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Human Immunodeficiency Viral DNA is Readily Found in Lymph Node Biopsies from Seropositive Individuals

Analysis of Fixed Tissue Using the Polymerase Chain Reaction

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Human immunodeficiency virus (HIV) DNA was detected in formalin-fixed, paraffin-embedded lymph node biopsies after* in vitro *DNA amplification by the polymerase chain reaction. Twenty-three of 25 biopsies from HIV seropositive individuals were positive for HIV DNA including 11 with follicular hyperplasia, six with follicular involution, two who were partially involved with Kaposi's sarcoma, one with granulomatous lymphadenitis, and three with non-Hodgkin's lymphoma. The remaining two biopsies from seropositive individuals lacking detectable HIV DNA also contained non-Hodgkin's lymphomas. An average of 0.0001 to 0.01 HIV DNA copies per cell was estimated to be present in biopsies with follicular hyperplasia or involution. The positive lymphoma biopsies contained approximately tenfold fewer HIV DNA. In contrast, 19 of 20 biopsies from seronegative or low risk individuals were negative for HIV DNA. The sole exception was a seronegative individual with chronic adenopathy from follicular hyperplasia and a history of prostitute contact. The studies demonstrated a high prevalence of HIV DNA in non-lymphomatous lymph node biopsies from HIV infected individuals. (Am J Pathol 1989, 135: 697–702)

Infection by human immunodeficiency virus (HIV) can result in lymphadenopathy.[1] This lymphadenopathy is often the result of follicular hyperplasia. Immunohistochemical and *in situ* hybridization techniques have detected HIV RNA[2,3] and antigens[4,5] in most such lymph nodes from HIV-infected individuals, although the frequency of cells expressing HIV RNA was very low (<0.00001 to 0.0001). Detection of HIV proviral DNA, the genomic template for HIV RNA synthesis, has been less successful. Only seven of 34 lymph nodes from HIV-infected individuals had HIV DNA detectable by Southern blot analysis.[6]

To improve the sensitivity of HIV DNA detection and further characterize the amounts of HIV provirus present in lymph nodes, therefore, we used *in vitro* DNA amplification of HIV-specific DNA sequences by the polymerase chain reaction (PCR).[7–12] To facilitate histologic correlations with the presence of HIV provirus, we used DNA prepared from formalin-fixed, paraffin-embedded, 10-μm lymph node sections as the HIV PCR substrate.[13] The PCR technique demonstrated HIV DNA in most lymph nodes from HIV-infected individuals.

Materials and Methods

Chart review allowed classification of the 48 patients examined into seropositive (25 patients), seronegative (nine patients), and serology not tested (14 patients) groups. The 14 patients not tested for HIV serology were further classified into a high-risk group (three patients with histories of homosexuality or intravenous drug abuse) and a low-risk group (11 patients). HIV serology was tested with a commercial ELISA assay (Abbott Laboratories, Chicago, Il).

Lymph node biopsies from the 48 patients were sliced to 2- to 3-mm thick sections and a portion placed in 10%

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neutral-buffered formalin. After overnight fixation, the sections were routinely processed and embedded in paraffin. The paraffin-embedded tissue (from 1986 to 1988) was cut on a microtome into 10-μm thick sections. One section was mounted and stained with hematoxylin and eosin (H&E) for histology. Standard criteria were used for histologic diagnosis with follicular hyperplasia and involution classified according to Ewing et al.[1] Follicular hyperplasia corresponds to the type I pattern with follicular and paracortical expansion, and involution corresponds to the type II pattern with absent to atrophic germinal centers. Another 10-μm section was processed for the PCR as previously described.[13] However, certain modifications were necessary. The section was deparaffinized with one wash of xylene and then two sequential washes with absolute ethanol. The section was desiccated (1 to 2 hours), yielding a white tissue pellet. Then 50 μl of digestion buffer (100 mM Tris HCl, 5 mM EDTA, pH 8.0) with 400 μg per ml of Proteinase K were added, and the mixture was incubated at 37 C overnight. The mixture was boiled for 7 minutes to inactivate the Proteinase K and centrifuged to pellet the residual tissue. Five μl or less of the tissue supernatant was used as the PCR substrate.

