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Principles of Laboratory Isolation and Identification of the Human Immunodeficiency Virus (HIV)

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Diagnosis of human immunodeficiency virus (HIV) infections has relied most frequently on detecting the presence of HIV antibodies in sera. In many situations, however, patient management can be significantly improved if the presence of HIV can be demonstrated in patients' specimens. In this review, the need and value of HIV isolation for confirming the diagnosis of HIV, for disease staging, and for monitoring the effectiveness of antiviral therapy are discussed. The steps involved in isolation of HIV, the cell systems permissive to HIV growth, and the procedures for virus identification are reviewed. Furthermore, methods available for the direct detection of HIV in patients' specimens are summarized. Although isolation of HIV is presently an elaborate procedure, as easier methods become available, it will play a large role in the management of HIV-infected individuals.

INTRODUCTION

The recent emergence of the acquired immune deficiency syndrome (AIDS) has profoundly challenged medical practitioners. But virologists in particular have been compelled to confront the challenge, since the etiologic agent has been identified as a retrovirus now called the human immunodeficiency virus (HIV). During the past five years, substantial progress has been made in understanding the molecular biology and pathogenesis of this virus. An important advance has been the establishment of laboratory methods for detecting the presence of HIV and antibodies to HIV. Because of the obvious public health significance, emphasis has been placed on procedures to screen blood and blood products for the presence of HIV antibodies.

While serodiagnosis is now used routinely for demonstrating exposure to HIV, none of the tests currently used is able to differentiate perfectly between infected and uninfected individuals. Isolation of HIV from clinical specimens is less frequently attempted and is performed primarily in specialized research centers. The inherent value of isolation and identification of HIV has been underestimated, despite the fact that there is a clear need for a test, not dependent on antibody detection, to confirm that a person is infected with HIV.

In this paper, the indications for use of HIV isolation in the laboratory are discussed. The principles of the procedures used for isolating and identifying HIV are outlined,

Abbreviations: AAF: 2-acetylaminofluorene AIDS: acquired immune deficiency syndrome CPE: cytopathic effect DNA: deoxyribonucleic acid ELISA: enxyme-linked immunosorbent assay HIV: human immunodeficiency virus HTLV: human T-cell leukemia virus IFA: indirect immunofluorescence assay RIA: radioimmunoassay RNA: ribonucleic acid

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TABLE 1
Indications for Isolation and Direct Detection of HIV

1. Confirmation of diagnosis of HIV infection
   a. Detection of HIV infection in individuals who have no detectable antibodies
      - because of recent acquisition of infection
      - because of severe immunosuppression
   b. Confirmation of HIV infection in cases where the significance of seropositivity is unclear
      - in infants born to HIV-infected mothers
      - in individuals with equivocal Western blot results
2. Disease staging
   a. Monitoring of asymptomatic antibody-positive individuals for the presence of HIV in blood
   b. Assessment of presence of HIV in cerebrospinal fluid in patients with central nervous system disease
3. Evaluation of effectiveness of antiviral therapy

and the host systems available for virus isolation are reviewed. In addition, the methods used for direct detection of the virus in clinical specimens are discussed.

VALUE AND INDICATIONS OF HIV IDENTIFICATION IN THE LABORATORY

There are situations where isolation of HIV in the laboratory is not a luxury but is instead mandated because such information facilitates proper management of HIV-infected patients (Table 1).

Confirmation of Diagnosis

HIV isolation may serve to detect HIV infection in antibody-negative individuals. In recently acquired HIV infection, infectious virus and HIV antigen may be present early, without detectable antibody [1,2]. Allain et al. [2] found in a study of hemophiliacs that the sequence of early markers of HIV infection were first HIV antigens, then antibodies to envelope proteins, and finally antibodies to core proteins. Antigenemia could be detected as early as two weeks after infection, whereas antibodies were not detectable until one month later [2]. Thus, demonstration of the presence of HIV may serve to detect early primary HIV infection and may allow the identification of antibody-negative individuals who are infectious. Antibody levels may decline to very low or undetectable levels in symptomatic or asymptomatic patients with HIV infections of longer duration. In these cases, identification of HIV in the patient's blood or other specimens may serve to define the sites involved in the infection.

