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Quantification of Baboon Thymopoiesis in Porcine Thymokidney Xenografts by the Signal-Joining T-Cell Receptor Excision Circle Assay

Aseda Tena, Prashanth Vallabhajosyula, Robert J. Hawley, Adam Griesemer, Kazuhiko Yamada, and David H. Sachs

Transplantation Biology Research Center, Massachusetts General Hospital, Boston, MA

Abstract

Background—Transplantation of vascularized donor thymic tissue along with a kidney transplant has markedly improved graft survival across the discordant pig-to-baboon xenogeneic barrier. To quantify the production of baboon T cells by the porcine thymic tissue, we recently developed an assay to measure the excised DNA products of baboon T-cell receptor (TCR) gene rearrangement (signal-joining TCR excision circles, sjTREC).

Methods—Initial polymerase chain reaction (PCR) analysis documented that TCR δ REC- ψ J α rearrangement occurs in baboons. Primers, specific to baboon sjTREC sequence were designed and used to quantify sjTREC molecules in peripheral blood mononuclear cells and thymic tissue using a quantitative PCR assay.

Results—sjTREC levels were higher in phenotypically naïve (CD3⁺CD45RA⁺⁺) T cells (650 copies/100,000 cells) than in phenotypically memory (CD3⁺CD45RA^{low}) T cells, with sjTREC below the limit of detection (40 copies/100,000 cells). Surgical removal of the native thymus in two baboons led to a significant decrease of sjTREC in peripheral blood (from 1104 and 920 copies to 184 and 190 copies/100,000 cells, respectively), confirming the role of the thymus in maintaining the peripheral T-cell pool. In two thymectomized baboons that received porcine thymokidney xenografts, sjTREC levels remained low in the peripheral blood (<40 copies/100,000 cells), but increased to 52 and 192 copies/100,000 cells in thymic biopsies, implying that baboon thymopoiesis had begun to occur in the porcine thymic xenografts.

Conclusions—Baboon sjTREC can be quantified by quantitative PCR using primers specific to baboon sequence. Initial results suggest that baboon thymopoiesis occurs in vascularized porcine thymus xenografts.

Keywords

Thymokidney; Thymopoiesis; Signal joint TCR excision circles (sjTREC); Xenograft

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Address correspondence to: David H. Sachs, M.D., Transplantation Biology, Research Center, Massachusetts General Hospital – East, Building 149-9019, 13th Street, Boston, MA 02129. david.sachs@tbr.mgh.harvard.edu.

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The use of animal organs could ameliorate the shortage of organs that currently limits the field of human transplantation. For many reasons, the pig is believed by most workers in this field to be the most likely potential source of such xenografts. Physiologically, porcine organs are similar to those of humans, but immunologically, they represent a discordant xenogeneic barrier that undoubtedly could not be overcome with a clinically acceptable amount of immunosuppression. For this reason, our laboratory has been exploring means of inducing tolerance to at least some of the most important xenogeneic antigens.

Transplantation of thymic tissue has been shown to be an effective means for inducing tolerance of porcine skin in rodents (1–3). Subsequent studies attempting to extend this methodology to large animals have shown that prevascularization of the xenogeneic thymic tissue is required for it to survive (4–6). In a pig-to-primate model, porcine composite thymokidney xenografts were shown to enjoy markedly prolonged survival over grafts without thymus (4,7). These grafts also induced donor-specific cellular hyporesponsiveness (7,8), although full tolerance has not yet been demonstrated.

Porcine thymic function in these studies was assessed indirectly, using antibodies specific to baboon CD4⁺ T cells and a CD45RA marker. In addition, persistence of porcine thymic tissue was demonstrated by immunohistochemical staining for cytokeratin-positive thymic epithelial cells (7,9). However, a direct demonstration of baboon thymopoiesis by the porcine thymic tissue was lacking. One reliable measure of such thymopoiesis would be the production of the baboon-specific signal-joining TCR excision circles (sjTREC) in newly generated T cells (10). Such TRECs are stable byproducts of T-cell receptor (TCR) rearrangement of the α locus and are contained episomally in the cell. Because sjTRECs do not replicate during mitosis of the cell, they are diluted by subsequent cell division. sjTREC analysis showing increasing values thus suggest thymic activity.

