Topographic and Quantitative Display of Integrated Human Immunodeficiency Virus-1 Provirus DNA in Human Lymph Nodes by Real-Time Polymerase Chain Reaction

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***In situ* polymerase chain reaction (isPCR) has been applied in many fields that require detection of a genomic marker in combination with its topographic localization in tissue. We describe here a novel approach that circumvents the major drawbacks of *in situ* PCR, ie, low sensitivity, leakage of DNA from cells, and inability to quantify the DNA input. Frozen sections of a lymph node from a human immunodeficiency virus (HIV)-1-infected patient were fixed on glass microscope slides, and the glass was scored into square fragments of 0.5-mm edge length using a diamond cutting device. Slides were then attached to adhesive, elastic plastic foil and finally broken, and the foil was extended to allow sorting of fragments into PCR microtiter plates. The material was tested for HIV-1 proviral DNA by a sensitive real-time PCR protocol. Subsequent sections were stained for follicular dendritic cells to identify follicles. The fragmentation process prevented leakage of amplified DNA to neighboring areas as often experienced with *in situ* PCR. Provirus was clearly associated with follicular areas, in which provirus-carrying cells represented an average of 0.8% of the total cell population (peak density, 3.1% of all follicular cells). The results of this method suggest that the high density of provirus-containing cells in follicles may be important for the persistence of proviral DNA in infected persons. (J Mol Diagn 2005, 7:219–225)**

Human immunodeficiency virus (HIV)-1 replicates in human CD4⁺ T lymphocytes and to a lesser extent in macrophages. The infection is maintained not only by circulating virus particles but also by proviral DNA integrated

into the chromosome of CD4⁺ lymphocytes.^{1–3} In contrast to virus particles in plasma, integrated provirus DNA is not efficiently eliminated from lymphatic compartments even after long highly active antiretroviral therapy (HAART).⁴ The detection of provirus in lymphatic tissue thus plays a crucial role in monitoring the success of attempts to eradicate the virus from the body.

Immunohistochemical methods have successfully been applied to detect HIV-1 p24 antigen or HIV-1 RNA in lymph node sections.⁵ The sensitivity of these methods, however, is not sufficient to display single molecules of integrated provirus that can give rise to new virus progeny after discontinuation of therapy. Better sensitivity can be achieved through *in situ* amplification of provirus DNA by the polymerase chain reaction (isPCR), but various properties render this technique problematic. First, leakage of amplified DNA into neighboring cells and nonspecific incorporation of labeled nucleotides into nuclear DNA decrease the specificity of isPCR.^{6–10} Second, formaline fixation required for isPCR is well known to reduce the efficiency of enzymatic amplification due to DNA crosslinking.^{6,11} Third, quantification of target DNA is not possible by isPCR. This would be beneficial in monitoring the elimination of a unique chromosomal property, eg, HIV-1 provirus, in experimental therapy.

In this pilot study, we describe a simple approach to avoid the mentioned problems of isPCR. Thin sections of solid tissue are fixed on microscope cover slides and cut with their support into small square-shaped fragments. Glass/tissue fragments are distributed into 96-well PCR reaction plates and amplified by quantitative real-time PCR. DNA copy numbers are plotted in a histogram representing the whole area of the tissue section, which can be viewed in different graphical output formats. Parallel layers of the same tissue section, eg, after cell-specific immunostaining, can be used to correlate the localization of PCR signals with anatomical compartments.

Using this technique, we have quantified the HIV-1 provirus DNA topographically along a thin section of a

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lymph node of an HIV-1-infected individual without antiretroviral therapy. The fractional and total amount of provirus DNA in different topographical areas of the lymph node was compared against the distribution of CD4⁺ cells as determined by immunostaining. HIV-1 provirus appeared to be strongly concentrated in the follicular compartment.

