Pathogenesis of Autoimmunity After Xenogeneic Thymus Transplantation

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Pathogenesis of Autoimmunity After Xenogeneic Thymus Transplantation

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Thymus transplantation is a promising strategy to induce xenotolerance, but may also induce an autoimmune syndrome (AIS). The pathogenesis of this AIS was explored using nude rats as recipients. Thymus grafts consisted of fetal hamster thymic tissue with or without mixing with fetal rat tissue such as thymus, thyroid, salivary gland, and heart. All hamster thymus recipients died of AIS within 2–3 mo. In most recipients of xenothymus mixed with rat tissues such as thymus, thyroid, and salivary gland, but not heart, AIS was prevented, indicating an insufficient presence of rat epithelial cell Ags within the xenothymus. AIS could be transferred to control nude rats by whole splenocytes or by splenocyte subpopulations such as CD3+, CD3−, and B lymphocytes, but not by non-T, non-B cells from AIS animals. This transfer could be suppressed by cotransferring either CD4+ or CD8+ lymphocytes from euthymic rats, but not by splenocytes from recipients of syngeneic or xenogeneic thymus mixed with rat tissue, indicating a defective generation of regulatory lymphocytes. As for CD4+ regulatory cells this defect was probably qualitative, because the percentages of CD4+CD25+ or CD4+CD45RClow populations were normal after xenothymus transplantation. As for the CD8+ regulatory cells, the defect was quantitative, as CD8+ cell levels always remained low. The latter was related to the nonvascularized nature of thymus grafts. In conclusion, AIS after xenothymus transplantation in nude mice is due to a combination of insufficient intrathymic presence of host-type epithelial cell Ags and a defective generation of regulatory T lymphocytes. The Journal of Immunology, 2003, 170: 5936–5946.

The shortage of organs for transplantation has stimulated the interest in xenotransplantation in recent years. Rejection of xenografts remains a formidable obstacle to be overcome and is the major hurdle to its clinical application. Xenoreactive immunity is very vigorous; hence, a profound form of immunosuppression and/or immunomanipulation will be needed to prevent rejection of xenografts. The first form of xenorejection, hyperacute rejection, occurs upon reperfusion of xenografts by the recipient blood because of binding of natural xenoantibodies to endothelial cells of the graft and activation of the complement system (1, 2). This can be overcome by interfering with the complement system, e.g., by using genetically modified donors (3). When hyperacute rejection is averted, acute vascular rejection (AVR)3 and T cell-mediated cellular rejection are the next major hurdles to the clinical application of xenotransplantation. It is generally believed that to safely and continuously prevent the latter types of rejection, the induction of xenotolerance will be needed. Mixed bone marrow chimerism and xenogeneic thymus transplantation have been explored as means of inducing xenotolerance (4, 5).

We have previously shown that T cell-independent B and NK cell xenotransplantation tolerance could be successfully induced in the hamster-to-nude rat heart transplantation model by using an immunosuppressive regimen (6) including donor xenoantigen infusion (day −14), temporary NK cell depletion (day −14), and 4-wk (days −14 to +14) treatment with a B lymphocyte immunosuppressant (lefunomide) to inhibit xenoantibody production. In the same model we recently achieved T cell xenotolerance using xenothymus transplantation (7). We showed that fetal hamster thymus grafts could survive permanently in T-deficient nude rats, leading to the generation of host-type T lymphocytes that did not reject concomitantly transplanted vascularized hamster heart grafts. However, in the latter model all the xenothymus recipients developed a lethal multiorgan autoimmune syndrome (AIS), characterized by the occurrence of wasting, thyroiditis, sialoladenitis, gastritis, lacrimal gland infiltration, and the production of autoantibodies (7). The present study aims at exploring the pathogenesis of AIS in this model in more detail. More specifically it is investigated whether AIS is due to the insufficient presence of rat epithelial Ags in the xenothymus and/or to a defect in the generation of regulatory cells.

Materials and Methods

Animals

Six- to 8-wk-old inbred congenital athymic PVGnu/nu male nude rats, purchased form Harlan CPB (Zeist, The Netherlands) and bred in a pathogen-free unit were used as recipients of thymus grafts or transfer experiments. Twelve- to 14-day-old fetal outbred Golden Syrian hamsters (AU/H6 Han Rj) were used as thymus donors. Seventeen- to 19-day-old fetal inbred PVG rats were used as donors of rat tissues, such as thymus, thyroid, salivary gland, and heart. Pregnant hamsters and rats received 9.5 Gy of total body irradiation 1 day before donation to prevent transfer of hamster lymphocytes to nude rat recipients that may provoke graft-vs-host disease (GVHD). Inbred euthymic adult (6- to 7-wk-old) PVG rats were also used as donors of vascularized thymus or thymic tissue, and inbred nude or euthymic PVG rats were used as donors of bone marrow cells.
Surgical procedures

Thymus, mixed thymus, and mixed hamster thymus/rat tissue transplantation. For syngeneic or xenogeneic fetal thymus transplantation, two lobes of a fetal PVG or of a fetal hamster thymus were grafted under the left kidney capsule in nude rat recipients via a subcostal incision. In case of the mixed fetal hamster thymus/rat tissue transplantation, rat tissue graft volume was comparable to the volume of fetal hamster thymus. These grafts were prepared by cutting the donor tissues into small pieces and mixing them extensively before transplantation under the left kidney capsule. All surgical procedures were performed under sterile conditions.

