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Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by *in situ* hybridization

(viral RNA/expression/^{35}S-labeled RNA probe)

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ABSTRACT **By using *in situ* hybridization methodology, we have directly examined primary lymph node and peripheral blood from patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex for the presence of human T-lymphotropic virus type III (HTLV-III) viral RNA. Mononuclear cell preparations were hybridized with a ^{35}S-labeled HTLV-III-specific RNA probe and exposed to autoradiographic emulsion for 2 days. HTLV-III-infected cells expressing viral RNA were detected in ≈86% (6/7) of lymph node and 50% (7/14) of peripheral blood samples studied. However, in all patient samples examined, labeled cells were observed at very low frequency (<0.01% of total mononuclear cells). The HTLV-III-infected cells exhibited morphological characteristics consistent with that of lymphocytes and expressed viral RNA at relatively low abundance (20–300 copies per cell). These results demonstrate that HTLV-III expression in lymph node and peripheral blood is very low *in vivo*. Furthermore, the lymph node hyperplasia observed in HTLV-III-associated lymphadenopathy is not directly due to proliferation of HTLV-III-infected lymphocytes.**

The acquired immunodeficiency syndrome (AIDS) presents a severe unexplained immune deficiency that involves reduction in the number of helper T lymphocytes (OKT4) (1–3). The disease is usually accompanied by multiple opportunistic infections and/or malignancies, the latter predominantly of the Kaposi sarcoma type (4). AIDS-related complex (ARC) encompasses milder forms and sometimes prodromal states of the disease, and it is characterized by other clinical manifestations, most frequently unexplained chronic lymphadenopathy or leukopenia involving helper T lymphocytes (1–4). Recent serologic and viral isolation studies have shown that the development of AIDS or ARC is due to infection with the human retrovirus, human T-cell lymphotropic virus type III (HTLV-III) (5–8). HTLV-III, a cytopathic virus, is included in the HTLV family because of multiple biological and structural properties in common with HTLV types I and II (reviewed in ref. 9), including: (*i*) tropism for lymphocytes; (*ii*) particular tropism for OKT4 helper T lymphocytes; (*iii*) magnesium-dependent reverse transcriptase of high molecular weight; (*iv*) induction of giant multinucleated cells in culture; (*v*) impairment of T-cell functions; (*vi*) immunological crossreactivity of some virally encoded proteins; (*vii*) double-spliced 3′ terminal mRNA; (*viii*) relatively small major core protein (p24/p25); (*ix*) unique juxtaposition of p24/p25 to the NH_2-terminal gag protein—i.e., absence of the *gag* gene-encoded phosphoprotein in this position; and (*x*) *trans*-acting transcriptional activity resulting from a specific protein encoded by a viral gene.

By using cloned HTLV-III probes (10, 11), analysis of fresh tissue from AIDS and ARC patients was carried out for detection of viral sequences. Southern blot experiments detected HTLV-III viral DNA at low levels in fresh lymphoid tissue from a number, albeit a minority, of patients with AIDS or ARC (12). At the same time, *in situ* hybridization experiments were initiated to detect HTLV-III viral RNA in primary tissue samples from AIDS or ARC patients. *In situ* hybridization offered several advantages as a detection method since it is direct, quantitative, and requires small tissue samples. Furthermore, *in situ* hybridization could be used to screen a wide variety of tissues for the presence of virus and could give information regarding the type(s) of cells infected with HTLV-III, as well as distribution of cells expressing viral sequences within a particular tissue.

In this paper, we directly demonstrate the presence of HTLV-III-expressing lymphocytes in primary lymph nodes and peripheral blood from AIDS and ARC patients using a highly sensitive *in situ* hybridization method that makes use of high specific activity RNA probes. HTLV-III-infected cells are present in both types of lymphoid tissue at very low frequency (<0.01% of mononuclear cells) and therefore cannot comprise the lymph node hyperplasia in HTLV-III-associated lymphadenopathy.

