

The effect of thymic inoculation to induce tolerance of allogeneic thyroid grafts in the outbred rabbit

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Summary. Many studies have demonstrated that allograft tolerance can be achieved in inbred rats and mice following intrathymic injection of donor cells or antigen and treatment with antilymphocyte serum (ALS). In outbred dogs, xenografts, and inbred rat strains with major MHC antigen difference, tolerance has not similarly been induced. The focus of this study was to determine whether allogeneic thyroid graft tolerance could be achieved in outbred rabbits. In the experimental group (n=5), recipients received an intrathymic injection of donor lymphocytes and a single treatment of ALS. Controls (n=5) received intrathymic cell culture medium and ALS treatment. Donor-recipient allogenicity was monitored with mixed lymphocyte culture (MLC) over 18 weeks. Donor thyroid tissue was placed into recipient gluteal muscle fibres one week following the last MLC measurement. A third group of rabbits (n=4) received thyroid autografts without any other treatment. There were no differences in MLC stimulation indices (SI) between the control and experimental group nor did MLC (SI) change within groups. All thyroid autografts survived the two week monitoring period and demonstrated normal appearing thyroid follicles on histologic examination. All thyroid allografts showed severe acute rejection reactions on biopsy within one week. Further studies using outbred animals to examine the role of thymic inoculation are required to determine whether similar techniques might be successful in the human.

Key words: Rabbit, Thymus, Tolerance, Thyroid, Lymphocyte

Introduction

Human allogeneic transplantation is a front-line treatment for a variety of incurable medical conditions most notably renal failure. By carefully matching HLA

(MHC) phenotypes, many grafts have survived for extended periods of time providing the recipient with an extended lifetime and also enhanced quality of life. For extended graft survival, the majority of patients require life long immunosuppressive therapy. There are significant direct and indirect costs associated with such therapy including treatment of related morbidities. Recent reviews of transplantation cost-effectiveness indicate that the cost of immune suppression per transplant is typically \$4,000/yr for Cyclosporine A (Evans and Mannine, 1988; Ramsey et al., 1995).

The use of intrathymic injection for developing immune tolerance to allografts would be a significant advance since it would reduce the annual costs incurred in transplantation and improve morbidity and mortality. Vojtišková and Lengerová (1965) provided the first experimental evidence that modifying the environment of the thymus could provide tolerance to alloantigens. Two strains of mice with an H-2 locus difference were used. Recipients had thymectomies followed by syngenic thymus grafts that had been injected with donor splenocytes. Groups that received the donor impregnated thymus grafts had extended survival of donor skin grafts. More recently, Remuzzi et al. (1991) isolated glomeruli from Brown-Norway (BN) rats and injected them intrathymically into Lewis (LEW) rats. Ten days following intrathymic injection, a bilateral nephrectomy was performed and a BN renal allogeneic graft was implanted. No post graft immune suppression was used. All control animals died with anuria within 6 to 8 days. Those that received the intrathymic injection of glomeruli lived for 70 days (experimental endpoint). Perico et al. (1991) later demonstrated that the tolerance effect was not dependent on cell type but was strain dependent. Using a slightly different approach, Posselt et al. (1992) gave neonatal BB rats subtherapeutic numbers of MHC compatible WF adult male rat islets (60-80 islets) intrathymically. The number of islets required for a therapeutic transplant is usually in the range of 1000-1500 islets. Controls received only intrathymic saline. No immunosuppression was given to either group. Rats that received the islets did not develop diabetes while 50% of the controls became diabetic by 120 days.

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Intrathymic treated rats remained normo-glycemic for greater than 70 days after removal of the thymus demonstrating that the islets in the thymus were not maintaining the normoglycemia. Native pancreatic islet cells were healthy on histologic examination in this group. These experiments indicate a deletion or inactivation of specific T-cell clonal population.

Many other studies have complimented the results of the earlier work. In most studies, rats and mice will accept allogeneic grafts provided they are pretreated with antilymphocyte serum and intrathymic inoculation of donor cells (Goss et al., 1992; Oluwole et al., 1993; Shen et al., 1994; Dono et al., 1995). In cases where there were major MHC mismatches between donor and recipient, graft survival was not extended (Shen et al., 1994). The intrathymic injection of nonimmunogenic cells does not induce tolerance in the rat model as demonstrated by Moore et al. (1995) and Ketchum et al. (1995). To better determine the factors involved in the induction of tolerance with intrathymic inoculation of donor cells more recent studies have refined the material used for injection. Natural or synthetic cell membrane antigens have been used as thymic inoculant in a number of studies, the results of which are essentially similar to previous successful studies (Qian et al., 1993, 1995; Ohakekwe et al., 1995; Shimomura et al., 1995).

