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Journal of Immunological Methods 237 (2000) 187–197

JIM
Journal of
Immunological Methods

www.elsevier.nl/locate/jim

Recombinant Technology

Detection of T cell receptor circles (TRECs) as biomarkers for de novo T cell synthesis using a quantitative polymerase chain reaction–enzyme linked immunosorbent assay (PCR–ELISA)

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Received 4 August 1999; received in revised form 22 December 1999; accepted 4 January 2000

Abstract

Currently, phenotypic markers that distinguish between recent thymic emigrants/de novo T cells and the rest of the peripheral T cell pool are lacking. This distinction is critical in studies aimed at evaluating immune reconstitution following intensive chemotherapy, in immunodeficiency-related therapies, or in the elucidation of the kinetics of thymic function. During V(D)J T cell receptor rearrangement, DNA extrachromosomal excision products are generated. These products, known as T cell receptor excision circles (TRECs), are not replicated during mitosis and are thus diluted with each round of cell division. Therefore, TRECs can be used as an indicator of recent thymic emigrants. Thus far, quantitative competitive–polymerase chain reaction (QC–PCR) and real time PCR were used to measure TREC levels. However, QC–PCR relies on radioactivity, is cumbersome when processing many samples at once and the cost of real time PCR does not make it a viable option for many laboratories. We describe here the development of a quantitative PCR–ELISA method for the measurement of coding joint TRECs generated from VαJα recombination. Our assay is ultra sensitive, relies on biotin labeling rather than radioactivity, is based on a 96-well format making multiple process sampling relatively easy, and is cost effective. Using this PCR–ELISA method, we evaluated thymic output among 22 normal subjects, ranging in age from 22–53 years, and among HIV-infected individuals following highly active antiretroviral therapy (HAART). We demonstrate that an inverse relationship exists between TREC levels and aging in normal individuals and that, among some HIV patients, HAART treatment leads to enhanced thymic output. Our assay has direct relevance in projects examining normal and abnormal thymic function and in immune reconstitution studies.

Keywords: T cell receptor excision circle (TREC); Thymopoiesis; Recent thymic emigrants; De novo T cells; PCR–ELISA

Abbreviations: PCR–ELISA, polymerase chain reaction enzyme–linked immunosorbent assay; TREC, T cell receptor excision circle

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0022-1759/00/$ – see front matter
PII: S0022-1759(00)00136-8

1. Introduction

The human thymus, a small organ located above the heart, is the primary site of T cell maturation and development. Shortly after birth, the thymus undergoes a life long process of involution whereby the organ is replaced by adipose tissue (reviewed in George and Ritter, 1996; Mackall and Gress, 1997; Steffens et al., 1999), suggesting that the adult thymus may not be functional. However, recent data suggest that despite thymic involution, pockets of active T cell synthesis (thymopoiesis) still exist (Bertho et al., 1997; Douek et al., 1998; Haynes et al., 1999; Poulin et al., 1999; Zhang et al., 1999). This finding can be exploited to reconstitute the immune system by enhancing thymic function in conditions that cause T cell depletion, such as the acquired immunodeficiency syndrome (AIDS) or intensive chemotherapy. Therefore, monitoring thymic function by measuring new T cell synthesis becomes a critical prerequisite in immune reconstitution studies. However, specific phenotypic markers that distinguish de novo T cells/recent thymic emigrants (RTE) from the rest of the peripheral T cell pool do not exist. Nevertheless, an alternative biomarker known as T cell receptor excision circle (TREC) can be used as a tool to monitor thymopoiesis. This approach utilizes the generation of DNA products during T cell receptor rearrangement that can be specifically correlated with de novo T cells (Douek et al., 1998; Poulin et al., 1999; Zhang et al., 1999).

TRECs are generated during V(D)J gene recombination, a process responsible for the diversity of the αβ and γδ T cell antigen receptor (TCR) repertoire. During this process, variable (V), diversity (D) and junctional (J) gene segments are rearranged in order to generate a functional β or δ TCR chain. Similar rearrangement events occur at the α and γ locus even if no D segments are present. This complex end-to-end fusion of gene segments is mediated by recombination-activating genes (RAG) 1 and 2 that recognize 'heptamer–spacer–nonamer' recombination signal sequences (RSSs) flanking each V, D and J gene segment. Generation of a coding TCR chain results in the excision of extrachromosomal DNA circles composed of the intervening genomic sequences present between both rearranged segments. The process of α and β TREC generation are illustrated in Fig. 1A and B (reviewed in Steffens et al., 1999), respectively. TRECs are stable, are not replicated during mitosis, and are thus diluted with each round of cell division (Livak and Schatz, 1996; Douek et al., 1998). Therefore, TRECs can function as a marker for the replicative history of a cell as well as an indicator of active thymopoiesis (Douek et al., 1998; Poulin et al., 1999).

TCR recombination events may generate numerous TRECs with no single TREC more indicative of thymopoiesis than another. Given that the TCRδ locus lies within TCRα locus (Fig. 1B), which must be excised before any TCRα gene recombination and because δRec- ΨJα represents approximately 67% of all of the δRec recombination events (Verschuren et al., 1997; Douek et al., 1998), δRec recombinations generating signal and coding joint TRECs are most often chosen as indicators of thymopoiesis (Douek et al., 1998; Zhang et al., 1999). Recently, a quantitative competitive (QC)–PCR assay (Douek et al., 1998) and a real time PCR assay with a molecular beacon (Lewin et al., 1999; Zhang et al., 1999) were used to detect signal and coding joint TRECs generated from TCRα gene recombination (Fig. 1B). However, QC–PCR relies on radioactivity, making evaluation of multiple samples difficult and posing an increased health risk to the researcher. Real-time PCR is expensive and requires specialized instruments that are not available to most laboratories. Therefore, we report here the development of an ultra sensitive quantitative PCR–ELISA for the evaluation of TRECs generated from VαJα gene recombination that does not require specialized equipment or radioactivity.