MATERIALS AND METHODS

Preparation of Cells. Fresh biopsy samples of lymph nodes from AIDS or ARC patients were minced and teased over sterile stainless steel screens, and mononuclear cell suspensions were obtained by centrifugation through lymphocyte separation medium (Litton Bionetics). Cells were rinsed twice with RPMI 1640 medium and resuspended at 10^6 cells per ml in medium containing 10% fetal calf serum. Cells were cytocentrifuged onto precleaned microscope slides, air-dried for 5 min, and then fixed according to Lawrence and Singer (13). For this, slides were immersed in 4% paraformaldehyde in phosphate-buffered saline for 1 min and then transferred to 70% ethanol. Preparations were stored at 4°C until used for hybridization.

Heparinized peripheral blood samples obtained from AIDS or ARC patients or normal individuals were centrifuged through lymphocyte separation medium and the mononuclear cells were resuspended, cytocentrifuged, fixed, and stored as described for lymph node cell suspensions.

Cell lines H9 and infected H9/HTLV-IIIB (5) were suspended in medium containing fetal calf serum, cytocentrifuged, fixed, and stored as described for lymph node cell suspensions.

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Abbreviations: HTLV, human T-lymphotropic virus; AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex; LTR, long terminal repeat; kb, kilobase(s).

Preparation of ^{35}S-Labeled RNA Probes. pBH10-R3 (designated R3) consists of the 9-kb HTLV-III insert of clone BH10 (11) inserted 3′ to 5′ in transcription vector pSP64 (Promega Biotec, Madison, WI) (constructed in our laboratory by Steven Josephs and Bruno Starcich). R3 DNA was digested with *Eco*RI, which cuts midway in the viral genome (11). One microgram of R3 template DNA was transcribed as suggested (Promega Biotec), except the reaction included 25 μM [^{35}S]UTP (1000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear), and 15 units of SP6 polymerase (New England Nuclear). After incubation at 40°C for 30 min, 15 additional units of SP6 polymerase was added, and unlabeled UTP was added to a final concentration of 500 μM (14). The reaction was allowed to continue for an additional 30 min at 40°C. The DNA template was then digested with DNase I and the ^{35}S-labeled RNA was purified (14). Typically, >80% of [^{35}S]UTP was incorporated; ^{35}S-labeled RNA exhibited a specific activity of 10^9 dpm/μg. Purified RNA was stored in aliquots at −70°C until used.

^{35}S-labeled control probe was transcribed from a transcription control template (Promega Biotec), which generates RNA molecules specific for bacteriophage λ. Transcription and RNA purification were carried out as described above.

^{35}S-labeled RNA transcripts were sized by electrophoresis through formamide/formaldehyde denaturing gels (15). ^{35}S-labeled RNA probes were typically found to be 1–2 kb long.

***In Situ* Hybridization.** Slide preparations were rinsed briefly in 2× SSC (0.3 M NaCl/0.03 M Na citrate) and acetylated in acetic anhydride/triethanolamine, pH 8.0 (16). Slides were rinsed briefly in 2× SSC and immersed in 0.1 M Tris·HCl, pH 7.0/0.1 M glycine for 30 min (13). Slides were rinsed in 2× SSC and then dehydrated in ethanol. Hybridization mixture contained ^{35}S-labeled RNA probe (10^8 dpm/ml; 100 ng/ml), 50% formamide, 2× SSC, 10 mM dithiothreitol, sheared salmon sperm DNA (1 mg/ml), *Escherichia coli* tRNA (1 mg/ml), and bovine serum albumin (2 mg/ml). The mixture was heated at 90°C for 10 min and then placed at 55°C. Hybridization mix was applied, coverslips were mounted and sealed with rubber cement, and hybridization was carried out at 50°C as suggested by Cox *et al.* (17) for 3 hr. Slides were then rinsed thoroughly in 50% formamide/2× SSC at 52°C, followed by several rinses in 2× SSC. Ribonuclease treatment with RNase A (100 μg/ml) (Sigma), RNase T1 (1 μg/ml) (Boehringer Mannheim) in 2× SSC was carried out for 30 min at 37°C. Slides were again rinsed in 50% formamide/2× SSC at 52°C, then rinsed in 2× SSC, and dehydrated in ethanol. Hybridized preparations were autoradiographed with NTB2 nuclear track emulsion (Eastman) diluted 1:1 with distilled water. After exposure for 2 days at 4°C, slides were developed with Dektol (18), dried, and stained with Wright's stain (Harleco). For each sample hybridized with the HTLV-III probe or control probe, analysis of 4–10 × 10^5 cells was carried out.

