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Specific B Cell Tolerance Is Induced by Cyclosporin A Plus Donor-Specific Blood Transfusion Pretreatment: Prolonged Survival of MHC Class I Disparate Cardiac Allografts

Chun-Ping Yang, Emma Shittu, and Eric B. Bell

Donor-specific blood transfusion (DST), designed to prolong allograft survival, sensitized recipients of the high-responder PVG-RT1\(^a\) strain, resulting in accelerated rejection of MHC-class I mismatched (PVG-R8) allografts. Rejection was found to be mediated by anti-MHC class I (A\(^a\)) alloantibody. By pretreating recipients 4 wk before grafting with cyclosporin A (CsA) daily (\(\times 7\)), combined with once weekly (\(\times 4\)) DST, rejection was prevented. The investigation explores the mechanism for this induced unresponsiveness. CD4 T cells purified from the thoracic duct of CsA/DST-pretreated RT1\(^a\) rats induced rejection when transferred to R8 heart-grafted RT1\(^a\) athymic nude recipients, indicating that CD4 T cells were not tolerized by the pretreatment. To determine whether B cells were affected, nude recipients were pretreated, in the absence of T cells, with CsA/DST (or CsA/third party blood) 4 wk before grafting. The subsequent transfer of normal CD4 T cells induced acute rejection of R8 cardiac allografts in third party- but not DST-pretreated recipients; prolonged allograft survival was reversed by the cotransfer of B cells with the CD4 T cells. Graft survival correlated with reduced production of anti-MHC class I (A\(^a\)) cytotoxic alloantibody. The results indicated that the combined pretransplant treatment of CsA and DST induced tolerance in allospecific B cells independently of T cells. The resulting suppression of allospecific cytotoxic Ab correlated with the survival of MHC class I mismatched allografts. The induction of B cell tolerance by CsA has important implications for clinical transplantation. The Journal of Immunology, 2000, 164: 2427–2432.

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D4 T cells play a central role in transplantation by activating one or more effector mechanisms that destroy the grafted tissue. Destruction of the graft may result from the action of CD8 cytotoxic T cells, a delayed-type hypersensitivity response or complement-mediated cytotoxic Ab (1, 2). In clinical transplantation, the rejection process was found to be effectively inhibited by cyclosporin A (CsA)\(^3\) (3), an immunosuppressive drug that blocks IL-2 gene expression in activated CD4 T cells by interfering with cyclophilin/calcineurin signaling (4–7). Although several groups have reported and explored the effects of CsA on B cells in vitro (8–13), little attention has been given to the possibility that an effect on B cells in vivo could influence allograft survival.

Increasing evidence from both clinical and experimental studies has highlighted the contribution that alloantibody makes during acute rejection (2, 14–19). Several transplant centers observed that the presence of panel-reactive Abs against MHC class I were strongly correlated with rejection episodes (20–22). In a well-characterized experimental model, it was shown that anti-MHC class I alloantibody was directly responsible for acute rejection of class I mismatched kidneys, hearts, and skin in the high responder PVG.RT1\(^a\) (RT1\(^a\)) rat strain (2, 14, 16).

In an attempt to prolong survival of MHC-mismatched organ allografts in this RT1\(^a\) strain, recipients were given a preoperative donor-specific blood transfusion (DST) (16, 23), a procedure that has been used successfully in both clinical and experimental settings (24–29). However, rather than inducing allograft acceptance, DST sensitized the recipients, resulting in production of anti-class I alloantibody and accelerated rejection (16, 23). Bradley and colleagues (23) went on to show that the hyperacute rejection of class I-mismatched kidney allografts could be prevented and a state of unresponsiveness induced by combining the preoperative blood transfusion with a short course of CsA. The present study has confirmed this effect for cardiac allografts in the same strain combination and has investigated the mechanism for prolonged allograft survival. We found that the CsA/DST preoperative treatment induced specific unresponsiveness not, as anticipated, among the CD4 T cell subsets, but rather within the allospecific B cell population.

