Transplant Int (1990) 3: 217–221

TRANSPLANT
International

Induction of mixed lymphocyte reaction nonresponsiveness after chimeric thymus transplantation

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Received May 3, 1990/Received after revision July 23, 1990/Accepted July 30, 1990

Abstract. Discussion still continues regarding whether self-tolerance is imparted within the thymus by the thymic epithelial cells (TEC) or by the bone marrow-derived (BMD) dendritic cells and macrophages. Many experiments suggest that BMD cells may be the major cells responsible for inducing tolerance within the thymus. In order to address this question and to see whether thymus chimerism could play a role in establishing transplantation tolerance, we looked in the present experiments at the induction of in vitro tolerance after transplantation of chimeric thymuses. Chimeric thymuses were constructed by injecting T-depleted C_3H (H_2k) bone marrow into lethally irradiated BALB/c (H_2d) mice and were thus composed of TEC of BALB/c and BMD cells of C_3H origin, something which was verified with immunoperoxidase staining. Subsequently, chimeric thymuses were transplanted into thymectomized BALB/c mice that were lethally irradiated and reconstituted with T-depleted syngeneic BALB/c bone marrow. In 8 out of 11 BALB/c mice this procedure was successful, and in all chimeric thymus graft-bearing mice, specific tolerance for C_3H (H_2k) antigens was documented in the mixed lymphocyte reaction (MLR). These experiments thus show that: (1) immunological tolerance can be imparted by BMD cells in the thymus and (2) the presence of chimerism within the thymus could be one of the mechanisms involved in the establishment or maintenance of transplantation tolerance.

Key words: Tolerance induction, thymus transplantation – Thymus transplantation – Chimerism, thymus role

A fundamental question in immunology concerns the mechanisms that induce tolerance to self components and whether these are also involved in transplantation tolerance. The widely accepted theory is that immature T cells are positively selected in the thymus for their capacity to bind to major histocompatibility complex (MHC) molecules [12, 13, 17, 24, 26, 27]. This theory also implies that cells with very high affinity and, hence, dangerous autoimmune potential can arise in the thymus. Therefore, a mechanism for eliminating self-reactive cells must also be operative [9, 10, 15, 16]. As far as the cells responsible for this so-called negative selection are concerned, discussion still persists. It is generally accepted that the cells of the dendritic/macrophage lineage – bone marrow-derived (BMD) cells – are responsible for this negative selection within the thymus [1–3, 8, 13, 21, 24]. If lymphohematopoietic cells are indeed able to impart tolerance within the thymus, the presence of donor-derived BMD cells within the host thymus could also play an important role in the establishment and maintenance of transplantation tolerance. However, the experiments indicating that thymic BMD cells induce tolerance are mainly based on experiments that show that after deoxyguanosine treatment, which selectively removes BMD cells from the thymus, transplantation tolerance to the thymus MHC antigens cannot be induced after thymus transplantation in nude mice [2, 3, 13, 21]. Yet, these experiments do not rule out the possibility that deoxyguanosine interferes with the function of other thymic (e.g., epithelial) cells.

In order to verify the role of the BMD cells in an alternative way, we have designed an experimental set-up using chimeric thymus transplantation. At the same time, these experiments could be relevant to the role of thymus chimerism in the induction of transplantation tolerance.

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Materials and methods

Animals

Two-to-six-month-old inbred BALB/c (H-2^d), C57BL/Ka (H-2^b), and C3H (H-2^k) mice were used in all experiments. Tylosine, a broad spectrum antibiotic for veterinary use, was added to the drinking water during and after completion of irradiation.

Thymectomy

Neonatal (day 2) thymectomy was performed by suction through sternotomy. The success was always verified at the time of sacrifice by serial histological sections.

