Acta Medica Okayama

Neonatal Intrathymic Splenocyte Injection Yields Prolonged Cardiac Xenograft Survival

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Abstract

Intrathymic (i.t.) injection of allogenic cells without administration of anti-lymphocyte serum (ALS) in neonatal recipients has induced donor-specific tolerance to subsequent cardiac allografts in rats. This study examines whether similar tactics can be successfully applied to a hamster-to-rat cardiac xenotransplantation model. Lewis neonates on their first day of life underwent i.t., subcutaneous (s.c.), intraperitoneal (i.p.), or intravenous (i.v.) injections of 5×10^{7} Golden Syrian hamster splenocytes. After six weeks, the rats underwent heterotopic cardiac transplantation of hamster hearts. Cyclophosphamide (CyP) was administered on the day before surgery and postoperatively to suppress antibody-mediated graft rejection. Rats given splenocytes with 80 mg/kg of CyP had the following graft survival times: 8 to 12 days for i.t. injection (mean, 9.4 days); 5 to 7 days for s.c. injection (mean, 6.6 days); 4 to 11 days for i.p. injection (mean, 7.4 days); and 4 to 13 days for i.v. injection (mean, 7.9 days). Only the extension of graft survival produced by i.t. injection was statistically significant in comparison with the rats given only CyP treatment (mean, 7.5 days; $P < 0.05$). Thus, it appears that i.t. injection of xenogenic splenocytes in neonatal recipients with administration of CyP, but without ALS, can prolong xenograft survival. This biological intervention may be most useful in pediatric xenotransplantation when combined with other immunomodulation techniques.

KEYWORDS: intrathymic injection, neonatal tolerance, xenografts

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Intrathymic (IT) injection of allogenic cells without administration of anti-lymphocyte serum (ALS) in neonatal recipients has induced donor-specific tolerance to subsequent cardiac allografts in rats. This study examines whether similar tactics can be successfully applied to a hamster-to-rat cardiac xenotransplantation model. Lewis neonates on their first day of life underwent IT, subcutaneous (SC), intraperitoneal (IP), or intravenous (IV) injections of 5×10^7 Golden Syrian hamster splenocytes. After six weeks, the rats underwent heterotopic cardiac transplantation of hamster hearts. Cyclophosphamide (CyP) was administered on the day before surgery and postoperatively to suppress antibody-mediated graft rejection. Rats given splenocytes with 80 mg/kg of CyP had the following graft survival times: 8 to 12 days for IT injection (mean, 9.4 days); 5 to 7 days for SC injection (mean, 6.6 days); 4 to 11 days for IP injection (mean, 7.4 days); and 4 to 13 days for IV injection (mean, 7.9 days). Only the extension of graft survival produced by IT injection was statistically significant in comparison with the rats given only CyP treatment (mean, 7.5 days; $P < 0.05$). Thus, it appears that IT injection of xenogenic splenocytes in neonatal recipients with administration of CyP, but without ALS, can prolong xenograft survival. This biological intervention may be most useful in pediatric xenotransplantation when combined with other immunomodulation techniques.

Key words: intrathymic injection, neonatal tolerance, xenografts

In 1953, Medawar *et al.* observed that fetal mice exposed to allogenic antigens of a prospective donor strain, during the time when their immune systems were still developing, could acquire permanent or long-lasting tolerance to a subsequent skin graft and described this phenomenon as "actively acquired tolerance" (1). Recently, this historic principle was successfully applied to a cardiac allograft model in rats by intrauterine exposure of prospective fetal recipients to allogenic cells (2) and later by intrathymic (IT) injection of allogenic cells in neonatal recipients (3). It has been demonstrated that these biological manipulations induce donor-specific tolerance to subsequent cardiac allografts without causing even transient immunosuppression. Although IT pretreatment has been shown to be effective in adult models, it requires administration of anti-lymphocyte serum (ALS) or other treatments to delete immunocompetent peripheral lymphocytes (4-6).

The success of producing donor specific tolerance in these allograft models has led some researchers to apply the same biological approaches to producing tolerance in animal xenographic cardiac transplant models. Sheffield *et al.* demonstrated that in a hamster-to-rat xenograft model, IT injection of xenogenic splenocytes in adult recipients produces prolonged cardiac xenograft survival when cyclophosphamide (CyP) is administered in addition to ALS (7). Although no extension of cardiac xenograft survival was induced by neonatal IT pretreatment alone (8), Sheffield *et al.*'s study suggests that the IT xenogenic cells can also alter the immune recognition process resulting in prolongation of graft survival.