The rabbit may be a more appropriate model with which to investigate clinically relevant transplant and immunology methods. The rabbit MHC Class II pattern of expression is close to that of humans (Sittisombut and Knight, 1986; Sittisombut et al., 1988). The rabbit express similar counterparts for human Class II gene regions (DR) (Laverriere et al., 1989), (DQ) (LeGuern et al., 1987), (DP, and DZ) in a variety of tested tissues including the spleen, bone marrow, appendix, and lymph nodes. Compared to the mouse, which has a more restricted expression of MHC II genes, the rabbit compares much more closely to the human expression or coexpression of genes from the three subloci (Sittisombut and Knight, 1986). Genetic homology by nucleotide sequences indicate the rabbit to human values for the DP $\alpha 1$ genes is approximately 89% (Sittisombut et al., 1986). Similarities between the human MHC Class I antigen expression and the rabbit have also been described in detail (Marche et al., 1985). In the mixed lymphocyte culture (MLC) technique for monitoring the allogenicity between donors and recipients, the rabbit RLD locus provides the necessary cellular control, identical to that in human cultures dependent on the HLA-D region (Lancki et al., 1975). The distribution of the rabbit 8AC8⁺ cells (helper and cytotoxic T-cells) and 8AC8⁻ cells (B-lymphocytes, null cells, granulocytes, and monocytes) is analogous to humans. Rabbit T-lymphocytes demonstrate close homology to human counterparts (Dubinski et al., 1988). The rabbit has been successfully used in traditional transplant models that rely on CsA for immune suppression (Thliveris et al., 1995).

This present study was designed to determine whether the development of immune tolerance by intrathymic inoculation of donor lymphocytes can be achieved in the outbred animal model. Rabbits were chosen since this species provides an immunologic model closer to humans compared to the rat or mouse. In addition, the rabbit was used to investigate whether this tolerance phenomenon could be demonstrated in a species other than the rat or mouse or whether its development was truly species specific.

Materials and methods

Animals

Six month old outbred New Zealand White rabbits (recipients) and California rabbits (donors) were obtained from Prairie Rabbitry (Manitoba) and treated prophylactically with enrofloxacin for *Pasteurellosis*. Recipient-donor pairs were assigned on a random basis and were maintained throughout the experiment. Rabbit pairs were divided randomly into experimental (n=5) and control (n=5) groups.

Protocol

Day 4, 7, 49, 124: Blood samples for MLC assay. Day 1: Injection of ALS. Day 0: Intrathymic injection (control: 1 ml of RPMI cell medium; experiment: 1 ml of RPMI with 5×10^7 donor lymphocytes). Day 132: Thyroid transplantation. Day 139: Biopsy of thyroid graft site. Day 174: Biopsy of thyroid graft site and euthanasia

Preparation of donor lymphocytes

Donor whole blood (10 ml) was collected by venipuncture of the marginal ear on jugular vein into a heparinized collection tube (Becton Dickinson, lithium heparin). The blood and an equal amount of RPMI-1640 (Sigma) cell culture media (22 °C) were added to a 50 ml conical centrifuge tube (Falcon Brand). Five milliliters of Histopaque-1077 (Sigma) were slowly added to the bottom of the tube. The layered solution was centrifuged at 400g for 30 minutes at 22 °C.

Mononuclear cells were removed and added to 20 ml of ice cold Hanks buffered saline solution (HBSS, Sigma). The cells were centrifuged at 400g for 10 minutes at 4 °C. The pellet was washed again with HBSS and centrifuged. The cells were resuspended in cold RPMI and maintained on ice until used for injection.

Intrathymic inoculation

Recipient rabbits received ketamine and xylazine as preanaesthetics. They were intubated and maintained with mechanical respiration and isoflurane for the remainder of the procedure. A 1.5 cm incision was made

Thymic inoculation in the rabbit

on the left chest wall between the second and third ribs beginning at the sternum. The incision was carefully carried through the intercostal muscles. The thymus was gently brought into the operating field using forceps and held in place during injection.

Intrathymic injection was completed using a 28g needle injecting into multiple sites. A small chest tube was placed into the pleural space and exteriorized to an air tight valve. Tissues were closed in layers and intermittent suction was applied to the chest tube. The tube was removed approximately 4 hours post operative once no further fluid or air could be aspirated. All animals received 48-72 hours of analgesia (torbugesic or buprenorphine) post operatively.