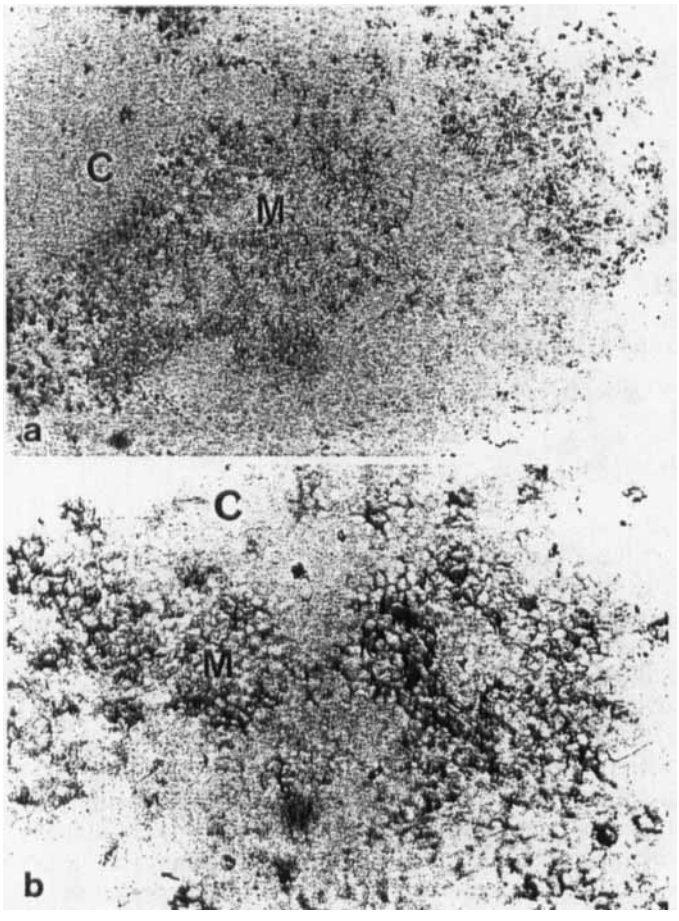

Fig. 1 a, b. Immune peroxidase staining of a chimeric ($C_3H \rightarrow ALB/c$) thymus with anti – I_Ak monoclonal antibody: **a** chimeric thymus [reticular staining only of the medulla *(M)* and not of the cortex *(C)*]; **b** the same under higher magnification

Irradiation

Total body irradiation (TBI) was carried out using gamma rays from a 60-cobalt source at a focus-skin distance of 100 cm and a dose rate of 0.35 Gy per minute.

Preparation of bone marrow cells

Bone marrow cells were prepared by flushing the femura and tibiae of donor mice (1–2 months old) with cold RPMI 1640 medium (Gibco) using a 25-gauge needle. Bone marrow plugs were then gently resuspended and washed twice. The viable cells were adjusted to the final desired concentration in RPMI 1640 medium.

T-cell depletion of bone marrow cells

T cells were removed from the bone marrow suspension by treating the cells with anti-Thy1-2 (Becton Dickinson, Mountainview, Calif.) and guinea pig complement (Behring Diagnostics, Brussels, Belgium). 0.5–1.0 µg anti-Thy1-2 per 1×10^6 bone marrow cells was used. Guinea pig complement at 1:10 final dilution was used for the depletion treatment. A two-step procedure was used. Cells (10×10^6/ml) suspended in RPMI 1640 medium containing 5% fetal calf serum (FCS) and antibody were incubated for 30 min on ice. After washing, the pelleted cells were resuspended in the same volumes of complement diluted 1:10 in RPMI-FCS and incubated for 40 min at 37 °C. Then the cells were washed and the treatment procedure repeated once. The cells were resuspended in RPMI 1640 medium at the final desired cell concentration. T-cell depletion from the bone marrow was verified by staining a sample of treated bone marrow cells with anti-Thy 1.2 (fluoresceinated; Becton Dickinson) and by analyzing the stained samples in a fluorescence-activated cell sorter (Becton Dickinson).

Preparation of spleen cells

Spleen cells were removed aseptically, and single cell suspensions were prepared by gently pressing the spleen fragments through a steel mesh into cold RMPI 160 medium. The cells were washed twice, suspended in 2% acetic acid, and counted. Cell viability was determined by trypan blue dye exclusion.

Preparation of TBI chimeras

10×10^6 T-cell-depleted C_3H bone marrow cells were injected intravenously into BALB/c recipients given 9 Gy TBI approximately 18 h prior to the infusion of the cells. Chimerism was determined by the presence of donor-type lymphocytes in the peripheral blood, as identified by cytofluorometry.

Detection of chimerism by flow cytometric method

Chimerism of peripheral blood leukocytes was scored using a sensitive flow cytometry assay [25]. Three to six months post bone marrow or thymus transplantation, the animals were bled retro-orbitally and peripheral blood leucocytes (PBL) were prepared. 0.5×10^6 – 1×10^6 PBL were incubated with polyclonal BALB/c anti-C_3H antiserum at 4 °C for 30 min. After the incubation the cells were washed twice and incubated with FITC-conjugated goat anti-mouse IgG (The Binding Site, Birmingham, UK) for 30 min at 4 °C. Cells were washed twice, resuspended in phosphate-buffered saline (PBS) and analyzed in Becton Dickinson FACS^{tar} PLUS. Chimerism was detected within the T-lymphocyte window, using Thy 1.2 on second labelling. This excluded false-positive staining through non-specific Fc binding (on monocytes) or anti-Ig binding (on B lymphocytes).

