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Total lymphoid irradiation, without intrathymic injection of donor cells, induces indefinite acceptance of heart but not islet or skin allografts in rats

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Abstract Allograft tolerance occurs in rodents given a dose of antilymphocyte serum (ALS) and intrathymic injection (ITI) of donor splenocytes (SC) 1–3 weeks prior to transplant (TX). The purpose of our study was to test total lymphoid irradiation (TLI) as an alternative to ALS in ITI tolerance induction to heart, islet, and skin allografts. Prepubertal Wistar Furth rats were recipients. ITI of donor (Lewis) SC was done at the end of the TLI course. Rats received either a heterotopic heart, a skin graft, or 2300 islets (diabetic recipients) intraportally from Lewis donors. TLI (without ITI) in a dose of 200 rads/day for 5 consecutive days, followed by TX in 3 weeks resulted in indefinite acceptance of heart (but not islet or skin) grafts in 60 % of the recipients. These data indicate that TLI by a dose schedule of 200 rads/day for 5 days should be tested for clinical relevance in large animal recipients of immediately vascularized grafts.

Key words Transplantation · Tolerance · Thymus · Irradiation · Rodents

Introduction

Systemic immunosuppression for organ transplantation often results in drug-specific toxicity and long-term immunosuppression is a major cause of morbidity and mortality [5]. These complications could be avoided if it were possible to achieve a state of donor-specific unresponsiveness without subsequent immunosuppression [2], and tolerance is the ultimate goal of transplantation.

It is known that thymocytes originate from bone marrow multipotential hematopoietic stem cells and mature in the thymus into antigen-reactive T-lymphocytes before migrating to the peripheral lymphoid organs [4]. A variety of protocols has been able to achieve donor-specific unresponsiveness to organ allografts in rodents [2]. One approach developed recently has involved depletion of mature T-cells followed by intrathymic exposure to alloantigen [3] or donor hematopoietic cells [7]. With such a protocol the recipient theoretically "relearns" self in the context of the new alloantigen, but this theory does not explain why the unresponsiveness may be organ-specific (e.g., heart accepted, skin or kidney rejected) [6]. In rodents, most protocols that used intrathymic injection (ITI) gave a single dose of antilymphocyte serum (ALS) at the time when donor cells were inoculated, 3 weeks prior to transplantation [3, 6, 7]. Whether it is important to use an anti-T-cell agent in ITI protocols, or whether a transient generalized immunosuppressant would work as well, has not been determined. The purpose of our study was to determine whether total lymphoid irradiation (TLI) can be used as an alternative to ALS in combination with ITI to induce tolerance to heart, islet or skin allografts in rats. A further rationale to test TLI results from the fact that anti-T-cell agents are not readily available for large animal recipients in which the intrathymic approach would ideally be tested in a preclinical model.

Materials and methods

Animals

For all experiments, the "Principles of laboratory animal care" (NIH publication 86-23, revised 1985) and the regulations of the University of Minnesota Animal Care Committee were followed. Lewis (LEW, RT1′) adult male rats were donors and Wistar Furth (WF, $RT1^u$) prepubertal (age < 6 weeks) male rats were recipients. Brown Norway (BN, $RT1^n$) adult male rats were used as third party for the in vitro studies.

TLI

WF rats were anesthetized with an i.m. injection of 60 mg/kg of ketamine and the skull, lungs, part of the pelvis, the hind limbs, and the tail were covered with lead shields. In some goups the thymus was also shielded (TS). A Phillips Rt-250 kV orthovoltage X-ray machine was used, and the dose rate was 139.9 rad/min. The rats received either 200 rad/day (a single or five consecutive doses) or 1000 rad/day in a single dose. Total lymphocyte count (LC) was monitored before the administration of TLI, after completion of TLI and before the transplant.

ITI

Donor LEW rats underwent splenectomy under ether anesthesia. The splenocytes were obtained by mincing and then passing the spleen through a 60-μm brass screen, and then lysing the erythrocytes with TRIS-NH_4Cl (0.83 %) at 37 °C. The remaining splenocytes were washed 3 times with Hank's balanced solution. After completion of TLI, WF rats were anesthetized with ether, the thymus was exposed through a partial median sternotomy, and a total of 30×10^6 LEW splenocytes were injected into the thymus (approximately 15×10^6 splenocytes in each lobe).