In the case of a syngeneic adult thymic tissue transplantation, a whole adult PVG thymus was cut into \( \frac{1}{2} \times 1 \times 1 \text{-mm pieces} \) and implanted under the left kidney capsule of nude rats.

The survival of the thymus grafts of the thymus grafts was examined by surgical inspection at 1, 2, or 3 mo after transplantation. For this purpose, recipients were anesthetized, a subcostal incision was made, and the presence of the graft was inspected under the kidney capsule and at the end of the experiments was confirmed histologically by removing the kidney bearing the graft.

Syngeneic vascularized thymus transplantation. Syngeneic vascularized thymus transplantation was performed using a composite heart/thymus graft as described previously (8). Adult (5- to 6-wk-old) PVG rats were used as donors. The donor procedure was as follows. Animals were anesthetized and heparinized, and grafts were perfused with chilled saline through the inferior vena cava after the main pulmonary artery was cut near its bifurcations. Both internal mammary arteries and veins and both carotid and subclavian arteries and veins were ligated near the chest wall so that both sides of the thymic arteries and veins were preserved. Then, the inferior vena cava, both sides of the pulmonary vessels, and the left aygys vein were ligated and cut. The trachea and esophagus were cut just below the thyroid cartilage. By pulling the cut end of the trachea, the graft was dissected from the thoracic spine. Finally, the thoracic aorta was divided so as to provide an optimal anastomotic site. Then the composite heart/thymus grafts were prepared in the recipient operation. The thoracic aorta and main pulmonary artery of the composite graft were anastomosed end-to-side to the recipient’s common carotid artery and the external jugular vein, respectively, using standard microsurgical suture techniques. Graft survival was monitored by observing heart beating.

Exclusion of the presence of hamster leukocytes in nude rats after transplantation of irradiated fetal hamster thymus

As no PE- or FITC-conjugated anti-hamster lymphocyte mAb was available for flow cytometry, indirect immunofluorescence methodology was used. Mouse anti-hamster IgM was raised in our laboratory in C57BL/6 nude mice rejecting hamster heart grafts and was used as the Ab1. FITC-conjugated goat anti-mouse IgM (STAR86, Serotec, Oxford, U.K.) was used as the Ab2. To eliminate the Ab2 cross-reaction with rat and hamster lymphocytes, Ab2 was consecutively incubated with nude rat and hamster splenocytes for 0.5 h each. This procedure was proved to eliminate cross-reactivity with rat and hamster tissues. Heparinized whole blood (200 \( \mu \text{l} \)) taken from xenotransplant recipients or from control naive nude rats or naive hamsters was depleted of RBC by a 0.83% NH\(_4\)Cl lysing solution and subsequently incubated with 5 \( \mu \text{L} \) of Ab1 for 30 min at 4°C. Samples were washed twice by PBS and then stained by FITC-conjugated goat anti-mouse IgM (Ab2; diluted 1/50) for another 30 min at 4°C. After two more washes, the final sample was adjusted to a volume of 1.0 ml and analyzed by a FACSort (BD Biosciences, Mountain View, CA). Ten thousand events were counted. Results were expressed as the mean channel fluorescence.

To determine the sensitivity of this assay, the mean fluorescence of samples from xenotransplant recipients was compared with that of positive and negative controls as well as with that of samples prepared by naive hamster samples that were diluted by naive nude rat samples in ratios of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, and 1/1024.

Monitoring of AIS

Thymus or mixed thymus transplant recipients were monitored for the occurrence of typical clinical signs of AIS, such as wasting, body weight loss, diarrhea as well as the occurrence of the sicca syndrome) or diarrhea as previously described (7). These symptoms usually occurred ~2 mo after xenotransplantation. The autoimmune nature of the syndrome was confirmed by the finding of anti-DNA autoantibodies and organ-specific autoantibodies (such as anti-thyroid, anti-salivary gland, and anti-stomach) and was confirmed by histology showing mononuclear cell infiltration and/or tissue architecture destruction in the thyroid, salivary glands, stomach, and lacrimal glands of sick animals.

Bone marrow transplantation

Nude euthymic PVG rat bone marrow cells were transferred to lethally irradiated (9.5 Gy) PVG rats. For bone marrow isolation (9), donor rats were sacrificed, and the femurs and tibiae were dissected free of surrounding tissue and collected in ice-cold RPMI 1640 medium. After cutting off one end of each bone, bone marrow cells were flushed out with ice-cold RPMI 1640, then passed through a nylon filter, washed, resuspended, and counted. The viability of the cells was always >98% as confirmed by trypan blue staining. Next, 40 \( \times 10^7 \) bone marrow cells were suspended in 1.5–2 ml of RPMI medium and injected via the penile vein to recipients that were lethally irradiated 24 h previously.

Monoclonal Abs and flow cytometric analysis

The following anti-rat mAbs were used for phenotyping: FITC-conjugated mouse anti-rat CD4 (OX-35), CD8b (341), and CD45RBC (MAC35F1); and PE-conjugated mouse anti-rat CD4 (G4.18), CD25 (IL-2R, α-chain, OX-39), and CD4 (OX35). All Abs were purchased from Serotec (Kidlington, U.K.).