RNA Blot Analysis of H9/HTLV-IIIB Cells. Poly(A)$^+$ RNA isolated from H9/HTLV-IIIB cells was blotted (0.5–10 μg per lane) and hybridized with ^{32}P-labeled BH10 insert DNA. Standard lanes consisted of HTLV-III RNA synthesized by transcription of clone R3 and loaded in the presence of 5 μg of total Molt-4 cell RNA at concentrations corresponding to 1, 5, 10, 50, 100, or 500 copies per cell.

RESULTS

Specificity of *in Situ* Hybridization for HTLV-III RNA. Specificity of the *in situ* hybridization method was shown by use of the T-cell line H9, a neoplastic aneuploid line significantly resistant to the cytopathic effects of HTLV-III (5). Cells from the uninfected H9 cell line exhibited essentially no grains when hybridized with the HTLV-III probe R3 (Fig. 1*A*). In contrast, highly significant labeling was observed over H9/HTLV-IIIB cells, a clone of H9 cells infected with HTLV-III and shown to be an active viral producer (5), when hybridized with the R3 probe. As shown in Fig. 1*B*, essentially 100% of cells in this line were highly labeled after 2 days of autoradiographic exposure. By microscopy, 50–200 grains per cell were observed on individual cells, while 200–500 grains were observed on each large multinucleated cell, a characteristic cell type observed after HTLV-III infection (5). However, when the H9/HTLV-IIIB cells were hybridized with the bacteriophage λ-specific control probe, again no