Heart transplantation

Donor (LEW) and recipient (WF) rats were anesthetized with an i.m. injection of 60 mg/kg of ketamine and an i.p. injection of 30 mg/kg of sodium pentobarbital. Heterotopic abdominal heart transplantation was performed using the modified technique of Ono and Lindsey [9]. Graft survival was assessed by daily transabdominal palpation. Rejection was defined by cessation of heart beat and confirmed by histological (hematoxylin and eosin staining) evaluation.

Islet transplantation

WF rats were made diabetic (blood glucose > 400 mg/dl for 2 consecutive days) by a single injection in the tail vein of 55 mg/kg of streptozotocin. Two LEW donors were sacrificed, their pancreata were harvested, and the islets were isolated and purified by a technique which has been previously described [11]. A total of 2300 islet, were transplanted into the portal vein of WF diabetic recipients. Graft survival was assessed by daily blood glucose count and rejection was defined as 2 consecutive days of blood glucose 0062 200 mg/dl. In addition, in order to test islet viability, diabetic LEW rats were transplanted intraportally with 2000 isogenic islets.

Skin transplantation

Donor LEW rats were sacrificed and abdominal skin grafts were harvested using the method of Billingham [1]. The skin grafts were then transplanted (sutured to the dorsal thorax after creating an integument deficit) to WF recipients which were under ether anesthesia. Graft survival was assessed by daily inspection and rejection was defined as graft necrosis and sloughing (> 90 %).

Mixed lymphocyte culture (MLC)

Responder splenocytes were harvested from transplanted WF rats. The spleens were dispersed into a single-cell suspension and washed 3 times in Dubecco's modified Eagle medium (DMEM) supplemented with sodium pyruvate, HEPES, 2-mercaptoethanol, L-glutamine, L-arginine, L-aspaginine, folic acid, penicillin, streptomycin, and 1 % heat-inactivated normal rat serum (HI-NRS, complete C-DMEM). Responder cells (2.5×10^5) were cultured in C-DMEM, 1 % HI-NRS with irradiated stimulator splenocytes (1×10^6 LEW, BN, or autologous splenocytes) in quadruplet 200-ul cultures, pulsed with ^{3}H-thymidine on days 3–7, and then harvested for scintillation counting. Proliferation was assayed by ^{3}H-thymidine incorporation.

Thymus histology

Four prepubertal naive WF rats were sacrificed and the thymus was harvested. The thymus was also harvested from prepubertal WF rats that were sacrificed after receiving 1×200 rad, 1×1000 rad and 5×200 rad (four animals in each group). The samples were stained with hematoxylin and eosin and the cortex/medulla ratio was assessed for the thymic lobules.

Statistical analysis

The Student's t-test was used to compare means. The chi-square test and, when applicable, the Fisher's exact test were used to compare categorical variables. Graft survival rates between groups were compared using the Wilcoxon test. For all statistical tests, a P value of < 0.05 was considered significant.

Experimental design

The heart allograft model is the one for which many previous ITI protocols have achieved good results [3, 6, 7] and in our study was used to assess the most successful TLI/ITI protocol: for each irradiation dose schedule recipients were transplanted either immediately after completion of TLI, or 3 weeks after completion of TLI with or without ITI. The protocols by which the highest rate of graft function > 100 days was achieved were then applied again to the heart allograft model, but with the thymus of the recipients shielded during TLI in order to further explore its role in induction of donor-specific unresponsiveness. In addition, for the same groups of animals with the highest rate of graft function > 100 days, the recipients received a skin transplant at 150 days in order to test their unresponsiveness to different grafts, and MLC studies were conducted (for animals with hearts functioning for at least 150 days) to test in vitro donor-specific unresponsiveness of those recipients. Finally, the protocols that gave the best results in the heart allograft model were tested for ability to prolong islet and skin allograft survival.