The PCR was performed as previously described[9] using a heat stable DNA polymerase (TAQ, Perkin-Elmer-Cetus, Norwalk, CT). Briefly, amplification primers for the HIV gag gene (SK38, SK 39[9]) and primers for a sequence expected in all human cells (either b-globin[14] or low density lipoprotein (LDL) receptor[15] were present during 50 PCR cycles. Only HIV DNA (and not RNA) would be detected with the assay. The human genomic primers served as a control for factors inhibitory to the PCR and for comparison of the relative amounts of human genomic to HIV DNA extracted from the tissue. For example, one lymph node biopsy was not included in the study because it did not give PCR amplification signals for HIV or human genomic sequences. Large amounts of DNA could be extracted from the nodal tissues. Often only 1 μl of the 50-μl tissue supernatant could be used for the PCR as greater amounts completely inhibited the amplification reaction. The reaction volume was 100 μl, and each primer was present at 50 pmol per reaction. Temperatures were 95 C during denaturation (30 seconds), 42 C during annealing (15 seconds), and 72 C during polymerization (60 seconds) on an automated thermal cycler (Perkin-Elmer-Cetus). Ten to 20 μl of the reaction products were placed on two separate dot blots and detected with ^{32}P-labeled oligomers specific for HIV or for human genomic DNA (LDL receptor or b-globin gene) as previously described.[13] Filters (Genetrans 45, Plasco, Woburn, MA) containing amplified HIV DNA were washed with 1× SSPE with 0.1% SDS twice at room temperature, and then in 2× SSPE with 0.1% SDS at 60 C for 10 minutes. Filters containing amplified human genomic DNA were washed twice with 2× SSPE with 0.1% SDS at room temperature and then in 5× SSPE with 0.1% SDS at 60 C for 10 minutes. Autoradiography (12 to 72 hours) was performed with Kodak X-AR film at −70 C with two intensifying screens. A positive control consisting of DNA purified from an HIV-infected cell culture was amplified by the PCR with each experiment. Water and an uninfected cell line (HeLa) served as negative controls.

The ratio of HIV DNA to cells was estimated by comparing the differential loss of PCR positivity of HIV *versus* genomic (LDL receptor or b-globin) sequences on serial dilutions of the DNA extracted from the paraffin sections (see below). Another PCR method to estimate the number of original HIV target sequences is to compare the relative amounts of PCR amplification products with known original standards.[10,12]

Results

HIV DNA was detected in virtually all the lymph node biopsies from seropositive patients (23 of 25) or at high risk for HIV infection (three of three). HIV DNA was present in all the lymph nodes with follicular hyperplasia or the involutional phases of adenopathy often associated with HIV infection. Two lymph nodes partially involved with Kaposi's sarcoma (70% and 95%) and one showing granulomatous lymphadenitis were also positive for HIV DNA. The two lymph nodes without detectable HIV DNA contained diffuse non-Hodgkin's lymphomas (immunoblastic, plasmacytoid, and large noncleaved cell), although three other diffuse non-Hodgkin's lymphomas (small noncleaved; two non-Burkitt's and one Burkitt's) were positive for HIV DNA. The results are summarized in Table 1 with representative results shown in Figure 1.

In contrast, HIV DNA was detected in only one seronegative patient. This patient had a history of prostitute contact and diffuse adenopathy for 8 months that showed follicular hyperplasia. Nineteen other low risk or seronegative patients did not have detectable HIV DNA in their lymph nodes.

The HIV PCR amplification autoradiographic signals were very strong suggesting the presence of large numbers of HIV DNA copies. To estimate the amounts of HIV DNA present in the sections, serial dilutions of the DNA extracted from 16 lymph nodes were performed before the PCR. As expected, the HIV signals decreased on dilution (Figure 2). One exception was with lymph node 2 in Figure 2A. In this instance, a less diluted specimen (1/50) was negative for HIV, whereas the more diluted specimen (1/500) was positive. This undoubtedly represented a mix-up during the dot blot procedure as a repeat experiment was positive for HIV at 1/50 and negative at 1/500. The experiments were repeated for several lymph nodes

Table 1. *HIV DNA Compared with Histology and HIV Status*

	Total	HIV PCR+	HIV PCR−
HIV seropositive			
FH	11	11 (1)	0
Involutional	6	6 (1)	0
Lymphoma	5	3 (3)	2 (1)
Kaposi's sarcoma	2	2 (2)	0
Granulomatous	1	1 (1)	0
Total	25	23 (8)	2 (1)
HIV seronegative			
FH	6	1	5
Lymphoma	1	0	1
Normal	2	0	2
Total	9	1	8
HIV serology not tested			
High risk group*			
FH	3	3	0
Low risk group			
FH	4	0	4
Lymphoma	5	0	5
Normal	2	0	2

* The high risk group includes patients with histories of homosexuality, intravenous drug abuse, or hemophilia.