Materials and Methods

Animals, pretreatment, and grafting

PVG-RT1\(^a\)-RT7\(^b\) (RT1\(^a\); A\(^B\)/D\(^C\)) PVG-RT1\(^a\)-mu/mu (RT1\(^a\) nude; A\(^B\)/D\(^C\)), PVG-RT7\(^b\) (PVG; A\(^B\)/D\(^C\)), and PVG-R8 (R8; A\(^B\)/D\(^C\)) rats were bred and raised under barrier conditions and maintained in a conventional environment. DA (A\(^B\)/D\(^C\)) rats were purchased from Harlan Olac (Bicester, U.K.). Using a previously described protocol (23, 30), euthymic RT1\(^a\) DST rats were transfused i.v. with 1.0 ml of freshly collected, heparinized donor blood from R8 strain rats syngeneic with the heart allograft or from third party PVG donors. Transfusions were administered on days −28, −21, −14, and −7 before grafting on day 0. CsA was administered by gavage (15 mg/kg in olive oil or in castor oil “Sandimun”, a kind gift of Sandoz/Novartis, Basel, Switzerland) daily for 1 wk (days −28 to −22). Pretreatment of unconstituted athymic nude rats was identical except that cardiac allografts were transplanted 1 or 2 days before lymphocyte transfer (day 0). Vascularized heart grafts were transplanted

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3 Abbreviations used in this paper: CsA, cyclosporin A; MST, median survival time; DST, donor-specific blood transfusion.

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A heterotopically by a standard microvascular technique employing an end-to-side anastomosis to the great vessels. Graft survival was monitored by abdominal palpation, and rejection was confirmed by ECG-determined loss of electrical activity.

**Cell separation**

Purified CD4 T cells were obtained as previously described (32). Briefly, thoracic duct lymphocytes were depleted of B cells and CD8 T cells using a mixture of mouse anti-rat mAbs grown in house as ascites or purchased from Serotec (Kidlington, Oxford, U.K.): OX12 (anti-Igκ), OX4 (anti-MHC class II), and OX8 (anti-CD8). Stained lymphocytes were removed by two or three rounds of magnetic adherence using anti-mouse Ig-conjugated immunomagnetic particles. The resulting population of cells was 95–98% CD4+. B cells were obtained from thoracic duct lymph of athymic nude rats; 80–85% of cells were Ig− and void of functioning T cells (33).

**Alloantibody determination by flow cytometry**

Sera from RT1a animals were serially diluted and mixed with RBC from R8 rats to detect anti-MHC class I (Aa) alloantibody. After incubation, the cells were washed and stained with FITC-sheep anti-rat IgG (Serotec) or with mouse mAb against rat IgG1 (MARG1-2), IgG2a (MARG2a-1), and IgG2b (MARG2b-3) (Serotec), followed by FITC-F(ab′)2 anti-mouse Ig (Dako, High Wycombe, U.K.) absorbed against solid phase rat Ig. The titer for each sample was determined as the reciprocal dilution (log3) at which the percentage of positively stained cells was >5% above that of a normal rat serum control.

**Cytotoxic Ab determination**

The procedure was adapted from that of Bradley et al. (34). Briefly, 50 µl of serially diluted test sera, in duplicate, was added to 96-well round-bottom microtiter plates (Alpha Laboratories, Eastleigh, U.K.), and 50 µl of 1/25 diluted rabbit complement (Serotec) was added, and the plates were incubated for 1 h at 37°C. Maximum release was obtained by the addition of 100 µl HCl in place of 7% FCS. The results indicated that CsA/DST treatment had not induced tolerance to cardiac allografts in RT1u nude recipients (day 0) engrafted 1 or 2 days before with R8 hearts.

**Statistics**

Means were compared using a nonparametric modification of Student’s t test.