HIV isolation can be used to confirm the diagnosis of HIV infection in infants who are born to HIV-infected mothers. Since newborns are not fully immunocompetent and because maternal antibodies cross the placenta, serologic methods are less useful as a means of rapidly detecting HIV infection in a newborn. Culture or direct detection of HIV may also be helpful in clarifying equivocal or negative Western blot results in an individual seropositive when tested by the enzyme-linked immunosorbent assay.

Disease Staging

Monitoring of asymptomatic antibody-positive patients for the presence of HIV may serve to predict a progression to active disease. HIV antigenemia, accompanied by a

TABLE 2
Sources of HIV Isolates

Sources
Blood mononuclear cells
Bone marrow cells
Brain tissue
Cerebrospinal fluid
Lymph nodes
Plasma
Saliva
Semen
Urine
Vaginal secretions

disappearance of antibody reactivity to the major HIV core protein p24, was found to precede the onset of AIDS in one adult and five children who were antibody-positive [3]. HIV culturing from cerebrospinal fluid may be useful to demonstrate active HIV infection in the brain of patients who develop central nervous system disease. In addition, the persistence or reappearance of HIV antigens in blood and cerebrospinal fluid has been found to correlate with clinical, immunological, and neurological deterioration [1].

Evaluation of Effectiveness of Antiviral Therapy

As promising antiviral chemotherapy becomes available, early recognition of active HIV infection in patients will permit institution of therapy early during the course of the disease and, it is hoped, will optimize effectiveness. Monitoring of treated patients for the presence of infectious virus is mandatory to monitor accurately effectiveness of antiviral compounds. Evaluation of the effectiveness of antiviral therapy depends crucially on the ability to quantify changes in HIV concentrations in specimens of treated patients. Presently, a positive blood culture is usually required for entry into clinical trials of anti-HIV therapy, and the effectiveness of drug treatment is evaluated by monitoring blood throughout therapy for changes in virus concentration [4].

VIRUS ISOLATION

Two observations have greatly facilitated the development of procedures for isolation of HIV. First, the discovery of T-cell growth factors for the continuous growth of human T cells *in vitro* [5,6]. Second, the observation that HIV can be propagated in human peripheral mononuclear cells [7–9]. In first reports, HIV was directly isolated from peripheral blood, bone marrow, or lymph node mononuclear cells obtained from AIDS patients and placed in cultures [7,8,10–12]. Subsequently, isolation of HIV was successfully accomplished when patients' specimens were inoculated in lymphocyte cultures obtained from normal human blood or umbilical cord blood [7,8,10,13] or in permissive T-cell lines [8,10,12]. Sites from which HIV has been isolated are listed on Table 2.

At present, isolation of HIV is an elaborate and labor-intensive procedure. The various steps involved in the isolation and identification of HIV, using fresh lymphocytes from normal donors, are outlined in Fig. 1. To prepare cultures of human lymphocytes from fresh peripheral blood of normal donors, mononuclear cells are obtained by Ficoll-Hypaque centrifugation and suspended in culture media containing a mitogen such as phytohemagglutinin and a T-cell growth factor such as interleukin-

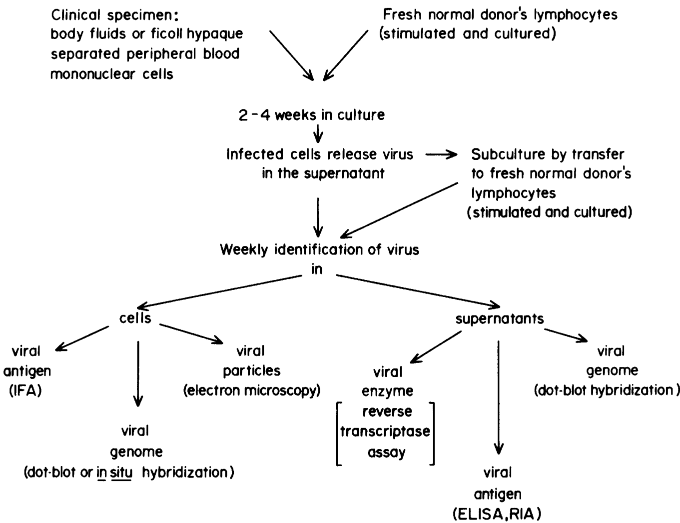

FIG. 1. Flow chart for isolation and identification of HIV.