Numbers in parentheses indicate the number of patients with AIDS. FH, follicular hyperplasia.

(1, 2, 6, 9, 11, and 16 in Figure 3) because the signals from the last positive dilution were weak. The results of these repeat experiments were identical except for some cases for which the presence or absence of a signal in the last (highest) positive dilution was not consistent between the trials. This kind of variation between experiments for the highest dilution samples is expected if in fact the average number of HIV molecules approaches one per tube because of the expected Poisson distribution.

Human genomic sequences were detected at greater dilutions than was the case for HIV sequences. In fact, human genomic sequences were generally detectable until the DNA from fewer than five cells (estimated visually from the stained sections at approximately 640,000 cells/cm^2/10-μm section and assuming 100% DNA extraction) was present. The ratios of HIV DNA copies to cells were estimated by assuming ideal conditions; ie, that a single HIV DNA copy could be detected and that 100% of the DNA was extracted from each section. These assumptions are likely to be in error. However, these errors (PCR less sensitive than expected and not all DNA extracted from the paraffin section) would underestimate the ratio of HIV DNA to cells visually present per lymph node section. The calculated ratios are presented in Figure 3.

The ratios of HIV DNA to cells per lymph node section were similar for the nodes with follicular hyperplasia or involution (0.0001 to 0.01 HIV copies per cell) and smaller for the nodes with lymphoma (0.00001 to 0.001 HIV copies per cell). The amounts of HIV DNA were similar between the seronegative (lymph node 1 in Figure 3) and seropositive nodes with follicular hyperplasia.

Discussion

HIV infects CD4-positive cells such as T helper lymphocytes and monocytes.[16,17] The total number of HIV DNA genomes present in lymph nodes and blood is small because HIV DNA is observed only in the minority of lymph node and blood specimens from HIV seropositive patients using the Southern blot technique.[6] However, using the PCR technique, HIV DNA was detected in most peripheral blood mononuclear cells from HIV-seropositive patients,[9,10] as well as high risk seronegative patients.[11] Similarly, in this study 92% (23/25) of seropositive patients had HIV detected in their lymph node biopsies.

Fixed lymph node biopsies were used because such specimens allow correlation with histology and are commonly stored. Because most of these biopsies were quite old, blood specimens for these patients were not available. A hybridization dot blot assay was chosen because it facilitates processing of multiple samples. In addition, specificity of PCR is improved with a hybridization step.[12] With our PCR assay conditions, HIV-specific bands were generally not visible after electrophoresis and ethidium bromide staining, but the expected 114 base pair product

Figure 1. *Typical autoradiograph (12 hours) of PCR reaction products. A, normal lymph node, low-risk group; B, follicular hyperplasia, low-risk group; C, follicular hyperplasia, HIV seropositive; D, Kaposi's sarcoma (70% involved), AIDS; E and F, lymphoma, low-risk group; G, DNA purified from HIV infected cell culture; H, water blank. An identical dot blot hybridized for b-globin, which was simultaneously amplified during the PCR, was positive for all the samples except for H, the water blank (not shown).*

