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Detection of HIV-1-Infected Cells From Patients Using Nonisotopic In Situ Hybridization

By Robert H. Singer, Kevin S. Byron, Jeanne B. Lawrence, and John L. Sullivan

We have demonstrated that a sensitive, nonisotopic in situ hybridization (ISH) assay can be used to detect HIV-infected cells from seropositive, asymptomatic individuals. Our assay is based on the detection of a biotinated HIV DNA probe hybridized to human immunodeficiency virus (HIV)-infected peripheral blood lymphocytes (PBL) using streptavidin and alkaline phosphatase to identify positive cells. This assay is rapid in that it can be performed within a day and is sensitive enough to unambiguously identify a rare, single, positive cell. Patient samples derived from HIV-seropositive hemophiliacs and HIV-seropositive infants were analyzed before and after coculture with normal PBL. The same samples were investigated using a Dupont

AQUIRED immunodeficiency syndrome (AIDS) has affected over 60,000 individuals in the United States over the past 10 years with a mortality rate of approximately 60%.¹ Based on surveys of seroprevalence, it has been estimated that 1 million to 1.5 million Americans have been infected with the human immunodeficiency virus type I (HIV 1).² Little information is currently available regarding the direct detection and quantitation of HIV infection in vivo. It is known that blood, semen, and vaginal secretions harbor HIV and represent the sources of spread of HIV from infected to noninfected individuals. Once HIV enters the host, the virus infects and replicates in a variety of cells, including blood and tissue monocytes/macrophages and T-helper (CD4) lymphocytes.³ Viral replication precedes the production of antibody by the host and may occur in the absence of symptoms. The recent studies of Ward et al⁴ suggest that as many as 460 individuals/year are infected with HIV because of HIV-contaminated blood products. These HIV-infected blood products are obtained from seronegative donors who are thought to be acutely HIV infected and viremic prior to the appearance of HIV antibody. While the risk of acquiring HIV infection through blood transfusion remains low (1 in 40,000), it is probable that continual spread in the heterosexual community will result in more blood donors being in the "window period" of HIV infection. The development of a rapid, sensitive, and convenient nonradioisotopic in situ hybridization assay to detect the viral genome directly in infected cells would increase the likelihood of detecting all HIV-infected individuals prior to the generation of a virus-specific immune response. A method to detect HIV directly would also be extremely useful in those situations when antibodies are not informative (eg, in infants of HIV-seropositive mothers where passively acquired maternal antibodies obscure the diagnosis⁵ and in those HIV-infected individuals who fail to mount an appropriate antibody response⁶ or lose the ability to maintain HIV-specific antibody following acute infection.⁷ Finally, a sensitive and quantitative method of detecting HIV-infected cells would be helpful in assessing the natural history of infection and in monitoring antiviral therapy or vaccine development.

In situ hybridization offers distinct advantages over immunologic methods, since it is able to detect infected cells whether or not viral proteins are expressed. Such hybridiza-

P24 antigen-capture kit. It was found that ISH always detected the same positive samples as antigen capture, often in shorter times of coculture. In situ hybridization detected over half of our HIV-infected hemophilia patient population as virus positive, whereas the antigen capture assay detected less than one fourth as virus positive. In situ hybridization detected positive cells directly, without coculture, in 12 out of 35 (34%) hemophiliacs and in three out of eight (37%) infants. The speed, sensitivity, and confidence of ISH and nonisotopic detection indicates that it will be useful as a tool for clinical research and diagnosis.

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tion was first used to detect HIV-infected cells in frozen brain tissue obtained from AIDS patients.⁸ Harper et al⁹ have published a further report demonstrating the detection of HIV-infected cells in the lymph nodes and peripheral blood of individuals with AIDS and AIDS-related complex (ARC) by in situ hybridization. Bush et al¹⁰ have shown that in situ hybridization can be used to detect virus-positive cells in cocultures of peripheral blood mononuclear cells from symptomatic patients at an earlier time of culture compared to any other viral detection technique. In these reports the in situ hybridization methodology¹¹ employed a radioisotopically (³⁵S) labeled probe. This report improves and applies nonisotopic in situ hybridization (ISH) to the detection of HIV-infected cells.