Materials and Methods

Frozen Sections

A lymph node was obtained from the axilla of a male patient 35 years of age with known HIV-1 infection who was admitted for initial antiretroviral therapy (ART). Plasma virus RNA concentration before ART was 8800 IU/ml. Frozen 4- μ m lymph node sections were obtained using a cryostat.

Fragmentation of Native Tissue Sections

Fragmentation and distribution of tissue sections were done as previously described.¹² In brief, a native frozen section was placed on a surface-treated cover slide (0.15 mm), and the slide was cut from the bottom side into a grid of 0.25-mm² squares with a guided diamond knife. The slide was then pressed onto an adherent plastic seal foil, the fragments were finally broken apart, and the foil was dilated isometrically into two directions. Each single fragment was picked from the dilated foil and transferred into a well of a microtiter PCR reaction plate. The position of each fragment within the whole lymph node section was recorded in a topogram as shown in Figure 3A.

Preparation of Cells Containing No HIV-1 Provirus

Vero cells were grown to subconfluence, trypsinized, pelleted, washed, and resuspended two times in phosphate-buffered saline. The suspension was quantified microscopically. DNA was extracted from the suspension using a DNA mini kit (Qiagen, Hilden, Germany) and tested using the real-time PCR protocol described below. No HIV-1 provirus DNA was detected.

Preparation of Single Cells Containing HIV-1 Provirus

ACH-2 cells, containing one copy of integrated HIV-1 provirus per cell,¹³ were cultured and prepared as single-cell suspensions. The suspension was diluted to a calculated concentration of two cells per 10 μ l in phosphate-buffered saline, and 10 μ l per well of the diluted suspension was distributed to Terazaki microtiter plates. The amount of cells per well was counted microscopically.

Immunostaining

The frozen sections were fixed first in acetone and then in chloroform (10 minutes each). Slides were immunostained according to the indirect immunoperoxidase method with diaminobenzidine development (Sigma, Munich, Germany), using the follicular dendritic cell (FDC)-specific monoclonal antibody R4/23 as primary antibody.¹⁴ Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Dianova, Hamburg, Germany) were chosen as secondary antibodies. Two secondary antibodies were applied to increase the intensity of staining. Before mounting, slides were counterstained in hemalum.

Amplification of HIV-1 Provirus DNA from Glass Slides by Real-Time PCR

Tissue fragments attached to 0.25-mm² glass slides were subjected to PCR amplification in a total reaction volume of 50 μ l without prior preparation of DNA. Reactions contained 1.25 U of FastStart TaqDNA polymerase (Roche, Mannheim, Germany), 10% (v/v) FastStart Taq reaction buffer concentrate, 2 mmol/L magnesium chloride, 200 μ mol/L of each dNTP, 400 nmol/L of primers LTR S4 (aagcctcaataaagcttgccttga, nucleotides 520 to 543 on HIV-1 strain HXB2 genome; GenBank accession number NC_001802) and LTR AS3 (gttcgggcgcgcactgtag, nucleotides 647 to 629 on HXB2) each, and 100 nmol/L probe LTRP1 (tctggtaactagatccctcagacc, nucleotides 580 to 605 on HXB2). The probe was labeled with 6-carboxy-fluorescein at its 5' end and 6-carboxy-N,N,N',N'-tetramethylrhodamine at its 3' end. Thermal cycling in a 7700 SDS instrument (Applied Biosystems, Weiterstadt, Germany) involved preincubation at 95°C for 8 minutes to denature the tissue and liberate DNA, 10 cycles of 95°C for 15 seconds and 62°C for 40 seconds, followed by 40 cycles of 95°C for 15 seconds and 56°C for 40 seconds. Fluorescence was measured automatically at 495 nm at the 56°C step in each amplification cycle.

HIV-1 Provirus Quantification Standard

A 127-bp DNA fragment was amplified from cDNA complementary to HIV-1 subtype B strain NL4-3, cloned in *Escherichia coli* by means of a pCR 2.1-TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany), sequenced, and termed pLTRw.