In the present study, we investigated whether neonatal IT injection along with administration of CyP would yield extension of cardiac xenograft survival. Results confirm

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this extension and suggest that the underlying mechanisms of neonatal tolerance induced in our xenograft model are similar to those in allograft models.

Materials and Methods

Animals.   Female adult Lewis rats (RT1[1]) at two weeks of gestation were obtained from Japan SLC (Hamamatsu, Japan). Their newborn male offspring were used as recipients at six weeks of age (130–180 g). Six-week-old male Golden Syrian hamsters (closed colony) were obtained from Japan SLC and served as donors at six to ten weeks of age (100–140 g). All animals were kept in pathogen-free animal facilities at Okayama University and were handled in a humane fashion in accordance with the university's modified version of the National Institute of Health guidelines.

Preparation of donor spleen cells.   Donor Golden Syrian hamster splenocytes were obtained by mincing the harvested spleen and passing it gently through a stainless steel mesh screen. The cells were washed three times with phosphate buffered saline (PBS), counted using crystal violet, and suspended in PBS at a concentration of $5.0 \times 10^7/0.1$ ml. The cells were injected soon after this procedure.

Pretreatment of graft recipients.   Neonatal rats on their first day of life were injected with either 5.0×10^7 hamster splenocytes or the PBS vehicle for control animals. A volume of 0.1 ml was injected into the thymus percutaneously with a 30-gauge needle under magnification via a caudally directed approach in the suprasternal location (3). Other neonatal rats on their first day of life had the same amount of hamster splenocytes injected either subcutaneously (SC), intraperitoneally (IP), or intravenously (IV). IV injection was accomplished without leakage via an external jugular vein using magnification. SC injection was performed at the suprasternal location aiming at the parathymic area following the same technique as the IT injection described above, so that no splenocytes were injected into the thymus. The neonates were placed back with their mothers until five weeks of age and then weaned. Heart transplantation was performed at six weeks of age.

Heart transplantation.   Hamster hearts were transplanted heterotopically using the modified technique of Ono and Lindsey (9). The donor organ was placed in the recipient's abdomen and anastomosed to the infrarenal aorta and inferior vena cava. Transplant viability was determined by daily palpation of the graft pulse through the abdominal wall. Rejection was diagnosed by the cessation of the heart beat and was confirmed through direct inspection and histopathology.

Histopathology.   After graft failure, the donor hearts were removed and fixed in 10 % buffered formalin and embedded in paraffin. Staining was carried out with hematoxylin and eosin. Slides were examined by light microscopy.

Cyclophosphamide and splenectomy.   CyP was suspended in normal saline at 8 mg/ml from 100-mg vials (Endoxan, Shionogi Pharmaceutical Co., Osaka, Japan). CyP was administered through IP injection 20, or 40 mg/kg on the day before the operation, followed by 20 mg/kg on days two, five, and eight of post-transplantation. Splenectomy was performed soon after the laparotomy for transplantation.

Dye study.   Lewis neonatal rats on their first day of life had 0.1 ml of India ink injected into the thymus percutaneously with a 30-gauge needle under magnification following the same technique of IT injection. Twenty-four hours later, the neonate was sacrificed. After subjecting the whole body to the same histopathology treatments described above, slides were examined by light microscopy.

Statistical analysis.   The groups were compared using the Mann-Whitney U nonparametric test.

Results

Survival of Golden Syrian hamster hearts in 67 Lewis rats is shown in Tables 1 and 2. Untreated Lewis rats rejected hamster hearts within 2.8 days (Group 1). Lewis rats of Groups 2 through 7 were administered a total of 80 mg/kg CyP (Table 1). Lewis rats that were given hamster splenocytes intrathymically (Group 4) had a prolonged graft survival of 8 to 12 days (mean, 9.4 days) compared to both control groups: one given CyP only (Group 2; mean, 7.5 days; $P < 0.05$) and one given PBS intrathymically (Group 3; mean, 7.2 days; $P < 0.05$). Lewis rats given splenocytes through SC injection at the parathymic area had a graft survival of 5 to 7 days (Group 5; mean, 6.6 days), which sharply contrasted with Lewis rats given splenocytes intrathymically ($P < 0.01$ vs Group 4). Lewis rats given splenocytes through IP and IV injections (Groups 6 and 7) had a graft survival of 4 to 11 days (mean, 7.4 days) and 4 to 13 days (mean, 7.9 days), respectively. The variability in efficacy of the

pretreatment that occurred in these groups was notable.