This PCR–ELISA approach is schematically presented in Fig. 2. Addition of digoxigenin (Dig) UTP allows its incorporation into the DNA during the amplification process, resulting in a PCR product that is Dig-labeled. Subsequently, the Dig-labeled PCR product is hybridized to a biotin-labeled probe corresponding to an internal sequence within the product. The biotin Dig-labeled product is then captured onto a streptavidin-coated plate. Anti-Dig antibody conjugated with peroxidase and 2,2-azino-di-[3-ethylbenzthiaxoline sulfonate] (ABTS) substrate permits the subsequent detection of the PCR product by a colorometric reaction that is read at a

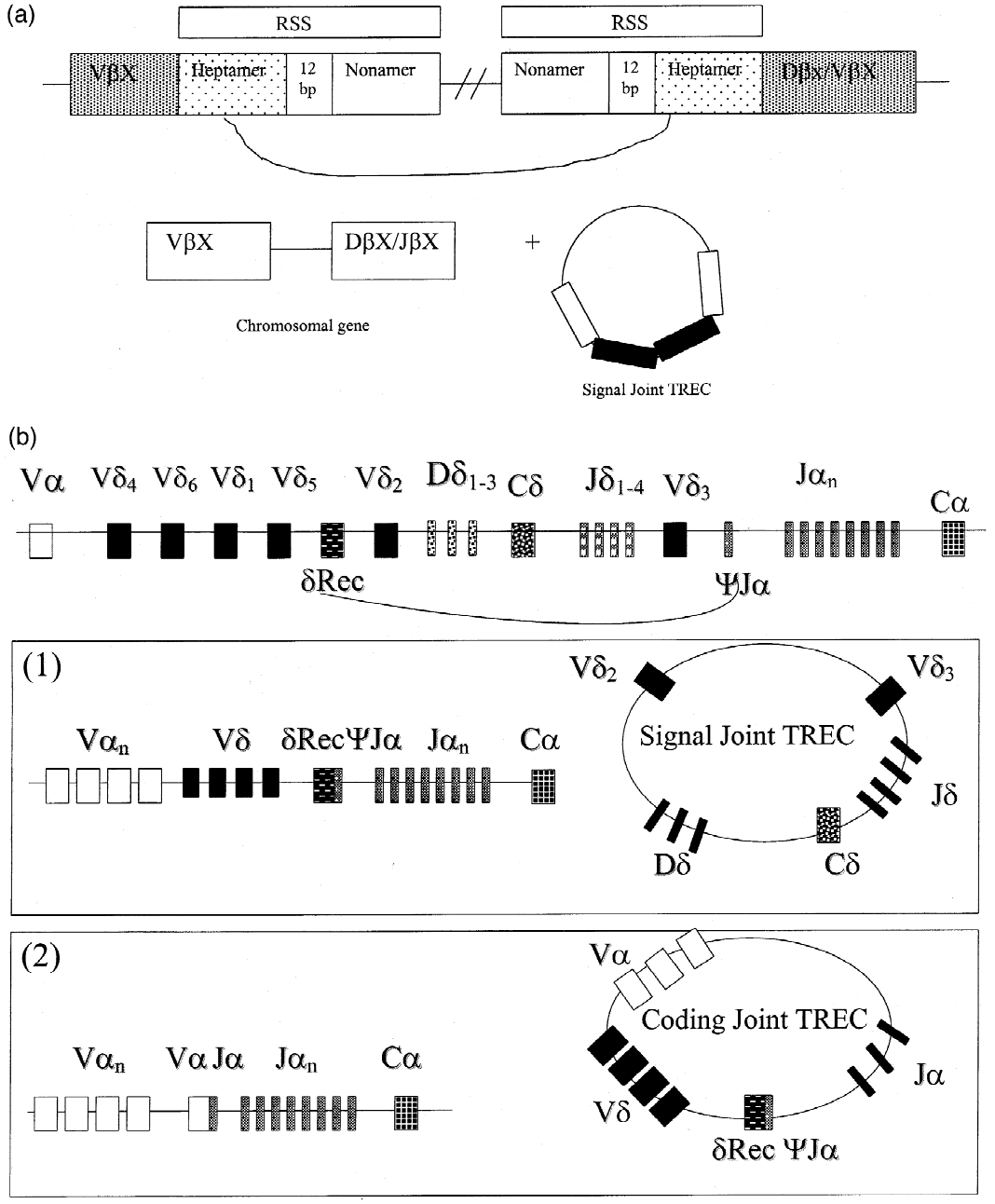

Fig. 1. Mechanism of TCRβ (A) and TCRα (B) TREC generation. (A) In TCRβ TREC generation, DJ recombination must occur prior to V recombination. A recombination signal sequence (RSS) flanks each gene to be recombined. RSS consists of a heptamer, a spacer sequence that is 12 or 23 bp in length, and a nonamer sequence. Recombination activating enzymes (RAG1/II) recognize RSS and cleave the DNA only between gene segments possessing 12 or 23 bp spacer sequence (the 12/23 rule). Cleaved sites form an extrachromosomal DNA circle, referred to as T cell receptor excision circle (TREC). In this case, the TREC consists of two adjacent heptamer sites flanked by the nanomer sequences and the intervening gene sequence of the spliced DNA. Removal of TREC permits the formation of a recombined chromosomal gene representing a specific VDJ repertoire. (B) Common to all TCRα germline DNA is an intervening sequence for the δ gene. In order to generate a VJ recombination event, the intervening δ gene is excised, in the same mechanism described for the β chain TREC, to initially generate a signal joint TREC. Recombination of VαJα will then generate a second TREC (coding joint TREC).

