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Distribution and persistence of antigen-presenting cells after intrathymic injection

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Abstract Intrathymic injection of donor immune cells has been shown by previous studies to prolong survival of rat allogeneic tissues. The aim of this pilot study was to assess the distribution and the persistence of intrathymically (IT) injected purified antigen presenting cells (APC) over a period of time in the rat model DA-to-WAG ($RT1^{av}$ to $RT1^{u}$) using a specific monoclonal antibody (Mab) with RT1Aa class I polymorphic specificity (R3/13 clone). Purified non-parenchymal cells (NPC) or dendritic cells (DC) were prepared from liver and spleen of DA rats with a purity of greater than 60% and 90%, respectively, shown by selected Mab staining methods. DA NPC (1×10^6) or DC (5×10^5) in 20 µl were injected into both lobes of the thymus of WAG rats with or without 1 ml antilymphocyte serum (ALS) intraperitoneally. Thymus tissue was removed on days 3, 5, 10, 20 and 30, and processed for frozen sections and immunohistochemical staining with R3/13. Numerous DA-positive cells were detected in the first 3–10 days post-IT inoculation in both NPC- and DC-treated rats, with or without ALS. After day 10, the proportion of positive cells decreased in all cases except in rats given NPC and ALS, where similar numbers of R3/13-positive cells were seen throughout. These DA-positive cells were mostly found in the medullary portion of the thymus at the corticomedullary junction in close proximity to thymic dendritic interdigitating cells. We concluded from this pilot study that the injected cells remained in the thymus for a limited period. However, the immunosuppressive effect of ALS promoted some degree of persistence of donor APC in the thymus beyond 30 days. Further studies are in progress to reveal the specificity of these cells.

Key words Intrathymic · Inoculation
Antigen-presenting cells
Immunohistochemistry

Introduction

Intrathymic (IT) inoculation of donor immune cells has been shown by previous experimental studies to be a successful model for the induction of specific unresponsiveness towards histoincompatible tissue without the need for chronic immunosuppression. Posselt et al. [1] have reported the indefinite survival of rat islet allografts transplanted into the thymus under cover of transient immunosuppression [antilymphocyte serum (ALS)].

Later, these studies were extended successfully to the BB rat [2] and to islet xenografts [3]. Remuzzi et al. [4] have demonstrated donor-specific unresponsiveness to kidney grafts after IT injections of isolated renal glomeruli and pretransplant immunosuppression. Perico et al. [5] have used unpurified white blood cells to induce tolerance to renal grafts without immunosuppression. In contrast, Nakafusa et al. [6] have reported donor-specific tolerance to cardiac but not renal allografts by IT donor type splenocyte inoculation. Significant prolongation of skin allografts in mice using disparate donor-recipient combinations involving class I and II differences have been reported by Ohzato and Monaco [7] after IT splenocyte injections under cover of transient immunosuppression. IT inoculation of bone marrow cells (BMC) renders recipients unresponsive to either donor type cardiac [8] or islet [9] grafts without the need for chronic immunosuppression. Unpurified cell supensions were used in most of these reports. Studies in our own laboratory [10] have demonstrated the induction of unresponsiveness to rat cardiac allografts after IT injection of liver non-parenchymal cells (NPC), consisting mainly of Kupffer cells and macrophages. Tolerance was tested in long-term survivors by donor type skin grafts that were rejected albeit in a delayed fashion, but the donor type stimulus did not seem to be strong enough to reject the heart grafts. A second donor type heart graft was also not rejected, whereas a third party heart graft was rejected in a normal fashion (unpublished observation), clearly demonstrating that organ-specific tolerance is induced by NPC's. In contrast, Campos et al. [11] have shown that both IT injection of parenchymal hepatocytes and non-parenchymal Kupffer cells fails to prolong orthotopic liver allograft survival compared to BMC, which induce indefinite liver graft survival. A possible explanation for the different results obtained with IT cell inoculation could be the relative content of a specific cell type and its survival within the thymus. Antigen-presenting cells (APC) are probably the cells most common in these cell inoculations. They are also represented, though in small numbers, in isolated islets or glomeruli preparations. We, therefore, evaluated the distribution and persistence of purified APC within the thymus after IT inoculation over a period of time using selected monoclonal antibodies (Mabs) and immunohistochemical staining techniques. The effect of ALS treatment was also considered. The effect of purified APC IT injections on tolerance induction were also tested in islet allografting studies [12].