Mixed lymphocyte culture method

We used a modification of the method described by Maske et al. (1994). Donor (DX) lymphocytes were irradiated with 2500 rads over 9 minutes using a γ -radiation (Beckman LS5801). An aliquot (typically 1 ml) of recipient (RX) lymphocytes was also irradiated for use as control cells. One hundred microlitres of untreated recipient (R) cells were mixed with 100 ml of DX cells in each of three wells (Falcon, 96 wells, flat bottom). Similarly control wells were set up containing 100 μ l of R and 100 μ l of RX cells. This same cell mixture was used for every donor-recipient pair. The plates were incubated at 37 °C, 5% CO₂ for 48 hour followed by addition of 30 μ l of ³H-thymidine (Amersham Life Science, 185 Gbq/mmol, 37 Mbq/ml) and a further overnight (18 h) incubation. The contents of each well was removed for cell collection on filter mats (Skraton) using a cell harvester (PHD 200, Cambridge Technology Inc). Six to eight washings were performed through the same filter. Filter mats were rinsed with 95% ethyl alcohol and dried for a minimum of 3 h at room temperature in individual scintillation vials. Before counting, 2 ml of scintillation fluid (Aquasol - Packard Ltd.) was added to each vial and the contents mixed. Each vial was counted for 1 minute. The mean count values for the triplicate samples were used in calculating the MLC stimulation indices.

Thyroid transplantation

The transplantation of thyroid tissue without the need for vascular microanastomosis provided an ideal model for use in this matched donor-recipient long term study. Simple implantation of thyroid tissue has had good clinical success (Skolnick et al., 1963; Swan et al., 1967; Wertz, 1974). Isolated follicle autografts have also been used successfully (O'Malley et al., 1993).

The donor was preanaesthetized with ketamine and xylazine and maintained on isoflurane by mask. After appropriate skin preparation, a midline incision was made over the anterior neck and the muscles retracted.

The thyroid was isolated from surrounding tissue and approximately one-half of one lobe was excised and placed into cold 0.9% saline. The thyroid tissue was diced into cubes approximately 1 mm³ and kept in the saline. The recipient was anaesthetized similarly. The skin covering the right gluteal area was incised and two locations of the gluteal muscle gently split. Five pieces of the donor thyroid were distributed into each cavity. The muscle was closed and a polypropylene suture was placed as a marker for biopsy locations and histological sections. Tissues were closed in layers. All animals received postoperative analgesia. Remaining thyroid tissue pieces were submitted for confirmatory histology. Four additional NZW rabbits received thyroid autografts using the same basic technique but were biopsied two weeks following grafting.

Histology

The muscle graft sites were excised *en bloc* under general anaesthetic. Tissues were fixed in formalin and processed for light microscopy. Sections (10 μ m) were cut every 100 μ m, stained with hematoxylin and eosin, and examined by two independent observers blinded to the experimental group.

Statistics

Statistical analysis was performed using SPSS (Chicago, IL). Wilcoxon Matched Pairs test was used to compare MLC values within one pair across time. Mann-Witney U testing was used to compare between control and experimental group MLC at each time interval. Only four pairs in the experimental group were included in the data analysis due to the late death of a matched pair donor.

Results

ALS Injection

Baseline lymphocyte counts (6 ± 0.9 cells $\times 10^9$ /l) decreased significantly ($p < 0.05$), 16 hours following ALS injection (3.1 ± 0.8 cells $\times 10^9$ /l).

MLC Stimulation indices

MLC stimulation indices for control and experimental animals over time are shown in Table 1. There was no statistical difference between controls or experimental animals. Comparisons of MLC indices between time periods within the same experimental group also demonstrated no statistical difference.

Thyroid graft survival

Animals that received autologous thyroid tissue demonstrated survival of the implanted tissue two weeks following graft placements. The follicular epithelial cells

Thymic inoculation in the rabbit

appeared normal, follicles were filled with colloid and there was no visible inflammatory reaction or evidence of graft necrosis (Figs. 1-3). All control and experimental rabbits demonstrated histological evidence of acute graft rejection in the biopsies of the heterotopic thyroid tissue one week following thyroid graft implant (Figs. 4, 5). Massive infiltration of lymphocytes and virtually complete destruction of follicular cells and intrafollicular matrix was noted. Control and experimental animal muscle biopsies showed continued histological evidence of chronic graft rejection and inflammatory of the thyroid graft six weeks following transplantation (Fig. 6). There was no evidence of graft

Table 1. Comparison of stimulation index (ratio of donor-recipient to donor control counts) between control and experimental recipients (mean \pm SD).