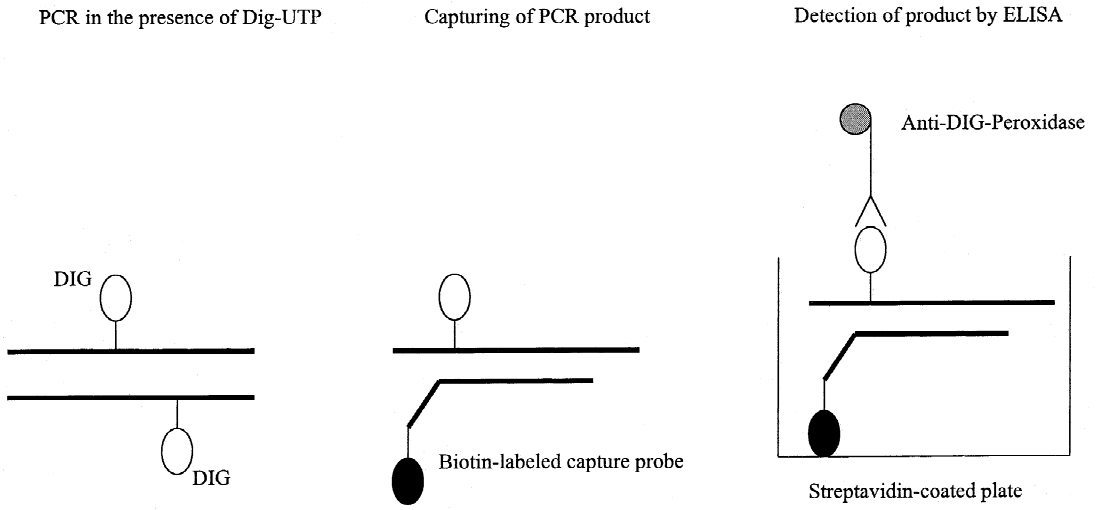

Fig. 2. A schematic diagram of PCR–ELISA. Genomic DNA is amplified in a PCR reaction in the presence of digoxigenin (Dig) UTP. The product is captured with a biotin-labeled probe recognizing the internal sequence of the PCR product. The biotin-captured product is placed on a streptavidin plate and an ELISA is performed using anti-Dig peroxidase antibody and ABTS colorometric substrate detection as specified by the manufacturer (Boehringer-Mannheim, Indianapolis, IN).

wavelength of 405 nm and reference of 490 nm (Boehringer Mannheim, Indianapolis, IN). To make this PCR–ELISA assay specific for the measurement of VαJα coding joint TREC (Fig. 1B), we cloned the VαJα coding joint TREC to generate a set of standards corresponding to various copies of the clone that were subsequently amplified within the linear range of the PCR. A standard curve is generated from which the amplified DNA can be quantified. While this approach is specific for the quantification of VαJα coding joint TRECs, it can be modified to apply to any gene of interest.

2. Materials and methods

2.1. DNA isolation from CD4$^+$ T cells

CD4$^+$ T cells were isolated from either venous blood or frozen samples of peripheral blood mononuclear cells (PBMC). For fresh samples, venous blood was collected in EDTA tubes and PBMC isolated by Ficoll-hypaque gradient centrifugation as previously described (Coligan et al., 1994). CD4$^+$ T cells were subsequently isolated from PBMC by positive immunoselection using Dynal CD4$^+$ beads and Detachabead, according to the manufacturer's instruction (Dynal Inc, Lake Success, NY) with a few modifications. Briefly, CD4$^+$ beads were added at a bead to target ratio of 4:1 and the final cell concentration was maintained at 3×10^6 cells/ml. The cell/bead mixture was incubated with gentile agitation at 4°C for 1 h. Using a magnetic particle concentrator (MPC, Dynal, Lake Success, NY), CD4$^+$ T cells attached to the beads were isolated and washed twice in 2% FBS–PBS (wash buffer). To detach the beads from the cells, the bead/cell mixture was resuspend in 100 μl wash buffer, 50 μl of CD4 Detachabead were added, and incubated for 1 h at room temperature. After MPC separation, the supernatant was removed (constituting pure CD4$^+$ T cells) and washed repeatedly in 1× PBS. The cells were counted by trypan exclusion assay and at least 400,000 cells were saved to perform surface staining for CD3/CD4 in order to determine the relative purity of the isolation procedure. DNA from CD4$^+$ T cells was isolated using DNAzol and the procedure performed according to the manufacturer's instructions (GIBCO-BRL, Grand Island, NY). The DNA was stored at −20°C until further use. Repeated freeze/thaw cycles were also avoided.

