Transpl Int (1996) 9 [Suppl 1]: S368–S371

D. C. Wahoff*
B. E. Papalois
L. A. Nelson
J. P. Leone
J. A. Everett
D. E. R. Sutherland

Allograft tolerance by intrathymic donor splenocyte transfer: an age-dependent, species-specific phenomenon?

D. C. Wahoff · B. E. Papalois · L. A. Nelson · J. P. Leone · J. A. Everett · D. E. R. Sutherland
Department of Surgery, University of Minnesota, Minneapolis, Minnesota, USA

D. E. R. Sutherland (✉)
Box 280 UMHC,
420 Delaware St. S. E.,
Minneapolis, MN 55455, USA
Ph + 1-612-625-7600
FAX + 1-612-625-8496

* Recipient of an American College of Surgeons research scholarship

Abstract Protocols that allow allograft survival without immunosuppression remain the ultimate goal in transplantation. Intrathymic injection of donor splenocytes into a transiently immunosuppressed recipient has induced tolerance to a variety of subsequently transplanted allografts in rats. The purpose of this study was to determine if recipient age is critical to intrathymic tolerance in light of age-dependent thymic changes, and if this protocol can be extended to an outbred, large animal model. Prepubertal and postpubertal Wistar-Furth rats underwent intrathymic injection of splenocytes from Lewis rats and antilymphocyte serum (ALS) intraperitoneally. On day 21, a heterotopic Lewis heart was transplanted, with graft survival evaluated by cardiac palpation. Graft tolerance (> 100 days) occurred in four out of five (80 %) of the prepubertal rats compared to two out of six (33 %) postpubertal rats. Tolerance was not demonstrated in rats receiving intrathymic injection of buffer only. In puppies, groups 1 and 2 underwent splenectomy with intrathymic injection of *allo* splenocytes. Control puppies (group 3) received intrathymic *auto* splenocytes. Groups 1 and 3 were given antilymphocyte gamma globulin (ALG) on days 7 to 0 with respect to the intrathymic injection. Group 2 did not receive ALG, but instead received cyclosporin A (CSA) on days 0–2. On day 21, all puppies underwent bilateral nephrectomy and single renal transplantation. No additional immunosuppression was given. Tolerance (creatinine < 7 mg/dl for 100 days) was not obtained by any dog in all three groups. There was no difference in graft survival between control and experimental dogs, with the longest surviving graft seen in a control dog (26 days). Our results suggest that thymic change during maturation may alter the ability to induce tolerance by intrathymic injection of donor cells in rats, and that the protocol is not easily adapted to large animals.

Key words Transplant tolerance · Intrathymic injection · Rodent · Canine

Introduction

Permanent acceptance of an allograft without long-term nonspecific immunosuppressant medications is the goal of transplantation research. The concept of induction of transplantation tolerance was pioneered in the 1950's by Medawar [1]; since then many protocols have successfully induced donor-specific unresponsiveness in selected inbred rodent donor-recipient strain combinations [4–8]. In other reports, cyclosporin (CSA), a common immunosuppressive agent, has been used as an adjunct to tolerance induction [2, 3]. It is thought that the

mechanism of action of CSA is that it interferes with secondary signals involved in cellular immunological interactions. Unfortunately, many of the experimental protocols described in rodents have either not been successful or have not been completely tested in outbred, large animal models [2–8].

One of the most recent and novel schemes to induce allograft tolerance in mice and rats as described by Posselt et al., involves injection of donor antigen or tissue into the thymus of a recipient transiently immunosuppressed with an antilymphocyte antibody prior to transplantation [6]. Previously published reports on variations of the Posselt protocol have used prepubertal rodents [4]. However, the thymus undergoes spontaneous involution at puberty in humans, possibly restricting the time period in which the thymus must be exposed to donor antigen. This possibility must be investigated prior to using the Posselt protocol in human beings. The purpose of this study was threefold: (1) to determine if allograft (heart) tolerance by intrathymic injection is equally successful in pre- and postpubertal rats, (2) to translate the protocol to prepubertal outbred dogs in a renal allograft model, and (3) to determine if the use of CSA rather than antilymphocyte antibody in the protocol could promote unresponsiveness in conjunction with intrathymic injection of donor cells.

Materials and methods

Rodent experiment

Animals

Prepubertal (< 6 weeks, 100 to 150 g) and postpubertal (> 8 months, > 400 g) Wistar-Furth (RT1, WF) rats were used as recipients. Prepubertal Lewis (RT1, LEW) rats were the donor animals for the splenocytes and heart allografts.