Lewis rats of Groups 8 through 11 were administered a total of 100 mg/kg CyP (Table 2). Lewis rats given splenocytes intrathymically had a graft survival of 7 to 12 days (Group 10; mean, 9.4 days) and 8 to 13 days (Group 11; mean, 9.4 days) for supplemental splenectomy, both of which did not reach statistical significance when compared to the corresponding control groups that were given only PBS intrathymically (Groups 8 and 9). Neither the increased dosage of CyP nor the supplemental splenectomy improved the IT pretreatment.

Histology of the grafts with short survival times of less than 4 days (as in Group 1) revealed diffuse vascular thrombosis with widespread hemorrhagic necrosis, which is a feature of acute humoral rejection. The grafts which showed longer survival times displayed, in amounts which increased in proportion to longer survival times, a significant amount of dense cellular infiltrate with edema, myofibril necrosis, and mild hemorrhage, consistent with

Table I   Survival of Golden Syrian hamster xenografts in Lewis rats after neonatal xenogenic splenocyte injection (CyP 80 mg/kg)

Group	Sp injection	CyP 80 mg/kg	Survival (days)	Number of rats	Mean (days)
I	—	—	2,3,3,3,3	5	2.8
2	—	+	7,7,7,8,8,8	6	7.5
3	IT(PBS)	+	6,7,7,8,8	5	7.2
4	IT	+	8,8,8,11,12	5	9.4*
5	SC	+	5,7,7,7,7,7	6	6.6
6	IP	+	4,5,7,7,8,8,9,11	8	7.4
7	IV	+	4,4,5,8,8,8,8,9,13	9	7.9

Sp: Splenocyte; CyP: Cyclophosphamide; IT: Intrathymic; PBS: Phosphate buffered saline; SC: Subcutaneus; IP: Intraperitoneal; IV: Intravenous. *Mann-Whitney U: $P < 0.05$ vs Groups 2 and 3; $P < 0.01$ vs Group 5.

Table 2   Survival of Golden Syrian hamster xenografts in Lewis rats after neonatal xenogenic splenocyte injection (CyP 100 mg/kg and splenectomy)

Group	Sp injection	CyP 100mg/kg	Splen-ectomy	Survival (days)	Number of rats	Mean (days)
8	IT(PBS)	+	—	7,7,9,10,11	5	8.8
9	IT(PBS)	+	+	7,8,8,8,10,10	6	8.5
10	IT	+	—	7,8,8,10,12	5	9.4[a]
11	IT	+	+	8,8,8,8,10,11,13	7	9.4[b]

Sp; CyP; IT; PBS: See legend to Table I.
a : NS (Not significant) vs Group 8; b : NS vs Group 9.
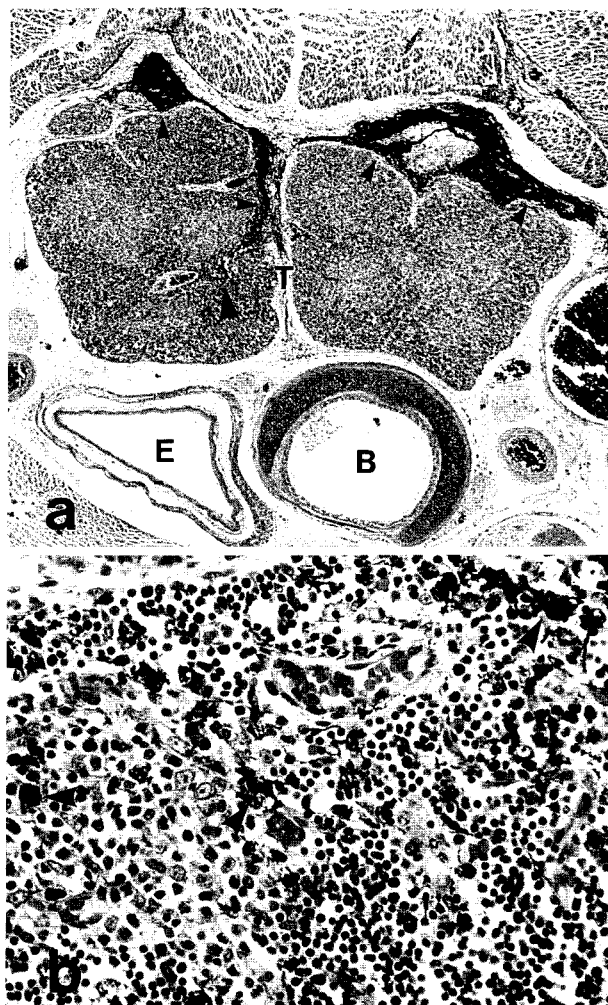
Fig. I   Photomicrographs showing the injected dye (India ink) in the thymus of a neonatal rat. The dye is visible in both lobes subcapsularly (small arrowheads) and in parenchyma (a large arrowhead) (a). High magnification view of the dye (arrowheads) injected in the parenchyma of the thymus (b). (Hematoxylin and eosin; a, × 30, b, × 300.) B: Bronchus; E: Esophagus; T: Thymus.