2.2. Cell surface staining and flow cytometry

Cell surface staining and flow cytometry analysis were performed according to a standard protocol (Coligan et al., 1994). Briefly, 200,000 cells were used for each antibody staining. All antibodies were purchased from Beckton Dickinson (San Jose, CA). Cells were stained for both IgG fluorescein isothiocyanate (FITC) and IgG phycoerythrin (PE) or CD3 FITC and CD4 PE by adding 5 μl of each conjugated antibody to the respective tube. Antibodies were incubated in the dark for 40 min and excess antibody removed by washing with PBS and centrifuging at 1200×*g* for 5 min. Cells were fixed using 1× methanol-free paraformaldehyde. The fixed cells were either analyzed by flow cytometry (FacsCaliber, Becton Dickinson, San Jose, CA) immediately or stored at 4°C for up to 2 days without a decline in signal intensity until further analysis.

2.3. Cloning of VαJα coding joint TREC

Primers, located approximately 300 bp adjacent to the recombination joint, were synthesized to specifically amplify a ~600 bp product of δRecΨJα recombination (sequence previously published by Verschuren et al., 1997). Genomic DNA (100 ng) from SCID-Hu thymocytes was amplified for 30 cycles using standard PCR conditions (1× PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 ng of each primer, 1 IU of Taq). The cycling reaction was performed for 30 s each at 90, 60, and 72°C. The PCR product was visualized with ethidium bromide on 1.25% agarose gels. The amplified fragment was purified from the gel (Qiagen, Santa Clarita, CA). The agarose-isolated product, corresponding to the coding joint TREC, was then cloned using blunt-end cloning into pBlueScript vector (Stratagene, LaJolla, CA). Both restriction enzyme and DNA sequence analysis were performed on the isolated clone to verify that no sequence mutation was introduced during the PCR-based cloning strategy.

2.4. Digoxigenin (Dig)-labeling and VαJα TREC amplification

Dig-labeling of the PCR product was performed using a Dig-labeling kit from Boehringer Mannheim (Indianapolis, IN). We optimized the labeling reaction for the amplification of VαJα TREC. Specifically, the PCR reaction was conducted in a final volume of 50 μl/reaction consisting of 1X PCR buffer without Mg^{2+}, 2.5 mM $MgCl_2$, 20 μM Dig-labeling mix (a mixture of Dig and dNTPs) 2 μM VαJα coding joint forward (CTAATAATAAGATCCTCAAGGGTCGAGACTGTC) and reverse (CCTGTTTGTTAAGGCACATTAGAATCTCTCACTG) primers, and 0.02 μ/μl Taq. Forward and reverse primers for the amplification of VαJα TREC were synthesized using a primer designer software based on a published sequence of TCRα locus (Verschuren et al., 1997). The primer designer software takes into account G/C ratio, sequence homology, primer-dimer formation, hairpin formation, and anchor sequences. The cycling reaction was performed at an initial denaturation step at 95°C/5 min and 25 cycles at 90°C/30 s, 60°C/30 s, and 72°C/30 s. A final extension step was added at 72°C/7 min.

With each PCR run, VαJα plasmid was also amplified to serve as a standard in the PCR–ELISA protocol at specific copies we determined to be within the linear range of the PCR (50,000, 5000, 500, and 0 copies). Genomic (experimental) DNA was routinely amplified at 1 μg, and 0.5 μg to ensure that the experimental DNA was also within the linear range of the assay.

2.5. General PCR precautions

To avoid PCR contamination, DNA isolation and amplicon analysis were performed in two different rooms with separate reagents and equipments. Aerosol-free tips were used throughout the PCR–ELISA protocol. Primers and the probe were diluted with DNase-free water, aliquotted at 50-μl each in Starstedt 1.5-ml tubes with screw caps, and frozen at −20°C until further use. Also, each PCR component from the PCR–Dig labeling kit (Boehringer Mannheim, Indianapolis, IN), including water were aliquotted and frozen at −20°C to avoid contamination and repeated freeze–thawing cycles. Finally, the biotin labeled probe to specifically capture the amplified product was generated to correspond to an internal sequence within the amplified product and

not to either one of the primers to avoid the detection of primer dimers.

2.6. Quantitation of VαJα TREC by PCR–ELISA

The ELISA protocol was performed according to the manufacturer's instructions with modifications specific for VαJα TREC detection (Boehringer Mannheim, Indianapolis, IN). Briefly, 10 μl of each PCR product and 20 μl of a DNA denaturation solution were added to a 96-well flat-bottom plate. The mix was incubated at room temperature for 10 min. Subsequently, 220 μl of a hybridization solution containing 7.5 pmol/ml of the biotin-labeled internal probe (biotin labeled at the 5′ end and designed to recognize an internal sequence of the PCR product, TCTGTGTCTAGCACGTAGCC) were added, mixed well, and 200 μl from this mix were added to a streptavidin-coated plate. The plate was sealed with an adhesive tape and incubated at 55°C for 3 h with gentle shaking. The plate was extensively washed using an ELISA washing solution and 200 μl of a 1/100 diluted solution of anti-DIG peroxidase was added to all wells except the designated blank well. Again, the plate was sealed and incubated at 37°C for 30 min with gentle shaking. After washing the plate as previously described, ABTS substrate solution was added to all wells, including the blank well. The plate was sealed, covered with foil, and incubated at 37°C for 30 min with gentle shaking. The absorbance was recorded using a plate reader at a wavelength of 405 nm and a reference of 490 nm employing a software program (SoftMax pro, Molecular Devices Corp, Sunnyvale, CA) that subtracts the blank well from all wells and generate a standard curve (absorbance versus copies of plasmid amplified). Values of experimental samples were determined from the standard curve and subsequently divided by the initial amount of DNA amplified to yield copies/μg present in each initial (pre-PCR) sample.