Heparinized whole blood was taken by heart puncture, depleted of RBC by a 0.83% NH\(_4\)Cl lysing solution, and double-stained for 30 min at 4°C with the following mAb to identity T cell subsets: 1) PE-conjugated anti-CD3 mAb together with FITC-conjugated anti-CD4 mAb or anti-CD8 mAb to identify CD3\(^+\) CD4\(^+\) or CD3\(^+\) CD8\(^+\) T cells, respectively; and 2) FITC-conjugated anti-CD4 together with PE conjugated anti-CD25 and FITC-conjugated anti-CD45RBC together with PE-conjugated anti-CD4 were used to identify CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD45RBC\(^+\) subsets of CD4\(^+\) T cells. Samples were washed twice, fixed by 1% paraformaldehyde, and analyzed by FACSort (BD Biosciences).

Cell purification

For cell population isolation, several magnetic beads, such as rat Pan T Cell, rat CD8a, and rat CD45RA (for rat B cells), were purchased from Miltenyi Biotec (Munich Gladbach, Germany).

CD3\(^+\) and CD3\(^+\) splenocytes were separated and purified by immuno-magnetic depletion as previously described (10). In brief, a single-cell suspension of splenocytes was prepared by mincing the spleen and passing it through a 70-μm pore size filter. RBC and dead cells were removed by density gradient centrifugation using Ficoll-Paque and washing the cells twice. Next, 20 μL of MACS Pan T cell microbeads were added per 10^7 total cells, mixed, and incubated for 30 min on ice. Subsequently, the cell suspension was passed over a column and washed by adding 10–20 times the labeling volume of buffer. Efficient cells were CD3\(^+\) cells; bound cells were CD3\(^+\) T cells. The latter were collected by removing the column from the magnet and eluting it with medium. The purity of CD3\(^+\) cells was usually >95% as confirmed by staining with PE-conjugated anti-CD3 mAb and analyzing by FACS. After positively selecting CD3\(^+\) cells, B cells from AIS animals were obtained from CD3\(^+\) splenocytes using CD45RA magnetic beads. The remaining cells were used as the non-B, non-T cell population.

CD4\(^+\) and CD4\(^+\) lymphocytes were also purified by magnetic beads as previously described (11). Briefly, CD3\(^+\) splenocytes obtained from normal euthymic adult PVG rats as described above were incubated for 30 min on ice with anti-CD8a magnetic beads. The cell suspension was then passed over a column and washed. The magnetically labeled CD8a\(^+\) cells were retained in the column while the unlabeled CD8a\(^+\) (CD3\(^+\) CD4\(^+\)) cells passed through. After removal of the column from the magnetic field, the magnetically retained CD8a\(^+\) cells were eluted with medium. The purity of the CD8a\(^+\) and CD4\(^+\) T cells was usually >90% as determined by flow cytometry.

Cotransfer experiments

To determine which subpopulation of cells was able to transfer the AIS, the following splenic subpopulations from nude rats with AIS were transferred to control nude rats: whole splenocytes (10^8), CD3\(^+\) splenocytes (2.5–3×10^7), CD4\(^+\) splenocytes (6–8×10^7), B lymphocytes (3–4×10^7), or non T-non B cells (3–4×10^7).

To determine whether transfer of the AIS could be suppressed by splenocyte subpopulations from control euthymic rats or from syngeneic or mixed thymus grafted nude rats without AIS, the following populations were cotransferred: whole splenocytes (10^8), CD4\(^+\) lymphocytes (2.5–5×10^7), or CD8\(^+\) lymphocytes (2.5–5×10^7) from euthymic control rats; whole splenocytes (10^8) from nude rats receiving syngeneic rat thymus grafts; and whole splenocytes (10^8) from nude rats receiving mixed hamster thymus/rat thyroid grafts.

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ELISA analysis of anti-DNA autoantibodies

Anti-DNA Abs were measured in the serum by ELISA as described previously (7). Briefly, microplates were coated overnight at 4°C with aliquots of 100 μl of DNA from calf thymus (Sigma-Aldrich, St. Louis, MO) diluted at 10 μg/ml and then washed with 0.005 Tween 20 in PBS (pH 9.6). After incubation with 3% BSA for 1 h at 37°C to block nonspecific binding, the wells were washed again. Aliquots of 100 μl of different dilutions (from 1/200 to 1/6400) of serum were then incubated for 1 h at 37°C. Next, the plates were washed, and alkaline phosphatase-conjugated rabbit anti-rat IgG or anti-rat IgG (Zymed, San Francisco, CA; diluted 1/1000) was added for 1 h at 37°C. The plates were washed, substrate solution (p-nitrophenyl phosphate; Sigma-Aldrich) was added, and absorbance (OD) at 405 nm was measured with an automated spectrophotometer (Bio-Tek, Winooske, VT). Results were expressed as the relative titer of OD, which was calculated as follows: relative titer = (OD of thymus-grafted rat sera − OD of negative control)/OD of naive rat sera − OD of negative control.

Histology and immunohistochemistry

Kidneys bearing thymus grafts, stomachs, salivary glands, thyroids, lacrimal glands, eyes, testes, lungs, livers, intestine, pancreas, kidneys, and skin were harvested from sacrificed experimental animals and fixed in 6% neutral formalin. Samples were embedded in paraffin, sectioned (5 μm), and stained with H&E for routine pathology.