Chimerism was scored absent when no significant shift of the mean fluorescence intensity was obtained after staining with the relevant alloantibody, as compared to staining with the syngeneic antibody. Positive and negative controls were always included. In control experiments we showed that this method was able to easily detect 0.5% chimerism.

Thymus transplantation

One lobe of a chimeric thymus was transplanted under the kidney capsule of experimental animals. Chimeric (C3H bone marrow→ BALB/c chimera) thymuses were used 2–3 months post bone marrow transplantation.

Light microscopy

Tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut at 5 µm. Sections were stained with hematoxylin eosin and with the periodic acid-Schiff stain.

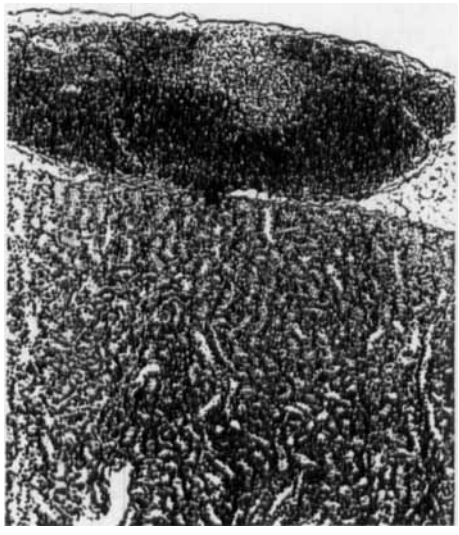

Fig. 2. Outgrowth of a chimeric (C3H→BALB/c) thymus under the kidney capsule of a thymectomized BALB/c mouse that was lethally irradiated and reconstituted with T-cell-depleted syngeneic bone marrow cells. Autopsy was performed 3 months after chimeric thymus transplantation

Immunoperoxidase staining

Five micron cryostat sections of thymuses were used for immunoperoxidase staining. They were fixed in 10% paraformaldehyde for 5 min and washed in PBS. The sections were then incubated with biotin-conjugated monoclonal antibodies (anti-I-A^k and anti-I-A^d; Becton Dickinson) at 37 °C for 30 min. After monoclonal antibody incubation, the slides were washed in PBS and horseradish peroxidase-conjugated avidin (Dako, Copenhagen, Denmark) was added and incubated at 37 °C for 30 min. Then the sections were rinsed in PBS and a substrate solution was applied to give a colored end-product. Finally, the sections were counterstained.

Mixed lymphocyte reaction (MLR)

MLR was performed 3 months after thymus transplantation. Responder (5×10^5) and stimulator (5×10^5) cells were cultured in a final volume of 0.2 ml/well, using flat-bottomed microculture plates (Greiner Labotechniek, Solingen, FRG). The stimulator cells were irradiated with 30 Gy from a 60-cobalt source. The cells were incubated in RPMI 1640 medium (Gibco, Ghent, Belgium), supplemented with 10% human AB serum, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes buffer, and 5×10^{-5} M 2-mercaptoethanol. Cells were incubated at 37 °C with 5% CO_2 air mixture in a humidified incubator for 120 h and then pulsed for 18 h with 1 µCi of [^{3}H]-thymidine (specific activity of 18–25 Ci/mmol; Amersham International, Buckinghamshire, UK). Cells were harvested onto glass fiber filter paper (Whatman International, Maidstone, UK) using a multiple automated sample harvester. The wet paper disks were placed into mini-vials and 0.1 ml of luma solve (Lumck, Landgraaf, The Netherlands) was added for 2 h. Then 4.5 ml of scintillation fluid (Lipa Luma; Lumck, Landgraaf, The Netherlands) was added and counted in a scintillation counter. The stimulation index (SI) was calculated as the ratio of counts per minute (cpm) from cultures containing allogeneic responders and stimulator cells to cpm from cultures countaining syngeneic responder and stimulator cells.