2. Cultures of normal donor lymphocytes can be inoculated with patients' specimens within three to four days after initiation of the cultures. At the time of specimen inoculation, the stimulated normal lymphocytes are treated with polybrene or DEAE Dextran to facilitate virus absorption [14]. It has also been suggested that addition of antibody to human α interferon to culture media increases the ability to recover HIV from *in vitro* cultures [7]. This antiserum neutralizes endogenous interferon, which is secreted by cells chronically infected by viruses—including retroviruses. The normal donor cell cultures inoculated with the clinical specimens are kept in culture for three to four weeks. They are monitored biweekly for cell counts and viability by the trypan blue exclusion method or by measuring ^{3}H thymidine uptake [15]. Tissue culture media are replaced with fresh media twice weekly and freshly prepared stimulated lymphocyte cultures from normal donors are added once a week.

Depending on the amount of virus present in the patient's specimen, infected cells will show, to various degrees, a characteristic cytopathic effect (CPE) consisting of multinucleated giant cells with ring formation. These multinucleated giant cells produce large amounts of virus and die over a period of a few days. As infection spreads, infected cells can be seen at all stages of development. In the early phase, giant cells have a clear, bubble-like cytoplasm that projects from the edges of clusters of growing lymphocytes. Subsequently, cell fusion occurs and numerous multinucleated giant cells, which are often masked by clusters of proliferating lymphocytes, are present. As CPE progresses, the cytoplasm is reduced and becomes granular before the cells disintegrate. A minor cell population may survive and give rise to another burst of virus production, followed again by cell death. Examples of CPE patterns seen in H9 cells infected with HIV are shown in Fig. 2.

In addition to evaluation of cell viability and cytopathology, the cell cultures are monitored at weekly intervals throughout the culture for virus expression and release (Fig. 1). One or more of the following assays can be utilized to identify the presence of HIV in supernatants of the lymphocyte cultures: *(1)* assay of particulate reverse transcriptase activity; *(2)* detection of HIV antigens by enzyme-linked immunosorbent assays or radioimmunosassays; *(3)* detection of viral genome by nucleic acid hybridiza-