For detecting organ-specific autoantibodies, cryostat section (5 μm) of thyroid, salivary gland, stomach, lacrimal gland, liver, testis, and pancreas of normal PVG rats were mounted on 3-aminopropyltriethoxysilane (Sigma-Aldrich)-coated slides and dried overnight at room temperature. The sections were fixed in methanol at 56°C for 3 min and sequentially incubated with positive serum from xenothymus recipients with AIS and with control nude rat serum used as a negative control at room temperature for 30 min. Different optimal dilutions (for thyroid, salivary gland, pancreas, and stomach, 1/20; for lacrimal gland, liver, and testis, 1/10) were used. Thereafter, FITC-conjugated rabbit anti-rat Ig (1/100 diluted; DAKO, Glostrup Denmark) was added. After incubation at room temperature for 30 min in dark conditions, 87% of glycerol was applied, and the sections were visualized using a Leica fluorescence microscope system.

Statistical analysis

Data analysis was performed using Student’s two-sample test. Differences with p < 0.05 were considered significant.

Results

Survival of various types of thymus grafts

Nude rat recipients received fetal thymus grafts from syngeneic and/or xenogeneic (hamster) donors as well as fetal hamster thymus tissue mixed with syngeneic fetal rat tissue such as thymus, thyroid, salivary gland, or heart. The mixed hamster thymus/rat tissue grafts were used to look at the influence on the development of autoimmunity (see below) of the presence in the xenothymus grafts of rat cells (epithelial cells), providing autoantigens potentially involved in the AIS.

In accordance with our previous findings (7), syngeneic thymus grafts (n = 5), xenothymus grafts (n = 12), as well as mixed rat/hamster thymus grafts (n = 8) could survive for >3 mo without any treatment. The same was true for the recipients of mixed hamster thymus/rat tissues (thyroid, n = 7; salivary gland, n = 5; heart, n = 5) grafts. The survival of these grafts was assessed surgically (see Materials and Methods) and was confirmed by histology at the end of the experiments.

T lymphocyte generation after thymus transplantation

T lymphocyte generation could be seen in the recipients of all types of thymus grafts (Fig. 1). In syngeneic fetal thymus graft recipients both CD4+CD3+ lymphocytes (Fig. 1a) and CD8+CD3+ lymphocytes (Fig. 1b) recovered better and faster than those in xenothymus or mixed rat/hamster thymus or mixed hamster thymus/rat tissue recipients. This may be because the fetal rat thymus volume is larger than that of fetal hamster thymus and may also be related to manipulation in the case of mixed thymus grafts. For the sake of clarity, on Fig. 1 only mixed hamster thymus/rat thyroid is shown as an example for mixed hamster thymus/rat tissue groups; the other subgroups (salivary gland, heart) were similar at all time points.

As found by Iwasaki et al. (12) after rat-to-mouse xenothymus transplantation and also by Zhao et al. (13) in thymectomized C57BL/10 mice receiving fetal pig thygus and liver tissue, the generation of CD8+ cells was proportionally always less good than that of CD4+ cells and always very low compared with that of euthymic control rats (Fig. 1b). As a result, the ratio of CD4:CD8 was 10:1 to 8:1 in all groups, including the fetal syngeneic thymus transplantation group, whereas in the euthymic PNG rats this ratio is between 5:1 and 4:1. The differences in CD4+ cells, CD8+ cells, or CD4:CD8 ratios among all xenothymus (with or without rat tissue) transplantation groups were not significant at any time point (p > 0.05).

Also, the CD4+ subsets that have previously been shown to have regulatory functions in autoimmunity, i.e., CD4+CD25+ (14, 15) or CD4+CD45RC− (16, 17), were determined in all experimental groups. At all time points, the percentages of these subpopulations were similar in all groups, independent of whether they would develop autoimmunity (the recipients of the xenothymus) or not (the recipients of the syngeneic or of the mixed thymus...
At 3 mo after thymus transplantation, the percentages of these two subpopulations approached those found in euthymic control (Fig. 2). As an example, Fig. 3 shows FACS profiles for these CD4\(^+\)/CD25\(^+\) subpopulations in euthymic controls or 3 mo after thymus grafting in recipients of xenogeneic thymus (developing AIS) or of mixed hamster thymus/rat thyroid (developing no AIS).

**Absence of hamster T cells that could mediate a xenogeneic GVHD-like syndrome in xenothymus recipients**

As GVHD, especially chronic GVHD can provoke multiorgan lesions resembling some seen in the AIS that will be described below, it was essential to prove that no hamster T cells were generated in the xenothymus recipients. Although this was very unlikely, as the thymus graft donors were always lethally (9.5 Gy) irradiated before donation, the presence of irradiation resistant hamster leukocyte precursors and their subsequent appearance in the periphery of nude rat recipients had to be excluded.