METHODS

Preparation of peripheral-blood lymphocytes and coculture. Blood was obtained by venous puncture and the lymphocytes separated by Ficoll-Hypaque (FH) centrifugation. Peripheral blood mononuclear cells were isolated from seronegative and seropositive individuals (3×10^6 cells) and cocultured with cells (3×10^6) from healthy seronegative donors that had been stimulated for 3 to 4 days with phytohemagglutinin (PHA) (2 μ g/mL, Wellcome Diagnostics, Research Triangle Park, NC). Cocultured cells were maintained in HEPES-buffered RPMI 1640 supplemented with 20% fetal calf serum (FCS), 2 μ g/mL polybrene (Sigma Diagnostics, St Louis,

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MO), 10% interleukin-2 (IL-2, Cellular Products Inc, Buffalo, NY), and 12.5 µg/mL gentamicin (GIBCO, Grand Island, NY) for 3 weeks. Cultures were split twice weekly and were supplemented with fresh PHA-stimulated normal mononuclear cells at 7-day intervals.

Immunofluorescence. Cells were mixed with a 1:100 dilution of patient serum (from a HIV-seropositive individual) in phosphate-buffered saline (PBS) and, after a series of washes in PBS, were detected with fluorescein-conjugated rabbit antihuman IgG (Cappel Laboratories, Organon Teknika, West Chester, PA). Cells were visualized under epifluorescence optics at 40 X objective.

Dot blot. Cellular pellets (2×10^6 cells) were washed and extracted three times with phenol chloroform after sodium dodecyl sulfate (SDS) (1%) treatment of the cells. Isolated nucleic acids were purified by ethanol precipitation, resuspended in high salt (0.3 mol/L Na acetate), and blotted onto nitrocellulose filters. Filters were hybridized with 32 P-labeled HIV genomic probe¹² (3×10^8 cpm/µg) overnight, washed, and exposed for 72 hours to radiographic film with intensifying screens.

Probe preparation by nick translation. An amount of 1.5 µL of 400 µmol/L biotin dUTP¹³ (BRL, Gaithersburg, MD) or the corresponding dATG mix for radioactive probes (or for radioactive probes, 35S-dCTP, Amersham 1,000 Ci/mmol/L, diluted 1:3 with unlabeled dCTP and used at a final concentration of 6 µmol/L), 1 µL of dACG mix (dATP, dCTP, dGTP 600 µmol/L each, PL Biochemicals, St Louis, MO), or dAGT mix for radioactive probes, 1 µL of 10X nick translation buffer (0.5 mol/L Tris Cl, pH 7.2; 0.1 mmol/L MgSO₄; 1 mmol/L dithiothreitol), 500 µg/mL bovine serum albumin (BSA, Pentax Frac V) 5-µL sterile glass distilled H₂O; 1 µL of 0.1 µg/µL HIV DNA (pJM [HIV-1]8.9[12] containing 8.9 kb of HIV-1 DNA), 1 µL of DNase (final 34 ng/mL: concentration determines the probe size; large probe sizes cause background), 1 µL of DNA Polymerase I (Boehringer). Incubation is 3 hours at 15°C, then 90 µL of 50 mmol/L EDTA and 1 µL of 10% SDS is added. Purification from incorporated nucleotides is with a sterile G50 sephadex "spin" column packed in a 1 mL disposable syringe.

In situ hybridization. The schematic in Fig 1 outlines the following methodology. Cells were washed in PBS, and 25×10^5 cells in 25 µL were applied to each well of a multiwell serologic slide (Celline, 5-mm wells). The excess fluid was immediately withdrawn by the pipette, leaving cells to be air dried (10 minutes), fixed in 4% paraformaldehyde in PBS (5 minutes), and stored in 70% ethanol at 4°C. From 1% to 4% (25,000 to 100,000; median 50,000) of the cells applied actually remain adhered to the slide. At appropriate times, slides stored in 70% alcohol are rehydrated in a 10-minute PBS, 5 mmol/L MgCl bath, then 10 minutes in 0.5% triton/0.5% saponin solution, washed in 2X SSC, treated for 10 minutes in 0.1 mol/L triethanolamine and 0.25% acetic anhydride, washed again in 2X SSC, and incubated for 2 minutes at 70°C in 70% formamide in 2X SSC before plunging into 70% ethanol and dehydrating through graded ethanol and air drying. This makes viral DNA as well as RNA accessible for hybridization. While the cells can be hybridized directly out of the 70% ethanol after rehydration in PBS, for a shorter pretreatment protocol, the signal is diminished. Forty nanograms of probe DNA is used, which can be obtained by aliquoting 40 µL of nick-translated probe into a microfuge tube and lyophilizing it with 4 µL of carrier nucleic acids (10 mg/mL of sheared salmon sperm and tRNA). Five microliters of deionized formamide is mixed with the lypholite and placed in a 90°C heating block for 10 minutes. Hybridization buffer is prepared by mixing 30 µL 20X SSC; 30 µL BSA, 60 µL dextran sulfate (50% solution, autoclaved in water) and 30 µL H₂O. Slides are then quickly used with the deposition of 5 µL of the heated probe mixed rapidly with an equal amount of hybridization buffer onto each serologic well (10 µL total), covered with a small strip of parafilm, and placed for 3 hours (or overnight as is