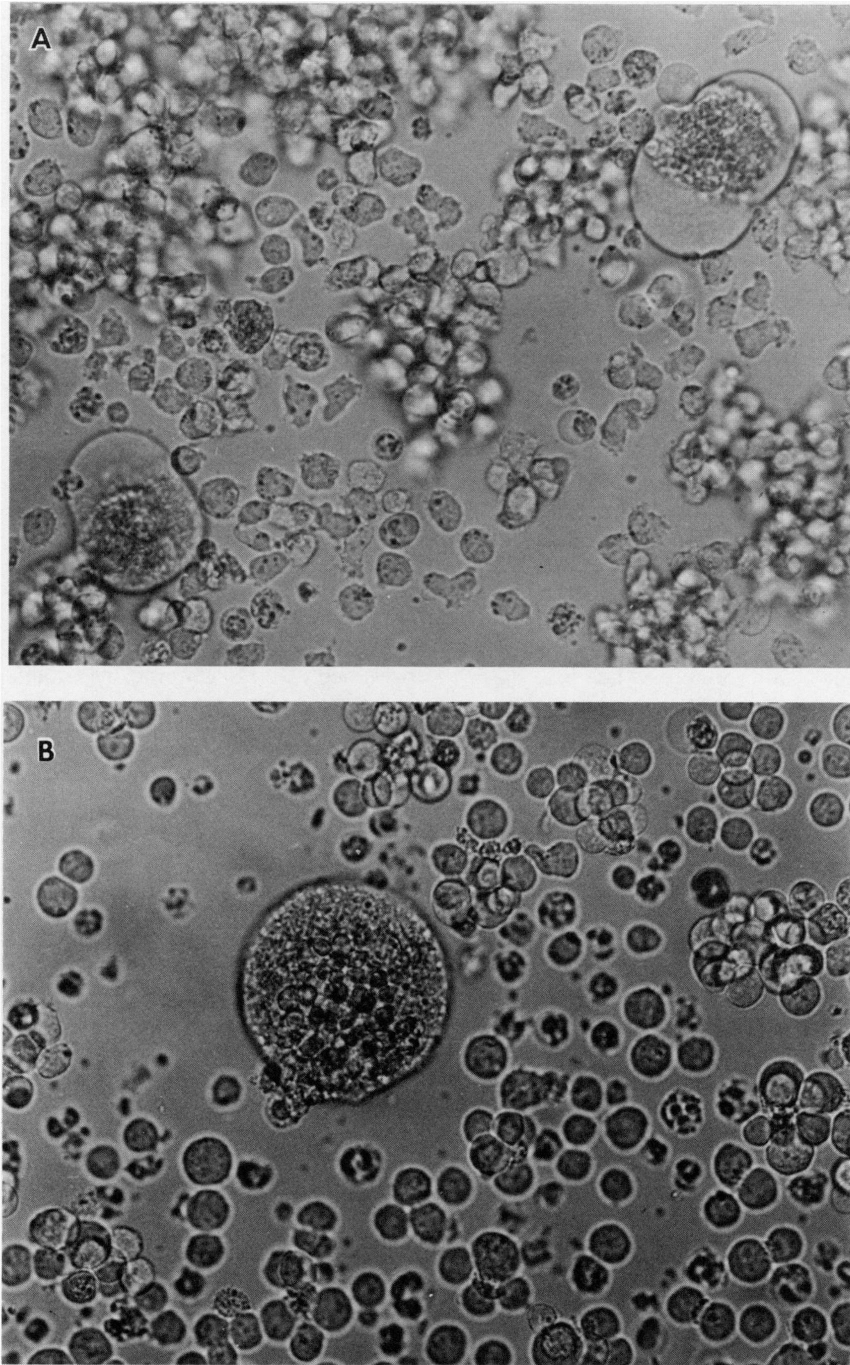

FIG. 2. Cytopathic changes in H9 cells, seven days post-inoculation with HIV (strain HTLV-IIIB). A. Cells with balloon-like formation (× 950). B. Multinucleated giant cell (× 950).

TABLE 3
Cells Frequently Used for HIV Growth

Cell Type	Cell Line	Cytopathic Effect[a]	HIV Production	References
Normal Donor Cells	Blood lymphocytes	+ + + +	Transient	[7,11]
	Umbilical cord lymphocytes	+ + + +	Transient	[7,8,13]
	Monocytes, macrophages	+ +	Continuous	[17,18]
Neoplastic T-Cell Lines	HT	+ +	Continuous	[8,10,21]
	H9	+ +	Continuous	[8,10,21]
	Hut 78	+ +	Continuous	[9,10,12,21]
	Molt 3	+ + + +	Continuous	[21]
	Molt 4	+ + + +	Continuous	[20]
	CCRF-CEM	+ +	Continuous	[19,20,21]
	A 3.01	+ + + +	Transient[b]	[15]
	MT2[c]	+ + + +	Plaque formation	[25–27]
	MT4[c]	+ + + +	Plaque formation	[25–27]
Monocytic Cell Lines	U937	+ +	Continuous	[16,20]

[a]Plus signs indicate extent of cytopathic effect: + + + + extensive, + + limited
[b]With possible reactivation
[c]These cell lines carry HTLV I.

tion. Infected cells can also be evaluated for *(1)* the presence of HIV antigen by an indirect immunofluorescence assay; *(2)* the presence of viral genome by nucleic acid hybridization; *(3)* the presence of viral particles by electron microscopy. To further confirm the presence of HIV in the clinical specimen, subculture of the newly isolated virus to uninfected cell cultures can be performed and the isolated virus identified as described above.