**Occurrence of autoimmunity after thymus transplantation (Table I)**

The occurrence of an AIS was diagnosed by the appearance of clinical symptoms such as body weight loss and the appearance of a hunched back and wasting and was confirmed by the histological finding of mononuclear cell infiltration and/or tissue destruction in the stomach, salivary glands, thyroid, and/or lacrimal glands as well as by the production of anti-DNA autoantibodies and rat splenocytes; see Materials and Methods). As expected, the mean channel fluorescence (MCF) in control nude rats is low (Fig. 4, panel 1; MCF = 13.0) and high in control hamster (Fig. 4, panel 2; MCF = 80.2). Fig. 4, panel 3, shows that the fluorescence (MCF = 13.4) in a nude rat receiving a xenogeneic thymus graft 3 mo previously and developing AIS is not essentially different from that in a control nude rat, excluding the presence of hamster leukocytes in this animal. Panels 4–6 show that this test was very sensitive, as up to a rat/hamster ratio of 1024:1 (panel 6) hamster cells could still be clearly demonstrated (MCF = 20.3), indicating a sensitivity for detecting chimerism of at least 0.1%. Hence, hamster chimerism leading to GVHD seems very unlikely as an explanation for the AIS seen in xenothymus-grafted nude rats.

**FIGURE 2.** Percentage of CD4\(^+\)/CD25\(^+\) (a) and CD4\(^+\)/CD45RC\(^{low}\) (b; regulatory) cell populations after syngeneic thymus transplantation (syn TTx), xenogeneic thymus transplantation (xeno TTx), mixed hamster thymus/rat thymus transplantation (mix T/TTx), and mixed hamster thymus/rat thyroid transplantation (mix T/Trd TTx) at 3 mo after transplantation compared with control euthymic rats. The last group is given as representative of the recipients of mixed hamster thymus/rat tissue such as thyroid, salivary gland, and heart, as all these types of mixed thymus grafts had the same capacity to generate these populations.

**FIGURE 3.** FACS profiles of CD4\(^+\)/CD25\(^+\) and CD4\(^+\)/CD45RC\(^{low}\) (regulatory) cells in euthymic control rats (left panels) or in nude rat recipients 3 mo after transplantation of xenogeneic thymus grafts (middle panels) or of mixed hamster thymus/rat thyroid grafts (right panels).
and organ-specific autoantibodies. After syngeneic thymus transplantation (Table I, group A) none of the animals developed AIS. In contrast, all xenogenic thymus-grafted rats (group B) developed symptoms of AIS characterized by tissue destruction and lymphocyte infiltration of the thyroid (see Fig. 5c), salivary gland (see Fig. 5e), stomach (see Fig. 5g), or lacrimal gland (see Fig. 5i). Organ-specific autoantibodies could be detected by immunostaining using serum taken from xenothymus recipients with AIS to stain control rat tissues (see Materials and Methods). These autoantibodies reacted against AIS target organs such as thyroid, salivary gland, stomach, and lacrimal gland (Fig. 6) and did not react with non-AIS target organs such as liver, pancreas, and heart (data not shown). Every sick animal presented at least one of the four histological symptoms, and several even two, three, or four. All sick animals showed significantly increased titers of anti-DNA autoantibodies and serious wasting syndrome. When a mixed hamster/rat thymus graft, a mixed hamster thymus/rat thyroid graft, or a mixed hamster thymus/rat salivary gland graft was transplanted, AIS was prevented in the majority of the animals (respectively, groups C, D, and E, Table I). This absence of AIS was confirmed by histology; as an example, Fig. 5, d, f, h, and j, shows the absence of histological signs of AIS in, respectively, the thyroid, salivary gland, stomach, and lacrimal gland of a rat receiving a mixed hamster thymus/rat thyroid graft (see Fig. 5b). In contrast, when rat tissue was taken from an organ that was not affected by the AIS, e.g., the heart, mixing this tissue with the hamster thymus was not able to prevent the AIS (Table I, group F).

Together these results indicate that whereas autoimmunity is seen in xenothymus-grafted nude rat recipients, it can be prevented by mixing xenothymus tissue with syngeneic thymus tissue or thyroid tissue or salivary gland tissue, but autoimmunity cannot be prevented by mixing the xenothymus with syngeneic heart tissue.

### Table I. Autoimmunity after thymus transplantation

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<th>Thymus Grains</th>
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<th>Clinical Symptoms</th>
<th>Anti-DNA IgG</th>
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<td>5</td>
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<tr>
<td>F. Mixed rat heart and hamster thymus</td>
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*a* Weight loss, diarrhea, hunched back, and wasting occurring within 2–3 mo after thymus transplantation.

*b* Anti-DNA titers were determined by ELISA at the time of appearance of clinical symptoms or at 3 mo after thymus transplantation in the case of the absence of clinical symptoms and was scored positive when significantly increased compared with healthy control euthymic or nude PVG rats.

Presence of mononuclear cell infiltration or tissue destruction in stomach, thyroid, salivary, or lacrimal glands (see Fig. 5) at the time of autopsy (performed in severely ill animals or at the end of the experiment 3–4 mo after transplantation). In group B six animals had autoimmune lesions in one organ examined, four animals in two organs, and two animals in three organs. In group C the only animal having autoimmune lesions in the thyroid was the same animal showing minor clinical and serological autoimmune.