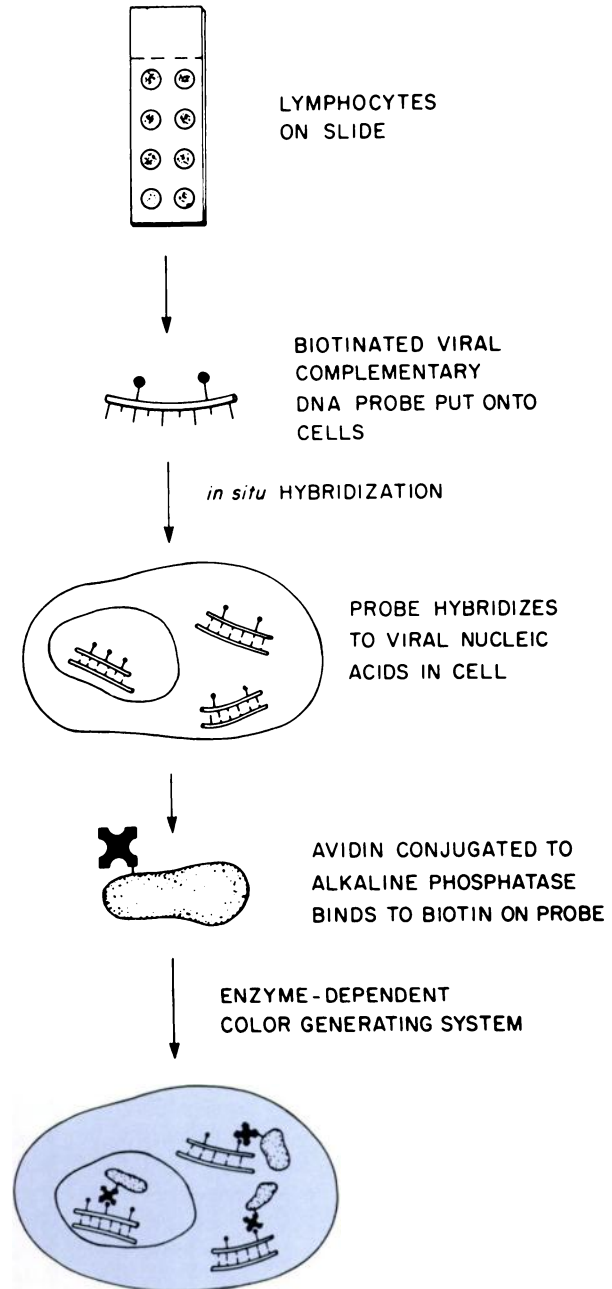


Fig 1. Schematic of the nonisotopic in situ hybridization and detection protocol.

convenient) in a humidified 37°C incubator. Slides are rinsed in 50% formamide in 2X SSC for 30 minutes at 37°C, then placed in 2X SSC for 30 minutes and finally in 1X SSC for 30 minutes.

Probes obtained from Molecular Biosystems, Inc (Dupont SNAP probe, San Diego, CA) were used at a concentration of 1 ng per hybridization (6 nmol/L concentration). Cells were hybridized in 5X SSC, 0.5% SDS, and 1% BSA at 50°C for 20 minutes in a humidified environment. Samples were then washed at 42°C for 5 minutes in 1X SSC at room temperature for 5 minutes each. The detection of the alkaline phosphatase was as described below.