2.7. Statistical analysis

The Pearson correlation coefficient was calculated between TREC and age for all 22 subjects as well as between each age group (20–29; 30–39; 40–49, and greater than 50) and TREC. TREC values (copies/μg) were transformed to $\log_{10}$ in all analyses. P values <0.05 were considered as statistically significant.

3. Results

3.1. Development of a PCR–ELISA to quantify VαJα coding joint TREC

To generate a quantitative assay to measure the amount of VαJα TREC, we cloned VαJα TREC using Scid-Hu thymocyte ($CD4^+CD8+$) DNA, which is enriched for TRECs. The cloned VαJα TREC was identified by both restriction enzyme analysis and direct sequencing (data not shown). This clone was used as the standard in the PCR–ELISA described here. Because PCR results in logarithmic amplification of DNA, initial experiments were set up to determine the linear range of the assay by amplifying a wide range of copies of the clone. Initially, 5×10^{10}, 5×10^{8}, 5×10^{6}, 5×10^{5}, 5×10^{4}, 5×10^{3}, 5×10^{2}, and 0 copies of the TREC clone were amplified and detected by PCR–ELISA. As seen in Fig. 3A, the PCR reaction as indicated by the ELISA was only linear below 5×10^{4} copies whereas values between 5×10^{10} and 5×10^{5} were not in the linear phase of the PCR reaction. Values lower than 500 copies did not always read above background (data not shown). Therefore, for this particular clone, the standards were used between 5×10^{4} and 5×10^{2} copies with a water control corresponding to 0 copies amplified. These values were then plotted using SoftMax software (Molecular Devices Corp, Sunnyvale, CA) to generate a standard curve representing absorbance vs. TREC copies. The assay using the set of standards indicated (50,000, 5000, 500, and 0 copies) were repeated several times and the values were similar between each PCR run performed (Fig. 3B). A photograph of a typical VαJα PCR–ELISA demonstrating the differential colorometric reaction is shown in Fig. 3C.

3.2. Quantification of VαJα TREC from genomic DNA

To detect coding joint VαJα TREC from genomic DNA, the PCR–ELISA was performed as described

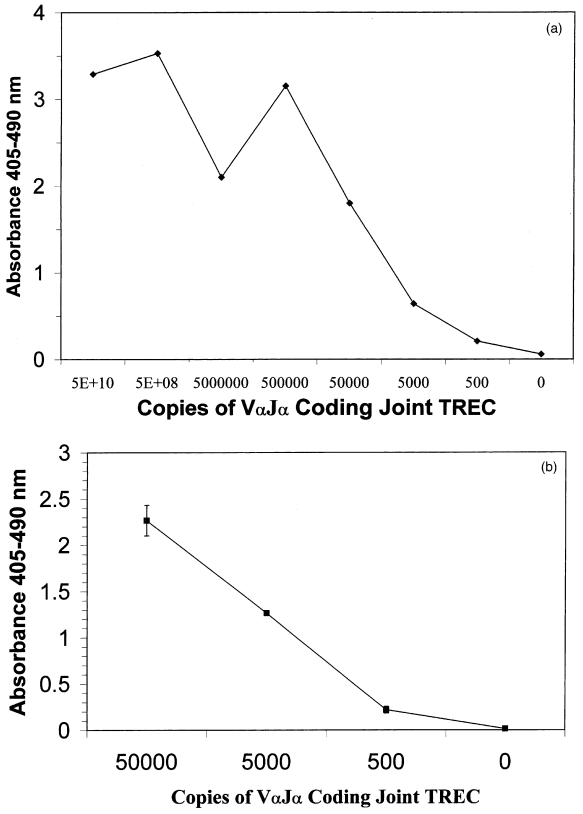

Fig. 3. (A) Amplification of a wide range of copies of the coding joint TREC. VαJα clone was amplified at copies ranging from 5×10^{10} to 5×10^{2} and ELISA performed to detect the TREC. (B) Determination of the linear range of the PCR–ELISA procedure. Copies of the coding joint TREC clone determined from A to be within the linear range of the assay were amplified and ELISA performed. Values are representative of three independent experiments. (C) Visualization of PCR–ELISA. 50,000–0 copies of VαJα cloned signal joint TREC were amplified and visualized by a colorometric ELISA reaction. Well A corresponds to a blank well, well B corresponds to 5×10^{4} copies, well C corresponds to 5×10^{3} copies, well D corresponds to 5×10^{2} copies, well E corresponds to 50 copies, and well F corresponds to 0 copies.

Fig. 3 (C).

above and SCID-Hu thymocyte ($CD4^{+}CD8^{+}$) DNA was also amplified. As seen in Fig. 4, VαJα coding joint TREC was detected from thymocytes, which are enriched for recent thymic emigrants, at 1×10^{6} copies/μg. As expected, TRECs were not detected from a Jurkat cell line, which have fully rearranged TCR loci but because these cells undergo continuous proliferation, the TREC signal is diluted and cannot be detected.