TIME	CONTROL RECIPIENTS	EXPERIMENTAL RECIPIENTS
Preoperative ^a	2 \pm 1 ^b	13 \pm 12
Week 1 postoperative ^c	8 \pm 12 ^b	7 \pm 9
Week 7 postoperative ^c	4 \pm 4 ^b	1 \pm 1
Week 18 postoperative ^c		

^a: preoperative MLC's were performed on triplicate samples; ^b: no significant difference between control and recipients ($p>0.05$); ^c: no significant difference between previous MLC test in control and experimental group.



Fig. 1. Sample of thyroid tissue used for graft implantation showing normal follicular cells and intact follicle content. x 40

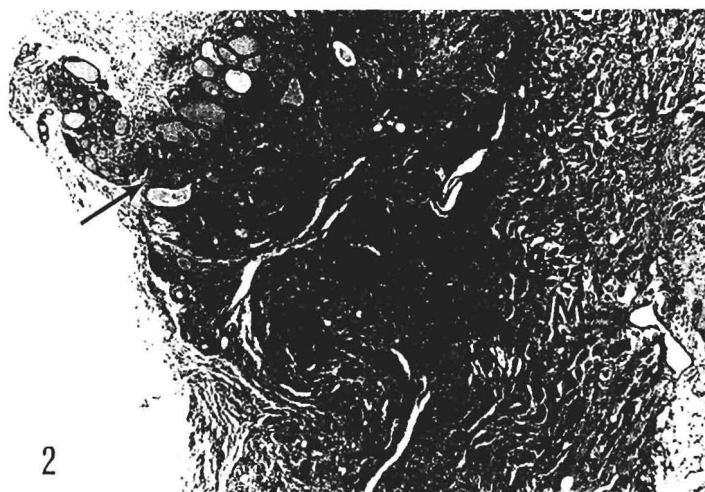


Fig. 2. Biopsy of implanted thyroid autograft (arrow) adjacent to recipient muscle tissue (M). There is normal follicle structure and absence of inflammation and necrosis. x 40

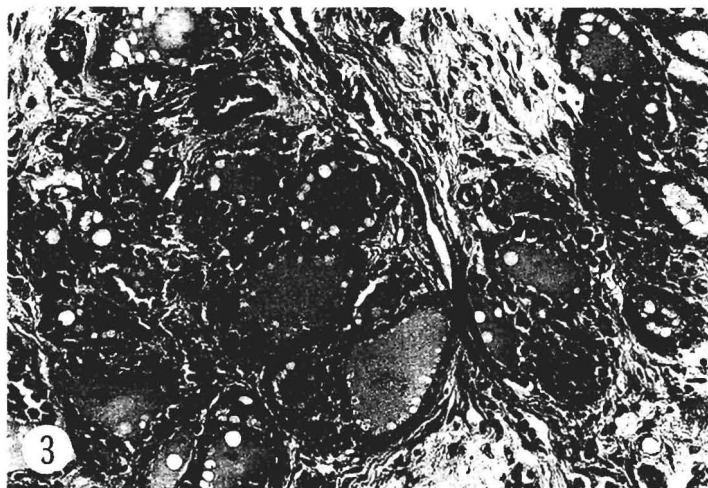


Fig. 3. High power view of implanted thyroid autograft showing normal follicle structure. x 200

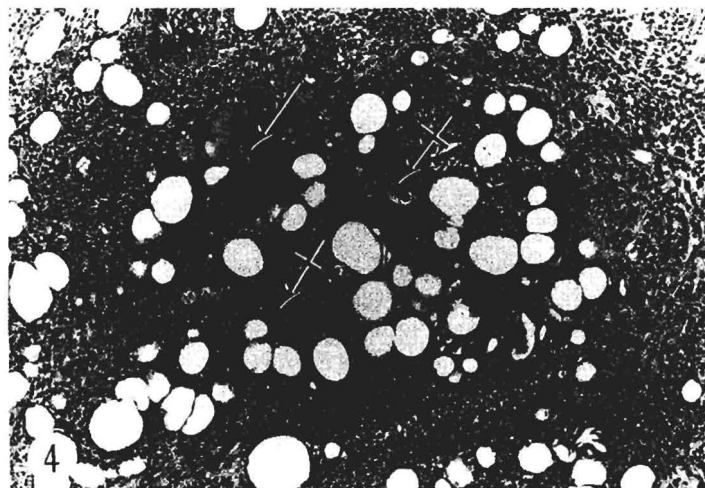


Fig. 4. One week biopsy of implanted allogeneic thyroid demonstrating marked mononuclear infiltrate (crossed arrow). Several follicles appear normal (arrow). x 40

necrosis in any specimens.