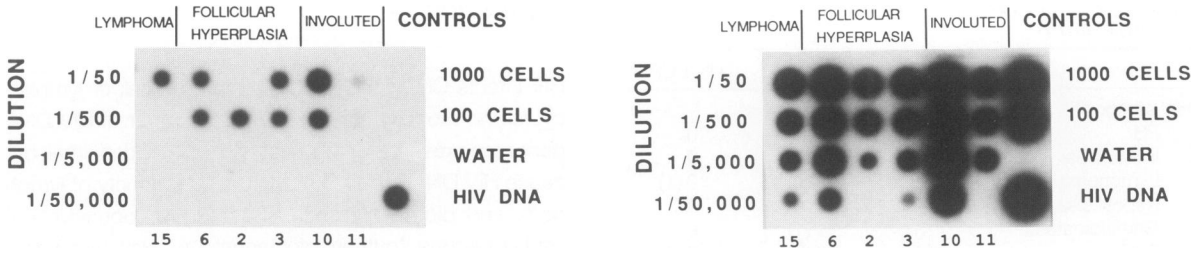

Figure 2. *Serial dilutions of HIV DNA-positive lymph nodes. The bottom numbers refer to lymph nodes as numbered in Figure 3. The PCR reaction was performed with primers for HIV and the LDL receptor genomic sequence (present in all cells) simultaneously present. The reaction products were placed on two separate filters and hybridized to an HIV-specific probe* **(left)** *or LDL receptor (genomic) specific probes* **(right)**. *Note that LDL* **(right)** *positivity was maintained at greater dilutions compared with HIV positivity* **(left)**. *The estimated number of genomes (assuming 100% DNA extraction from each tissue slice) present in the 1/50,000 dilution row is, from left to right, 7, 7, 4, 5, 10, and 4. For comparison, the amplification products from 1000 and 100 uninfected boiled cells are shown in the control column. The node (2) with follicular hyperplasia negative for HIV at 1/50 but positive at 1/500 was positive only at 1/50 when the experiment was repeated (see Results).*

band could be detected after Southern blot analysis (not shown).

The high sensitivity of the PCR requires precautions to prevent false-positive reactions due to contamination. In this study, multiple lymph nodes from seronegative or seropositive patients were studied and the PCR results correlated with the serologic data. The sole example of a possible false-positive reaction was the seronegative patient with follicular hyperplasia. In this instance, the PCR was repeated with the same results from new sections from different tissue blocks. Because this patient is no longer available for follow-up, we cannot conclusively distinguish between a false-positive PCR assay or a false-negative antibody test. In addition, the DNA extracted from this and other positive specimens could be diluted and still retain positivity. Blanks consisting of no added sample were included with every experiment and were consistently negative for HIV and the control genomic sequences.

Immunohistochemical studies for HIV antigens and *in situ* hybridization with HIV RNA as a target showed evidence for HIV in most lymph nodes showing follicular hyperplasia from HIV-infected patients.[2-5] The numbers of cells involved, however, were low and the evidence of HIV infection was diminished in lymph nodes showing the terminal or involutional stages of HIV-associated adenopathy. In contrast, in this study HIV DNA was as prevalent