Table 1 Heart allograft survival. $P \leq 0.001$ for II versus I, III and V, $P \leq 0.005$ for III versus I, and V, $P \leq 0.04$ for IV versus I and V, $P \leq 0.02$ for IV versus I, III and V, $P \leq 0.02$ for IX versus I, III, IV, and V, $P \leq 0.04$ for X versus I, III, IV, V, VII, VIII, XII, and XIII, $P \leq 0.03$ for XI versus I, II, III, IV, V, VII, and VIII, $P = 0.02$ for XIII versus V. (*ITI* Intrathymic injection, *TS* thymus shielding, *TLI* total lymphoid irradiation)

Group	*n*	Treatment	Transplant	Graft survival (days)	Median
I	4	None		8, 9, 9, 10	9
II	7	ITI	In 3 weeks[a]	14, 16, 27, 31, 53, 55, 130	31
III	6	1 × 200 rad	Immediately[b]	11, 11, 12, 14, 17, 22	13
IV	6	1 × 200 rad	In 3 weeks	9, 9, 12, 15, 23, 62	13.5
V	5	1 × 200 rad + ITI	In 3 weeks	5, 5, 8, 8, 11	8
VI	5	1 × 1000 rad	Immediately	15, 22, 30, 174, 192	30
VII	6	1 × 1000 rad	In 3 weeks	4, 6, 19, 22, 79, 88	21
VIII	6	1 × 1000 rad + ITI	In 3 weeks	4, 10, 13, 38, 39, 99	25.5
IX	7	5 × 200 rad	Immediately	23, 27, 30, 37, 57, 65, 348[c]	37
X	10	5 × 200 rad	In 3 weeks	15, 16, 20, 65, 108, 184, 195, 198[d], 221[d], 269[d]	> 100
XI	11	5 × 200 rad + ITI	In 3 weeks	6, 35, 38, 81, 84, 118, 137, 145, 214[d], 241[d], 353[d]	> 100
XII	6	5 × 200 rad + TS	In 3 weeks	4, 9, 24, 29, 41, 45	26.5
XIII	6	5 × 200 rad + TS + ITI	In 3 weeks	7, 12, 23, 24, 42, 100	23.5

[a] Transplant immediately after comletion of ITI, TLI, or TLI + ITI
[b] Transplant in 3 weeks after completion of ITI, TLI, or TLI + ITI
[c] Sacrificed with a functioning graft because of old age
[d] Sacrificed with a functioning graft for the in vitro studies

Results

TLI was effective in decreasing peripheral blood LC, particularly when 200 rad was given in five consecutive doses. The mean (± SD) reduction of the total LC (cells/mm^3) was 7000 ± 1900 for 1 × 200 rad (the LC for all rats after TLI was between 39 % and 48 % of the LC before TLI), 7600 ± 1400 for 1 × 1000 rad (the LC for all rats after TLI was between 37 % and 40 % of the LC before TLI), and 17266 ± 2100 for 5 × 200 rad (the LC for all rats after TLI was between 0 and 17 % of the LC before TLI), ($P = 0.01$ for the mean ± SD reduction of the LC for 5 × 200 rad versus 1 × 200 rad and 1 × 1000 rad). The total LC reverted to normal range 3 weeks after completion of TLI and regardless of the TLI dose and the induction or not of ITI. There were no apparent side effects after completion of TLI with either the 1 × 200 rad or 5 × 200 rad dose schedule. All the rats that received 1 × 1000 rad developed mild diarrhea and weight loss after completion of TLI.

The results for the heart transplants are presented in Table 1. One protocol (group X, 5 × 200 rad, transplantation in 3 weeks after TLI) was effective in prolonging median heart allograft survival > 100 days without ITI, and heart allograft survival was > 100 days in 60 % of the recipients in that group. All the other TLI protocols (group III, 1 × 200 rad, transplant immediately after TLI; group IV, 1 × 200 rad, transplant 3 weeks after TLI; group VI, 1 × 1000 rad, transplant immediately after TLI; group VII, 1 × 1000 rad, transplant 3 weeks after TLI; and group IX, 5 × 200 rad, transplant immediately after TLI) were less effective in prolonging heart allograft survival (median graft survival was 13 days in group III, 13.5 days in group IV, 30 days in group VI, 21 days in group VII, and 37 days in group IX). However, in two (40 %) recipients in group VI and in one recipient (14.3 %) in group IX, graft survival was prolonged > 100 days ($P = 0.6$ for X versus VI and $P = 0.1$ for X versus IX).