Materials and methods

DA ($RT1^{av1}$) were used as donors for the isolation of APC. NPC were purified from liver as described previously [10] and dendritic cells (DC) were purified from the spleen by a modification of the technique described by Klinkert [13]. Briefly, single spleen cell suspensions were made by gently massaging splenic tissue through a metal sieve. Mononuclear cells were prepared by BSA gradient centrifugation. Adherent cell populations, e.g. macrophages, were removed by overnight tissue culture and the remaining cell suspension was centrifuged at 1100 *g* for 20 min over a 13.68% metrizamide gradient. Cells were harvested at the interphase, washed and assessed for viability by phase contrast microscopy.

NPC and DC cell numbers were adjusted to 1×10^6 and 5×10^5 in 20 µl and injected by our standard technique into both lobes of the thymus of young WAG ($RT1^u$) rats weighing between 140 and 200 g. Immunosuppressive treatment with ALS (Accurate Chemical Scientific Corp, Westbury, N. Y.) 1 ml i. p. was given immediately after the cell inoculation in half the animals (NPC ± ALS; DC ± ALS; $n = 5$ in each group). The thymus and, if possible, the parathymic lymph nodes were removed on days 3, 5, 10, 20 and 30 post inoculation in all groups, snap frozen, processed for frozen sections and analysed for donor type cell content and distribution by immunohistochemical staining procedures using Mab R 3/13 (RT1 Aa class 1 polymorphic specificity) specific for DA donor type cells. Thymus sections from WAG-to-WAG syngeneic NPC or DC IT inoculations were used as controls.

Each NPC and DC preparation was assessed for the relative content of NPC or DC by immunohistochemical staining of cytospin slides using a series of Mabs (all Mabs were obtained from Serotec UK.) against rat surface antigens: ED1, 2 and 3 specific for macrophages and Kupffer cells, MRC OX 6 specific for rat class II antigens on DC and B cells, MARK-1 for B cells, OX-52 for T cells, MRC OX 43 for endothelium and MRC OX 42 for macrophages and DC. STAR 21 A (rabbit anti-rat IgG F $(ab')_2$ peroxidase conjugated) was used as a second antibody at 1:100 dilution in phosphate-buffered saline (PBS) plus 5% non-immune rat serum. Slides from each thymus were stained with either the first or second antibody alone and served as background controls. A few scattered cells were stained with the second antibody in control sections of non-injected, normal rat thymus and syngeneic and allogeneic IT-injected thymi.

Immunohistochemical staining was performed using the immunoperoxidase technique described by Barclay [14]. Briefly, frozen sections were fixed with acetone for 10 min, air-dried, washed in PBS and incubated with the appropiate dilution of the primary Mab for 60 min, followed by extensive washing in PBS, and 60-min incubation with the secondary Mab. After extensive washing in PBS the slides were reacted with diaminobenzidine DAB plus H_2O_2 for 10 min, counter-stained with Harrison's haematoxylin and embedded in DPX. The score from negative (+) to few (++), medium (+++), to many (++++) DA-positive cells per field was obtained by scanning serial sections of the respective thymus, thus assessing the distribution of the inoculum throughout the thymus, using control slides as comparison.