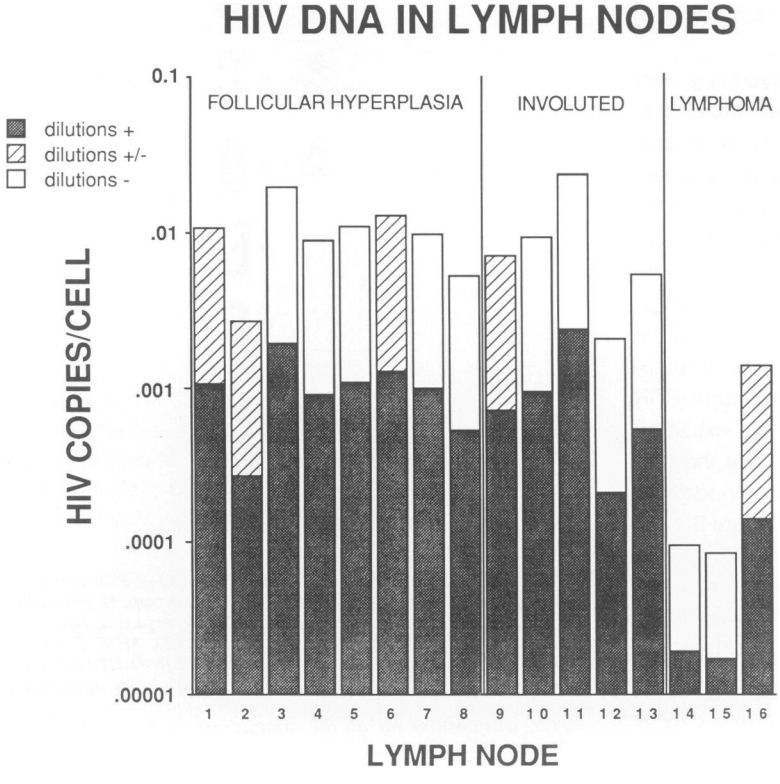

Figure 3. *The DNA extracted from the lymph nodes was serially diluted before the PCR (see Figure 2). The HIV PCR remained positive (dark bar) at early dilutions. By determining the dilutions at which the HIV PCR positivity disappeared (clear bar) or was not consistently positive on duplicate experiments (cross-hatched bar), the ratio of HIV DNA to cells visually present in each section was estimated. Lymph node number 1 was from the seronegative, HIV DNA-positive patient with adenopathy.*

in the involutional stages as in the follicular stages, and the ratios of HIV DNA to cells per section were similar.

Dilution studies demonstrated approximately 0.0001 to 0.01 HIV DNA copies per cell, which, as discussed in the results, may be an underestimate. Approximately half of the specimens contained more than 0.001 HIV DNA copies per cell (Figure 3). Our data are consistent with the data obtained by Southern blot analysis that estimated the HIV DNA content of lymph node specimens to be fewer than one copy per ten cells.[6] The relatively poor sensitivity of the Southern blot analysis, however, did not allow a more detailed estimate and, in addition, could not detect HIV DNA from most of the lymph node specimens.

Because multiple HIV DNA copies may be present in an infected cell,[6] the proportion of infected lymphoid cells cannot be accurately estimated. If, however, there is one HIV proviral copy per infected cell, 60 to 6000 HIV-infected cells per square centimeter of lymph node section would be expected. The number of cells expressing measurable HIV RNA was approximately 1 to 10 per lymph node section.[3] It seems likely that some infected cells may not express detectable HIV RNA. Consistent with this idea is that HIV DNA was detected with greater frequency (94%) compared with HIV RNA (64%) in peripheral blood specimens using the PCR.[10] Therapies against HIV must control not only the small numbers of cells expressing HIV RNA but also infected cells containing transcriptionally "silent" proviral DNA.

This high prevalence of HIV DNA in lymph nodes regardless of histology demonstrates that HIV-infected cells are not exclusively associated with the follicular hyperplasia commonly present in seropositive patients. HIV DNA was detected in lymph nodes showing histologies not usually directly associated with HIV infections. HIV DNA was detected in two of two nodes with Kaposi's sarcoma and in three of five nodes with lymphoma. Because the neoplastic cells of Kaposi's sarcoma[6] or AIDS-related lymphoma[18–20] are not infected with HIV, the associated lymphocytes and monocytes were most likely the cells harboring HIV. The small numbers of CD4 cells present in lymphomas may have precluded their detection in the two HIV DNA negative lymphomas. The ratios of HIV DNA to cells per HIV-positive lymphoma node section were approximately tenfold lower compared with follicular or involuted histologies. The average presence of 0.00001 to 0.001 HIV DNA copies per lymphoma cell makes infection of the tested high-grade lymphoma cells unlikely.

The one seronegative patient with a positive PCR analysis had a risk factor of prostitute contact, and his lymph node biopsy showed follicular hyperplasia. He had chronic diffuse adenopathy for 8 months with weight loss. His lymph node contained HIV DNA in amounts similar to those found in seropositive individuals with follicular hyperplasia. Although as discussed above this individual might have been a false-positive by the PCR test, he appears to be similar to other seronegative patients with latent HIV infections documented by culture,[21,22] PCR,[11] or antigen, antibody[23] assays.

In conclusion, we demonstrated the presence of HIV DNA in most lymph nodes from HIV-infected patients. The ease with which DNA sequences are detected using PCR in commonly processed tissues, even that up to 40 years old,[24] should facilitate both prospective and retrospective analysis of this epidemic.

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Note Added in Proof

Since this paper was accepted, an article was published (Schnittman SM, Psallidopoulos MC, Lane HC, Thompson L, Baseler M, Massari F, Fox CH, Salzman NP, Fauci AS. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science 1989, 245:305–308) that demonstrated by PCR that the frequency of HIV-1 infected peripheral blood CD4+ T cells was at least 1/100 cells in AIDS patients.