Detection of biotinylated probes. Originally streptavidin followed by biotinylated alkaline phosphatase after the method of Singer et al¹⁴

Table 1. Detection of HIV-Infected Cells From Seropositive and Seronegative Individuals: Comparison of Antigen-Capture ELISA With In Situ Hybridization

Patient Population (N)	p24 Antigen Capture Positive (%)	In Situ Hybridization Positive (%)
Coculture*		
Seronegative hemophiliacs (2)	0	0
Seropositive hemophiliacs (34)	8 (24%)	18 (54%)
Seronegative normal controls (8)	0	0
Seropositive infants (15)	5 (33%)	8 (53%)
Direct detection*		
Seronegative normal controls (7)	0	0
Seropositive hemophiliacs (35)	4 (11%)	12 (34%)
Seropositive infants (8)	2 (25%)†	3 (37%)

*At four intervals over 21 days, the culture was tested, and the sample was considered positive if any one of those days was positive. For in situ hybridization, 1 cell per well (average 50,000 cells) was sufficient for a positive determination. For the P24 antigen capture ELISA, the samples were assayed as described by Dupont: 200 μ L of supernatant was tested, and readings corresponding to 40 pg/mL of p24 antigen or greater were considered positive. For direct detection, patient mononuclear cells were put directly on slides and processed as described previously. Serum samples were tested for the presence of p24 antigen on each patient studied by direct detection.

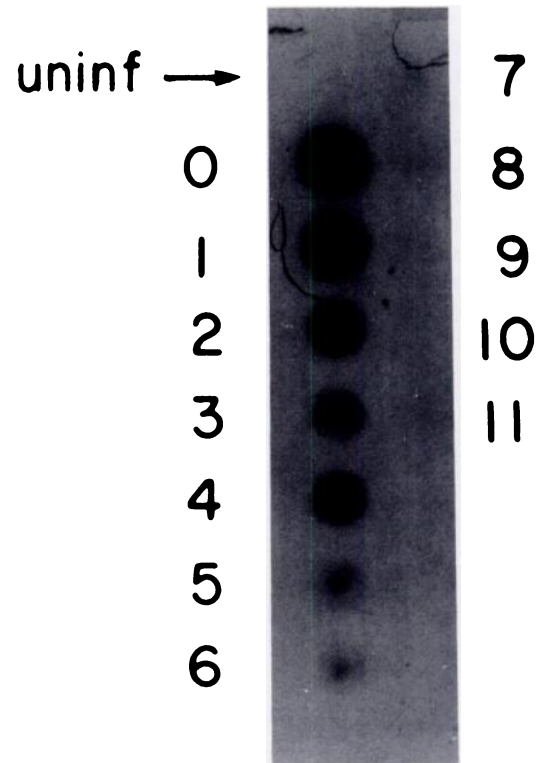
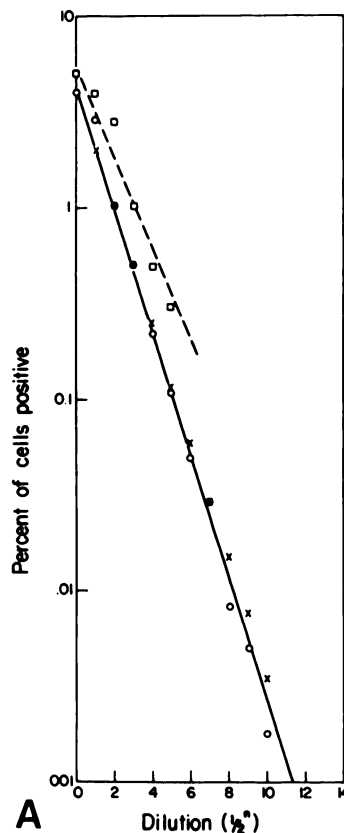
†Both infants with detectable serum p24 antigen had symptomatic HIV infection, one each with AIDS and progressive generalized lymphadenopathy.

was used for the data presented in Figs 2 and 3. The clinical results reported in Table 1 were obtained using streptavidin-alkaline phosphatase conjugate (Dakopatts, Santa Barbara, CA), which removes a step in the detection protocol and improves the background. The conjugate is used after dilution 1:250 into 4X SSC with 1% BSA and exposed to the hybridized cells for 30 minutes. After washing in 4X SSC (three washes for 10 minutes each), the cells were put into a pH 9.5 solution (0.1 mol/L Tris, 0.1 mol/L NaCl, 50 mmol/L MgCl₂) for color development with nitroblue tetrazolium (BRL, Gaithersburg, MD; 1:230 dilution) and bromochloroindolyl phosphate (BRL, Gaithersburg, MD; 1:300 dilution; developed 20 to 30 minutes). Isotopic detection was by standard autoradiographic technique; development was for 3 to 5 days.