Assays for TREC have previously been reported for mice (11), human (12), rhesus (13), and sooty mangabeys (13). To date, however, there have not been reports of TREC in baboons. Therefore, we have developed a baboon-specific sjTREC assay as a marker of thymic activity in baboon recipients of porcine thymic grafts. We report here both the development of this assay and its application to the assessment of thymic function in baboon recipients of porcine thymokidney transplants.

RESULTS

Baboon sjTREC Assay

Primers to amplify the baboon signal-joint TREC were designed by using the published sjTREC sequence of humans and of rhesus macaques (12, 13). Primers were selected in the area of highest homology for δ Rec and ψ Ja regions of both rhesus macaque and human. These primers were then used to amplify a putative polymerase chain reaction (PCR) product from the baboon δ Rec/ ψ Ja regions. The expected 1170 base-pair PCR band was then cloned and its sequence analyzed by alignment with the published sequences of human and rhesus macaque TCR A/D locus (Fig. 1A).

While similar recombination processes were seen in all three species, some differences were observed including a two base-pair deletion and some nucleotide mismatches (Fig. 1). Despite these differences, more than 97% overall sequence homology was seen between these species. Sequences of the recombination signal sites were conserved in all three species. The 1170 base-pair baboon sjTREC was further aligned with swine sjTREC to design primers specific to the baboon sjTREC. Quantitative PCR (qPCR) probe and primer pairs were selected in the area which was less conserved (Fig. 1B).

A standard curve of the sjTREC molecules for qPCR analysis was derived from a known number of plasmid DNA molecules which was titrated by 10-fold serial dilutions to obtain a standard curve that could be used to determine between 10 and 10,000,000 copies of sjTREC (see **Figure, Supplemental Digital Content 1**, <http://links.lww.com/TP/A371>).

sjTREC Assay Within Fluorescence-Activated Cell Sorted Baboon Peripheral Blood Mononuclear Cell

To determine the cell specificity of the baboon signal-joint TREC, we purified phenotypically distinct lymphocytes by fluorescence-activated cell sorting (FACS) from young baboon peripheral blood mononuclear cells (PBMCs) and used qPCR to detect the signal-joint sequence. sjTRECs were detected within phenotypically naïve baboon cells CD3⁺CD45RA^{high} at a level of 650 copies/100,000 cells. The TREC levels in the CD3⁺CD45RA^{low} cells were below the level of detection (40 copies/100,000 cells; Fig. 2). This experiment validated our approach by demonstrating detectable sjTREC in naïve (including recent thymic emigrants [RTEs]) and not in antigen-experienced T cells.

sjTREC Distribution With Age

sjTREC levels have been reported to decline with age, concomitant with thymic involution (11, 14). To confirm that the sjTREC levels we were measuring in baboons also declined with age, three baboons aged 3, 5, and 20 years were studied. sjTREC levels were assessed within thymus, PBMC, spleen, and lymph node (Fig. 3). The levels of sjTRECs were found to decline with age in all four tissues, most markedly in the thymus. In this experiment, we also observed that the second highest tissues containing sjTREC molecules were the spleen and the lymph node. This finding suggests that naïve T cells home to the secondary lymphoid organs after maturation in the thymus. Similar results have been observed in the mouse and other non-human primates (13).

Effect of Thymectomy

Because the thymus is the primary organ of new T-cell generation, we examined the effect of thymectomy in two young baboons. sjTREC levels were analyzed in PBMC before and 4 weeks after thymectomy (Fig. 4). The sjTREC levels in athymic primates fell to as low as 190 copies/100,000 cells 4 weeks after thymectomy, indicating the importance of a functioning thymus to maintain the peripheral T-cell pool.

Specificity of TREC Assay for Baboon Thymopoiesis

To assure that the TREC assay we had designed was specific for baboon TREC and did not measure TREC in porcine T cells, we next tested for potential cross-reactivity of the baboon qPCR primers with porcine DNA. Primer specificity was tested in a qualitative PCR reaction using DNA from a young baboon and porcine PBMC and thymic tissue (Fig. 5A). No amplification was observed with porcine thymic or PBMC DNA.

