Transplant Int (1989) 2: 13-15

TRANSPLANT
International

A requirement for continued graft presence in the maintenance of systemic tolerance induced by cyclosporin A (CyA) in rats

Susan M. L. Lim and David J. G. White

Department of Surgery, Level 9, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK

Abstract. We report on a requirement for the continued presence of a heart graft in the maintenance of systemic tolerance induced by cyclosporin A (CyA). Hearts from DA rats (RT1a) were grafted into PVG (RT1c) recipient. CyA-induced long-term survivors (LTS) bearing functioning heart grafts are systemically tolerant, as demonstrated by the significant prolongation (> 50 days) of donor-strain skin grafts. In contrast, donor-strain skins grafted 3 weeks after the removal of heart grafts from LTS are acutely rejected (median survival 10.0 days; $P < 0.005$). Using an adoptive transfer assay, 5×10^7 splenic lymphocytes obtained from LTS 3 weeks after the removal of the heart grafts fail to mediate adoptive tolerance (median survival 17.0 days), in contrast to the tolerance achieved by splenic lymphocytes obtained from LTS with functioning heart grafts (median survival > 100 days; $P < 0.005$).

Key words: Cyclosporin A - Heart graft - Tolerance - Suppressor cells.

We have previously shown that short-term cyclosporin A (CyA) treatment can produce indefinite survival of fully allogeneic heart grafts in rats [6, 8]. Using M.H.C. class I disparate heart grafts, we found that the retention of a graft alone, in the absence of initial immunodepressive treatment, was inadequate for the induction of systemic tolerance [7]. The findings of other studies [1, 5] suggest that the mechanisms which may be responsible for this tolerant state include suppressor cells, enhancing alloantibodies, and graft adaptation. The systemic nature of CyA-induced tolerance, as reported previously [6, 8], implies a major role for either suppressor cells or enhancing antibodies. Indeed, suppressor lymphocytes have been demonstrated in the spleens of CyA-treated, heart-grafted rats [5]. The recent work of Hall and colleagues [2] suggests that there is a requirement for the presence of a graft in the perpetuation of these cells. Here, we report on the mechanism underlying CyA tolerance in rats and investigate a requirement for both the presence of the graft and CyA in the maintenance of this systemic tolerance.

Offprint requests to: S. M. L. Lim, Department of Surgery, National University Hospital, Lower Kent Ridge Road, Singapore 0511

Materials and methods

Generation of tolerant rats bearing heart grafts

Donor-acceptor (DA) (RT1a) donors (females) and PVG (RT1c) recipients (males) were used. Donors weighed between 100 and 150 g, recipients between 200 and 250 g. Both rat strains were obtained from Bantin and Kingman (Hull, UK). Accessory cervical heart transplantation was performed according to the technique described by Heron [3]. CyA (a gift from Sandoz, Basle, Switzerland) was dissolved in olive oil at a concentration of 25 mg/ml. It was administered IM at a dose of 15 mg/kg per day for 17 days (days 0-6, 21-27, and 42-44 post-grafting) using an intermittent schedule designed for tolerance induction [8]. Rats bearing functioning heart grafts more than 100 days were classified as long-term survivors (LTS).

Experimental design

Functioning heart grafts were removed on day 100 by ligating and dividing both the external jugular vein and common carotid artery. The use of portex cuffs for the vascular anastomoses facilitated the complete, intact removal of all original donor tissue. Hemostasis was essential to ensure the continued survival of these animals following transplantectomy. A requirement for the presence of the graft in the maintenance of systemic tolerance was investigated using two assays. First, DA skin grafts were sutured into full-thickness beds on LTS 3 weeks following the removal of heart grafts. Second, spleens were harvested from LTS 3 weeks

Table 1. Skin graft survival in CyA-tolerant rats (LTS) with and without heart grafts (HG)

Group	Heart graft survival (days)	Median survival (days)
Naive	8, 8, 8, 9, 9, 9, 9, 10, 10, 10	9.0
LTS - HG present	38, 45, > 50 (× 8)	> 50.0
LTS - HG removed	8, 9, 10, 10, 10, 10, 10, 11, 12, 12	10.0

Table 2. Splenic lymphocyte (SL) activity in CyA-tolerant rats (LTS) following heart graft (HG) removal, using a 550-rad adoptive transfer assay (reconstituting dose = 5×10^7 cells)

Group	Heart graft survival (days)	Median survival (days)
Irradiated controls (no cells)	11, 11, 16, 16, 17, 17, 18, 18, 20, 24	17.0
SL (naive)	13, 13, 13, 14, 14, 14, 14, 15, 15, 16	14.0
SL (LTS) - HG present	> 100 (× 8)	> 100.0
SL (LTS) - HG removed	13, 15, 15, 16, 18, 22, 28, 28	17.0

after heart graft removal, and the ability of splenic lymphocytes to transfer tolerance adoptively was investigated. Appropriate controls were included.

Skin grafting

Skins were raised as full-thickness grafts (2 × 1 cm) from the ventral abdominal wall of DA donors and sutured into full-thickness beds on recipient flanks ($n = 10$). Dry dressings, secured with 2-inch elastoplast (Smith and Nephew, Hull, UK), were removed after 5 days and grafts were inspected daily thereafter. Rejection was diagnosed when more than 50% of the graft surface had become raised, necrotic, or scabby.

Preparation of splenic lymphocytes

Splenic lymphocytes were prepared by mincing and expressing the spleens through a stainless steel sieve into RPMI 1640/5% FCS (medium). The cell suspension was layered on ficoll isopaque (specific gravity 1.077 g/l) and centrifuged at 350 *g* for 40 min at room temperature. The lymphocyte layer was removed from the interface, washed twice with medium, and finally made up to the required concentration of 5×10^7 cells/ml.