Discussion

Many studies have demonstrated that allogeneic graft tolerance can be induced in inbred rats and mice by inoculating the recipient thymus with donor cells/antigen before transplantation.

ALS Treatment

Most successful tolerance induction experiments in rats and mice have required the use of ALS which depletes the peripheral T-cell pool signaling the bone marrow to release additional stem cells for re-establishment of circulating T-cells. The stem cells must pass through the thymus inoculated with donor antigen. In this way a new population of recipient T-cells tolerant to donor antigen may be produced. In the current experiments, ALS was given the evening before thymus inoculation with donor lymphocytes. Our results demonstrate a 66% reduction in the levels of peripheral lymphocytes. B-cells typically represent 20% of peripheral lymphocytes which would translate to an approximate 80% reduction in peripheral total T-cell counts. The relative reduction in T-cells is similar to that reported in successful rat models. To determine if further decreases could be obtained in lymphocyte counts, two non-grouped non-paired rabbits were given a second injection of 0.5 ml ALS approximately 24 hours following the first injection. Within minutes of injection, both animals displayed symptoms of cardiovascular shock and died, apparently a result of severe immune reaction.

Intrathymic inoculation of donor lymphocytes

A majority of previous studies in the rat have utilized splenocytes as the inoculant. The spleen provides a simple matrix from which a mixture of T and B lymphocytes as well as macrophages can be collected. The cells provide both MHC Class I and II antigens. Other groups have inoculated with T-cells (Chowdhury et al., 1993), leukocytes (Perico et al., 1991), glomeruli (Remuzzi et al., 1991), bone marrow cells (Posselt et al., 1992), whole blood (Hamano et al., 1990), and islets (Gerling et al., 1992) with similar successful induction of tolerance. The number of lymphocytes injected into the thymus (5×10^7) was chosen empirically after review of other published successful experiments in rats. Oluwole et al. (1994) defined a specific weight of donor antigen for inoculation that resulted in tolerance induction, however the results were never described in terms of cell counts or dose per unit weight of thymus tissue. In our experimental design, in order to ensure donor viability, the maximum single blood withdrawal for lymphocyte harvesting was 10 ml, considering that blood was also required for MLC assay. It is possible that the larger mass of the rabbit thymus compared to the rat did not allow exposure of new pre-T-cells to a sufficient concentration of donor antigen.

Timing of intrathymic inoculation with graft implants is also highly variable in published reports. Successful tolerance induction has been seen with IT injection over a range of seven days post grafting to 13 weeks pre grafting (Light et al., 1995). Our experimental protocol resulted in grafting of the thyroid tissue 19 weeks after thymic inoculation. It is possible that although a new T-cell population, non-reactive to donor tissues, was created within the first few weeks following inoculation,



Fig. 5. High power view. One week biopsy of implanted allogeneic thyroid demonstrated mononuclear infiltrate between follicles and within the colloid (arrows). x 200

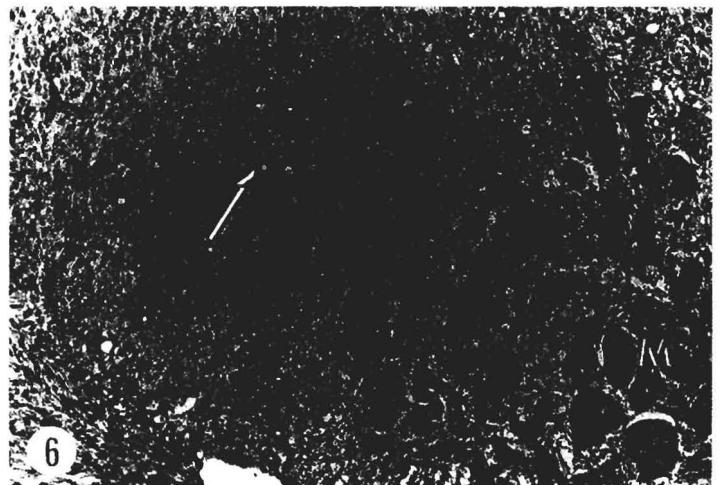


Fig. 6. Six week biopsy of implanted allogeneic thyroid graft demonstrating complete loss of thyroid tissue organization and presence of marked mononuclear infiltrate (arrow). M: recipient muscle. x 40

enough of this peripheral population did not survive until the time of grafting. However, it has been shown that non-activated T-cells remain in the peripheral circulation for months to years.