Quantification of HIV-1 Provirus DNA on Glass Slide Fragments

Plasmid pLTRw was amplified in real-time PCR following the protocol given above at concentrations of 10,000, 1000, 100, and 10 copies per reaction. Three replicate reactions were conducted at each plasmid concentration. In addition to reagents and plasmids, each reaction

contained 1300 Vero cells as well as a 0.25-mm² microscope cover slide fragment to mimic the standard assay conditions (1300 cells were determined to represent the amount of tissue normally attached to a cut tissue/glass fragment). The threshold cycle (C_t) values, representing the real-time PCR cycle at which the fluorescent DNA probe signal could be distinguished from the background noise for the first time, were recorded for each reaction. The mean of three C_t values per plasmid concentration was plotted against the logarithmic input copy numbers of plasmid, and the resulting linear equation was determined by regression analysis using the Statgraphics 5.0 software package (Statistical Graphics, Jena, Germany). The equation was $\text{Log}_{10}\text{copies}(r) = 5.67 - 0.18 \times C_t(r)$, in which r is a given real-time PCR reaction. The provirus concentration in an unknown sample would be determined by inserting its C_t value into the formula.

Graphic Display of Provirus Density in Tissue Sections

Three 96-well plates were used to amplify all tissue/glass fragments derived from one lymph node section. After real-time PCR, C_t values for each well were copied into a Microsoft Excel V 7.0. spreadsheet in a composition representing the two-dimensional arrangement of cover slide fragments before cutting. All C_t values were then transformed into absolute DNA copy numbers using the equation given above. Copy numbers were plotted directly from the datasheet into a histogram using the "surface" mode of histogram wizard function in Excel.

Results

Primers and probes were selected within a conserved region of the 5'-long terminal repeat (LTR) element of HIV-1. To precisely determine the limit of detection of the optimized assay, realistic assay conditions were simulated and results were evaluated by statistical means. ACH-2 cells were diluted to the single-cell level, microscopically counted, and amplified in multiple replicate real-time PCR reactions. Each reaction was supplemented with 1300 Vero cells to simulate the expected background of cells present on a cover slide fragment (see below). Eleven of these reactions contained one single ACH-2 cell. Fifteen, five, three, and one reaction, respectively, contained two, three, four, and six cells. Twenty reactions with buffer only and 20 reactions with an average of 20 cells served as negative and positive controls, respectively. C_t values obtained in real-time PCR were recorded, and values less than 40 were counted as positive results. Qualitative results were subjected to Probit regression analysis as shown in Figure 1. An amount of 2.96 cells per reaction was determined to yield a detection probability of 95%. According to the Poisson distribution formula, this corresponded very closely to an 100% test efficiency [$P(a) = e^{-m}(m^a/a!)$], where P is the probability of a occurrences per test at an average of m

Figure 1. Probit analysis of the positivity rates in replicate PCR reactions containing microscopically counted ACH-2 cells (11 reactions containing one cell each, 15 reactions with two cells each, five with three, three with four, and one with six cells; controls: 20 reactions with 0 and 20 reactions with 20 cells). **x axis:** Cells per reaction. **y axis:** Expected proportion of positive results according to a Probit model (dose-response curve).

objects per volume unit: $m = 2.99$ if $P = 0.05$ and $a = 0$.]. We therefore assumed that our test was capable of detecting one HIV-1 provirus-containing cell per reaction.

To evaluate the precision of provirus quantification by real-time PCR, we determined the provirus copy number in end-point diluted and microscopically counted ACH-2 cell suspensions. In each of 29 suspensions containing one to six cells, the provirus DNA concentration was determined on the recorded C_t values. Figure 2 shows that the differences between absolute cell counts and provirus copies were close to zero, reflecting a good correlation between provirus quantification and microscopic cell counting.