3.3. Thymic output and age

To determine the relationship between thymic output and age, we isolated $CD4^{+}$ T cells from 22 normal individuals ranging in age from 22–53 years. TREC levels were measured by the PCR–ELISA method described here. As seen in Fig. 5, a significant correlation exists between age and TREC levels (Pearson correlation coefficient (r)=−0.534; P=

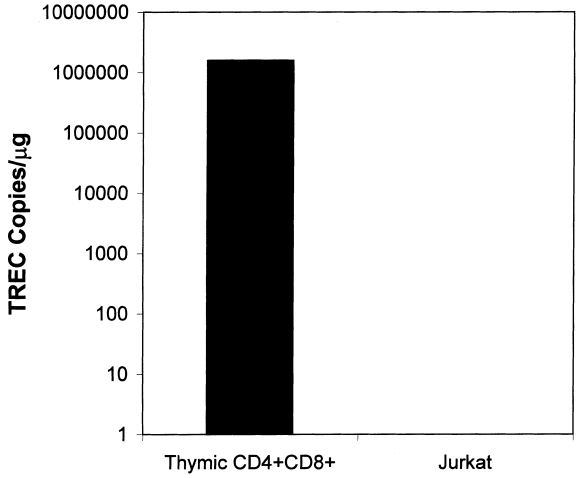

Fig. 4. Quantification of coding joint TREC from fetal SCID-Hu thymocytes and a Jurkat T cell line. DNA from $CD4^+$ T cells from SCID-Hu thymocytes ($CD4^+CD8^+$) or from Jurkat T cells were amplified along with the standard (coding joint TREC clone) and ELISA performed. Values represent TREC levels measured as copies/μg of DNA amplified.

0.011) whereby an inverse relationship exists between age and thymic output. We also analyzed this data by grouping the subjects into four categories based on age (22–29; 30–39; 40–49, and >50) and still demonstrated a significant correlation between $\log_{10}$ TREC and age group ($r=-0.526$; $P=0.012$).

3.4. Quantification of TREC from HIV-infected patients

Given that HIV can infect the thymus and lead to thymic dysfunction (Bonyhadi et al., 1993; Calabro et al., 1995; McCune, 1997; Gaulton, 1998), we evaluated thymic function as measured by VαJα TREC PCR–ELISA from HIV positive patients in early stage disease (CD4 >500 cells/μl) both before and after potent suppression of HIV using highly active antiretroviral therapy (HAART). We found that following 48 weeks of HAART, in at least 2/8 patients, thymic output increased as shown by an approximately 1.5 log increase in VαJα TRECs (Fig.

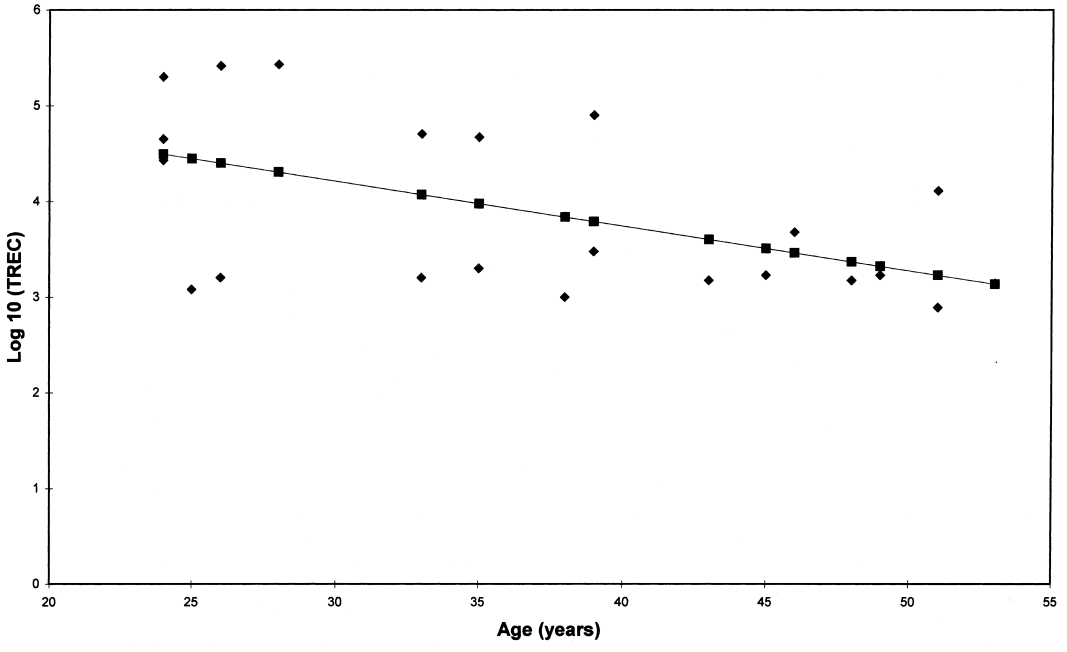

Fig. 5. Relationship between age and TREC levels. $CD4^+$ T cells were isolated by positive immunoselection from 22 normal subjects, ranging in age from 22–53 years. TREC levels were quantified using VαJα PCR–ELISA and values (copies/μg) were transformed to $\log_{10}$. The Pearson correlation coefficient was calculated ($r=-0.534$; $P=0.011$) and a regression line is indicated in the figure.

6). These data indicate that suppression of HIV may lead to enhanced thymic output and that our PCR–ELISA procedure can be used to quantify changes in TREC levels post-therapy.