Adoptive transfer assay

Suppressor cell activity was tested using an adoptive transfer assay. PVG rats received a brief, sublethal dose of radiation (550 rads) and were reconstituted with 5×10^7 splenic lymphocytes on day −1. These irradiated and reconstituted rats were heart-grafted with DA hearts the following day and were monitored for up to 100 days post-transplant. The ability of the adoptively transferred inoculum of cells to prolong heart graft survival was then determined. Four groups of adoptive recipients were used: (1) an irradiation control group not receiving any cells ($n = 10$), (2) a reconstituted naive control group receiving splenic lymphocytes from naive PVG rats ($n = 10$), (3) a tolerant control group receiving splenic lymphocytes (SL) from LTS bearing heart grafts ($n = 8$), and (4) a test group reconstituted with splenic lymphocytes from LTS whose heart grafts had been removed ($n = 8$).

Results

DA skins were acutely rejected in naive PVG rats (median survival 9.0 days; see Table 1). When grafted onto LTS bearing functioning heart grafts, donor-specific skin survival was prolonged to a median of more than 50 days ($P < 0.005$). Skins grafted 3 weeks after the removal of long-surviving heart grafts no longer enjoyed prolonged survival (median survival 10.0 days; $P < 0.005$).

Studies of suppressor cell function in LTS by adoptive transfer yielded the following results (Table 2). In the 550-rad-irradiated, but unreconstituted group, DA hearts were rejected by day 25 (mean survival time 16.8 days). The adoptive transfer of 5×10^7 splenic lymphocytes from normal PVG rats restored heart graft rejection times to near-normal (median survival 14.0 days). In contrast, splenic lymphocytes obtained from LTS bearing heart grafts adoptively transferred tolerance in all cases (median survival > 100 days; $P < 0.005$). However, splenic lymphocytes obtained 3 weeks after the heart grafts had been removed failed to adoptively transfer tolerance (median survival 17.0 days; $P < 0.005$).

Discussion

Earlier studies, performed by Kasahara et al. [4], have shown that the presence of a heart graft between days 14 and 33 post-transplant (i.e., during the induction phase) is important for the prolongation of donor-strain heart graft survival. We, however, using an intramuscular route, have demonstrated that immunosuppressive levels of CyA, administered for 2 weeks at 15 mg/kg per day, are still detectable in the blood [6]. Therefore, a direct role for the presence of a graft in the maintenance of systemic tolerance to other donor-strain grafts cannot be determined during this time. Furthermore, our failure to demonstrate adoptive tolerance from CyA-treated, heart-grafted rats during this time period (S. M. L. Lim, D. J. G. White, in preparation) renders studies on the role of the graft in the perpetuation of suppressor cell mechanisms difficult.

The studies described here support the results of Hall and colleagues [2] in demonstrating a requirement for the presence of the graft in the maintenance of systemic tolerance. Here, we confirm the presence of stable systemic tolerance in greater than 100-day LTS by the significant prolongation (> 50 days) of donor-strain skin grafts and by the consistent transfer of adoptive tolerance by 5×10^7 splenic lymphocytes. Removal of the graft resulted in a loss of systemic tolerance to donor-strain antigens. This was manifested by the rejection of donor-strain skin grafts in first set fashion, despite the fact that these animals had previously been grafted with hearts from the same rat strain. Thus, although tolerance was lost as a result of removing the heart grafts, subsequent immunity, as reflected by second set rejection, did not develop. In addition, splenic lymphocytes obtained from LTS 3 weeks after the removal of the graft failed to mediate adoptive tolerance.

In contrast, suppressor cells are consistently detectable by adoptive transfer assays in LTS still bearing heart grafts. The ability of suppressor cells to mediate adoptive tolerance in the absence of CyA implies that once these cells have been induced, CyA is no longer necessary. In these animals, the loss of suppressor activity as early as 3 weeks after graft removal substantiates a requirement for a continuous, dynamic interaction between suppressor cells and antigen. Indeed, Hall et al. [2] have also reported the loss of suppressor cell activity 1 week after removal of heart grafts. Whether it is the graft itself or the antigens released from the graft that maintains the tolerant state could not be determined from these experiments.

References

1. Barber WH, Hutchinson IV, Morris PJ (1985) Mechanisms of kidney allograft maintenance in rats treated with cyclosporine. Transplant Proc 17: 1391-1393
2. Hall BM, Gurley K, Dorsch SE (1987) Specific unresponsiveness in rats with prolonged allograft survival is dependent upon the graft and suppressor T cells. Transplant Proc 19: 495-496
3. Heron I (1971) A technique for accessory cervical heart transplantation in rabbits and rats. Acta Pathol Microbiol Immunol Scand [A] 79: 366-372
4. Kasahara K, White DJG, Calne RY (1982) Antigen dependence of cyclosporin A-induced allograft acceptance. Transplantation 34: 216-218
5. Kupiec-Weglinski JW, Lear PA, Strom TB, Tilney NL (1983) Population of cyclophosphamide-sensitive T suppressor cells maintain cyclosporine-induced allograft survival. Transplant Proc 15 [Suppl 1]: 2357-2363
6. Lim SML, White DJG, Calne RY (1987) Identifying a susceptible period following cyclosporine A induced tolerance of heart allografts in the rat. Transplant Proc 19: 4218-4220
7. Lim SML, White DJG (1988) Long term residence of a graft is an insufficient stimulus for the induction of tolerance. J Exp Med 168: 807-810
8. Lim SML, White DJG, Calne RY (1988) Cyclosporine coverage during the risk period leads to 100% long term graft acceptance in the rat. Transplant Proc 20 [Suppl 3]: 1013-1015