To obtain information on the distribution and quantity of provirus DNA in histological material, a frozen section of an axillary lymph node of an HIV-1-infected individual was fragmented and analyzed with our method. Figure 3A shows a flow chart representation of the procedure as well as the data output formats obtained. Fragments ($17 \times 21 = 357$) were analyzed by PCR. As determined by comparison with a superimposed subjacent layer of the same thin section, 114 fragments did not carry tissue (compare Figure 3A). Amplification of these negative control fragments did not yield provirus signals, except in one fragment that was located directly adjacent to the border of the section (compare top right corner of stained section and histogram in Figure 3B). Of 243 fragments containing tissue, 98 yielded a provirus PCR signal. The C_t values of these signals were used to calculate the provirus copy numbers per glass fragment. Copy numbers per fragment ranged from 0.5 to 40.2 copies.

To demonstrate the reliability of topographic real-time PCR, the whole procedure including staining, fractionation, and real-time PCR was repeated after 3 months of

Figure 2. Differences between microscopically determined cell count and PCR-quantified provirus content in 29 cell suspensions containing one to six single ACH-2 cells each. The mean difference ($n_{\text{cells}} - n_{\text{provirus}}$) was 0.626.

storage at -70°C with a different section layer of the same lymph node. Essentially the same topographic distribution of real-time PCR signals, again adhering to the location of germinal centers, was observed (Figure 4). This time, the determined copy numbers per glass fragment ranged from 0.5 to 33.1.

Microscopic examination of a section layer before final cutting yielded an average cell content of 2600 cells per 0.25 mm^2 glass fragment. Because lymphocytes with a diameter of $10\text{ }\mu\text{m}$ represent the main cell type in a lymph node, about one-half of their cytoplasmic and nuclear material would be present in a frozen section of $5\text{-}\mu\text{m}$ thickness, or the equivalent of 1300 cells. With the determined provirus copy numbers, between 0.04% (0.5/1300) and 3.1% (40.2/1300) of cells thus contained provirus. As Figure 3 shows, high provirus density was clearly associated with areas of high FDC content and hence with lymphatic follicles. The provirus copy numbers in three large follicular areas (compare Figure 3B, areas marked with an "x") ranged at an average of 9.9 copies per 1300 cells or a density of provirus-carrying cells of 0.8%.

Discussion

In this report, we describe a simple method for quantitative and topographic detection of HIV-1 provirus DNA in lymph node sections. Fragmenting tissue along with a supporting glass cover slide is a proven method for producing micro immunofluorescence slides, eg, for automated autoantibody diagnostics.¹² In our setting, it min-

imizes the risk of carry over of DNA because mechanic manipulation occurs only on the backside of the slide, which does not carry tissue. Real-time PCR amplification has been used numerous times to detect DNA and accurately quantify the target gene at the same time.¹⁵⁻¹⁷ Combining both methods facilitates the determination of target gene contents in defined topographic units of a tissue section. Fragmenting, distribution of glass slides to reaction vessels, and real-time PCR can be accomplished altogether within 1 working day, with the distribution of glass fragments being the most labor-intensive step (about 30 minutes). Automating this step by use of a robotic pipetting platform is currently under way.

To determine the detection limit of our assay, we had to apply an experimental model that included microscopic determination of the average number of cells present on each glass slide. This background of cells was then represented in sensitivity tests by counted culture cells. Although the model did only provide an indirect measure of sensitivity, its outcome was in good concordance with results from quantifying single cells (see below). The determined detection limit was extraordinarily low, with one copy of provirus DNA being detectable in a background of 1300 cells. It can thus be assumed that the small amount of tissue introduced into PCR in our procedure generally does not interfere with amplification. Single-molecule sensitivity is required in settings in which the complete elimination of a genomic marker, eg, in HIV-1 provirus, has to be proven.^{4,5,8} Because formaline fixation used in current *in situ* PCR procedures is well known to interfere with the amplification efficiency of PCR,¹¹ such a high sensitivity is difficult to achieve by *in situ* PCR.