RESULTS AND DISCUSSION

In this work we describe the application of in situ hybridization followed by enzymatic, nonisotopic detection¹⁴⁻¹⁶ to the detection of HIV in a variety of cellular samples. Since the report of Harper et al,⁹ it was apparent that the application of the approach to HIV detection in clinical samples required that it be sensitive, convenient, and yet permit a signal to be evident in an extremely rare infected cell with no background contributed by negative cells. A progressive approach to the improvement of methodologies for rapid in situ hybridization and detection was used. Initial experiments used the producer lines of HIV (CEM or H9) in which a majority of cells are highly positive to optimize protocol conditions. For instance, conditions that provided optimal retention of cells on slides were monitored by microscopy. Fixation of the cells, treatments prior to probe application,

Fig 2. Detection of serially diluted infected cells: in situ hybridization versus dot blot or immunofluorescence. CEM cells chronically infected with HIV were serially diluted 1:1 with uninfected CEM cells, and aliquots were taken for microscopy. Methods of detection used an indirect immunofluorescence assay and in situ hybridization. In addition, dot blots were made from RNA isolated from aliquots of the same cells. (A) Dilution curve (O-O): percent infected cells (average of 10 fields). (□-□) Identical samples measured by immunofluorescence. (X-X) The expected percent of infected cells determined by calculation at each dilution. Since the undiluted sample contained 7% positive cells, as determined by in situ hybridization, this provided the numerator in the serial dilutions. (B) Samples in (A) taken for dot-blot analysis.



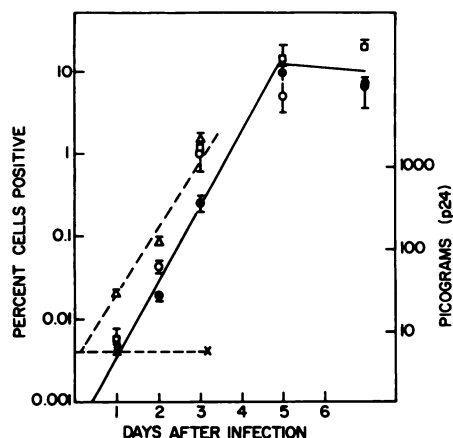


Fig 3. Kinetics of infection of normal T lymphocytes: Detection of infected cells by in situ hybridization of antigen capture. Normal T lymphocytes were cultured in vitro for 3 days with IL-2 and PHA and then were infected with HIV-1 at low multiplicity of infection (MOI). At daily intervals, including just after exposure to the virus (day 0), cell samples were taken for in situ hybridization. Three methods of in situ hybridization were used: isotopically labeled probe (open squares), nonisotopically labeled probe (open circles), and conjugated oligonucleotides (●-●). In addition, p24 was measured in the supernatant of the culture using an ELISA method (open triangles) (Dupont first-generation kit). Control, uninfected cells were processed at days 0, 1, and 3 for the ELISA assay for p24 (x---x). For the in situ hybridization, the results were 0 cells positive on day 0 and day 3.

and hybridization conditions, all of which influence probe penetration¹¹ and accessibility to the target molecules, were monitored by isotopic detection using ³²p-labeled probe followed by scintillation counting or detection nonisotopically. Finally nonisotopic detection was evaluated by variation in chromogen development time or conditions of streptavidin-alkaline phosphatase incubation and washing. After color development, quantitative data were obtained by counting the positive cells under the microscope and expressing them as a percent of total cells. Once the protocols for detection of HIV were established, comparison of in situ hybridization with conventional methods of virus detection such as RNA dot blot¹⁷ and antigen capture methods¹⁸ was possible. By providing information as to numbers of cells infected in situ, hybridization provides a means to evaluate sensitivity, where we have defined sensitivity as the ability to detect the rare cell. We evaluated this form of sensitivity by a mixing experiment in which positive cells were diluted (to less than one in 10⁵ cells). Virus-positive cells were serially diluted by uninfected cells and the percent positive cells at each dilution determined by in situ hybridization. Aliquots were also collected for dot-blot filter hybridization or the indirect immunofluorescence. Approximately 7% of the cells in the initial cell culture were positive (1 in 14 cells), and these positive cells could be accurately detected by nonisotopic in situ hybridization when diluted to one infected cell per 14,000 (Fig 2A). The false-positive rate was determined on an uninfected population of cells and was found to be zero (no false positives seen in 100,000 cells). Because of the visual requirements for characterizing a cell as positive (the cell must be completely colored, indicating significant