Detection of Baboon TREC After Thymokidney Xenotransplantation

PBMC and thymokidney tissue biopsy samples from two thymectomized baboon recipients of porcine thymokidneys were tested for baboon sjTREC levels. These levels were below level of detection (40 copies/100,000 cells) at day 54 and 81 in PBMC of both animals. However, the biopsy samples of thymic tissue from the thymokidney transplants showed 52 copies/100,000 cells (day 81) in one animal and 192 copies/100,000 cells in the second animal (day 54; Fig. 5B). Controls consisting of baboon kidney tissue and porcine thymus both showed undetectable levels of sjTREC, confirming that the baboon sjTREC molecules were specifically detected in porcine thymus.

DISCUSSION

During intrathymic differentiation, progenitor cells undergo rearrangement of TCR genes to become mature T cells, leading to the formation of extrachromosomal DNA excision circles, with one of them being the sjTREC. These newly developed CD3⁺ T cells have been characterized by phenotypic markers such as CD45RA and CD62L. However, the CD45RA marker is not an unequivocal marker for the RTE (14). Another assay, using PCR to quantify sjTREC molecules, has been described as a more reliable method for detection of newly emigrating T cells in both humans and mice (11, 12, 17). A comparable assay has not previously been described for baboons.

Our laboratory has demonstrated that vascularized thymokidney allografts can induce tolerance across fully disparate major histocompatibility complex barriers in miniature swine (15, 16). The composite thymokidney has also been shown to prolong organ survival and function in a xenogeneic model (pig to baboon) (7). However, other functional properties of the transplanted porcine thymus have yet to be evaluated. Therefore, we sought to develop the baboon-specific sjTREC assay to characterize baboon thymopoiesis in the porcine thymus.

For development of the baboon sjTREC assay, we first derived baboon sjTREC primers from the published sjTREC sequence of the human and rhesus macaque. These primers amplified a putative baboon sjTREC sequence which was confirmed by sequence analysis. As expected, further PCR assays showed that baboon α/β T cells undergo specific recombinations during thymic differentiation, enabling detection of the sjTREC. Next, we developed a quantitative PCR TREC assay, capable of detecting from 10 to 106 copies of baboon sjTREC molecules (see **Figure, Supplemental Digital Content 1**, <http://links.lww.com/TP/A371>). A qualitative PCR assay confirmed that our assay was baboon specific, thus assuring that the assay could detect baboon thymopoiesis reliably in porcine thymokidneys.

Additional confirmation of the reliability of this assay was obtained by FACS sorting of baboon CD3⁺CD45RA^{high} T cells and demonstration of higher copies of TREC molecules in these naïve T cells than in their CD45RA^{low} counterparts. The fact that mature T cells showed undetectable levels of sjTREC molecules is consistent with the expectation that these molecules are diluted out during cellular proliferation (13). These findings also confirm the fact that baboon sjTREC primers amplify these molecules only in naïve T cells.

During the assay development, we also observed that TREC molecules were higher in the secondary lymphoid organs (spleen and lymph node) than in the periphery (PBMC). This observation is consistent with the fact that under steady state conditions, RTE tend to home to secondary lymphoid organs rather than circulating in the periphery, as observed in humans and non-human primates (12, 13). TREC molecules markedly declined 4 weeks after the surgical removal of the thymus and more slowly thereafter, undoubtedly reflecting differences in the rate of peripheral turnover of T cells with age and substantiating the continuing significant contribution of the thymus to T-cell homeostasis throughout life.

In the porcine thymokidney biopsies taken from two baboon recipients, we detected baboon sjTREC molecules in the thymic tissue, while no baboon TREC molecules were detected in the porcine kidney tissue. Also, FACS analysis of the thymokidney tissue confirmed the presence of baboon double-positive CD4⁺CD8⁺ (i.e., newly formed) T cells in porcine thymus (18). Thus, the fact that sjTREC molecules were low in the PBMC of baboons may reflect the low peripheral T cell counts at the time of the analysis, and the fact that newly generated T cells would most likely undergo lymphopenia-induced proliferation leading to loss of the sjTREC molecules. We suspect that with more prolonged xenograft survival, we

may be able to detect baboon sjTRECs in the periphery of baboon recipients. Although these findings are not yet conclusive because of the small number of animals studied, they are certainly suggestive and will hopefully be confirmed as more animals are studied in ongoing experiments.