Results

Pattern of BMD antigen expression in chimeric thymuses

Chimeric thymuses were constructed by infusing 10×10^6 T-cell-depleted C3H bone marrow cells into lethally (9 Gy) irradiated BALB/c recipients. All of these BALB/c mice became fully allogeneic chimeras as phenotyping of their peripheral blood leukocytes and splenocytes using the sensitive flow cytometry method showed more than 99% donor type (C3H) cells. The presence of allogeneic BMD cells within the thymus and the presence and distribution of donor-derived class II MHC antigens at 2–3 months post bone marrow transplantation was verified by immunoperoxidase staining. Immunoperoxidase staining of the chimeric thymuses was performed with monoclonal biotin-conjugated anti-I-A^k (anti-C3H) or anti-I-A^d (anti BALB/c) antibodies as described earlier. Normal C3H thymus staining with anti-I-A^k gave a confluent medullary and cortical staining, indicating the expression of class II MHC antigens in both the cortex and medulla (data not shown). Normal BALB/c thymus staining with anti-I-A^k was always negative (data not shown). When the chimeric thymuses (C3H→BALB/c) were stained with anti-I-A^k, a positive reticular staining pattern was observed only in the medulla (Fig. 1). Anti-I-A^d staining of the chimeric thymus gave a positive pattern similar to that observed in normal control BALB/c mice (data not shown). These results clearly show that in the chimeric thymuses, donor-derived class II MHC expression is detectable and confined to the medulla.

Table 1. Induction of specific mixed lymphocyte reaction (MLR) nonresponsiveness in thymectomized BALB/c (H_2^d) mice receiving chimeric (C_3H BALB/c) or syngeneic (BALB/c) thymus transplants

Thymus transplant (no. of mice)	Stimulator cells	Percent response[a]
Chimeric (8)	C3H (H_2^k)	8 (0–10)
Chimeric (8)	C57BL (H_2^b)	37 (29–45)
Syngeneic (3)	C57BL (H_2^b)	41 (34–48)

[a] As compared to untreated control BALB/c mice, mean (range)

Table 2. Specific mixed lymphocyte reaction (MLR) nonresponsiveness after chimeric thymus transplantation

Responder cells	Stimulator cells	[^{3}H] Thymidine incorporation (mean cpm ± SE)	Stimulation index
BALB/c control	BALB/c	6163 ± 339	–
BALB/c control	C3H	90857 ± 3589	14.8
BALB/c control	C57BL	75796 ± 3339	12.3
Experimental animal with chimeric thymus transplant	BALB/c	5600 ± 177	–
Experimental animal with chimeric thymus transplant	C3H	5493 ± 234	1.0
Experimental animal with chimeric thymus transplant	C57BL	46101 ± 2123	8.3

Outgrowth of chimeric thymus grafts in thymectomized, irradiated, and reconstituted BALB/c recipients

Thymectomized BALB/c mice were used as recipients. These mice received lethal TBI (9 Gy) and were reconstituted with T-cell-depleted syngeneic bone marrow cells (10×10^6 per recipient). Within 18 h after the bone marrow transplantation, these animals were transplanted with a chimeric thymus graft under the kidney capsule. Chimeric thymuses were taken from lethally irradiated BALB/c mice that had received T-depleted C3H bone marrow cells 2–3 months earlier. Immunoperoxidase staining with an anti-C3H monoclonal antibody showed that BMD cells of C3H origin were present within the medulla of the chimeric thymuses. All of the animals were observed for the presence of a chimeric thymus graft between 3 and 6 months post-transplantation, both grossly and microscopically.

Despite the multiple manipulations with donor thymuses and recipient mice and despite the fact that we were dealing with adult thymus transplantation, which is technically more difficult than neonatal thymus transplantation, a normal outgrowth of chimeric thymuses was observed in 8 out of 11 mice. Figure 2 shows one of the chimeric thymuses obtained at 3 months post-transplantation.

Induction of specific MLR nonresponsiveness in thymectomized BALB/c mice receiving chimeric thymus transplants

Three months after thymus transplantation, we investigated whether the thymus graft-bearing BALB/c mice were tolerant in the MLR to the BMD cell type alloantigens (C_3H) expressed in the chimeric thymuses (Table 1). Spleen cells from chimeric thymus-transplanted animals gave no substantial response (zero or less than 10%) against BMD (C_3H) cell type stimulators as compared to control BALB/c mice but gave a significant mean response of 37% against third party (C57BL) stimulator spleen cells. The subnormal antithird party response was probably due to the various experimental manipulations. Indeed, thymectomized mice receiving TBI followed by syngeneic bone marrow infusion and transplantation of a syngeneic unmanipulated thymus were also unable to mount a normal MLR response (mean 41%) as compared to untreated controls. Table 2 shows details of the MLR of one of the tolerant mice. These experiments show that chimeric thymus transplant-bearing mice are specifically nonresponsive in MLR against the alloantigens exposed by the allogeneic BMD cells in the chimeric thymus graft.