amounts of viral RNA in the cytoplasm), the possibility of a cell being a false positive is negligible. In several million uninfected cells viewed over many months, we have never seen a cell that we would characterize as positive. With background levels this low, any signal is significant, and a single positive cell represents sufficient signal to score a sample as positive. This does not mean that samples are negative if no cell is seen, since a low level of positive cells may result in sampling variations that eliminate positive cells. A single positive cell could be detected with confidence in 50,000 negative cells in many of our samples.

On equivalent samples it was found that the limit of sensitivity of dot-blot filter hybridization of cellular DNA was equivalent to one infected cell per 900 (Fig 2B). The sensitivity of indirect immunofluorescence was limited due to the higher magnification required by epifluorescence and the tendency to identify weakly fluorescent cells as false positive. Hence, detection of infected cells by immunofluorescence was also accurate in our hands to only about one infected cell per 1,000.

Another model to assess comparative sensitivity is to follow the infection of a culture with time where the amount of viral nucleic acids per cell is increasing, eventually to as many as tens of thousands of copies of HIV RNA per cell.¹⁹ Hence the increase in the number of detectable cells in a population should represent the increase in viral nucleic acid per cell as well as an increase in infected cells. Normal PHA-activated T lymphocytes were infected with HIV (HTLV-III B) and sampled at daily intervals for 1 week for HIV detection by in situ hybridization or p24 antigen capture by ELISA assay (Dupont, Wilmington, DE). In situ hybridization was done using isotopically labeled probes detected by autoradiography, biotinylated probes detected by streptavidin and alkaline phosphatase, and oligonucleotide probes conjugated to alkaline phosphatase directly (SNAP probes) in a system where fewer copies of HIV per cell are expressed when compared with producer cell lines. Both the isotopic and nonisotopic detection of in situ hybrids using the nick-translated genomic probe were of comparable sensitivity (Fig 3). The mixture of three different enzyme-linked oligonucleotides detected, on the average, 25% of the cells detected using nick-translated probes, and only after an overnight exposure to the chromagen. We have recently tested a mixture of 20 enzyme-linked oligonucleotides (kindly provided by MBI DuPont, Billerica, MA) and found that the time required for sufficient color development to detect the positive cells was decreased approximately proportional to the increase in the mixture complexity.

The antigen capture ELISA test was used on the same samples as in situ hybridization. It was found to be the less sensitive method as a result of background levels (about 5 pg/mL). Since results from both methods were determined on equivalent samples, it is possible from Fig 3 to determine how many positive cells (defined by in situ hybridization) are required for a positive determination by antigen capture ELISA assay. We used 40 pg/mL as a confident positive determination. This level of p24 expression would correspond to approximately 1 positive cell per 2500 negative cells. A lower limit of detection (10 pg/mL) would correspond to approximately 1 cell positive per 11000 (this value may be

more compatible with the more sensitive tests now available from Abbott or DuPont). The background level for in situ hybridization in this experiment was not detectable (less than 1 cell per 100,000 uninfected cells). From these considerations there is an order of magnitude improvement in sensitivity in detecting cells actively undergoing viral replication when using in situ hybridization relative to the best available p24 antigen-capture tests. It should be emphasized that these tests measure different parameters of infection. Antigen is detected in the supernatant of the cell cultures, presumably as a result of lytic infection. These cells would not be detected by in situ hybridization. Conversely, cells undergoing viral replication or expression would be detected by in situ hybridization but not necessarily by antigen capture. The direct measurement of virus-positive cells therefore becomes less comparable with the ELISA as antigen accumulates in the media with increasing time in culture. Therefore these two approaches should be seen as providing complementary information.