ITI injection by itself (group II) significantly prolonged heart allograft survival (median 31 days) compared to controls but only one recipient (14.3 %) had graft function > 100 days ($P = 0.5$ for II versus VI, $P > 0.9$ for II versus IX, and $P = 0.1$ for II versus X). An additive or synergistic effect of ITI with TLI could not be demonstrated when ITI was combined with TLI protocols that were partially effective (group V, 1 × 200 rad + ITI, transplantation 3 weeks after TLI + ITI; group VIII, 1 × 1000 rad + ITI, transplantation 3 weeks after TLI + ITI). When ITI was combined with the most effective TLI protocol (group XI, 5 × 200 rad + ITI, transplantation 3 weeks after TLI + ITI), the median graft survival was > 100 days and prolongation of graft survival > 100 days occurred in 54 % of the recipients ($P = 0.1$ for XI versus II, $P > 0.9$ for XI versus VI, $P = 0.1$ for XI versus IX, and $P > 0.9$ for XI versus X).

The importance of thymus irradiation was demonstrated when the protocols of groups X and XI were modified by TS during TLI. Median graft survival was only 26.5 days in group XII (5 × 200 rad + TS, transplantation 3 weeks after TLI) and 23.5 days in group XIII (5 × 200 rad + TS + ITI, transplantation 3 weeks after TLI + ITI) with no grafts functioning > 100 days in both groups. The skin grafts that were transplanted to the animals in groups X and XI with hearts functioning > 150 days were rejected at 7–15 days while the hearts continued to beat.

In the in vitro studies (Fig. 1), the mean ± SD (mean for four different counts for each animal and for three

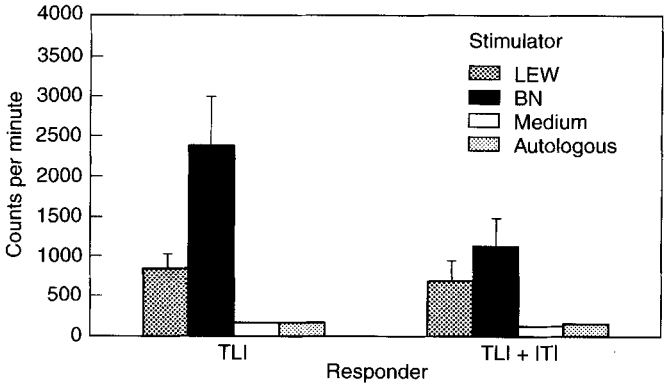

Fig. 1 Effect of total lymphocyte irradiation *(TLI)* and intrathymic injection *(ITI)* on mixed lymphocyte culture. Responder splenocytes were harvested from three Wistar Furth rat (WF) recipients in group X (5×200 rad, transplantation 3 weeks after TLI) and three WF recipients in group XI (5×200 rad + ITI, transplantation 3 weeks after TLI + ITI) with heart allografts functioning > 150 days. Responder cells (2.5×10^5) were cultured with irradiated stimulator splenocytes [1×10^6 Lewis *(LEW)*, Brown Norway *(BN)*, or autologous splenocytes]. Proliferation was assayed by ^{3}H-thymidine incorporation. When comparing the mean ± SD counts per min (proliferation on day 5) for different stimulators in group X, the *P* values were: 0.08 for LEW versus BN cells, 0.03 for LEW versus autologous cells or medium, 0.02 for BN versus autologous cells and medium, and 0.4 for autologous cells versus medium. For the same comparison in group XI, the *P* values were: 0.4 for LEW versus BN cells, 0.1 for LEW versus autologous cells or medium, 0.6 for BN versus autologous cells, 0.052 for BN cells versus medium, and 0.4 for autologous cells versus medium. There was no difference between groups X and XI when comparing response to LEW ($P = 0.7$), BN ($P = 0.2$), autologous stimulator cells ($P = 0.6$), and medium ($P = 0.09$)