**Results**

**Experimental model**

Using a published protocol for prolonging the survival of kidney allografts in the high responder RT1a strain of rat (23), we determined whether the same preoperative treatment was effective in enhancing survival of cardiac allografts. Pretreating RT1a recipients with weekly injections (×4) of MHC class I-mismatched R8 strain blood (days −28, −21, −14, and −7) combined with a 7-day course of CsA, starting day −28 (CsA/DST) induced delayed rejection (Fig. 1), and 50% of the heart allografts survived indefinitely. Others showed that pretreating rats with CsA alone had no effect on allograft survival (23). A single injection of R8 blood 2 wk before transplantation and in the absence of CsA had, as expected (2), the opposite effect, inducing accelerated rejection (Fig. 1). Furthermore, this group of recipients produced high levels of anti-MHC class I (Aa) cytotoxic Ab on day 6, i.e., 3 days after rejection (log3 titer = 8.4 ± 0.5). In contrast, three rats from the CsA/DST group, with allografts that were still beating 15 mo after grafting, had very low levels of cytotoxic Ab (log3 titer = 3.0 ± 2.0) when tested at 8 mo. These observations were consistent with earlier work demonstrating that rejection of R8 allografts in RT1a recipients was mediated by anti-Aa Ab (2, 14, 16).

**Are CD4 T cells tolerant?**

To determine whether CsA/DST treatment had rendered CD4 T cells unresponsive, highly purified CD4 T cell populations were adoptively transferred into cardiac-allografted athymic nude recipients. Earlier work showed that CD4 T cells were both necessary and sufficient to induce rejection of MHC class I-mismatched R8 allografts in RT1a nude recipients (2). CD4 T cells were purified from the thoracic ducts of 1 untreated control donors, 2 syngeneic donors pretreated in the standard way with CsA and R8 blood, or 3 syngeneic donors given CsA and third-party blood transfusions. The results showed that 20 × 106 CD4 T cells from all three sources induced acute rejection (Fig. 2). There was no significant difference in allograft survival between the CsA/DST group and the CsA/third party group (median survival time (MST) = 17 and 16 respectively); rejection in the control group occurred earlier (MST = 8).

An examination of Ab production in CD4 T cell-injected nude rats indicated that the onset of rejection in the control group corresponded with production of cytotoxic Ab (Table I). When Ab production was assessed instead by using the flow cytometric assay, specific anti-Aa IgG was not detected until day 21, and titers were consistently lower. An analysis of IgG subclasses was not informative. CD4 T cells from both the CsA/DST and CsA/third party donors also induced substantial levels of cytotoxic alloantibody (log3 titer of 8.9 ± 1.47 and 9.1 ± 1.10, respectively) that persisted for many weeks after rejection. Taken together, the results indicated that CsA/DST treatment had not induced tolerance in the CD4 T cell population, leaving unresolved the mechanism by which cardiac allografts were able to survive.

**FIGURE 1**. Survival of MHC class I mismatched (R8) cardiac allografts is prolonged in the high responder RT1a strain by pretreating recipients with CsA plus DST. RT1a rats were given once weekly transfusions of R8 blood (×4) on days −28, −21, −14, and −7 and a 1-wk course of CsA (15 mg/kg given daily by gavage on days −28 to −22) (□, CsA/DST, n = 6). Control groups were untreated (○, Control, n = 4) or received an R8 blood transfusion alone on day −14 (△, DST, n = 5). Recipients received an R8 heart allograft on day 0.

**FIGURE 2**. CD4 T cells are not tolerized by CsA/DST treatment. CD4 T cells were purified from the thoracic duct of donors that were untreated (○, Normal control, n = 12), given the standard 4-wk CsA/DST treatment (□, CsA/DST, n = 6), or given CsA and third-party (PVG) blood (△, CsA/third party, n = 6). CD4 T cells (20 × 106) were transferred i.v. to RT1a nude recipients (day 0) engrafted 1 or 2 days before with R8 hearts.
Are B cells tolerant?