Results

Assessment of NPC and DC inoculum

Inoculated NPC suspensions showed a purity of over 60% for Kupffer cells, 30% positive for macrophages

Table 1 Persistence of DA donor cells inoculated into WAG thymus with or without ALS (*ALS* antilymphocyte serum, *NPC* non-parenchymal cells, *DC* dendritic cells)

Days post inoculation	DA-positive cells in WAG thymus			
	NPC		DC	
	+ALS	−ALS	+ALS	−ALS
3	++++	++++	+++	+++
5	++++	++++	+++	+++
10	+++	++	++	++
20	+++	++	++	+
30	+++	++	++	+

and were contaminated by some T and B cells with very few hepatocytes and endothelium cells. DC were 90–98% positive for MRC OX6 and contaminated by some B cells also positive for MARK-1. Viability of the cell suspensions were over 90% and 98%.

Effect of ALS on the thymus

The treatment of rats with 1 ml ALS i.p. had a profound effect on the thymus but not on the histological appearance of parathymic lymph nodes that were recovered from some animals. The architecture of the thymus was in general, disturbed, the corticomedullary regions were undefined and the medulla was greatly depleted of thymocytes on days 3 and 5. By day 10 most thymi had recovered with normal histological appearance and restoration of the thymocyte pool.

Distribution and persistence of NPC and DC inoculum

Staining of the thymus inoculated with DA NPC with Mab R3/13 specific for DA class I alloantigens revealed that DA-positive cells were found within all thymi throughout the observation period (Table 1). On day 3, positive cells were detected around the injection site, thereafter, positive cells were seen to be more uniformly distributed throughout the thymi. By day 10, the number of positive cells was reduced but remained stable thereafter. They were detected mainly in the medullary area of the thymus at the corticomedullary junction in close proximity to thymic dendritic interdigitating cells. In rats given ALS at the time of inoculation, the persistence of positive cells was similar from day 3 to day 30, through in general with a reduced number of cells from day 10 onwards. But, unlike animals without ALS, no further depletion in positive cells was observed.

The cell number of DC injected into the thymi was two-fold lower (5×10^5) compared with NPC (1×10^6) inoculations and, thus, the detection of positive cells was more difficult. At days 3 and 5, similar numbers of positive cells were observed. Reduction occurred after day 5, and by day 30 only very few positive cells were seen, similar to background staining. Again, in ALS-treated rats, positive cells were slightly more numerous.

Discussion

Most of the cell preparations used for IT inoculations to induce tolerance to allogeneic extrathymic organ or islet grafts were composed of unfractionated white blood cells usually prepared from splenic or liver tissue or bone marrow containing variable amounts of APC. In our pilot study, purified APC, mainly Kupffer cells and macrophages isolated from donor liver or highly purified DC from the spleen were used for the inoculum and their distribution and persistence was followed by immunoperoxidase staining of the donor cells. NPC at 1×10^6 per inoculum were chosen because these cell numbers resulted in tolerance induction to cardiac allografts reported by us previously [10]. DC cell numbers were at two-fold lower concentration at 5×10^5 per inoculum, because these cells are highly efficient in antigen presentation and occur normally in very low numbers in the splenic, blood, liver, islet or other tissues and, thus, would be more comparable to most published IT inoculation protocols. Moreover, in order not to disrupt the thymic microenvironment, these lower cell concentrations were also injected suspended in 20 µl, in contrast to published figures of more than 100 µl.

Donor chimerism was observed in both NPC- and DC-inoculated thymi throughout the observation period, though at lower levels by day 10 and thereafter. Because the initial number of DC injected was much lower, cells positive for DA alloantigen seemed to decline more rapidly, and by day 20 sections appeared to look similar to control staining. ALS i.p. injections altered the morphological appearance of the thymus during the 1st week after the injection, but by day 10 most thymi seemed to have recovered and appeared normal again. Immunosuppression with ALS supported the survival of donor type cells to a larger extent than in animals without transient immunosuppression. However, the thymus as a privileged site (thymus blood barrier) also protected the implanted cells from destruction, permitting residence of allogeneic NPC for over 30 days without ALS treatment. The manifestation of the donor cells at a particular site, the corticomedullary junction, needs to be investigated further.

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