In group D one animal had autoimmune lesions in stomach and one in the salivary glands; both animals had positive clinical and serological autoimmune. In group E one of five animals showed a clinical autoimmune syndrome with lesions in stomach and thyroid, and in group F all animals developed AIS, with one animal showing autoimmune lesions in one organ, two animals in two organs, and two animals in three organs.
Generation of regulatory T cells in nude rats after various types of thymus transplantation

As it is well known that immune regulatory T cells play a pivotal role in the pathogenesis of autoimmunity (16–20), cotransfer experiments were performed to investigate whether regulatory T cells were involved in the pathogenesis of the AIS (Table III). When splenocytes from normal, euthymic PVG rats were cotransferred together with splenocytes from xenothymus recipients with AIS, the AIS did not occur (group A). In contrast, cotransfer of splenocytes from nude rats that received syngeneic rat thymus (group B) or mixed hamster thymus/rat thyroid graft (group C) 3 mo previously were not able to suppress the AIS despite the fact
that these animals themselves did not develop AIS. These data indicate that all thymus-grafted nude rats, regardless of whether they developed AIS, lacked regulatory cells able to suppress autoimmune responses that are normally found in euthyemic control rats. As for the phenotypes of the control splenocytes able to suppress the transfer of AIS, both CD4 and CD8 lymphocytes had the capacity to suppress the transfer by either CD3⁺ or B lymphocytes taken from animals with AIS (groups F–I, Table III).

Pathogenesis of insufficient generation of CD8⁺ lymphocytes after fetal thymus transplantation

As CD8⁺ lymphocytes from euthyemic control rats could suppress the occurrence of AIS (Table III), and as this subpopulation is poorly generated after thymus transplantation, the pathogenesis of the defect of CD8⁺ lymphocytes after thymus transplantation was further explored. First it was examined whether it could be due to a defect in nude rat bone marrow precursors to generate CD8⁺ lymphocytes. To explore this possibility, both nude rat and euthyemic rat bone marrow cells were transferred to lethally irradiated (9.5 Gy) euthyemic rats. It was shown that nude rat bone marrow cells had the same capacity as euthyemic bone marrow cells to generate either CD3⁺CD4⁺ (Fig. 7a) or CD3⁺CD8⁺ (Fig. 7b) lymphocytes such that at ~3–4 mo after transplantation they both reached the level of control euthyemic rats (respectively, ~40 and 10%). Next, it was examined whether the impaired generation of CD8⁺ cells after thymus transplantation may be due to the nonvascularized nature of the thymus grafts. Therefore, syngeneic vascularized (see Materials and Methods) or nonvascularized adult thymus grafts were transplanted to nude rats. Adult vascularized thymus grafts were able to very rapidly generate CD3⁺CD4⁺ (Fig. 8a) and CD3⁺CD8⁺ (Fig. 8b) lymphocytes that they almost reached control levels after 1 mo. In contrast, nonvascularized adult thymus tissue grafts only sufficiently supported the generation of CD3⁺CD4⁺ cells, whereas only very low percentages of CD3⁺CD8⁺ cells were generated even after 3 mo. The fact that for technical reasons a syngeneic heart graft was transplanted at the same time of the thymus did not influence the generation of T cells, as a concomitant syngeneic heart transplantation did not improve lymphocyte generation in the recipients of the nonvascularized thymus grafts (n = 6 data not shown).

Discussion

The present study was undertaken to further explore the pathogenesis of a multorgan AIS that we previously described to occur in nude rats after transplantation of a hamster thymus (7). The occurrence of a serious AIS has previously been reported by other investigators as well in nude mice after transplantation of thymus grafts from various donors, including rat, rabbit, hamster, guinea pig, swine, and cow (21, 22), as well as in a minority of T/NK-depleted, thymectomized mice after transplantation of fetal pig thymus and liver tissue (13). However, in other experimental situations, e.g., after transplantation of xenogeneic fetal thymus and

Table II. Transfer of the AIS to control nude rats by splenocytes or by splenocyte subpopulations from nude rats receiving hamster thymus 2–3 mo previously and developing an AIS or from control euthyemic 
PVG rats

<table>
<thead>
<tr>
<th>Cells Transferred</th>
<th>n</th>
<th>Occurrence of Autoimmunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Splenocytes from control euthyemic PVG rats</td>
<td>3</td>
<td>0/3</td>
</tr>
<tr>
<td>B. Splenocytes from nude rat recipients of PVG thymus grafts</td>
<td>3</td>
<td>0/3</td>
</tr>
<tr>
<td>C. Splenocytes from nude rat recipients of hamster thymus</td>
<td>5</td>
<td>5/5</td>
</tr>
<tr>
<td>D. Purified CD1⁺ splenocytes from nude rat recipients of hamster thymus grafts</td>
<td>8</td>
<td>7/8</td>
</tr>
<tr>
<td>E. CD3⁺ splenocytes from nude rat recipients of hamster thymus grafts</td>
<td>5</td>
<td>5/5</td>
</tr>
<tr>
<td>F. Purified B cells from nude rat recipients of hamster thymus grafts</td>
<td>6</td>
<td>6/6</td>
</tr>
<tr>
<td>G. Non-T, non-B splenocytes from nude rat recipients of hamster grafts</td>
<td>6</td>
<td>1/6</td>
</tr>
</tbody>
</table>

*Number of cells used for adoptive transfer experiments: whole splenocytes, 10 × 10⁷; CD3⁺ cells, 2–3 × 10⁵; CD1⁺ cells, 6–8 × 10⁷; purified B cells, 3–4 × 10⁷; non-B non-T cells, 3–4 × 10⁷.