In the absence of an apparent effect on CD4 T cells, we asked whether the CsA/DST treatment could instead be affecting B cells. Nude rats develop a normal B cell population despite the absence of functioning T cells. Therefore, nude recipients were treated before CD4 T cell reconstitution with the standard 1 wk course of CsA (day −28 to −22) and given a weekly injection of R8 blood (days −28, −21, −14, and −7). As a specificity control, third party blood was substituted for the R8 transfusion. As a further control to determine whether CsA was essential for the effect, nude recipients were pretreated with R8 blood (days −28, −21, −14, and −7) but in the absence of CsA. Nude recipients received an R8 heart graft on days −2 or −1 and were reconstituted with normal CD4 T cells (18−20 × 10⁶) from untreated euthymic donors on day 0. In contrast with the acute rejection observed in untreated nude recipients (see Fig. 2), CsA/DST pretreated nude recipients showed prolonged R8 allograft survival (Fig. 3), and one third of the R8 hearts survived indefinitely. This suggested that B cells in the nude recipients (before CD4 T cell transfer) had been tolerized by CsA/DST treatment. The unresponsiveness was apparently specific, for pretreating nude recipients with CsA plus third party blood had no effect, i.e., acute rejection was observed. Furthermore, CsA was necessary to induce unresponsiveness since R8 blood transfusions in the absence of CsA were unable to prolong allograft survival. Although blood samples of nude recipients receiving DST alone showed a low level of donor A⁺ chimerism (mean 0.29% ± 0.21), cardiac allograft rejection was unaffected. Nude rats possess an active NK cell response that rapidly removes allogeneic lymphocytes (35). As an additional control to show that normal CD4 T cell-mediated rejection had not also been disrupted, CsA/DST pretreated nude recipients were engrafted with DA (RT1⁺) hearts, i.e., mismatched at MHC class II (B/D⁺) and class I-like (C⁺) loci in addition to class I (A⁺). Earlier work showed that alloantibody was ineffective in destroying the class II disparate allograft (16), indicating that rejection of a class II difference was cell mediated, requiring functioning CD4 T cells. The rejection of DA allografts (Fig. 3) confirmed that CD4 T cells functioned normally against MHC class II- and I-like differences in the CsA/DST-pretreated nude recipient.

To determine whether the unresponsiveness could be reversed (i.e., rejection restored), nude recipients pretreated with the standard 4-wk CsA/DST protocol were reconstituted on day 0 with a mixture of CD4 T cells (20 × 10⁶ from normal euthymic donors) and B cells (70 × 10⁶ cells obtained from the thoracic duct of untreated nude rats). The addition of B cells restored rejection (Fig. 4). Seven weeks after engraftment, serum samples were collected from the recipients in Fig. 4 to assay for cytotoxic Ab. Grafts that were rejected by 35 days in either the “CD4 T cell” or “CD4 + B cells” group had high levels of cytotoxic Ab (Fig. 5). In contrast, three recipients from the CD4 T cell group that retained their allografts indefinitely had low levels of cytotoxic Ab. Taken together, the results indicated that unresponsiveness induced by CsA/DST lay within the B cell compartment.

### Table 1. Anti-MHC class I (A⁺) Ab production in R8 heart-grafted, CD4 T cell-reconstituted RT1⁺ nude rats

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<td>Cytotoxic</td>
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<td>IgG</td>
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 workflow: ① A comparison of cytotoxic and flow cytometric-based assays. Values are mean log₃ dilutions ± SD of five or six samples. R8 heart grafts were rejected between days 7 and 15 (MST = 8).
  ② No binding at a dilution of 1/3.
  ③ Not tested.

![FIGURE 3](The Journal of Immunology) Pretreating T cell-deficient RT1⁺ nude recipients with CsA/DST before CD4 T cell reconstitution prevents subsequent rejection. RT1⁺ nude rats were given the standard 4-wk CsA/DST treatment, engrafted with R8 (class I A⁺-mismatched) hearts ( ), Nu-CsA/DST, DA ( ), and 1 or 2 days later, i.e., day 0, injected i.v. with 18−20 × 10⁶ CD4 T cells from normal untreated donors. Other groups of nude recipients were pretreated with a 4-wk course of R8 blood alone ( ), Nu-DST, n = 6) or with CsA and third party (PVG) blood (Δ, Nu-CsA/3rd party, n = 7), and given R8 heart grafts and 20 × 10⁶ CD4 T cells on day 0. †, Recipients died with surviving allografts.