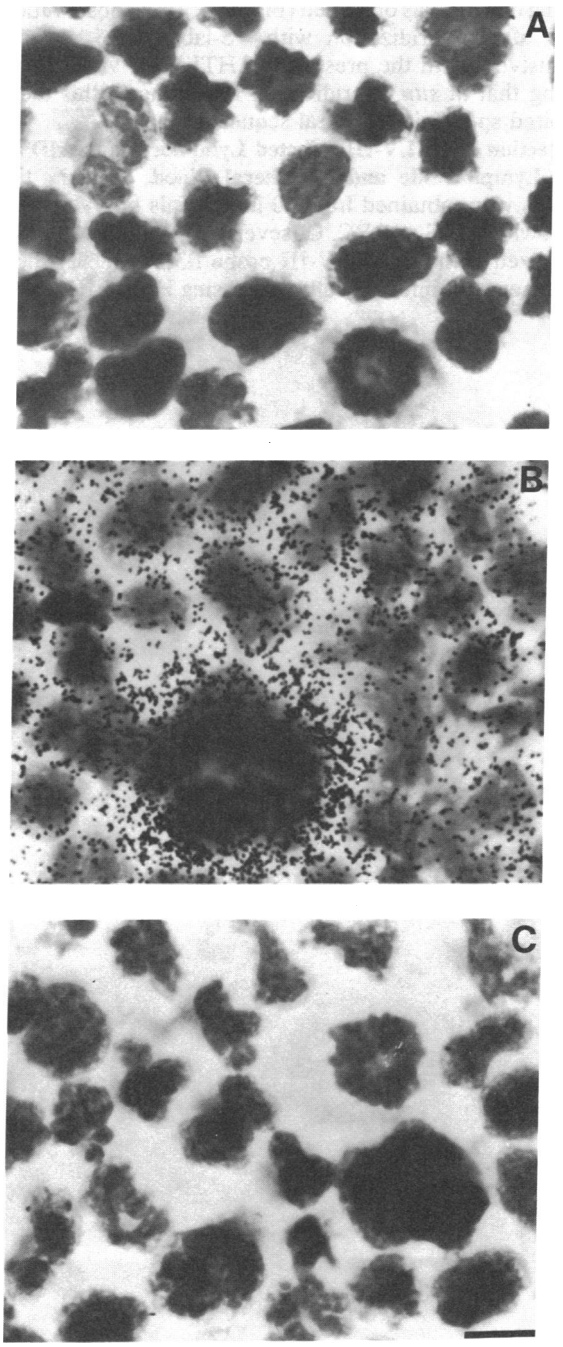

FIG. 1. Specificity and sensitivity of *in situ* hybridization of the HTLV-III probe to uninfected and infected H9 cell lines. (*A*) Uninfected H9 cells hybridized with HTLV-III probe. (*B*) H9/HTLV-IIIB cells, which are active viral producers, hybridized with HTLV-III probe. Note that since grains are positioned at various levels within the emulsion, some grains are not observed or appear out of focus in photograph. (*C*) H9/HTLV-IIIB cells hybridized with bacteriophage λ-specific control probe. (Bar = 10 μm.)

labeling of cells was observed (Fig. 1*C*). Thus, observation of grains after hybridization with ^{35}S-labeled R3 correlated conclusively with the presence of HTLV-III virus, demonstrating that *in situ* hybridization according to this method exhibited specificity for viral sequences.

Detection of HTLV-III-Infected Lymphocytes in AIDS and ARC Lymph Node and Peripheral Blood. Primary tissue samples were obtained from 20 individuals previously diagnosed with AIDS or ARC. Of seven lymph node suspensions hybridized with the HTLV-III probe R3, six (86%) showed the presence of infected cells expressing HTLV-III RNA, as demonstrated in Fig. 2 *A* and *B* (Table 1). Labeled cells were very rare, constituting <0.01% and, in some cases, <0.001% of the cell populations. Hybridization of probe R3 to peripheral blood mononuclear cells also resulted in labeling of very rare cells that represented HTLV-III-expressing cells (Fig. 2 *C* and *D*; Table 1). However, a lower percentage of the peripheral blood samples studied were positive for labeled cells (7 of 14, or 50%) as compared to lymph node. Although quantitation between various tissues and samples was difficult because of the low numbers observed, positive peripheral blood samples generally exhibited labeled cells at a