*Clinical, serological, and histological symptoms (see Materials and Methods) occurring within 4 mo after transplantation.
fetal liver tissue in SCID mice, this syndrome has not been reported (23). Hence, the pathogenesis of this AIS remains unclear and needs to be explored, especially when thymus transplantation would be used as a mean to induce xenotolerance in clinic organ transplantation.

The AIS described in the present study is unlikely to be due to a GVHD-like syndrome caused by xenogeneic chimerism. Firstly, thymus donors were always lethally irradiated before transplantation. This procedure can be expected to eliminate the majority of lymphocytes or hemopoietic cells in the thymus grafts. Second, to formally exclude the possibility that a few donor lymphocytes may survive the irradiation and subsequently expand in the recipients, a sensitive flow cytometry method was developed, which could exclude the presence of a significant level of donor chimerism. Hence, GVHD seems to be a very unlikely explanation for the syndrome seen after xenothymus transplantation. Finally, typical symptoms of GVHD, such as occurring in liver, skin, tongue, lymphoid organs, or gut, were not found (data not shown).

As the AIS affected the thyroid, salivary gland, stomach, and lacrimal gland, but not other organs such as heart, liver, pancreas, kidney, small bowel, and skin, a possible explanation is that the presentation of some self-peptides is deficient in a xenogeneic thymus environment. As bone marrow-derived APCs were shown to be very important for the elimination of autoreactive clones in the thymus (24, 25), a possibility was that host-type APC were absent in the xenogeneic thymus. However, we previously showed that host-type APC could be clearly found in the xenothymus early after transplantation (7). Hence, this hypothesis could be excluded. As transplantation of a hamster thymus with rat tissues from organs that are affected by this AIS, such as thyroid and salivary gland, was able to prevent AIS, but not from an organ unaffected by this AIS, such as heart tissue, this suggested an insufficient presence of some rat epithelial Ags that normally are needed to tolerate rat T cells for some organ-specific Ags. The reason of mixed hamster thymus/rat thymus preventing the AIS is compatible with the recent findings that thymic medullary epithelium can express >30 tissue- or organ-specific genes (26–29). Analyzing the expression pattern of these genes showed a strong expression by a few cells rather than a widespread low level expression by many cells (28, 29). Although the correlation between the expression of these genes and clonal deletion remains enigmatic, highly efficient mechanisms, i.e., protein expression by very few thymic epithelial cells, may mediate complete tolerization of a broad Ag-specific T cell repertoire may. As in our experiments, the transplantation of fetal rat thyroid or salivary gland tissue together with hamster thymus was able to prevent not only thyroiditis and sialoadenitis, but also gastritis and lacrimal adenitis, this suggests that fetal thyroid or fetal salivary gland express Ags that are shared by the stomach and lacrimal glands. Clearly, these shared autoantigens are not expressed on rat heart tissue, which explains why heart is neither a target for the AIS nor able to prevent it when present in the thymic environment. We very recently found that irradiated fetal rat liver tissue also could prevent the AIS when mixed with hamster thymus (Y. Yan, unpublished observation). At first sight, this may be surprising, as adult rat liver itself was not affected by AIS and hence could be expected to lack the relevant autoantigens. However, a recent study demonstrated that fetal liver tissue consists of cells in epithelial-to-mesenchymal transition (30), which express epithelium molecules such as CK-8, CK-18, CK-19, and E-cadherin, also shown to be expressed on the salivary gland, lacrimal gland, thyroid, and stomach (31–34). However, these Ags disappear from the liver at the end of gestation. We are presently investigating whether adult rat liver as opposed to fetal rat liver may be incapable to prevent the AIS. If confirmed, these

FIGURE 7. Generation of CD4+ (a) or CD8+ (b) lymphocytes in lethally irradiated euthymic PVG rats after transplantation of 40 × 107 bone marrow cells from nude rats or from euthymic PVG donors (n = 6 in each group).
FIGURE 8. Generation of CD4⁺ (a) or CD8⁺ (b) lymphocytes in nude rat recipients after transplantation of vascularized (n = 6) or nonvascularized (n = 6) adult PVG thymus grafts.

experiments could further support the hypothesis that the lack of a sufficient presence of some autoantigen that are targets for autoimmune effector cells may result in the AIS after xenothymus transplantation.

Defective clonal deletion of autoreactive cells is not the only factor involved in the occurrence of the AIS after thymus transplantation. Indeed, transfer of the AIS by splenocytes of AIS animals could be prevented by the cotransfer of splenocytes from normal control euthymic rats, but not from mixed thymus graft recipients and not even from syngeneic thymus graft recipients despite the fact that the latter two groups themselves did not develop autoimmunity. This indicated that a defect in regulatory cells was another contributing factor as well. There are many data showing that regulatory T cells, especially CD4⁺ cells, play a pivotal role in preventing AIS in models of xenothymus transplantation (14–17, 35). Also in our experiments cotransfer of control CD4⁺ cells could suppress the transfer of AIS by splenocytes from sick animals. A quantitative defect of CD4⁺CD25⁺ or CD4⁺CD45RClow regulatory cells previously described to be involved in autoimmunity seems unlikely in our experiments, as the percentage of these cells was almost normal after xenothymus transplantation. A qualitative defect in these subpopulations as a consequence of insufficient exposure to autoantigens in the thymus is also unlikely, as cotransfer of splenocytes from recipients of mixed hamster thymus/rat thymus grafts did not prevent the AIS (Table III, group C). Hence, another qualitative defect or a lack of an as yet unidentified other phenotype of regulatory CD4⁺ cells may be involved. We are presently trying to determine this by studying the phenotypes and functions of CD4⁺ lymphocytes from normal compared with thymus-grafted rats in more detail.