![FIGURE 4](The Journal of Immunology) CsA/DST-induced tolerance is broken in RT1⁺ nude recipients by adoptive transfer of normal B cells. RT1⁺ nude rats were given the standard 4-wk CsA/DST treatment, engrafted with R8 hearts, and 1 or 2 days later, i.e., day 0, injected with 18 × 10⁶ CD4 T cells alone ( ), data from Fig. 3) or with 20 × 10⁶ CD4 T cells together with 70 × 10⁶ B cells obtained from the thoracic ducts of untreated RT1⁺ nude rats (Δ, CD4 + B cells, n = 6).
Discussion

Previous studies showed that, in those donor/recipient combinations where primary allograft rejection was mediated by allograft, giving recipients a prior blood transfusion sensitized rather than tolerized the recipients (2, 15, 16, 23). As a result, subsequent transplants underwent accelerated destruction. Thus, in the high responder RT1<sup+a</sup> strain anti-MHC class I alloantibody was induced following transfusion of DA (A<sup+a</sup>/B<sup+d</sup>/C<sup+c</sup>) or R5 (A<sup+a</sup>) mismatched blood. Earlier studies showed that combining the pretreatment transfusion with a short course of CsA suppressed alloantibody formation (30, 36) and reversed the whole rejection process (23); class I-mismatched kidney allografts were now accepted long-term and anti-A<sup+a</sup> alloantibody was greatly reduced. The present investigation confirmed these observations for class I-mismatched cardiac allografts and studied the basis for the apparent tolerance.

Contrary to expectations, CD4 T cells were not tolerized by the CsA/DST pretreatment; CD4 T cells from CsA/DST-treated donors induced acute rejection when adoptively transferred to cardiac-allografted nude recipients. Instead, the evidence suggested that prolonged allograft survival was due to tolerance in the specific B cell compartment. When nude recipients were pretreated with the standard CsA/DST protocol in the absence of T cells, the subsequent injection of CD4 T cells from normal donors failed to induce prompt rejection, and a third of the nude recipients retained their cardiac allografts indefinitely. This failure to reject correlated with a reduction of alloreactive IgG cytotoxic Ab. Furthermore, the effect was Ag specific and CsA dependent: substituting donor with third party blood did not prevent rejection in pretreated nude recipients, and DST pretreatment of nude recipients in the absence of CsA resulted in acute rejection. Tolerance was broken by coculturing normal B cells together with normal CD4 T cells, a combination that also restored high level production of allospecific anti-class I cytotoxic Ab.

The results indicated, somewhat unexpectedly, that the unresponsiveness induced by CsA/DST was finely balanced and in some animals was lost with time. Perhaps new B cells emerging from bone marrow stem cells restored the alloreactive repertoire. Certainly deliberate injection of nontolerant B cells reinstituted both alloantibody synthesis and destruction of the heart graft. It is worth considering whether periodic CsA treatment could be used to tolerize newly emerging B cells, in which case the allograft itself might substitute for the alloantigens initially provided by the DST.

CsA and a similar drug, FK 506, have major effects on a calcineurin-mediated signal transduction pathway in T cells (7). CsA complexes with cyclophilin that binds to and prevents Ca<sup2+</sup>-dependent activation of calcineurin (37). In turn, the transcription factor NF-AT, normally dephosphorylated by calcineurin, fails to initiate transcription of IL-2 (4, 6). Although most investigations have explored CsA’s effect on T cells, CsA was also shown to inhibit PB cell proliferation (9, 11), to reduce Ab production initiated in vitro or in vivo (8, 10, 38, 39), and to block NF-κB activation (12, 13). The present study provides strong in vivo evidence that CsA, when combined with a specific blood transfusion, induces tolerance in those B cells that normally elicit a thymus-dependent Ab response. Aside from an incidental observation (40), the possibility that CsA could mediate B cell tolerance has not been recognized before. However, earlier studies clearly showed that CsA could affect B cells. It was found that CsA alone had no effect on B cell proliferation in vitro, but in combination with anti-IgM, not only inhibited B cell proliferation (9) but increased the level of B cell death (11). Whether CsA has a primary role in regulating B cell survival is controversial; several groups have shown that CsA could prevent apoptosis in immature T and B cells (41, 42). However, more recent evidence suggested that CsA had a direct role in mediating programmed cell death in mature lymphocytes (43). The sequence in which B cells were exposed to CsA relative to Ag may be important. Calcineurin-mediated dephosphorylation of NF-AT depends on free Ca<sup2+</sup> normally released from intracellular stores by cross-linking the B cell receptor. When B cells (and T cells) were “sensitized” first by CsA and then stimulated by ionomycin to activate intracellular Ca<sup2+</sup>, apoptosis was induced (43). Apparently, a combination of Ca<sup2+</sup> and TGF-β1, produced by the CsA-activated B cell, was needed to initiate the apoptotic event. Giving the blood transfusion after the CsA, the sequence used in the present study, could have been instrumental in inducing the B cell tolerance. Interestingly, using the RT1<sup+a</sup> model, it was observed that giving a single transfusion before the course of CsA had little effect in prolonging allograft survival (23). Clearly, further work is needed to establish the critical factors leading to CsA-induced tolerance of B cells.