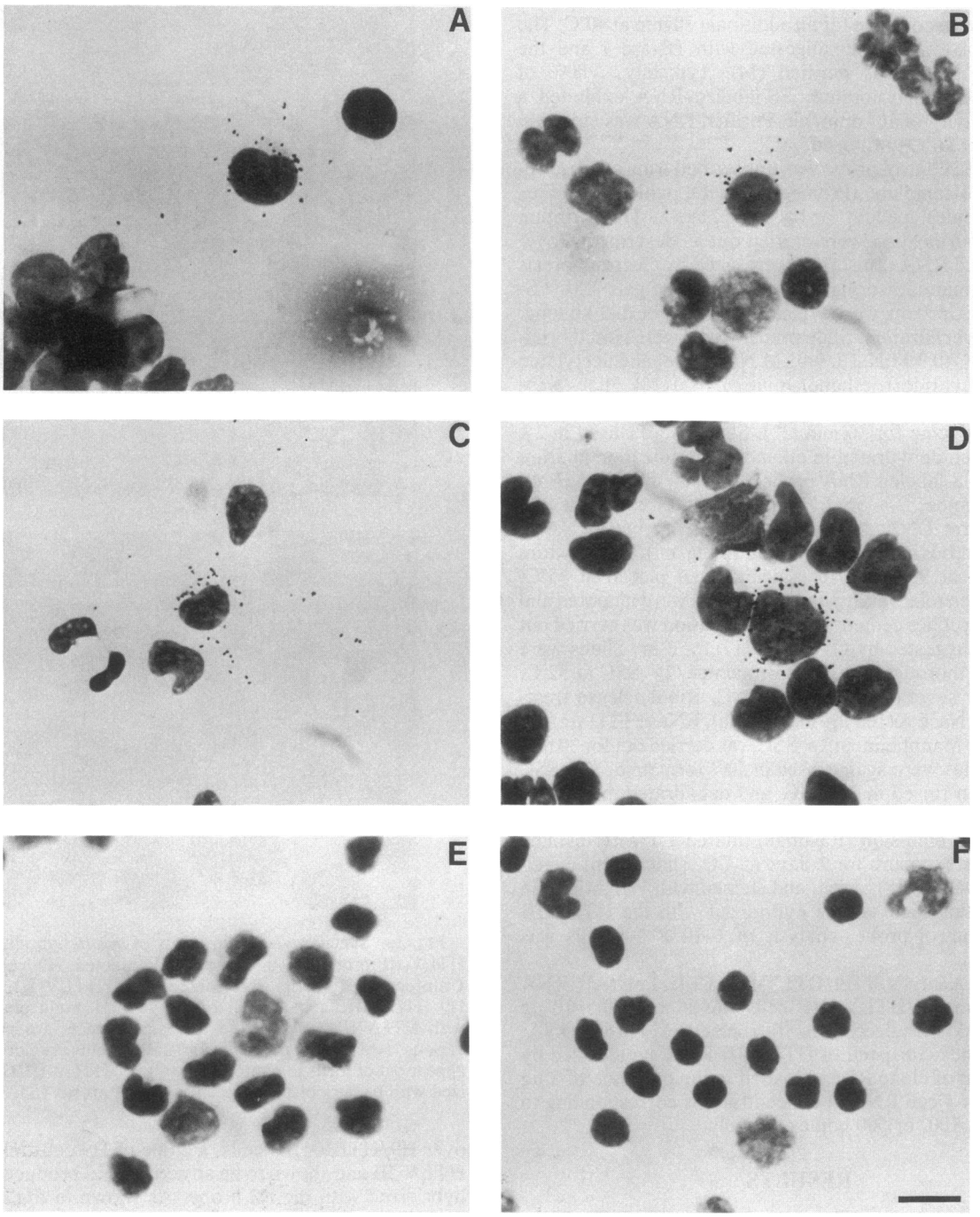

FIG. 2. Detection of HTLV-III RNA in primary lymph node and peripheral blood lymphocytes from AIDS and ARC patients by *in situ* hybridization. (*A* and *B*) Lymph node mononuclear cell preparations from representative ARC and AIDS patients, respectively, hybridized with HTLV-III probe, illustrating rare lymphocytes expressing HTLV-III RNA. (*C* and *D*) Peripheral blood mononuclear cells from representative AIDS and ARC patients, respectively, hybridized with the HTLV-III probe, illustrating lymphocytes expressing HTLV-III RNA. (*E*) Peripheral blood mononuclear cell preparation from the same ARC sample as in *D* but hybridized with bacteriophage λ-specific control probe. (*F*) Peripheral blood mononuclear cell preparation from normal individual hybridized with HTLV-III probe. (Bar = 10 μm.)

Table 1. Fresh tissue specimens from AIDS or ARC patients analyzed by *in situ* hybridization

Tissue tested	Clinical diagnosis	No. of patients	
		Tested	Positive
LN	ARC	4	3
	AIDS	3	3
PB	ARC	2	2
	AIDS	12	5

A total of 21 samples from 20 patients were studied. LN, lymph node; PB, peripheral blood.

frequency similar to that of lymph node (<0.01% and usually <0.001%). Longer exposure periods (up to 5 days) did not alter the frequency of labeling in either type of tissue.