4. Discussion

Given that cell surface markers specific for recent thymic emigrants/de novo T cells do not exit, we developed a quantitative PCR–ELISA method to detect a biomarker known as T cell receptor excision circles as an indicator of de novo T cells. Our assay is highly sensitive with a lower limit of detection at 500 copies. Unlike previously reported assays, this procedure does not rely on radioactivity as the described QC–PCR for TREC detection (Douek et al., 1998) nor does it require highly specialized equipment such as that for real-time PCR (Zhang et al., 1999). This assay can be adapted into any laboratory with relative ease. Also, using samples from older subjects, which have been shown to contain fewer de novo T cells, we only require 0.5 μg of input DNA to detect the coding joint TREC in comparison to 1–3 μg required by the QC–PCR protocol. Likewise, we detected the thymocytes at an order of magnitude higher than that reported by Douek et al. (1998), suggesting that this assay is more sensitive. The assay we developed for VαJα TREC detection is quantitative, reproducible, cost effective, and both PCR and ELISA can be performed in less than 7 h.

To evaluate the usefulness of the developed VαJα TREC PCR–ELISA method, we evaluated the relationship between age and TREC levels among 22 subjects, ranging in age from 22–53 years. We demonstrated a correlation between age and TREC levels, indicative of thymopoiesis. Older individuals had less measurable VαJα TRECs than younger individuals, a relationship recently established by

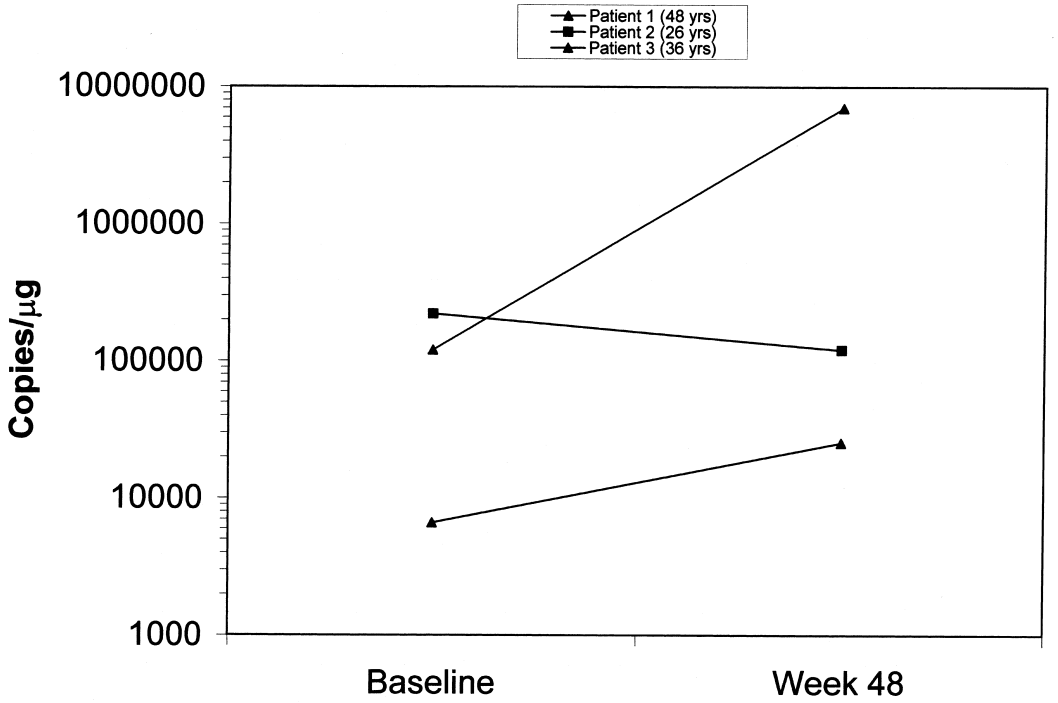

Fig. 6. Effect of HAART on thymic function. $CD4^+$ T cells were isolated from eight patients at baseline and 48 weeks post-HAART. Patients were matched for CD4 cell count and all experienced a reduction in viral load to undetectable levels. Values represent TREC levels measured as copies/μg of DNA amplified from two of the patients demonstrating an increase in TREC and one patient that had no change in TREC.

Douek et al. (1998) using QC–PCR and Zhang et al. (1999) using real-time PCR. This indicates that the PCR–ELISA procedure we developed for the quantification of TREC levels yields results which are comparable to those generated by the two alternative methods (QC–PCR and real-time PCR). However, we noted that although a general inverse relationship exists between age and TREC levels, some older individuals have a TREC profile indicative of a younger person. For example, a 53-year-old female possessed TREC levels similar to those of an individuals between the age of 22–29, suggesting that enhanced thymic output may also be found in a few individuals despite progressive age. We also examined the thymic output, as measured by VαJα TREC PCR–ELISA, among HIV-infected individuals before and after HAART. We demonstrated that enhanced thymic output can be achieved after HAART in some patients. Although we only examined a total of eight HIV-infected patients, we did not observe a relationship between enhanced thymic output after HAART and impaired levels of TRECs at baseline, as reported by Zhang et al. (1999). This variation may be due to studies on different patient populations. While our finding was based on a small yet well defined cohort of HIV-patients in early stage disease (CD4 $>$500 cells/μl, treatment naïve, under the same antiretroviral regime, and all achieving undetectable viral load post-HAART; unpublished data), the findings by Zhang and colleagues was based on a mix of patients in acute or chronic stages of HIV infection and under different antiretroviral regimes including with or without IL-2-based therapy. Also, the two-thymic responders (the two patients that demonstrated a remarkable increase in TREC levels post therapy) were older, a 48- and a 36-year old. This preliminary finding indicate that levels of induced/accelerated thymopoiesis after virus suppression in some patients may exceed that seen in HIV-seronegative-age matched group (unpublished data), suggesting enhanced thymic output in some individuals in response to therapy.