Although we found that production of anti-class I Ab as measured by the cytotoxic assay correlated with allograft rejection, when Ab was assessed by flow cytometry, the assay was less sensitive and the link with rejection was inconsistent. Additional cytomagnetic analysis of IgG1, IgG2a, and IgG2b subclasses shed no further light on the discrepancy. It may be relevant that the flow cytometric assay relies only on the ability of Ab to bind; it does not necessarily reflect Ab function in vivo.

The effect of CsA on B cells has been largely overlooked in clinical transplantation for a variety of reasons. First, in vitro studies showed that B cells were less sensitive than T cells to CsA at therapeutic doses (8, 38). Second, the link between CsA and B cell-induced Ab synthesis is indirect. Although T-dependent alloantibody production is profoundly inhibited by CsA treatment (44), this effect has been attributed primarily to CsA’s well-known action on CD4 T cells. Third, acute rejection has long been associated with CD4 T cells, shown to be necessary and sufficient to

FIGURE 5. B cell reconstitution restores anti-MHC class I alloantibody production in CsA/DST-treated RT1<sup+a</sup> nude recipients. Serum collected from nude recipients 7 wk after CD4 T cell reconstitution and with or without B cell injection (see Fig. 4 legend for details) were assayed for the presence of cytotoxic anti-A<sup+a</sup> alloantibody. Recipients of the “CD4 T cell” group were subdivided between those that had or had not rejected their R5 allografts as indicated. Values are means ± SD (n = 3, 3, and 5 respectively).
induce allograft rejection (45–47); for example, athymic nude animals that lack functioning T cells but express a full complement of B cells retain allografts indefinitely (33, 48). Consequently, B cells have tended to be relegated to a secondary, nonessential role. Fourth, a majority of animal models strongly supported the concept that acute rejection was cell and not Ab mediated (1). The literature contains numerous accounts of the failure to induce acute rejection by passive Ab treatment (49–52). More recent experiments have now rectified earlier misconceptions; the importance of anti-class I Ab in evoking acute rejection has been clearly demonstrated in experimental models (2, 14, 16, 23) and closely reflects clinical experience (18, 20, 22, 53).

One of the reasons for withdrawing prior blood transfusion from clinical use was the inadvertent induction of alloantibody, rendering patients unsuitable for subsequent transplantation (54, 55). From another perspective the DST-induced sensitization may be less detrimental than initially perceived. Transfusion-induced alloantibody production serves to identify those patients whose immune system is already poised for an aggressive anti-graft response should a transplant be given (56). The remainder (non-Ab producers) benefit from DST, a treatment that appears to be effective in preventing T cell-mediated rejection (16). In those patients in which allograft rejection is T cell mediated, it is not clear whether CsA pretreatment would preserve (or destroy) the tolerance-inducing properties of prior DST; this needs to be investigated. If the tolerance-inducing properties were to be preserved, it may be possible to benefit both “Ab” and “non-Ab” producers by routinely combining pretransplant transfusion with CsA therapy as described here.

Parenthetically, it is interesting to reflect on the reasons for the success of calcineurin-blocking drugs in clinical transplantation. The unexpected influence of CsA on Ag-specific B cells suggests that the effect of CsA on B cells in preventing organ allograft rejection may have been underestimated. Clearly, there is scope for developing immunosuppressant drugs that primarily target activated B cells.

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