HOST CELL SUSCEPTIBILITY

As with other viruses, the availability of susceptible cells is crucial for the successful isolation of HIV. The first recognition of the presence of HIV was based on its *in vitro* toxic and cytopathic effect on T helper cells from patients with AIDS or at risk for AIDS [7–9]. The major target of HIV has been demonstrated to be T4 cells [11,16] and cultures of T helper cells prepared from blood, bone marrow, and lymph node have grown HIV [8,10–12]; however, short-term cultures of monocytes/macrophages and B lymphocytes can also be infected with HIV [17–20].

The types which have been frequently used for HIV growth are listed in Table 3. In addition to cultures prepared from normal human peripheral blood mononuclear cells or umbilical cord lymphocytes, neoplastic T-cell lines and some macrophage cell lines can efficiently propagate HIV *in vitro*. While some neoplastic B-cell lines have been found to be permissive to HIV [19,20,23], they have not been used for HIV growth. Transmission of HIV to an established T-cell line, H9, was first achieved by Popovic et al. [10]. In addition to this cell line, a number of other T-cell lines have been found to be susceptible to HIV [11,16,21,22]. In contrast to freshly prepared short-term lymphocyte cultures derived from normal donors, cells from neoplastic lines have the advantage that, when chronically infected, they continue to grow, show little CPE, and produce large amounts of virus [9,12,20,21]. These neoplastic T-cell lines express the T4 cell surface marker which is believed to be associated with susceptibility to the

virus. Because differences in T helper cell antigen expression in established cell lines have been obtained in various laboratories, differences in susceptibility of some cell lines to HIV, for example, the Molt 4 cell line, have been noted [10,20].

Primary cultures prepared from normal donors have been reported to be more sensitive than neoplastic T-cell lines for the isolation of HIV from patients' specimens [24]. Virus is produced earlier during the course of the *in vitro* infection, and the amount of virus released is often higher in freshly prepared lymphocyte cultures than in established cell lines. Attempts at developing sensitive and useful cell lines have been made. One cell line cloned from the CEM cell line was found to mimic primary lymphocyte cultures from normal donors in that virus production was transient and CPE was seen as early as three to five days after infection and resulted in significant cytotoxicity [15]; however, this cell line was less sensitive to infection by HIV than normal mononuclear cell cultures [15]. A variety of other cell lines have been tested without success [20]. Plaque assays which are used to quantitate virus infectivity cannot be readily performed in cells that grow in suspension. Therefore, two neoplastic T-cell lines (MT2 and MT4) that carry the HTLV I genome and grow in monolayers have been used in a plaque assay developed by Harada et al. [25–27].

METHODS OF VIRUS IDENTIFICATION

Methods of HIV identification rely frequently on the demonstration of viral proteins in infected cells or in supernatants of infected cells.

Detection of Reverse Transcriptase Activity in Supernatants of Infected Cells

The measurement of reverse transcriptase is broadly utilized as the primary method for the detection and quantitation of HIV infectivity. The assay is based on identifying the enzyme's capacity to assemble DNA from a RNA template. In general, it takes 7 to 21 days of co-cultivation of HIV-infected specimens with susceptible cells to accumulate adequate virus for detectability in the supernatant.

The following steps take place during the reverse transcriptase assay. Virus is precipitated from cell-free supernatant by polyethylene glycol. The pellet containing virus is then disrupted by a detergent. The disrupted virus is mixed with ^{3}H-labeled deoxythymidine triphosphate, $MgCl_2$, a primer, and either of two synthetic templates, polyribo(A) and polydeoxyribo(A). The reaction takes place at 37°C for one hour. The two templates used allow for the distinction between the viral reverse transcriptase that uses RNA as a template and the activity of related cellular polymerases that exploit DNA as a template. The labeled DNA synthesized by the reverse transcriptase is precipitated by trichloracetic acid and the amount of incorporated radioactivity is determined by scintillation counting. A combination of good response with polyribo(A) and a poor response with polydeoxyribo(A) is indicative of viral reverse transcriptase activity.