APPLICATION TO CLINICAL SAMPLES

If the in situ hybridization approach is to become useful clinically, it must be able to detect the rare virus-positive cells from infected individuals.⁹ To determine the ability of nonisotopic in situ hybridization to detect HIV in the blood of HIV-infected patients, we hybridized patient mononu-

clear cells after coculture with normal, stimulated, human T lymphocytes and also performed direct hybridization of patient mononuclear cells without coculture. As in the previous experiment, we compared all samples with results of the p24 antigen-capture assay. Two different HIV-seropositive patient populations were studied: one population consists of hemophiliacs who are seropositive but asymptomatic, the other of infants of seropositive mothers. Results are shown in Table 1. In situ hybridization detected virus-positive cells in 53% to 54% of both groups during a 3-week coculture with normal, stimulated T lymphocytes. The p24 antigen-capture ELISA test detected 24% to 33% of these samples as positive, all of which agreed with the in situ hybridization results. Repeated samplings gave similar results. While in situ hybridization can detect positive cells with shorter times of culture,¹⁰ improvements in both viral culture as well as the p24 ELISA are also expected to decrease the time needed for coculture. The isolation of HIV from 98% of HIV seropositive hemophiliacs using an improved coculture technique with a larger inoculum (10×10^6) of patient mononuclear cells has been recently reported.²⁰ Furthermore, both Dupont and Abbott have produced more sensitive p24 detection that allows decreased culture time. Preliminary comparisons on similar samples indicate that these improvements increase the percent of samples scored as positive. As described previously, in situ hybridization continues to provide quanti-