Preparation of donor splenocytes

Donor LEW splenocytes were obtained by dispersion of the pulp through a 100-μm plastic screen followed by erythrocyte lysis with Tris-NH_4Cl (0.83 %) at 37 °C. The splenocytes were then washed three times with Hank's balanced salt solution (HBSS, Celox, Hopkins, Minn.) and stored at 4 °C until injection.

Intrathymic injection and immunosuppression

The thymus of the LEW recipients was exposed through a partial median sternotomy and either a total of 3×10^7 splenocytes or HBSS was injected into both lobes with a 30-guage needle. After intrathymic injection, a single 1-ml dose of rabbit anti-rat antilymphocyte serum (ALS, Accurate Chemical Corp, Westbury, N.Y.) was injected intraperitoneally.

Heterotopic cardiac transplantion

Twenty-one days after intrathymic injection, heterotopic abdominal cardiac allografts were performed by the technique developed by Ono and Lindsey [5]. Graft survival was determined by daily palpation. A palpable heartbeat indicated graft function; cessation of a heartbeat along with histologic rejection defined graft failure. Tolerance was defined as graft survival > 100 days

Canine experiment

Animals

Outbred puppies (< 3 months old and weighing 5–10 kg) were used as both the recipients and donors.

Intrathymic injection and immunosuppression

On day 0, all puppies underwent splenectomy and intrathymic injection of 2×10^9 donor allo- or autosplenocytes. Splenocytes were isolated by straining through a 100-μm screen after tissue was minced and gently crushed at 37 °C in Tris-NH_4Cl (0.83 %) buffer. In groups 1 and 3, antilymphocyte gamma globulin IV (ALG; 40 mg/kg per day) was administered intravenously from day – 7 to day 0 (with respect to the day on which the splenocyte injection was performed). Group 2 puppies received oral CSA (20 mg/kg per day) for 3 days from day 0 to day 2 following splenocyte transplantation. This group did not receive ALG, and CSA was not given following kidney transplantation. Instead of allosplenocytes, the control puppies (group 3) received an intrathymic injection of autologous splenocytes following splenectomy.

Renal transplantation

Twenty-one days after splenocyte injection, the puppies underwent bilateral nephrectomies followed by single kidney allotransplantations from the previous splenocyte donors for groups 1 and 2, except for the control group puppies (group 3) who received an injection of their own splenocytes. Surgically, the renal artery was anastamosed end-to-end to the external iliac artery and the renal vein was anastomosed to the external iliac vein in an end-to-side fashion. The ureteral-bladder anastomosis was done by pulling the ureter into the bladder through a puncture on its underside and then fixed in place with a simple suture. No postoperative immunosuppression was given, and rejection was monitored by measuring urine creatinine levels daily. Graft failure was defined by a creatinine level > 7 mg/dl and was histologically confirmed to be rejection. Tolerance was defined as graft survival > 100 days.

Statistics

The Mann-Whitney *U*-test was used for comparison of independent samples with nonuniform sampling distributions to determine the probability that differences between graft survivals were due to chance alone. Fisher's exact test for comparison of two proportions in independent samples was used to compare differences in tolerance between treatment groups.

Animal care

For the animal experiments, the *Principles of laboratory animal care* (NIH publication No. 86-23, revised 1985) were followed, as well as the regulations required by the USDA Animal Welfare Act and the University of Minnesota animal care committee.

Results

Rodents (Table 1)

In the prepubertal rodents four out of five (80 %) animals were tolerant (grafts functioned > 100 days), while in the postpubertal group two out of six (33 %) animals achieved tolerance (Fisher's exact $P = 0.24$). Prepubertal ($n = 2$) and postpubertal ($n = 4$) WF rats that received buffer only intrathymically plus ALS did not become tolerant (mean graft survival 14 ± 2 days). No animals died due to intrathymic injection of splenocytes, intraperitoneal injection of ALS, or technical complications of the cardiac transplant.

Canine (Table 2)

Tolerance was not achieved in the large animal model (Table 2). In the experimental group that received allosplenocytes intrathymically, ten out of ten grafts failed before 26 days; the longest duration of function was seen in the three controls who received an intrathymic injection of autosplenocytes. The addition of a short course of CSA following allosplenocyte injection did not prolong renal allograft survival. No animals died due to intrathymic injection of splenocytes, nor did ALG or CSA therapy result in death. Three allografts failed due to technical failure (i.e., urine leak or thrombosis), and the dogs were not included in analysis.