Detection of HIV Antigens in Supernatants of Infected Cells

Because the protocols for sample pretreatment and for measurement of the reverse transcriptase are cumbersome and labor-intensive, and because the assay is not specific for HIV, a number of methods have been developed to measure more efficiently and specifically virus release in supernatants of cell cultures. Radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) have been utilized and are both based on the immunological detection of the p24 major structural HIV core protein.

The principle of the RIA test is based on the competition between a radioactive and non-radioactive antigen for a fixed number of antibody binding sites. The precipitation of ^{125}I-labeled affinity purified p24 by a reference monospecific rabbit polyclonal antiserum is competed with clinical samples containing unlabeled HIV antigen. In the antigen capture sandwich ELISA, polyclonal antibodies [28] or monoclonal antibodies that recognize epitopes of the HIV core protein [29] are coated to beads or plates and used to catch the antigen. A positive reaction is identified with a probe anti-HIV antibody and a labeled secondary antibody. The capture ELISA has been found to compare well with the reverse transcriptase assay and requires twentyfold less supernatant material [28,29]. In addition, the ELISA and RIA for the viral core protein were more sensitive than the reverse transcriptase in detecting HIV in dilutions of chronically infected H9 cultures and allowed for the detection of HIV in supernatant of H9 and PHA-stimulated peripheral blood leukocytes earlier during the course of the infection [30].

Detection of HIV Antigens in Infected Cells

The presence of HIV antigens in cultured cells can be demonstrated rapidly by an indirect immunofluorescence assay (IFA). Cells from HIV-infected cultures and control uninfected cells are placed on welled slides and fixed. They are reacted with a primary HIV-positive antiserum. A primary HIV-negative serum is used as control. A fluorescein-labeled antihuman IgG antibody is used as the secondary antiserum. The assay is simple to perform but needs to be controlled carefully because cells used as target contain many antigens that might possibly react nonspecifically with antibodies. For example, class II histocompatibility DR proteins have been identified in preparations of HIV made in H9 cells and represent a ready source of antigen which can cause false-positive reactions [31]. IFA has been used to monitor HIV infection in a number of neoplastic T-cell lines including CEM, H9, and Hut 78 [32–34]. In H9 cells, three patterns of HIV-specific reactivity have been observed: a peripheral pattern seen frequently during early log phase, a star pattern, and a clumped pattern dominating in cells fixed in late log phase of replication [33]. In HIV-infected Hut 78 cells, two different staining patterns were observed: a diffuse cytoplasmic pattern and a cytoplasmic focal pattern [34].

Detection of HIV Nucleic Acids in Supernatants of/or in Infected Cells

Various methods based on the detection of HIV nucleic acids have been used as a means to identify HIV. A cytoplasmic dot-blot technique has also been developed to detect viral RNA sequences in infected cultured cells [35]. In this assay, cytoplasmic RNA prepared from cultured cells is denatured, spotted on nylon filters, and detected with a ^{32}P-labeled DNA clone of HIV. When compared to the reverse transcriptase assay and IFA, the RNA dot-blot method allowed for the detection of HIV several days earlier during the course of the infection. A procedure using 2-acetylaminofluorene (AAF)-modified DNA as hybridization probe for HIV nucleic acids spotted on nitrocellulose filters has also been utilized [36]. AAF was used to covalently modify guanine residues in a 6.5 kb fragment of cloned HIV DNA. Rabbit antibody to AAF was added and the AAF-DNA antibody complex was detected with a fluorescein or alkaline phosphatase tagged secondary antibody [36]. A recently developed oligonucleotide-directed procedure which provided a 10^5-fold *in vitro* amplification of DNA templates could identify infected cell lines that were not actively releasing virus particles as judged by absence of reverse transcriptase activity [37].