In summary, these results are very encouraging for the goals of our study, as they show: (1) the utility of the sjTREC assay in detecting baboon thymopoiesis; and (2) that baboon thymopoiesis can occur in porcine thymic stroma after thymokidney transplantation. The latter finding represents a key element of the experimental approach to xenograft tolerance that is currently under development in this research center (7).

MATERIALS AND METHODS

PBMC and Tissue Isolation

PBMC were prepared from freshly collected, heparinized, whole blood diluted approximately 1:1 with Hanks balanced salt solution (HBSS; GIBCO BRL, Grand Island, NY). Mononuclear cells were obtained by gradient centrifugation using lymphocyte separation medium (Organon, Teknika, Durham, NC), washed once with HBSS, and contaminated red cells were lysed using ACK buffer (B&B Research Laboratory, Fiskeville, RI). Cells were then washed with HBSS and resuspended in tissue culture medium. An aliquot of 5×10^6 cells was used for total DNA isolation. DNeasy Qiagen kit was used for DNA isolation according to manufacturer's guidelines. A Nanodrop spectrophotometer was used to measure DNA concentration in each sample.

Baboon lymph node, thymus, and spleen samples were obtained from operative biopsies. Twenty-five milligrams of each tissue was weighed and lysed with 50 $\mu\text{g}/\text{mL}$ proteinase K at 56°C for 4 hr. DNA extraction was performed by using the DNeasy tissue kit isolation using the manufacturer's recommended protocol (Qiagen).

A section of the transplanted composite thymokidney organ was obtained at the time of graftectomy or autopsy. The thymic tissue sample was slowly dissected from the kidney portion to assess thymopoiesis. Total DNA was isolated as above. All animals were cared for according to the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Sequence of Baboon $\delta\text{Rec-}\psi\text{Ja}$ Recombination Sites

Nucleotide sequence analysis of the TCR $\delta\text{Rec-}\psi\text{Ja}$ recombination sites was used to identify regions of similarity between baboon, human, and rhesus macaques. The 1170-bp baboon signal joint fragment was obtained by using 10 pmol of $\delta\text{-rec}$ primer: 5'-TTTCTTACCATCTGCTGCCATCTAGTGG-3', and 10 pmol of ψJa primer: 5'-TCTTCATTAGGGGGTAGCATAATTCCTGG-3', 250 ng of thymic DNA, 0.5 μL Hot start Taq polymerase (Qiagen), 1 μL of 10 mM dNTP, 5 μL of 10 $\times$ buffer, in a 50 μL total reaction volume. PCR conditions were: 95°C for 15 min, followed by 35 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 60 sec. A final extension step of 6 min at 72°C was added. All PCR reactions were run using a PTC-100 Programmable Thermal Controller (MJ Research, Inc). The PCR reaction product was run on a 2% agarose gel, and the DNA was isolated from the gel using a GeneClean spin kit (Q-BIOgene). The purified PCR band was cloned into pCR2.1 TOPO TA cloning vector (Invitrogen) according to the manufacturer's protocol. Six plasmid clones containing the 1170-bp insert fragment were sent for sequencing analysis.

Quantitative PCR and Baboon sjTREC Analysis

qPCR primers were designed from the 1170-bp baboon sequence containing the recombination signal sites of the δ -rec and ψ J α regions. Each 25- μ L reaction included 800 nM of ψ J α forward primer: 5'-GGTGTCTCTGTCAACAAAGTTGATGC-3', with 800 nM of δ -rec reverse primer: 5'-ATGACAAGTTCAGCCCTCCATGTC-3' with 200 nM of labeled probe: 5'-/56-FAM/CCCTGTCTGCTCTTCATTACCATTCACG AG/36 - TAMSp/-3'.

Other reagents included 0.25 μ L. Hot start Taq polymerase (Qiagen), 2 μ L 10 mM dNTPs, 150 ng (25,000 cells at 6 pg of DNA per cell) of DNA template, 2.5 μ L of 10 $\times$ Hot start Taq buffer. The cycling conditions were as follows: 95°C for 15 min, followed by 50 cycles of the steps: 94°C for 30 sec, 55°C for 60 sec, and 72°C for 30 sec. All qPCR reactions and quantitative analysis were performed using a Strategene Mx3005 machine.