Table 1 Lewis cardiac allograft survival in prepubertal (Pre) and postpubertal (Post) Wistar-Furth (WF) rats after receiving Lewis splenocytes intrathymically (IT) and antilymphocyte serum (ALS)

Group	*n*	Treatment	Graft survival time		Tolerance[b] (%)
			days[a]	median	
Pre	5	IT Lew SC + ALS	15, > 100 × 4	100	4/5 (80)
Post	6	IT Lew SC + ALS	9, 9, 10, 53, > 100 × 2	37.5	2/6 (33)

[a] Mann-Whitney $P = 0.14$
[b] Fisher's Exact – $P = 0.24$

Table 2 Renal allograft survival in prepubertal dogs after intrathymic (IT) injection of auto- or allosplenocytes with antilymphocyte gamma globulin (ALG) or cyclosporin (CSA) immunosuppression (*SC* splenocytes)

Group	Treatment	*n*	Graft survival time		Tolerance (%)
			days[a]	median	
1	T allo SC + ALG	10	3, 5, 6 × 3, 7, 8, 15 × 2, 25	6.5	0/10 (0)
2	IT allo SC + CSA	5	7, 7, 8, 8, 11	8.0	0/5 (0)
3	IT auto SC + ALG	3	7, 7, 26	7.0	0/3 (0)

[a] Mann-Whitney 1 vs 2 $P = 0.46$; 1 vs 3 P 0.31; 2 vs 3 $P = 0.88$

Discussion

Many investigators have shown that donor-specific tolerance can be induced in rodents after intrathymic injection of donor alloantigen followed by subsequent transplant of various allografts [4–7]. In humans the thymus undergoes spontaneous postpubertal involution. Therefore, thymic change may alter the effect of induced tolerance by donor intrathymic transfer in animals. In our study, tolerance was more frequent in prepubertal rats after intrathymic injection of allosplenocytes and ALS therapy. However, further studies using larger numbers of rats are needed to determine if the differences are statistically significant.

When a similar protocol of intrathymic injection of alloantigen and ALS for donor-specific tolerance was transferred to large animals, puppies were chosen as the experimental model based on the improved results in the prepubertal rats. With our protocol, puppies receiving intrathymic allosplenocytes and ALS prior to renal allograft transplantation failed to demonstrate graft survival consistent with tolerance as defined in this study. The substitution of CSA as an adjunct to intrathymic injection of splenocytes was also unsuccessful in prolonging graft survival. Thus, a protocol designed to inhibit antigen recognition and sensitization via secondary signals at the time of antigen presentation did not alter time to graft rejection in this model.

Failure to replicate long-term survival by intrathymic transplant in the dog model implies that this phenomenon may be species specific. Thus, tolerance by intrathymic donor splenocyte injection, like many other tolerance schemes, may be unique to young, inbred rodents. Variations in lymphocyte depletion, intrathymic tissue type and dose, timing of the allograft placement, and the duration and dose of adjunct therapies (i.e., total lymphoid irradiation, immunosuppressive drugs, ultraviolet irradiation of donor tissue, etc.) need to be studied further if allograft unresponsiveness is to be obtained in higher mammals.

References

1. Billingham R, Brent L, Medawar P (1956) Actively acquired tolerance of foreign cells. Nature 172: 603–605
2. Calne RY, Watson CJE, Brons IGM, Makisalo H, Metcalfe SM, Sriwatanawongsa V, Davies HS (1994) Tolerance of porcine renal allografts induced by donor spleen cells and seven days' treatment with cyclosporine. Transplantation 57: 1433–1435
3. Hartner WC, Markees TG, De Fazio SR, Shaffer D, Van der Werf WJ, Gilchrist B, Yatko C, Monaco AP, Gozzo JJ (1995) Effect of early administration of donor bone marrow cells on renal allograft survival in dogs treated with antilymphocyte serum and cyclosporine. Transplantation 59: 131–155
4. Nakafusa Y, Goss JA, Mohanakumar T, Flye MW (1993) Induction of donor-specific tolerance to cardiac but not skin or renal allografts by intrathymic injection of splenocyte alloantigen. Transplantation 55: 877–882
5. Ono K, Lindsey ES (1969) Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 57: 225
6. Posselt AM, Barker CF, Thomaszewski JE, Markmann JF, Choti MA, Naji A (1990) Induction of donor-specific unresponsiveness by intrathymic islet transplantation. Science 249: 1293
7. Remuzzi G, Rossini M, Imberti O, Perico N (1991) Kidney graft survival in rats without immunosuppressants after intrathymic glomerular transplantation. Lancet 337: 750–752
8. Vriens PW, Nisco SJ, Hoyt EG, Lyu S-C, Pierre P, Reitz BA, Clayberger C (1994) Tissue-specific differences in the establishment of tolerance: tolerogenic effects of lung allografts in rats. Transplantation 57: 1795–1798