Detection of Viral Particles by Electron Microscopy

Electron microscopy does not play a large role in the identification of HIV isolates despite the fact that it allows for the direct detection of HIV in infected cells and that the ultrastructure of HIV is significantly different from that of other retroviruses. Studies of the ultrastructural development of HIV have indicated that most virus is found in extracellular spaces and that cytoplasmic inclusions or nucleocapsid precursors are usually not detected [38,39]. Figure 3 shows typical HIV particles as seen under the electron microscope.

DIRECT DETECTION OF HIV ANTIGENS OR GENOME IN CLINICAL SAMPLES

Methods for detecting HIV directly in body fluids and cells from infected individuals do not involve prior *in vitro* amplification in cell cultures and thus are rapid and less cumbersome, but also less sensitive. The previously described sandwich ELISA for detecting HIV core antigen in supernatants of cultured cells has been utilized to detect HIV antigen in serum and cerebrospinal fluid [1–3]. Antigenemia was found to precede the onset of AIDS [3] and could be demonstrated as early as two weeks after infection, one month prior to the appearance of HIV antibodies [2]. In patients enrolled in a placebo controlled trial of azidothymidine, a significant decrease in HIV core antigen was demonstrated in the serum of patients treated with azidothymidine, as compared to placebo-treated controls [4]. Direct detection of HIV antigen expressed *in vivo* on the surface of human lymphocytes has also been accomplished with monoclonal anti-HIV antibodies using a flow cytometer [40].

Immunological reagents directed to viral antigens may not permit detection of the virus during some stages of disease, particularly during persistent infection when only small quantities of virus particles may be actively produced. A strategy for viral detection in this case would require the identification of viral nucleic acids in infected cells. Techniques for detecting HIV nucleic acids in infected individuals have been developed in a number of laboratories. HIV infection was demonstrated in mononuclear cells of a newborn by *in situ* hybridization with a HIV-specific cDNA probe [41]. Harper et al. utilized ^{35}S-labeled HIV-specific RNA probes to detect lymphocytes expressing HIV in lymph nodes and blood from infected individuals by *in situ* hybridization [42]. In another report, HIV RNA was detected in peripheral blood mononuclear cells by hybridization on nitrocellulose filters, using probes prepared from proviral DNA and labeled with ^{32}P [43]. Results obtained from these studies all clearly suggest that, in uncultured lymph nodes or peripheral blood cells from infected individuals, only very few lymphocytes express HIV RNA.

CONCLUSION

Since the initial reports of AIDS cases over five years ago, much effort has been expended in attempts to improve methods for the diagnosis of HIV infection. Over the next decade, with the expected progress in antiviral therapy and knowledge of the disease, the need for an accurate and rapid diagnosis of HIV infection will undoubtedly become more acute. Currently, HIV isolation remains a time-consuming and cumbersome procedure. Although significant improvement in methods of direct HIV detection has been made, it appears that only a very small number of cells are infected *in vivo*. Therefore, culture of HIV in permissive cells, which results in amplification of initial virus input, is likely to continue to play a crucial role in diagnosis of HIV in the