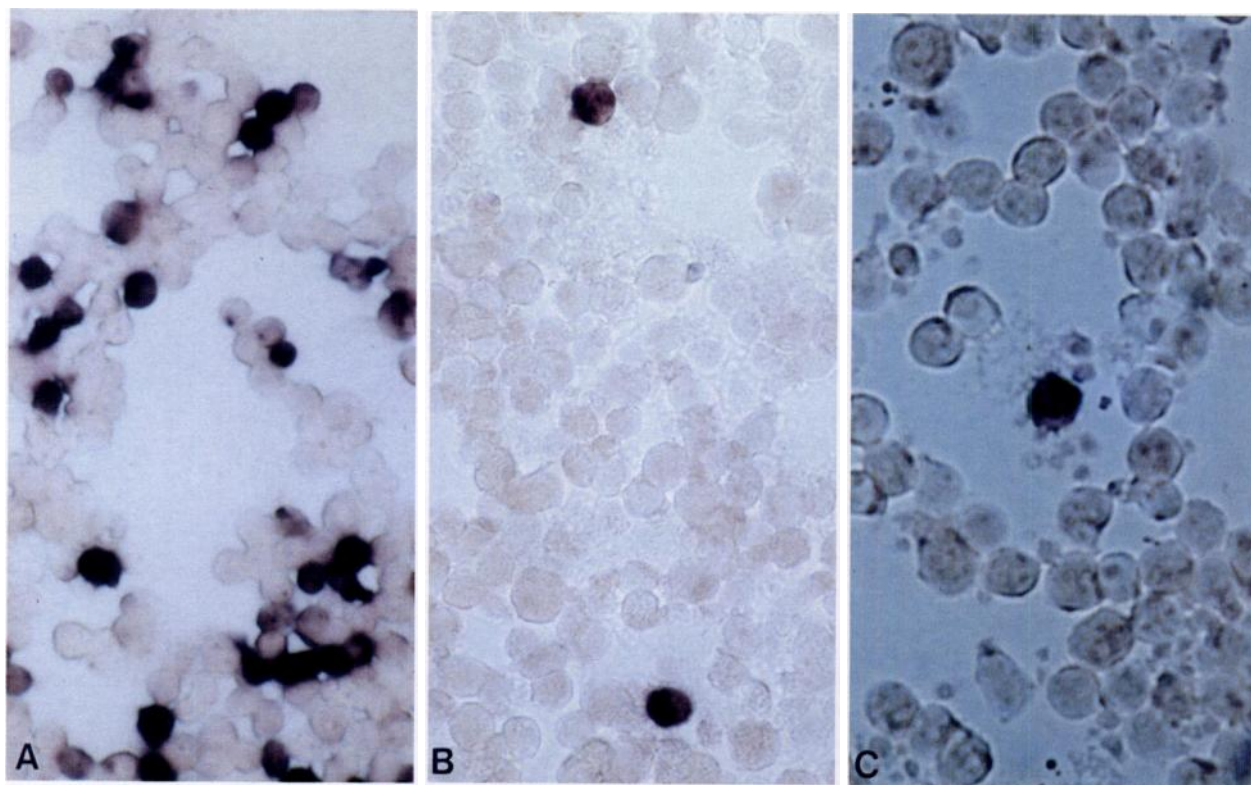


Fig 4. Examples of detection of viral nucleic acids in positive cells using in situ hybridization. (A) Cells from a normal lymphocyte culture were infected in vitro with HIV-1 and cultured for 7 days. At this point 16% of the cells were positive and were clearly distinguishable from the uninfected cells. (This constitutes a positive control slide used in conjunction with all our patient detections.) (B) Positive cells from a seropositive individual after a 7-day coculture with normal lymphocytes. Positive cells were 0.1% of total cells. (C) Direct detection of positive cells from patient similar to (B). Positive cells were 0.01% of total cells. (Note that phase microscopy increases the contrast of negative cells.)

tative cellular information complementary to these other techniques.

The ability of in situ hybridization to detect rare, positive cells makes it immediately applicable to patient peripheral-blood lymphocytes. Cells from the same adult patient population investigated with coculture above were tested directly without coculture. Approximately two thirds of the number of patients detected after coculture were detected positive without use of coculture (34%). The number of virus-positive cells detected in these patients varied between 1 cell to 10 cells per well (average 50,000 cells/well). The antigen-capture assay was positive on 11% of these patients' serum. The CD4-positive cells of these patients were also assessed, but no obvious correlation was found between CD4 lymphocytes and number of viral-infected circulating mononuclear cells. Figure 4 shows representative, nonisotopic detection of HIV nucleic acids in patient samples following coculture and by direct detection using freshly isolated mononuclear cells.

Peripheral blood monocytes, splenic mononuclear cells, bone marrow, and cells in culture infected by a sample of cerebrospinal fluid (CSF) were screened in a preliminary evaluation of direct testing of other cell types. One seropositive patient was found to have a single infected cell in a direct preparation of spleen cells. Coculture of this patient's blood mononuclear cells produced a culture with 100 infected cells per 10^5 cells by day 14 of culture, increasing the confidence of the detection considerably. Stimulated T cells inoculated with CSF from a HIV-seropositive individual gave large numbers of virus-infected cells after 7 days. Several bone marrow samples were also investigated (isotopically); and positive cells were found in these patient samples as well, as were positive cells found in monocyte preparations isolated and cultured from seropositive donors. It is presumed that the in situ approach will also prove important for evaluation of cytologic aspects of the viral infection within tissue sections obtained by biopsy or autopsy.^{8,9,21} While the work just described is currently anecdotal, it serves to illustrate further applications for this methodology.

Further increases in sensitivity and convenience will

improve nonisotopic in situ hybridization; for instance, work in progress using a protocol developed for fluorescent detection of a single copy of viral DNA²² can detect a single copy of integrated HIV DNA. As an alternative or complement to other means of examination of patient samples such as dot blot,¹⁷ antigen capture,¹⁸ or polymerase chain reaction (PCR),²³⁻²⁵ there are some immediate applications of the approach described in this work. One of the major contributions of in situ hybridization is the single-cell nature of the data, which allows a quantitative assessment of absolute numbers of cells containing viral nucleic acid. This allows a direct evaluation of viral load in the patient and can be used to test the effects of therapeutic drugs. If some patients are found to contain significant numbers of circulating infected lymphocytes, their response to therapy could be monitored using this approach (in vitro or in vivo). Second, the approach can be used, as shown above, to detect virus-positive cells in infants of seropositive mothers, where maternal antibodies may confuse the diagnosis; or in seronegative individuals to confirm suspected infection.²⁶ Third, it provides a rapid, quantitative, and convenient complement to reverse transcriptase or p24 assays in conjunction with virus culture or serves as an alternative to culture in individuals with detectable, circulating infected cells. The time required to complete the assay directly on patient lymphocytes is less than 1 day and could be decreased considerably. Finally, it requires no specialized equipment other than a microscope, nor does it require specialized training.

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