Also, a quantitative defect of CD8⁺ lymphocytes may be involved in the pathogenesis of AIS, as all thymus-grafted recipients showed poor generation of CD8⁺ cells and cotransfer experiments demonstrated that CD8⁺ T lymphocytes from control euthymic rats were able to suppress the transfer of AIS by whole splenocytes, CD3⁺ cells, or B cells taken from xenothymus recipients with AIS.

CD8⁺ T cells play a very important role in prevention of autoimmunity in both experimental models (36–38) and clinic diseases (39, 40). More recently, a number of groups demonstrated that the immunoregulatory functions of CD8⁺ T cells can be mediated by direct lysis or apoptosis of specific CD4⁺ T cells, including autoreactive CD4⁺ cells, and/or by secretion of particular cytokines such as TGF-β, IL-10, IL-4, or IFN-γ, which act directly on the target CD4⁺ cells as a suppressive or differentiation factor (41–44). In addition, CD8⁺ regulatory cells can function in an indirect manner, by modifying the behavior of APC, which, in turn, influence the development and/or function of other effector cells (45, 46). In vitro culture experiments (47) showed that CD8⁺ cells can kill autoreactive B cells directly.

As for the pathogenesis of impaired CD8⁺ T cell generation in thymus graft recipients, which has been found by other investigators as well (12, 13), it was not related to the species of the donors of thymus grafts, because CD8⁺ T cell generation was impaired in both xenogeneic and syngeneic thymus transplantation. It was also not due to a defect of lymphocyte precursors in nude rat bone marrow, as the latter had the same capacity as autologous euthymic bone marrow to restore T cell repertoires in lethally irradiated euthymic recipients. Another explanation could be that the extrathymic environment of nude rats was deficient in supporting CD8⁺ cell expansion. Cytokines and some peptides are known to play a critical role in driving homeostatic expansion of lymphocyte subsets. In the presence of IL-2 (48), human umbilical cord lymphocytes expand, especially in the CD8⁺ direction, after in vitro culture. Also IL-7 (49) and IL-12 (50) have well-documented effects on CD8⁺ T cell proliferation and differentiation, whereas TGF-β may dampen CD8⁺ lymphocyte expansion (51). Hence, the balance among various cytokines may determine homeostatic expansion of CD8⁺ cells. The fact that the syngeneic vascularized adult thymus grafts in nude rats were able to restore both CD4⁺ and CD8⁺ T cells to the levels of autologous euthymic rats, whereas this was not the case with nonvascularized adult or fetal thymus grafts excluded a defect of the extrathymic environment in nude rats as a cause of the defect in the generation of CD8⁺ lymphocytes. Rather, the impaired CD8⁺ T cell generation was related to the nonvascularized nature of the thymus grafts. Intrathymic T cell maturation is governed by sequential interactions of thymocytes with different stromal cell types during the migration of T cells from the outer cortex to the central medulla (52, 53). Although it is not exactly clear to what extent these sequential selection processes are dependent on the intrathymic microenvironment, it was shown (54) that T cell maturation from CD4⁺CD8⁻ to CD4⁺CD8⁺ depends on the development and normal organization of thymic medullary epithelium. Most reports (55, 56) indicate that positive selection for CD4⁺ and CD8⁺ lymphocytes is promoted by, respectively, MHC class II and class I expression on thymic epithelial cells, whereas negative selection is largely mediated by
bone marrow-derived dendritic cells. Nevertheless, positive selection by thymic dendritic cells has been described as well. As the level of MHC I protein is 10-fold higher on thymic dendritic cells than on thymic epithelial cells (57), the ratio between thymic epithelial to dendritic cells may determine the MHC class I distribution and hence may play an important role in forming the repertoire of mature MHC I-restricted CD8+ T cells. We are presently exploring in more detail to what extent the thymic architecture and composition are different in vascularized compared with nonvascularized grafts.

In our model autoreactive B cells and autoantibody production played a very important role in the pathogenesis of the AIS, as 1) anti-stomach, anti-thyroid, and anti-salivary gland organ-specific autoantibodies and anti-DNA IgG have been found by immunohistology or ELISA; 2) B lymphocytes from AIS animals were able to transfer the AIS to control nude rats; and 3) splenectomy in AIS animals was shown to be able to attenuate the autoimmune syndrome (data not shown). This Ab production seemed T dependent, as CD3+ lymphocytes taken from animals with AIS can transfer this AIS to control nude rats, and cotransfer CD4+ or CD8+ lymphocytes from control euthymic rats together with either CD3+ lymphocytes or B cells from animals with AIS can prevent the occurrence of AIS in nude rat recipients.

In conclusion, autoimmunity after xenogeneic thymus transplantation in nude rats is due to a combination of an insufficiency of mature MHC I-restricted CD8+ T cells. We are presently exploring in more detail to what extent the thymic architecture and composition are different in vascularized compared with nonvascularized grafts.

References