Although the approach we describe here is specific for the quantification of coding joint VαJα TREC, this approach can be devised for any gene of interest following the steps described here. Specifically, the gene of interest should be first cloned and PCR amplification optimized. Subsequently, the clone can be amplified at various copies to establish the linear range of the PCR reaction. Once a standard curve is generated, the reproducibility of this reaction should be checked, experimental DNA samples can be amplified along with the standards, and finally values calculated from the standard curve. These values should be divided by the amount of DNA amplified to determine the concentration of the gene expressed as copies/μg of DNA. Our approach is highly specific since capturing the product with an internal biotin labeled probe is equivalent to performing a Southern blot hybridization with each amplification. The PCR–ELISA procedure we developed for the quantification of VαJα TREC will permit future studies aimed at defining true cell surface markers indicative of recent thymic emigrant, for studies examining the effect of conditions leading to thymopoietic dysfunction, and further elucidating the effect of aging on thymic output.

Acknowledgements

This work was supported by a grant from the American Foundation for AIDS research (amFAR) Grant # 02634-26-RGI. We thank Dr Shande Chen (Section of Biostatistics, Department of Preventive Medicine, Rush Medical College, Chicago, IL) for assistance with statistical analysis.

References

Bertho, J.M., Demarquay, C., Moulian, N., Van Der Meeren, A., Berrih-Aknin, S., Gourmelon, P., 1997. Phenotypic and immunohistological analyses of the human adult thymus: evidence for an active thymus during adult life. Cell. Immunol. 179, 30.

Bonyhadi, M.L., Rabin, L., Salimi, S., Brown, D.A., Kosek, J., McCune, J.M., Kaneshima, H., 1993. HIV induces thymus depletion in vivo. Nature 363, 728.

Calabro, M.L., Zanotto, C., Calderazzo, F., Crivellaro, C., Del Mistro, A., De Rossi, A., Chieco-Bianchi, L., 1995. HIV-1 infection of the thymus: evidence for a cytopathic and thymotropic viral variant in vivo. AIDS Res. Hum. Retroviruses 11, 11.

Coligan, J., Kruisbeek, A., Margulis, D., Shevach, E., Strober, W., Coico, R., 1994. Current Protocols in Immunology, Wiley, New York.

Douek, D.C., McFarland, R.D., Keiser, P.H., Gage, E.A., Massey,

J.M., Haynes, B.F., Polis, M.A., Haase, A.T., Feinberg, M.B., Sullivan, J.L., Jamieson, B.D., Zack, J.A., Picker, L.J., Koup, R.A., 1998. Changes in thymic function with age and during the treatment of HIV infection. Nature 396, 690.

Gaulton, G.N., 1998. Viral pathogenesis and immunity within the thymus. Immunol. Res. 17, 75.

George, A.J., Ritter, M.A., 1996. Thymic involution with ageing: obsolescence or good housekeeping? Immunol. Today 17, 267.

Haynes, B.F., Hale, L.P., Weinhold, K.J., Patel, D.D., Liao, H.X., Bressler, P.B., Jones, D.M., Demarest, J.F., Gebhard-Mitchell, K., Haase, A.T., Bartlett, J.A., 1999. Analysis of the adult thymus in reconstitution of T lymphocytes in HIV-1 infection. J. Clin. Invest. 103, 921.

Lewin, S., Kostrikis, L., Karanicolas, R., Skulsky, E., Vesanen, M., Hurly, A., Ramrantam, B., Kost, R., Markowitz, M., Ho, D.D., 1999. The impact of HIV infection on the number of recent thymic emigrants in blood and the effect of HAART. In: Proc. 6th Conf. on Retroviruses and Opportunistic Infections, January 31–February 4, Chicago, IL.

Livak, F., Schatz, D.G., 1996. T-cell receptor alpha locus V(D)J recombination by-products are abundant in thymocytes and mature T cells. Mol. Cell. Biol. 16, 609.

Mackall, C.L., Gress, R.E., 1997. Thymic aging and T-cell regeneration. Immunol. Rev. 160, 91.

McCune, J.M., 1997. Thymic function in HIV-1 disease. Semin. Immunol. 9, 397.

Poulin, J.-F., Viswanathan, M.N., Harris, J.M., Komanduri, K.V., Weider, E., Ringuette, N., Jenkins, M., McCune, J.M., Sekaly, R.-P., 1999. Direct evidence for thymic function in adult human. J. Exp. Med. 190, 479.

Steffens, C., Marchetti, G., Landay, A., Al-Harthi, L., 1999. The human thymus: a new perspective on thymic function, aging, and HIV infection. Clin. Immunol. News Lett. 19, 65.

Verschuren, M.C., Wolvers-Tettero, I.L., Breit, T.M., Noordzij, J., van Wering, E.R., van Dongen, J.J., 1997. Preferential re-arrangements of the T cell receptor-delta-deleting elements in human T cells. J. Immunol. 158, 1208.

Zhang, L., Lewin, S., Markowitz, M., Lin, H.H., Skulsky, E., Karanicolas, R., He, Y., jin, X., Tuttleton, S., Vesanen, M., Spiegel, H., Kost, R., Lunzen, J., Stellbrink, H., Woliinsky, S., Borkowski, W., Palumbo, P., Kostrikis, L., Ho, D.D., 1999. Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals and after effective therapy. J. Exp. Med. 190, 725.