cellular rejection. Any difference that IT pretreatment made was not visible upon histological examination.

India ink was not identified in the thymus in 2 out of 9 neonatal rats given the dye intrathymically while in the other 7 (78%), varying small amounts of dye were observed subcapsularly and in the parenchyma (Fig. 1).

Discussion

Now that human neonatal transplantation has become a reality, some researchers are beginning to explore the

possibility of performing pediatric heart transplants in the brief "window of opportunity" which exists during the first few days or weeks of life (10). However, the need for donor hearts is particularly desperate, especially for the newborn infants with severe congenital cardiac anomalies who quite often die while waiting for hearts. In light of this, we believe that advances in xenotransplantation, using animal hearts in conjunction with currently developing immunosuppressive therapies, might be of tremendous value to this population. However, these therapies require such massive doses of immunosuppressive agents causing significant morbidity and mortality, that clinical application is not advisable (11, 12). A reliable method to produce graft tolerance would mitigate complications by reducing the dosage of, or eliminating one or two elements of these regimes. Our study demonstrates that IT pretreatment could play such a roll as a substitute for cyclosporine (CsA) or FK-506 that suppress T-cell immunity specifically.

When Medawar et al. (1) published their startling discovery of "actively acquired tolerance", they thought that the immune system of the fetal period was special because exposure to alloantigens in adult life produced "immunity", whereas early in fetal life, it produced "tolerance". The immune system of the neonatal period is believed to provide a transition into the phase of the adult immune system. As far as the neonatal and adult immune systems are concerned, these two apparently different immune systems are remarkably similar with the following exceptions: According to Streilein (13), one important difference is that immunocompetent lymphocytes occupy the peripheral lymphoid organs in adults, whereas few immunocompetent T-cells are present in the periphery of neonates because export of mature T-cells from the thymus has barely begun at the time of partition. Consequently, the introduction of allografts into adult recipients produces a predictable destructive immune rejection, whereas in neonates, allografts are readily accepted. Streilein maintains that another important difference is the rapidity with which chimerism is established and the rapidity with which tolerogen-specific T-cells are purged from the neonatal thymus as compared to the adult thymus (clonal deletion). This difference implies that the blood-thymus barrier of the neonate is immature, at least with regard to the entry of some bone marrow and spleen cells. In this comprehensive understanding of the two immune systems, it is essential for an adult immune system to be administered ALS before IT pretreatment

which deletes the immunocompetent peripheral lymphocytes, whereas in the neonatal immune system this is not necessary. Furthermore, exposure to allogenic cells by an extrathymic route induces tolerance.

In a xenograft model, no extension of graft survival was induced by in utero fetal or neonatal pretreatment of graft recipients with inoculation of xenogenic cells through IP or IT (8) injection. We hypothesize that these xenogenic cells might have desensitized T-cell response by entering the thymus with resultant clonal deletion, but did not desensitize B-cell response, resulting in rejection of the xenograft at the usual speed. If the B-cell response was suppressed by CyP, it should reveal the true effect of those pretreatments. The results of the present study favor this hypothesis. It appears that the extrapolation of the hypothetical explanation of neonatal tolerance by Streilein (13) to xenograft models is acceptable as far as the T-cell response is concerned. If such is the case, the mechanisms leading to host unresponsiveness following IT injection observed in our xenograft model would be similar to those in allograft models.