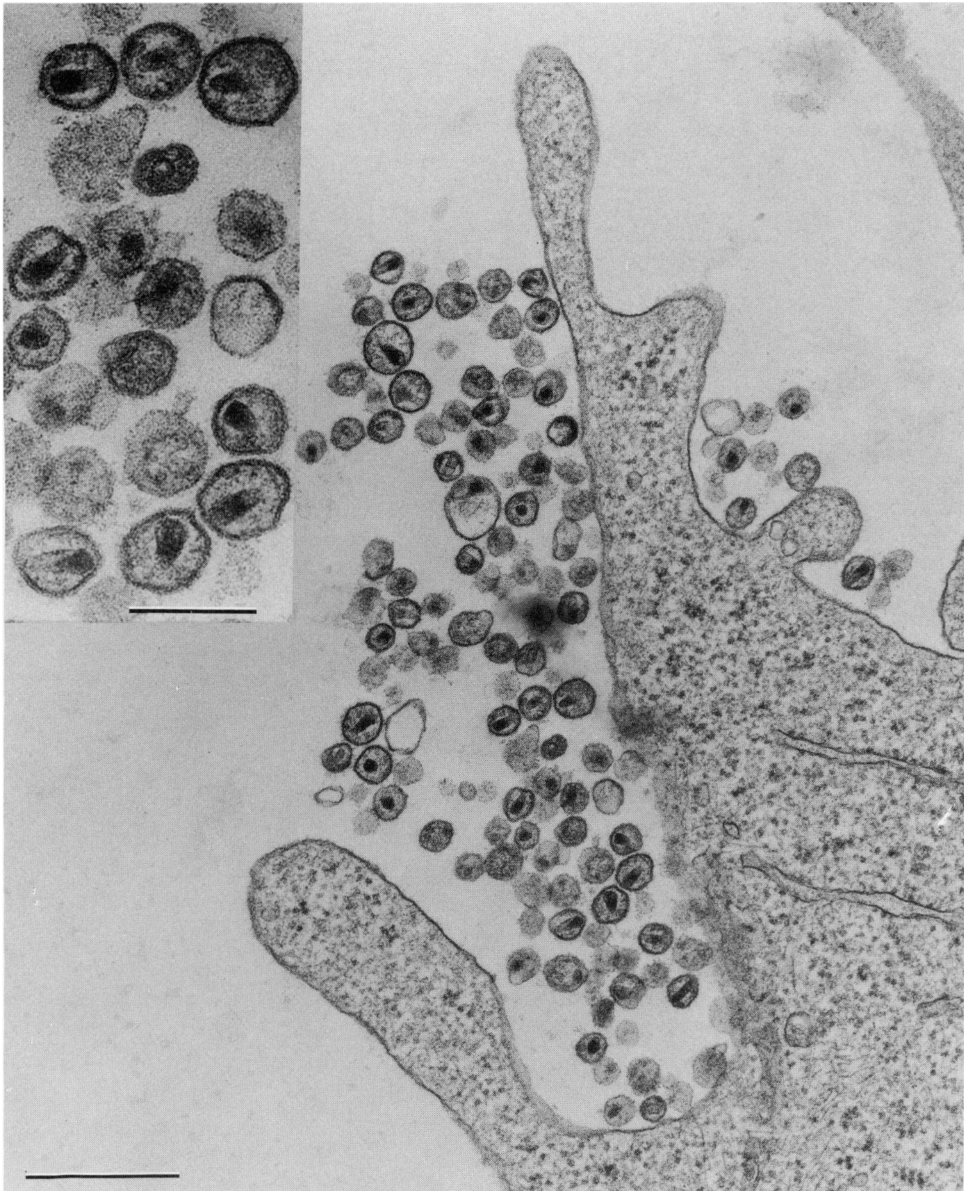

FIG. 3. Electron micrograph of an HIV-infected H9 cell, ten days post-inoculation with HIV (strain HTLV-IIIB). Typical HIV particles are seen in extracellular spaces (bar represents 0.5 μ). *Insert* shows detail of HIV particles (bar represents 0.2 μ).

laboratory. To arrive at an accurate identification of HIV, results obtained need to be interpreted carefully in light of the specimen, the cell system, and the methods used. Some of the neoplastic cell lines that contain endogenous retroviruses may yield mixtures of HIV and other HTLV types. Other retroviruses such as the human retrovirus associated with adult T-cell leukemia virus also show syncytia formation [44], and dual infections with human T-cell leukemia virus and HIV have been reported [45,46]. In addition, tests used commonly to identify HIV, such as the reverse

transcriptase assay, are not specific for HIV, since other retroviruses also yield reverse transcriptase activity. The virus isolation process may also be further complicated by differences in the replicative ability of different clinical isolates [12,13,20,47,48]. Furthermore, some cases of AIDS have been found to be caused by other biotypes of HIV [49–51]. These newly isolated viruses differ in some of their antigenic and genomic characteristics from previous HIV isolates but have similar growth and cytopathic properties. As more is learned about HIV-cell interactions *in vitro*, the development of less elaborate procedures for HIV isolation can be anticipated. Undoubtedly, this will result eventually in the incorporation of HIV isolation and identification methodology into the repertoire of many clinical virology laboratories.

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