Labeled cells from lymph node or peripheral blood mononuclear preparations generally exhibited 20–100 grains per cell. These grains were located over the nucleus and cytoplasm, and with many cells they were also located in the immediate periphery, possibly because of leakage of cell material. HTLV-III-positive cells exhibited morphological detail consistent with that of lymphocytes, including round or slightly indented nuclei and clumped chromatin that stained intensely with Wright's stain. Mononuclear cell preparations from the same patients hybridized with the bacteriophage λ-specific control probe were consistently negative, as shown in Fig. 2*E* by hybridization to the same ARC peripheral blood previously found positive and shown in Fig. 2*D*. Cell preparations hybridized with this probe typically exhibited no grains; rare labeled cells generally exhibited 1–2 grains with a maximum of 4 grains per cell. Similarly, mononuclear cells from four normal individuals were negative for HTLV-III RNA when hybridized with probe R3 (Fig. 2*F*).

Specificity of Hybridization for RNA. Specificity for viral RNA, as opposed to DNA, was demonstrated by hybridization of probe R3 to H9/HTLV-IIIB cells after digestion with either RNase (RNase A at 100 μg/ml; RNase T1 at 1 μg/ml) or DNase I (20 μg/ml) at 37°C for 18 hr. Cells treated with DNase prior to hybridization exhibited significant label at a level similar to that described above for slides not exposed to enzyme pretreatment. However, cells treated with RNase before hybridization exhibited essentially no label (results not shown). These observations indicate that, under the hybridization conditions used, grains were specific for viral RNA and not unintegrated HTLV-III DNA, shown previously to be present in H9/HTLV-IIIB cells by Southern blot hybridization (11).

Sensitivity of *in Situ* Hybridization Method. To quantitate the number of RNA copies per cell detected with the *in situ* hybridization method, H9/HTLV-III cells from one culture were analyzed by *in situ* hybridization as well as by RNA blot hybridization. Cells hybridized *in situ* with ^{35}S-labeled R3 under identical conditions to that used above again averaged 100–150 grains per cell after 2 days of autoradiographic exposure. RNA blot analysis of isolated RNA from the same culture, hybridized with ^{32}P-labeled BH10 insert, indicated the presence of 250–500 copies per cell of HTLV-III RNA in this population of H9/HTLV-IIIB cells (M.E.H., L.M.M., R.C.G., F.W.S., and S. Arya, unpublished data). Therefore, each grain observed after a 2-day exposure represented 1–3 copies of RNA per cell, and the HTLV-III-infected lymphocytes observed in primary lymphoid tissue likely contained 20–300 copies of RNA per cell.

DISCUSSION

In situ hybridization was used to detect cells expressing HTLV-III RNA in uncultured lymph node and peripheral blood mononuclear cell samples from AIDS and ARC patients. Histopathological studies of lymph nodes from patients with generalized lymphadenopathy, which frequently accompanies or precedes other manifestations of AIDS, have described explosive reactive hyperplasia and involution of follicles (19, 20). More recent immunohistochemical studies suggest a continuing process of follicular fragmentation and atrophy, including infiltration of follicles by T cells, eventually leading to follicular depletion and clinically manifest AIDS (21). Our observation of very rare lymphocytes containing a low abundance of HTLV-III RNA in the majority of lymph nodes analyzed suggests that expression *in vivo* occurs at a very low level. Previous Southern blot hybridization experiments showed the presence of HTLV-III-specific DNA in only 7 of 34 (21%) lymph nodes analyzed. Furthermore, signal intensities were weak, indicating the presence of HTLV-III DNA at a level of <1 copy per 10 cells (12). If this DNA is contained in multiple copies per cell, analogous to that found in several infected cell lines (12), the total number of HTLV-III-containing cells would be low—e.g., 1 in 1000 if there are 100 copies per cell. Moreover, the frequency of HTLV-III DNA-positive cells is likely even lower in the 79% of samples that were negative by Southern blot hybridization. Since the *in situ* hybridization method detects RNA, we cannot determine what proportion of infected cells may be expressing virus at a given time. However, these results do indicate that the proliferative syndrome of lymphadenopathy patients is not due to proliferation of HTLV-III-infected lymphocytes.