different animals in each group) counts per min (proliferation on day 5) in group X (5×200 rad, transplantation in 3 weeks after TLI) was not different when comparing response to LEW (allo-specific) versus BN (third party) stimulator cells ($P = 0.08$), or when comparing response to autologous stimulator cells versus medium ($P = 0.4$). There was a significant difference when response to LEW stimulator cells was compared to autologous cells or medium ($P = 0.03$ in both cases), and when response to BN stimulator cells was compared to autologous cells or medium ($P = 0.02$ in both cases). In group XI (5×200 rad + ITI, transplantation in 3 weeks after TLI + ITI), there was no significant difference when comparing response to LEW versus BN stimulator cells ($P = 0.4$), or when response to autologous cells was compared to medium ($P = 0.4$). In group XI (in contrast to group X) there was no significant difference when response to LEW stimulator cells was compared to autologous cells or medium ($P = 0.1$ in both cases), or when response to BN stimulator cells was compared to autologous cells ($P = 0.6$) or medium ($P = 0.052$). There was no difference between groups X and XI when comparing response to LEW ($P = 0.7$), BN ($P = 0.2$), autologous stimulator cells ($P = 0.6$) and medium ($P = 0.09$).

The histological findings of the heart allografts after cessation of beating (Fig. 2) revealed either rejection with massive lymphocyte infiltration, or necrosis with trophic calcification. Both ot those types of histological findings were present in all groups and no difference in the incidence of one type versus the other was observed in a specific group. Of note, heart allografts functioning > 150 days from animals that were sacrificed for the in vitro studies also had some histological evidence of either lymphocyte infiltration or necrosis.

The results for islet and skin allograft survival are presented in Tables 2 and 3, respectively. The diabetic LEW rats, which were transplanted with isogenic islets in order to test islet viability, were normoglycemic on the first posttransplant day and remain normoglycemic > 100 days. When the two most effective protocols in prolonging heart allograft survival (5×200 rad ± ITI, transplantation 3 weeks after TLI ± ITI) were applied to islet and skin allograft recipients, there was no prolongation of survival of either islet or skin allografts.

The cortex/medulla ratio (Fig. 3) in the lobules of normal rats was 1:1. The cortex/medulla ratio was decreased to 1:1.5 in the animals that received 1×200 rad, 1 : 2 in the animals that received 1×1000 rad, and 1:1.5 in the animals that received 5×200 rad.

Discussion

Different TLI protocols gave different results in the heart allograft model. A total dose of 1000 rad fractionated in five consecutive doses of 200 rad/day and followed by transplantation in 3 weeks was the most effective TLI protocol in depleting the lymphocytes (without any apparent side effects) and it was also the most successful in prolonging graft survival even without ITI. However, recipients treated with 5×200 rad ± ITI (transplantation in 3 weeks after TLI ± ITI) with hearts functioning > 100 days, rejected the skin grafts that were transplanted to them at 150 days while the hearts continued to beat. That indicates that they were not tolerant to the donor and that their allo-responsiveness was reduced only to heart allografts. However, the results of histology for heart grafts of the animals that were sacrificed for in vitro studies (while their heart grafts continued to beat) demonstrated some evidence of lymphocyte infiltration or myocardial necrosis. These histological data indicate that the recipients were rather hyporesponsive than unresponsive to heart allografts, since those allografts were sensitive to rejection-effective mechanisms.

ITI by itself did not have a synergistic effect in vivo with either the less effective TLI protocols or with the most successful TLI protocol of 5×200 rad followed by

