Transpl Int (1996) 9 [Suppl 1]: S379–S381

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Induction of specific inhibition of alloreactivity in beagle dogs by intrathymic injection of donor splenocytes

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Abstract The aim of this study was to investigate whether intrathymic injection (ITI) of donor splenocytes in dogs might lead to specific immunomodulation as assessed by mixed lymphocyte culture (MLC) tests. Two groups of five beagles each were used. Group 1 contained animals that were 2 years old, group 2 consisted of animals that were 6 months old. One animal was splenectomized per experimental group and 1×10^9 splenocytes were injected into the thymic lobes or thymic remnant of the four remaining dogs. On the day of ITI the dogs were treated subcutaneously with a single dose of 2 ml/kg anti-lymphocyte serum (ALS). In group 1 the thymus of all dogs was found to be atrophic. ITI in this group did not result in a decreased immunoreactivity but rather in an enhanced immune response. In group 2 the thymus was still clearly present and ITI was easy to perform. ITI induced a significant reduction of specific MLC reactivity at 1 week after treatment. The effect was transient and not significantly further diminished at week 2. These results indicate that ITI is a technically feasible procedure in a preclinical animal model. It may induce temporary sensitization as well as immunosuppression, possibly depending on the age of the recipient.

Key words Intrathymic · Unresponsiveness · Dogs

Introduction

Posselt et al. [1, 2] were the first to show in rats that intrathymic injection (ITI) of pancreatic islets could lead to induction of specific unresponsiveness. Extension of this original finding by many groups in various rat and mouse models revealed also that ITI of lymphocytes or purified alloantigens, combined with a brief course of immunosuppression, was able to evoke donor-specific unresponsiveness of subsequent allo- and xenografts [3–8]. The question remained whether this new way of inducing unresponsiveness was just a typical rodent phenomenon or also would hold for preclinical animal models and even might be extrapolated to man. Recently, it was claimed by Une et al. [9] that ITI of islets in NIH minipigs, in conjunction with a short course of anti-lymphocyte serum (ALS), did induce donor-specific inhibition of cell-mediated cytotoxicity shortly after inoculation. In some cases, this reactivity even became undetectable after 1 month. In contrast, Merhav et al. [10] failed to produce prolonged survival of cardiac or renal allografts in mongrel dogs by donor-specific ITI of splenocytes and transient immunosuppression. The aim of the present study was to further investigate this issue in a well-defined preclinical animal model. Beagle dogs of different ages were used to study the feasibility of ITI. The capacity to induce unresponsiveness by ITI of spleen cells was monitored by performing mixed lymphocyte culture (MLC) tests.

Materials and methods

Animals

Two groups of male and female beagle dogs (Harlan, Zeist, The Netherlands), each containing five animals, were used. Group 1

consisted of adult dogs, which were about 2 years old, group 2 consisted of young dogs, which were 6 months old.

DLA matching and MLC monitoring

Tissue typing for class I and II antigens of the dog MHC (DLA) was achieved by serology and MLC tests, as described previously [11]. For both groups of experimental animals, only dogs were selected that were unrelated and DLA-incompatible. MLC tests for monitoring of immune reactivity were performed in a similar manner as for DLA class II typing [12]. The results were expressed as stimulation index (SI). To control for inter-assay variations during time, changes in donor-specific MLC reactivity after ITI were assessed by using normalized SIs. Therefore, a ratio was calculated by dividing the experimental SI by the mean SI of controls, being the results of mutual MLCs between the four ITI dogs. The SI ratio (SIR) is given as a percentage.

Antilymphocyte serum

Goat anti-dog lymphocyte serum (ALS) was produced by immunizing a goat 3 times subcutaneously (s.c.) with beagle mesenteric lymph node cells. The serum was collected 1 week after the last immunization. The lymphocytotoxic titer of the product was 1:126.

Experimental design

The beagle in each group of five dogs showing the highest stimulatory capacity in MLC against the four other dogs was selected to undergo splenectomy. Spleen lymphocytes were prepared in RPMI by standard methods and divided into four aliquots, each containing about 1×10^9 cells. The cells were cryopreserved in dimethyl sulfoxide (DMSO) employing programmed, stepwise freezing, and stored at −196 °C until used for ITI. ITI of splenocytes in the four remaining dogs of the group was performed under general anesthesia. Via a lateral incision in the neck, access was gained to the thymic region, after which the thymus (young animals) or its atrophic remnant (adult animals) was exposed. Biopsies were taken to ensure that the appropriate region was explored. The spleen cells were then injected into the two lobes of the thymus or into the thymic remnant, in a volume of 0.5 ml per lobe. After ITI, a single injection of ALS was given s.c. at a dose of 2 ml/kg body weight. The number of peripheral blood leukocytes was determined regularly and MLCs were performed at weekly intervals to monitor specific alterations in immune reactivity. The criteria for a significant change in immune reactivity were twofold: (1) specific SIs in a given assay had to be significantly different from control SIs; and (2) SIRs had to be significantly different from the baseline SIR.

Results

Feasibility of ITI

In all four adult dogs of group 1, the thymus was found to be atrophic. Histology revealed that small thymic remnants were still present. Injection of spleen cells into the atrophied tissue was feasible but leakage could not be prevented. In the young animals of group 2, the two thymic lobes were easily identified and injection of cells in a volume of 0.5 ml/lobe was easy to perform. Leakage of cells into surrounding tissue did not seem to occur.