Although it was not possible to quantitate between patients at various stages of the disease because of the low numbers of virus-expressing cells, no significant differences in labeling frequency were noted among the samples studied. The exact relationships between the locations of the HTLV-III-expressing cells and the histopathology of the lymph node can now be investigated by *in situ* hybridization to lymph node sections. We have recently used this approach to confirm the presence of very rare HTLV-III-infected cells expressing viral RNA in the lymph nodes of infected individuals and to sublocalize the expressing cells within the lymph node structure (M.E.H., L.M.M., R.C.G., F.W.S., and K. Chayt, unpublished results).

Hybridization of the HTLV-III probe R3 to AIDS or ARC peripheral blood mononuclear cell preparations also resulted in detection of lymphocytes expressing HTLV-III at very low frequency: <0.01% of mononuclear cells. Although quantitation between the two types of lymphoid tissue was not possible, our observation that fewer peripheral blood samples were positive compared to lymph node (50% vs. 86%) suggests the presence of fewer infected cells in peripheral blood. These results are also supported by other studies to determine the presence of HTLV-III in peripheral blood of AIDS and ARC patients. In particular, Southern blot hybridization detected HTLV-III DNA in only 1 of 22 peripheral blood samples from AIDS or ARC patients and with weak signal intensity, suggesting the number of genomic copies to be very low (12). By using virus isolation techniques, HTLV-III has been cultured from ≈60% of AIDS or ARC peripheral blood samples studied to date (6, 22). Thus, the *in situ* hybridization results, in conjunction with previous studies, suggest that HTLV-III-infected lymphocytes are, in general, present in peripheral blood of AIDS and ARC patients, but within each individual they are present at very low frequency. Negative results by *in situ* hybridization could be due to poor preservation of RNA during cell processing and slide preparation, to inadequate sampling, or to the extremely low frequency of cells expressing HTLV-III RNA.

In these experiments, HTLV-III RNA was detected by using an HTLV-III-specific RNA probe transcribed from DNA clone pBH10-R3. The 9-kb insert from this clone comprises almost the entire HTLV-III genome, lacking 180 base pairs of 5′ long terminal repeat (LTR) sequence (11).

Formamide/formaldehyde gel electrophoresis of the ^{35}S-labeled RNA transcripts indicated an average probe length of 1–2 kb. Therefore, transcription of clone R3 initiated within the 3′ LTR of the viral genome and generated probe RNA specific for the LTR and the *env* region, as well as the purine-rich sequence preceding the 3′ LTR (11, 23). Quantitation of HTLV-III RNA copy number of H9/HTLV-IIIB cells by RNA blot analysis and comparison with *in situ* hybridization of cells from the same culture indicated that 1–3 copies of RNA were represented by each grain when slides were exposed for 2 days. High sensitivity of detection was made possible by several improvements in methodology, including use of high specific activity ^{35}S, RNA probes obtained by transcription of specific DNA fragments subcloned into the vector pSP64 (17, 24), fixation of cells with paraformaldehyde (13), and hybridization pretreatment of cells to prevent nonspecific binding (13, 16).

HTLV-III has been shown to exhibit tropism for T4 helper lymphocytes (5). The current studies demonstrate the presence of rare lymphocytes expressing HTLV-III RNA in uncultured lymph nodes and peripheral blood from infected individuals. However, the possibility that HTLV-III infects other types of cells cannot at present be excluded, and it will be necessary to screen a variety of tissues from infected individuals to determine the extent of cells and tissues infected. In this regard, *in situ* hybridization to frozen brain sections from AIDS patients has shown the presence of cells infected with and expressing RNA specific for HTLV-III (25). These cells do not exhibit the cellular morphology of lymphocytes. Continued use of *in situ* hybridization, in conjunction with immunocytochemistry for detection of specific cellular antigens, will help to further define and characterize cells that are infected with HTLV-III, including "reservoir" cells, which may continue to express the virus during progression of the disease.

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