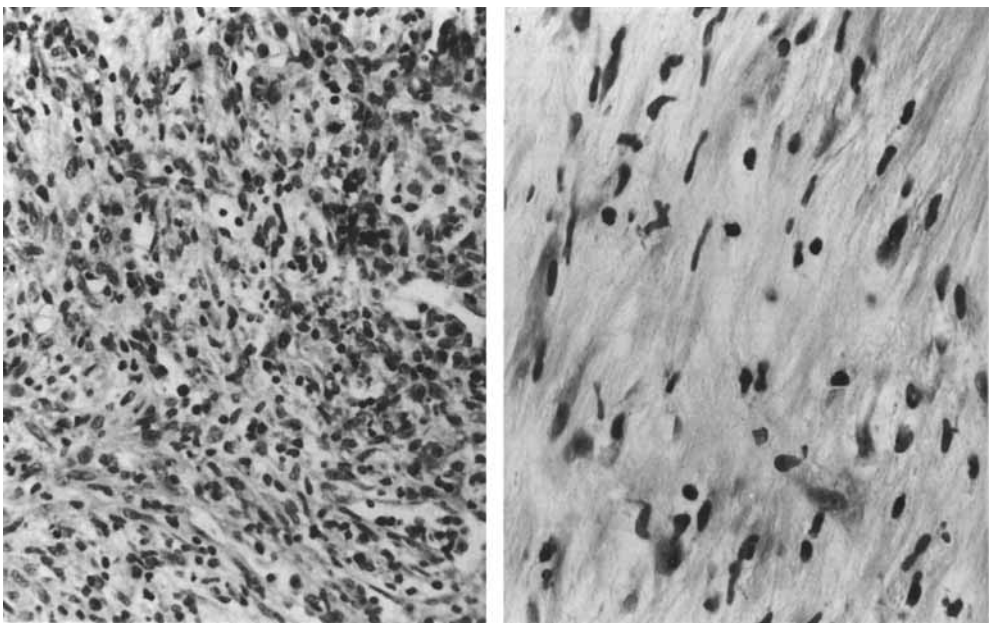

Fig. 2 The histological findings of the heart allografts after cessation of beating revealed either rejection with massive lymphocyte infiltration *(left)*, or necrosis *(right)* with trophic calcification. Both of these types of histological findings were present in all groups and no difference in the incidence of one type versus the other was observed in a specific group

Table 2 Islet allograft survival. $P = 0.2$ for I versus II and III

Group	*n*	Treatment	Transplant	Graft survival (days)	Median
I	4	None		0, 0, 1, 3	1
II	12	5 × 200 rad	In 3 weeks[a]	0, 0, 0, 0, 0, 0, 0, 1, 1, 4, 5, 6	0
III	13	5 × 200 rad + ITI	In 3 weeks	0, 0, 0, 0, 0, 0, 0, 1, 1, 1, 1, 1, 3	0

[a] Transplant in 3 weeks after completion of TLI or TLI + ITI

Table 3 Skin allograft survival. $P = 0.5$ for I versus II and III

Group	*n*	Treatment	Transplant	Graft survival (days)	Median
I	3	None		13, 15, 15	15
II	11	5 × 200 rad	In 3 weeks[a]	9, 10, 10, 13, 14, 15, 15, 15, 16, 17, 19	15
III	9	5 × 200 rad + ITI	In 3 weeks	11, 11, 13, 15, 17, 17, 17, 20, 21	17

[a] Transplant in 3 weeks after completion of TLI or TLI + ITI

transplantation in 3 weeks. This demonstrates a difference with the protocols that used ALS + ITI for inducing donor-specific unresponsiveness where ITI had a significant additional effect to the use of ALS only, and indicates that TLI (by the protocols used) is not a substitute for ALS. However, the 5 × 200 rad protocol (followed by transplantation in 3 weeks) can replace both ALS and ITI in inducing indefinite acceptance of heart allografts in rats. Nevertheless, there is some evidence in our in vitro data that recipients treated with ITI and TLI (5 × 200 rad, transplantation in 3 weeks after TLI + ITI) had lower responsiveness to donor and third party stimulator cells compared to recipients that were treated only with TLI. These data indicate that pretransplant treatment with ITI could possibly have had a positive effect in prolonging heart allograft survival in the long run–that is if the recipients had not been sacrificed for in vitro studies. However, since only three animals were used in each group for the in vitro experiments, further in vitro studies are necessary to verify these results.

The 5 × 200 rad TLI protocol (without ITI) was more effective when transplantation was performed 3 weeks after completion of TLI (when the total LC had reverted to normal range) than immediately after completion of TLI (total LC reduced between 0 and 17 % of the LC before TLI). This might indicate that what is really important for prolonging heart allograft survival by using TLI is not the generalized immunosuppressive effect of TLI but the correct timing between lymphocyte depletion and transplantation. The presence of the heart allograft could be considered as donor-specific antigen presentation to the recipient (having the role that ITI is supposed to have in making the recipient relearn self)