Discussion

The major finding of the present study is that when a chimeric thymus transplant can grow out in TBI-treated mice that are syngeneic to the thymus epithelium and that are reconstituted with T-depleted syngeneic bone marrow, this results in specific tolerance towards the MHC antigens expressed by the allogeneic cells present within the chimeric thymus grafts.

The chimeric thymus contains two populations of allogeneic cells: the bone marrow-derived (BMD) macrophages/dendritic cells and thymocytes. It is very unlikely that the thymocytes are responsible for the induction of tolerance, as it is known that after thymus transplantation thymocytes totally disappear from the thymus within 2 weeks [11]. Another argument that the thymocytes do not confer immunological tolerance for class II MHC antigens as detected in the MLR reaction is that we found that in immunoperoxidase experiments BMD-type class II MHC antigen expression was only expressed by medullary BMD cells and not by the thymocytes. Other investigators [11, 22] have already shown that thymocytes do not express MHC class II antigens by themselves but rather seem to pick these up from the allogeneic thymic epithelial cells. Indeed, small intracellular vesicles, which are Ia-positive, can be seen in the lymphocyte cytoplasm adjacent to the thymic epithelial cell processes. These small vesicles are occasionally continuous with the lymphocyte surface. Immunoelectromicroscopic examination of thymic lymphocyte suspensions reveals that about 35% of the thymic lymphocytes can be stained with anti-Ia antibodies [5]. Flow cytometry analysis of thymocytes also indicates that a significant population of thymic lymphocytes expresses small amounts of Ia antigens [6]. Thymocytes most likely adsorb Ia antigens synthesized by the nonlymphoid cells in the thymus. Indeed, when these Ia antigens are removed enzymatically, they are not re-expressed [23].

As tolerance against class II C_3H MHC antigens was demonstrated in our BALB/c mice and class II C_3H MHC antigens were only expressed by BMD cells in the chimeric thymuses, it is thus very likely that tolerance is imparted by the BMD cells. The possibility that chimeric thymus transplantation led to tolerance through the induction of microchimerism was ruled out as in none of the thymus-bearing animals could chimerism be demonstrated with a very sensitive flow cytometry method [6] able to detect levels of chimerism of less than 0.5%.

Recently, we investigated three mice 6 months after chimeric thymus transplantation (data not shown). At that time, all BMD cells of C_3H origin were replaced by BALB/c BMD cells, and no MLR nonresponsiveness against C_3H antigens could be demonstrated anymore. This once again suggests that it is the C_3H BMD cells that impart tolerance. Our in vivo results are in agreement with the findings of Matzinger and Guerder [19]. By coculturing fetal thymuses and allogeneic dendritic cells, these investigators found a specific disappearance of the capacity of the T cells maturing in these thymuses to mount cytotoxic responses against targets expressing the same MHC antigen as the allogeneic dendritic cells.

This does not mean that the thymic epithelium is not involved at all. On the contrary, there are several recent experiments suggesting that the epithelium may play a role as well [4, 14, 18]. As it has been shown that thymic epithelium can bind and present various antigens to lymphocytes [14], and as it has also been shown that MHC antigens can be picked up and presented by allogeneic

cells [20], one could imagine that in our model BMD cells secrete allo-MHC antigens that subsequently are picked up by the thymic epithelial cells. It can however, still be stated that the presence of BMD cells in the thymus can result in immunological tolerance.

Our experiments are not only of theoretical importance but also have important practical implications, mainly for clinical bone marrow transplantation. They mean that after allogeneic bone marrow transplantation, the induction of mutual tolerance between donor and recipient may depend upon the presence of BMD cells of both donor and recipient origin in the thymus. The recent finding of excellent results after mixed syngeneic allogeneic bone marrow transplantation [7], which probably leads to thymus chimerism, may be partially explained by such a mechanism. Moreover, it may also be of interest to investigate whether after solid organ transplantation the migration of hematopoeitic cells from the graft to the thymus during the 1st days after transplantation (when immunosuppression is maximal) may be responsible for the induction or maintenance of transplantation tolerance.

Acknowledgements. We thank Mr. O. Rutgeerts, Mr. C. Segers, and Ms. R. Van Laer for their valuable technical assistance and Ms. D. Brabants for her preparation of the manuscript. This work has been sponsored by the National Fund for Scientific Research of Belgium.

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