac allografts after RIB-5/2 mAb treatment. Interestingly, however, the nature of donor-specific tolerance in both recipient groups was strikingly different. First, pretransplant ATX prevented the development of operational infectious tolerance, as evidenced by the inability of spleen cells from the original ATX + CD4 mAb-treated hosts to confer the tolerant state to new generations of test recipients. The immune mechanisms involved in thymus-dependent induction of infectious tolerance and the generation of regulatory T cells remain unclear. Perhaps, by migrating from cardiac allografts to the thymus, dendritic cells may become responsible for a central component in tolerance induction. Alternatively, thymic migrants may be instrumental for inducing peripheral tolerance to alloreactive cells in the graft. Indeed, several lines of evidence have emerged that refute the dogma of one-way trafficking through the thymus. In fact, our own studies have documented the recirculation of immunoresponsive thymocytes from the thymus to cardiac grafts and then back to the thymus in this accelerated rejection model (12). Moreover, passive transfer of thymocytes from rats rendered tolerant to vascular organ transplants into secondary naive hosts can lead to prolongation of test graft survival (23, 24). The thymus has also been identified as a source of natural suppressor cells in mice (25).

While the thymus was important for the induction of infectious tolerance in CD4 mAb-treated hosts, its presence in the maintenance phase was not mandatory. Indeed, ATX performed at >100 days posttransplant neither influenced otherwise permanent allograft survival nor affected the ability of adoptively transferred spleen cells to tolerize new sets of test rat recipients. This suggests that recent thymic migrants were sufficient to ensure the development of infectious tolerance in this model. Moreover, unlike in the original CD4 mAb-treated rats, thymus was not required to maintain the infectious tolerance pathway in new cohorts of test animals. Perhaps, once generated in CD4 mAb-treated rats, the regulatory T cells do not require the thymus to express their "infectiousness" and to overcome allorecognition properties of naive cells, which otherwise are fully capable of triggering rejection in test allograft recipients. Indeed, this was the case in Waldmann's infectious tolerance model, in which naive cells were transferred into allograft-containing tolerant transgene-marked recipient mice (2). Moreover, others have shown that even in the absence of the thymus, T cells capable of transferring donor-specific tolerance may emerge in the periphery (26). We favor the notion that the infectious phenomenon represents the natural host immune mechanism, independent of the initial immunomodulation, and our ongoing experiments suggest that the ability of regulatory T cells to maintain the infectious tolerance pathway may be strictly cell dose dependent (K. Onodera et al., manuscript in preparation).

The infectious-permissive environment may be maintained by a specific cytokine milieu, resulting from the peripheral distribution of helper cell types. Our RT-PCR analysis has revealed that intragraft IL-2, IL-4, and IL-10 gene expression was diminished in both euthymic and ATX tolerant hosts. However, grafts from ATX animals showed significant IFN- γ gene expression, suggesting a less efficient down-regulation of Th1-like cells in the absence of regulatory cells. Because down-regulation of Th1- and Th2-type cytokines represents one of the cardinal features of clonal anergy in vivo (1), we then asked whether addition of exogenous IL-2 may break the tolerant state in our model. Indeed, a course of rIL-2 or infusion of freshly

alloactivated spleen cells recreated cardiac allograft rejection in ATX but not in euthymic hosts. Thus, unlike in euthymic rats, a state of cytokine-responsive immune anergy represents the prime mechanism responsible for the "noninfectious" form of tolerance in ATX hosts. In the remainder of ATX animals that rejected their transplants in an acute rather than an accelerated manner, the microenvironmental factors associated with non-specific immune activation and cytokine release disturbing a delicate immune balance may be involved.

Alloantibody responses and isotype switching involve complex interactions between T cells and B cells, which may be regulated by cytokine networks (27). This study extends our previous findings (6, 18, 28–31) by demonstrating that ATX did not influence CD4 mAb-mediated effects upon host alloantibody networks in this model. As prevention of the switch from IgM to IgG alloantibody response may be critical for long-term allograft acceptance or tolerance (6, 28–31), no correlation between serum IgM/IgG alloantibody levels and allograft survival in ATX recipients could be found. In fact, both graft rejection and tolerance were accompanied by equally diminished host alloantibody responses, suggesting that ATX + CD4-targeted therapy erased preexisting sensitization, which otherwise triggers a mixed cellular and humoral immune response, culminating in an accelerated allograft loss in sensitized rats. This study confirms our previous findings (6) by demonstrating that systemic IgM and IgG alloantibody responses are virtually abolished in euthymic + CD4 mAb-treated tolerant animals. It remains to be determined, however, to what extent the depression of humoral alloreactivity contributes to the absence of histopathologic abnormalities pathognomonic of posttransplant arteriosclerosis and chronic rejection in the infectious tolerance pathway.

A continuous presence of alloantigen has been identified as a critical factor in the development and maintenance of unresponsiveness both to MHC (3, 32) and non-MHC (33, 34) Ags. While the graft function is maintained, donor alloantigens are shed into the periphery, where they may be able to inactivate newly emerging T cells. The results of our retransplantation studies corroborate other published reports (35) by demonstrating that normal LEW rats rejected LBNF₁ hearts despite such hearts having been parked for >100 days in CD4 mAb-conditioned LEW hosts. Hence, this tolerant state does not result from "graft adaptation," and regulatory T cells restricted for the original alloantigen are exposed to its continuous stimulation. In addition, our results from both graft-free and adoptive transfer studies document that effective memory for suppression in the infectious tolerance pathway depends upon persistent donor-specific alloantigen stimulation; it wanes at about 3 wk after its removal. Similarly, regulatory T cells lost their tolerizing potential in murine recipients, if hearts were engrafted 14 days after adoptive transfer (5). Moreover, because a functional graft was required to maintain operational tolerance in our studies, microchimerism via passenger donor-type cells or donor-derived peptides may not be sufficient for tolerance maintenance. Putative mechanisms leading to the loss of tolerance in an alloantigen-free environment may include inactivation of regulatory T cells or a failure of infectious tolerance to divert naive cells toward tolerance.

In summary, the induction of infectious transplantation tolerance in original hosts under the cover of CD4-targeted therapy is thymus dependent, consistent with the role of thymus in generating tolerance-mediating regulatory T suppressor cells. These regulatory T cells remain critically alloantigen dependent and may then "spread" tolerance in an infectious manner into a

thymus-free environment of new cohorts of test recipients. A rather unstable level of clonal anergy may replace regulatory T cells in ATX long-term hosts. Hence, both central and peripheral immune mechanisms, orchestrated by the tolerizing donor-type alloantigen, contribute to the development of infectious tolerance in CD4 mAb-treated engrafted rats. This study should add further substance to the current discussion of why all clinical attempts to induce transplantation tolerance in adult patients have been so far unsuccessful.

Acknowledgments

We thank Drs. M. Lehmann for providing RIB-5/2 mAb and T. B. Strom for helpful discussions and Mrs. Sigrid Koenig for excellent technical assistance.

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