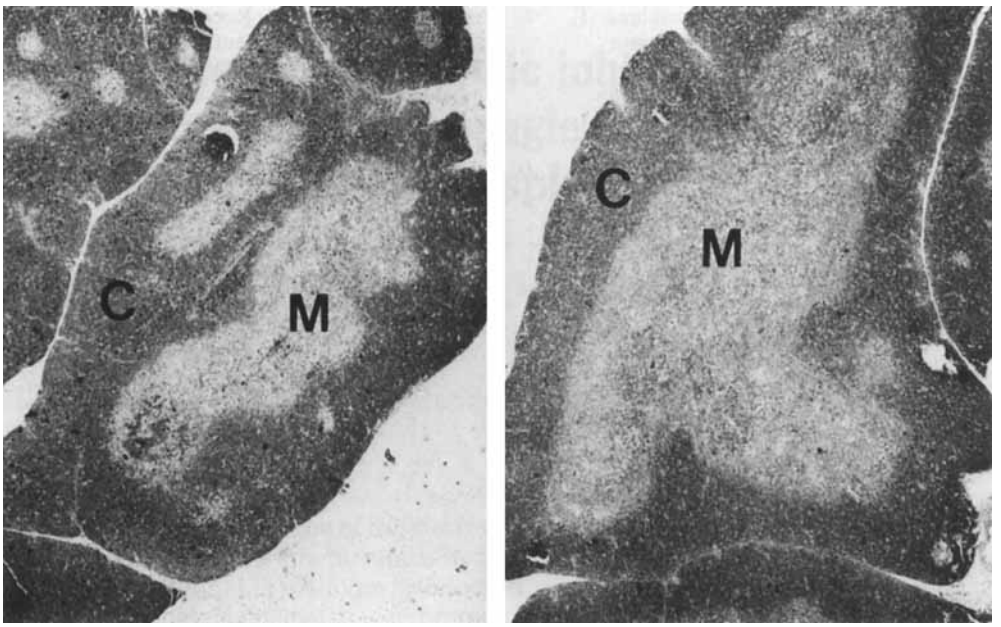

Fig. 3 The cortex *(C)*/medulla *(M)* ratio in the lobules of the thymus of normal rats was 1:1 *(left)*. The cortex/medulla ratio after TLI was decreased to 1:1.5 in the animals that received 1 × 200 rad, 1:2 in the animals that received 1 × 1000 rad *(right)*, and 1:1.5 in the animals that received 5 × 200 rad

and 3 weeks post-TLI might be the optimum time for the new immature T-cells to "meet" the donor antigens in the peripheral blood and become hyporesponsive to them. This hypothesis is in accordance with similar observations for development of donor-specific unresponsiveness by using peritransplant immunosuppression and i. v. injection of donor cells [8].

The 5 × 200 rad protocol (with or without ITI, followed by transplantation in 3 weeks) was effective only when the thymus was exposed to irradiation, indicating that depeletion of the T-cells that exist in the thymus (and have already learned what is self and non-self) is probably important for inducing donor-specific hyporesponsiveness.

TLI altered the histology of the thymus, decreasing the cortex/medulla ratio from 1:1 before to 1:1.5 after 1 × or 5 × 200 rad and 1:2 after 1 × 1000 rad. The histological picture after TLI resembled that of an old, nonfunctioning thymus [10] and further supports the hypothesis that donor-specific hyporesponsiveness develops in the periphery, and the heart allograft by itself serves for donor-specific antigen presentation to the recipient. However, the in vitro results that suggest donor and third party hyporesponsiveness in recipients treated with TLI + ITI versus TLI alone is consistent with at least partial function of the thymus after TLI.

The fact that prolongation of islet or skin allograft survival was not achieved by using the two most effective protocols in prolonging heart allograft survival (5 × 200 rad ± ITI, transplantation in 3 weeks after TLI ± ITI) indicates that the effect of TLI ± ITI in inducing donor-specific hyporesponsiveness is organ-specific, as is the effect of the use of ALS in combination with ITI [6]. These results also indicate a difference between immediately (heart) and later (islets, skin) vascularized grafts.

In conclusion, TLI in a dose schedule of 200 rad/day for 5 consecutive days followed by transplantation in 3 weeks was effective (even without ITI) in inducing indefinite acceptance of heart but not islet or skin allografts in rats. Thus, TLI by this scheme should be tested for prolonging survival of immediately vascularized grafts in a preclinical large animal model.

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