Normal Lewis rat serum contains antibodies (IgM > IgG) that bind to hamster leukocytes and endothelial cells (14). This combination is considered weakly discordant but the anti-hamster antibodies do not cause hyperacute rejection. Transplanting a hamster heart to a rat results in a release of hamster lymphoid cells from the graft, which lodge in the recipient spleen where recipient T- and B-cell populations initiate DNA synthesis within one day (14, 15). A profound anti-donor IgM spike occurs resulting in antibody deposition in the graft, complement fixation, and graft rejection in four days (14). CyP, a purine antimetabolite, suppresses the anti-graft antibody production by depleting splenic marginal zone B-cells specifically (16) and thus extends xenograft survival. This demonstrates that this rejection is mediated by the classic pathway of complement, not by an alternative pathway characteristic of discordant rejection. Monotherapy is insufficient to control the T-cell mediated graft damage so that the rejected grafts express a dense cellular infiltrate typical of cellular rejection and histologically similar to allograft rejection (7).

On the other hand, monotherapy with CsA or FK506, both of which suppress T-cell immunity selectively, does not extend xenograft survival since the anti-graft antibody response includes T-cell independent as well as T-cell dependent components (17). This is supported by the fact that nude rats, those that are T-cell

deficient but have B-cells, reject hamster hearts at the usual pace (18). It appears that the inability to extend xenograft survival with IT pretreatment alone (8) correlates with the inability to extend xenograft survival with CsA or FK506 alone. The combined therapy of CsA or FK506 and CyP controls synergistically both arms of the immune system resulting in excellent xenograft survival (11, 12). Hasan et al. (11) demonstrated that the anti-graft antibody response could be inhibited by a short pulse of CyP and continuous CsA therapy. This, when combined CyP and CsA, was capable of producing long-term survival with total absence of rat anti-hamster antibodies for the duration of CsA therapy. The investigators maintain that these two events are causally linked. However, this excellent long-term survival is accomplished at a cost of high recipient mortality. They demonstrated that lowering the dosage of CyP to as low as a total of 80 mg/ kg in the combined therapy with CsA (10 mg/kg/day) results in excellent graft survival, reduced mortality, and total absence of rat anti-hamster antibodies for the duration of CsA therapy.

In our study, we administered CyP in accordance with Hasan et al.'s regime expecting IT pretreatment to control T-cell immune response as a substitute for CsA. However, our results were not as conclusive as theirs. One factor could be leakage. Another could be sensitization of a small amount of peripheral immunocompetent T-cells that may have been exported from the thymus before the neonatal pretreatment. We expected that either an increased dosage of CyP or the supplemental splenectomy would act favorably to improve the IT pretreatment. However, the results were negative. It seems that these measures cannot abrogate the fierce rejection response caused by the second set of xenografts.

In neonatal models, it is difficult to assure that the injected splenocytes indeed entered the thymus because of the blind technique in neonatal IT injection. The dye study showed that much of the injected dye directed at the thymus leaked out. However, at least a small amount of the dye was observed subcapsularly in 78 % of the neonatal rats that received IT injection of the dye. Furthermore, comparison of SC with IT injection revealed the true effect of the IT splenocytes. This, combined with the results of the dye study, demonstrates that IT splenocytes, which may be quite small in number, produce prolonged cardiac xenograft survival. Other routes such as IP or IV seemed also effective in inducing tolerance, but may have produced strong sensitization at

the same time. It appears that some splenocytes entered the thymus through the blood-thymus barrier resulting in clonal deletion just as in allograft models, while other splenocytes sensitized the immune system somewhere else in peripheral lymphoid organs. Thus, the best route would be IT injection without leakage since this technique yields maximum tolerance in T-cells and sensitizes the immune system least. IT injection under direct vision might improve the treatment, but excessive human handling leads to abandonment by the maternal rat (3), a phenomenon observed in our preliminary studies. A more precise technique to inoculate cells into neonatal thymus would improve this treatment in the xenograft model.

In conclusion, it was demonstrated that IT injection of xenogenic splenocytes in a neonatal recipient without administration of ALS produced prolonged xenograft survival when used with CyP. A more precise technique to inoculate cells into the neonatal thymus without leakage is needed to improve this treatment. However, our study showed that xenographic transplantation, when paired with immunomodulation techniques, holds tremendous promise, especially in the field of neonatal medicine. For possible clinical application, similar experiments should be undertaken on larger animals, so that the technical problems that were encountered in the rat model would be lessened.

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