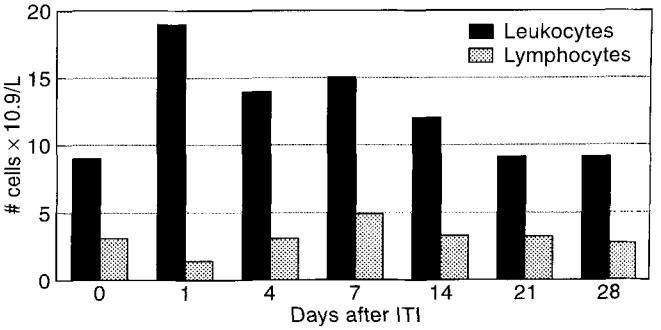

Fig. 1 Mean number of peripheral blood leukocytes and lymphocytes ($\times 10^9$/l) after a single subcutaneous injection of 2 ml/kg goat anti-dog lymphocyte serum ($n = 4$). The number of leukocytes is significantly increased on days 1, 4, and 7; the number of lymphocytes is significantly decreased on day 1. (*ITI* Intrathymic injection)

Effect of ALS on lymphocytes

Total leukocyte and differential lymphocyte countings were performed in the four dogs of group 1. The results are given in Fig. 1. The number of leukocytes was elevated significantly during the first week after ITI, possibly due to the surgical procedure or to ALS-induced leukocytosis. ALS produced a transient lymphopenia (less than 50 % of normal) on day 1 after ITI, after which the number of lymphocytes normalized again rapidly.

Immune reactivity after ITI

The results are depicted in Fig. 2. In group 1, monitoring of MLC reactivity was only performed once, at 1 week after ITI. Surprisingly, it was found that the mean SIR of the four dogs against the spleen cell donor was not diminished but, rather, had increased from 179 ± 82 % to 700 ± 538 % (Wilcoxon: $P = 0.05$). The results in group 2 were quite different. One week after ITI the mean SIR had dropped from 260 ± 86 % to 50 ± 39 % ($P = 0.02$); 1 week later the mean SIR was 68 ± 28 % (not significant); at 4 weeks the mean SIR was normalized to 103 ± 19 %.

Discussion

Three major conclusions can be drawn from the current experiments: (1) ITI in dogs is a feasible procedure with no morbidity; (2) ITI in adult dogs with an atro-

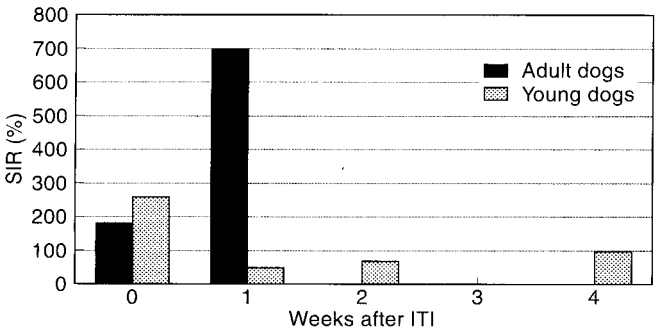

Fig. 2 Changes in specific immune reactivity as assessed by mixed lymphocyte culture (MLC) reactivity after intrathymic injection *(ITI)* of donor spleen cells into the thymus of four adult dogs (group 1) and four young dogs (group 2). MLC reactivity was calculated as the stimulation index (SI), which was normalized to a ratio (*SIR* in %) by dividing the mean donor-specific SI per group by the mean SI of controls. One week after ITI the mean SIR of group 1 was significantly increased ($P = 0.05$), whereas the mean SIR of group 2 was significantly decreased ($P = 0.02$)

phied thymus may lead to specific immunization; and (3) ITI in young dogs with an intact thymus leads to a transient form of specific immunosuppression. The latter result implies that ITI-induced unresponsiveness in not just a rodent phenomenon but also applies to a preclinical model. Although less striking, our results are in agreement with those of Une et al. [9] obtained in minipigs. Following ITI of islets, these authors found a profound and durable suppression of MLC reactivity, whereas we observed a moderate and transient suppression. A major reason for this difference in efficacy of ITI may have been the quality or dose of the ALS used. The goat anti-dog ALS employed by us may have been of moderate quality, evidenced by the finding that it only induced a short-term reduction in the number of peripheral blood lymphocytes. On the other hand, the fact that we did find a significant degree of specific unresponsiveness may cast doubt on the dogma that depletion of peripheral lymphocytes at the time of ITI is essential in larger animals; it may even be counterproductive. We recently observed in a specific rat model that ITI of spleen cells, in combination with a single dose of ALS, did not lead to prolonged acceptance but to accelerated rejection of heart allografts [13]. The current findings in adult dogs that ITI may lead to sensitization are very reminiscent of these observations.

It is obvious that the reduced MLC reactivity observed in our young dog model is only a remote parameter for the ultimate aim of ITI: prolonged graft survival. In this respect, the first transplantation results obtained by Merhav et al. [10] in dogs are very sobering. They indicate that the clinical road to tolerance may, as ever, not be as easy as the rodent's.

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