MLC assay

The MLC assays demonstrated no change from preinoculation through to the period of thyroid graft placement. The ideal method for monitoring changes in MLC would have been to use cryogenically maintained deposited blood obtained from a wide selection of donor rabbits. This could then be used as a standard to which individual donor-recipient pairs could be compared. Such a pool was not available. In addition, the use of MLC testing for monitoring allogeneic acceptability has recently come into question (Mickelson et al., 1993) where it appears that up to 40% of MLC tests can have questionable results.

Rabbits

The rabbits used in this study were outbred New Zealand Whites and Californians. It is not known what specific genetic immune difference exists between them. Not a single graft survived beyond the first week after transplant. When donor and recipient incompatibility in both major and minor MHC existed in rat experiments there was no extended survival of grafts (Shen et al., 1994; Dono et al., 1995; Klatter et al., 1995). Similar results were found in the xenograft (Zeng et al., 1995; Shen et al., 1996) and outbred canine models (Merhav et al., 1995), where one would also expect significant MHC differences. These differences would also be found in non-related live or cadaveric human donor-recipient combinations. It would appear that in rats, the thymic inoculations may provide a form of tolerance that resist minor differences in MHC and that is overwhelmed in situations of major antigen differences.

Many medications and biological stressors have been found to result in changes in thymus size and activity (Kendall, 1980). Drugs used in this experiment included anaesthetic agents (ketamine, xylazine, and isoflurane), analgesics (torbugesic and buprenorphine), and antibiotics (enrofloxacin). Buprenorphine increases thymic and lymph node weights during chronic administration of high doses to young rats, which might indicate some immunotoxicity (Van Loveren et al., 1994). It is not known what effects if any would be seen with acute usage in rabbits. Chronic cephalosporin administration in mice cause increases in IgM levels but does not appear to affect host resistance or cell-mediated immunity (Furuhama et al., 1993). There is no data on immune functional changes with the acute use of the other drugs from this study.

Thyroid transplant

Thyroid tissue was chosen for transplantation in our

model because of the technical simplicity of grafting. In two studies in rats using intrathymic inoculation of either antigen (Nakafusa et al., 1993) or splenocytes (Goss et al., 1993) and ALS, survival of one tissue type was contrasted by rejection of another though both groups received the identical tolerance developing treatment. In the study by Nakafusa et al. (1993), mismatched rats demonstrated long term acceptance of heterotopic heart grafts, yet the same protocol did not provide acceptance of renal or skin grafts. The higher number of passenger lymphocytes in the latter two tissues might have caused an enhanced reaction to the tissues. Biopsy of normal thyroid tissue does not demonstrate an abundance of lymphocytes. It is possible that normal thyroid possesses potential antigens or autoantigens that would not be expressed by donor lymphocytes inoculated into the rabbit thymus. T-cell clones reactive to any thyroid specific antigen would not have been deleted within the thymus resulting in immune reaction at the graft site. Genetic differences in outbred rabbits could result in recognition of autoantigens (such as those associated with Hashimoto's thyroiditis) on the thyroid implants not compensated for by any tolerance induced by intrathymic inoculation.

In contrast to a significant body of published rat model research, inoculation of donor lymphocytes with concomitant treatment with ALS did not result in tolerance to allogeneic thyroid grafts in outbred rabbits. This study is in agreement with other studies in outbred dogs (Merhav et al., 1995) and xenografts (Zeng et al., 1995; Shen et al., 1996). The reasons for failure of tolerance induction in this model remain unclear but may be due to insufficient donor lymphocyte inoculation, immune peculiarities of the thyroid, insufficient recipient induction lymphopenia, timing between inoculation and grafting, drug interactions with the immune system, or large donor-recipient MHC incompatibilities that could not be overcome with simple thymic inoculation treatment.

Further studies focusing on evaluating the dose of donor antigen per mass of recipient thymus, the relationship of recipient infections, and the survival of other graft types need to be performed in order to determine the feasibility of this method for use in humans.

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