FACS Sorting

PBMC were separated by FACS. In this experiment, isolation of CD3⁺CD45RA^{high} cells was performed using FITC- anti-CD3 mAb (Becton Dickinson) and PE-anti-CD45RA mAb (Becton Dickinson) to stain for 30 min. After washing, cells were separated into CD3⁺CD45RA^{high} and CD3⁺CD45RA^{low} populations using a modified FACStarPlus (Becton Dickinson, San Jose, CA) connected to MoFlo electronics (Cytomation, Fort Collins, CO). Purity of sorted populations was more than 98% as determined by postsort flow cytometry.

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FIGURE 1.

(A) Nucleotide sequence comparison between Rhesus macaque, baboon, and human sjTREC sequence across the TCR A/D recombination regions. Nucleotide similarity is depicted by "...," deletions are depicted by "--" and the location of the signal joint is shown by "//." (B) Nucleotide alignment between baboon and swine sjTREC. *Underlined* and in *green* are the primer sequences used in the quantitative polymerase chain reaction assay. The probe used in the assay is italicized and *underlined in red*. "|" sign depicts nucleotide similarity between the two sjTREC sequences. TCR, T-cell receptor; sjTREC, signal-joining TCR excision circles.

FIGURE 2.

Cell specificity of baboon signal-joint in control animals. (A) Naïve T cells from a young animal were sorted by using CD3 and CD45 staining. (B) Purity of the CD3⁺CD45RA^{high} cells is shown. (C) Purity of the CD3⁺CD45RA^{low} is also shown. (D) Quantitative polymerase chain reaction (qPCR) analysis of naïve (CD3⁺CD45RA^{high}) versus memory (CD3⁺CD45RA^{low}). DNA was isolated from each cell type and quantified by qPCR. sjTREC molecules are expressed as copy numbers per 100,000 cells. The *solid line* represents the detection limit (40 copies of sjTREC/100,000 cells). The naïve T cells contain higher numbers of sjTREC molecules compared with the memory phenotype, which fall below the threshold. The assay was repeated twice, and each sample was run in triplicates. TCR, T-cell receptor; sjTREC, signal-joining TCR excision circles.

FIGURE 3.

Baboon sjTREC distribution in lymphoid organs. sjTREC levels within two younger and one older baboon were analyzed in thymus, spleen, lymph node (LN), and peripheral blood mononuclear cells (PBMC) and expressed per 100,000 cells. The highest copy numbers of sjTREC were detected in the thymus of the two younger baboons. A decline in thymopoiesis was observed in the thymus of the older primate. TCR, T-cell receptor; sjTREC, signal-joining TCR excision circles.

FIGURE 4.

The thymus is required for generation of sjTREC + cells. sjTREC levels were assessed in peripheral blood mononuclear cells (PBMC) of two young thymectomized baboons. Analyses were performed within PBMC before and 4 weeks after thymectomy. A marked decline in sjTREC levels/100,000 cells was observed after thymectomy. All samples were run in triplicates. TCR, T-cell receptor; sjTREC, signal-joining TCR excision circles.

FIGURE 5.

sjTREC analysis reveals baboon thymopoiesis. (A) Baboon primers were baboon specific. The baboon sjTREC quantitative polymerase chain reaction (qPCR) primers were tested with porcine peripheral blood mononuclear cells (PBMC) and thymic DNA. In the gel electrophoresis, there was no polymerase chain reaction (PCR) amplicon detected within porcine DNA. This experiment confirmed the specificity of the primers to amplify only the baboon sjTREC region. (B) Baboon thymopoiesis detected in porcine thymus. qPCR analyses were performed within PBMC and tissue biopsies in two different young and thymectomized baboon recipients of thymokidney (TK) xenografts 54 and 81 days after transplantation. The thymic tissue was dissected from the kidney and tested for sjTREC molecules. The kidney tissue analysis was also included for control. All samples were run in triplicates. TCR, T-cell receptor; sjTREC, signal-joining TCR excision circles.