The good accuracy of our quantification method has been demonstrated by comparing the microscopic cell count against the PCR-determined provirus content in suspensions of cells that contain one provirus per genome. Even in a very low concentration range of one to six cells per reaction, the average deviation of the two procedures was less than one cell. We could thus determine the absolute provirus count per glass/tissue fragment or a provirus density in terms of a fraction of cells containing provirus for each topographic unit.

Protection against carry-over contamination was demonstrated by co-processing and co-testing of 114 glass fragments surrounding the fixed lymph node section, containing no tissue. Only one of these negative fragments yielded a low virus signal, and this fragment was located directly next to the border of the section adjacent to a follicular area. This signal is likely to be associated with a slightly different shape of the lymph node in the parallel section layer actually analyzed.

Comparison of the chief localization of provirus signals within lymph node compartments showed that provirus was clearly associated with follicular areas. Three large areas of follicular tissue yielded an average of 0.8% of provirus-carrying cells, with a maximum of 3.1%. We know from fluorescence-activated cell sorting experiments that HIV provirus DNA in lymph nodes is almost exclusively found in the CD4^{+} cells.¹⁸ These cells can account for up to 80% of cells outside the follicular area

Figure 3. A: A frozen section of a lymph node (**top left**) was cut into square fragments of 0.5-mm edge length (**middle**) and distributed automatically to the wells of a 96-position PCR reaction plate. The resulting C_t values for each reaction were plotted into an Excel spreadsheet according to the position of the tissue/glass fragment of each reaction within the section. C_t values were transformed into absolute provirus copy numbers using the calibration curve formula, and copy numbers were plotted into a surface histogram (**bottom**). A parallel section layer of the same lymph node was immunostained with a monoclonal antibody against human follicular dendritic cells (FDC) to identify follicular regions (**top right**), and the two section layers were compared. **B:** Comparison of localization of PCR signals and FDC (**top**). Areas marked with an "x" have been used to determine an average provirus load in three FDC-rich areas (refer to text). Display of the absolute provirus quantity as determined by real-time PCR in each tissue/glass fragment (**bottom**; z-scale gives number of provirus molecules per 1300 cells present on one glass fragment).

but contribute only about 10% to the cell population of follicles where B cells predominate. Provirus would therefore be present in an average of 8% of follicular $CD4^+$

cells, with a maximum of 38%. These results are in good agreement with earlier findings in which follicular $CD4^+$ cells exhibiting activation markers ($CD57^+$) have been

Figure 4. The same analysis as shown in Figure 3 was repeated after 3 months of storage of the lymph node at -70°C . **A:** Stained section parallel to that used for analysis. **B:** Surface histogram. **C:** Three-dimensional display of provirus concentrations in glass fragments.

analyzed. Here, an up to 10-fold higher frequency of infected cells was found in the $\text{CD57}+\text{CD4}^{+}$ germinal center T cells compared with $\text{CD57}-\text{CD4}^{+}$ T cells. About 10% of the $\text{CD57}+\text{CD4}^{+}$ T cells had provirus, which correlates well with our calculation.¹⁸

The combination of tissue fragmentation on glass support and real-time PCR provides an uncomplicated way of topographically detecting target genes. The approach eliminates low sensitivity, DNA leakage, and nonspecific incorporation of labeled nucleotides, which are all associated with common *in situ* PCR methods.^{6–10} Due to the possibility of using real-time PCR detection, the method also quantifies the target DNA with high accuracy. The only relevant drawback of our procedure is its low topographic resolution. In its current format, it only displays the topographic association of DNA species with functional compartments of organs. A resolution of 0.25 mm^2 is not sufficient for analyzing single cells. Because in our current approach, slides have to be cut mechanically and distributed into PCR reaction vessels, a certain minimum size of fragments is required to facilitate manipulation. A much better resolution will therefore require a different principle of fractionation. Future modifications of the procedure will involve direct separation of tissue material by pressing it into a miniaturized grid of PCR reaction vials, eg, a gridded microscope slide. Real-time PCR could then be performed and fluorescence could be monitored on-site, eg, by a temperature controlled microarray fluorimeter.

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