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Intracellular viral RNA and proteins expressed during the in vitro propagation of human immunodeficiency virus

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INTRACELLULAR VIRAL RNA AND PROTEINS EXPRESSED DURING THE IN VITRO PROPAGATION OF HUMAN IMMUNODEFICIENCY VIRUS

by

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THESIS

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CHAPTER I

INTRODUCTION

A. Epidemiology of Acquired Immunodeficiency Syndrome

Acquired immunodeficiency syndrome (AIDS) was initially recognized as a distinct clinical entity in 1982 (1). The incidence of the disease has increased at an exponential rate in high-risk groups and the disease appears to be spreading to the low-risk heterosexual population (2,3,4). Recent CDC data indicate that over 25,000 cases have been reported in the United States, and 49% of these patients have died (5).

It is now clear that AIDS is an infectious disease. Most cases of AIDS appear to have been transmitted through intimate sexual contact, blood contamination of intravenous needles and blood products. The disease is most prevalent in homosexual and bisexual men, intravenous drug users, recipients of transfusions and blood products (such as factor

VIII and factor IX), newborn children of mothers from one of these risk groups, and Haitians and Central Africans (8).

AIDS presents as a severe, unexplained immune deficiency that involves reduction in the number of a subset of lymphocytes, the helper/inducer OKT4+ lymphocytes (6). This immune deficiency is manifested by multiple opportunistic infections or malignancies, the latter predominantly Kaposi's sarcoma or B cell lymphomas (7). AIDS-related complex or conditions (ARC) encompasses prodromal states of the disease, and is characterized by milder clinical manifestations, most frequently unexplained chronic lymphadenopathy or leukopenia involving helper T cells (6,7). Studies have indicated that of people infected with HIV, 5% to 20% develop AIDS within the first 2 to 5 years after becoming infected. More than half of those infected remain asymptomatic for a prolonged period, and these people appear to serve as latent carriers of HIV.

B. Etiology of AIDS

The etiologic agent of AIDS was first isolated from the lymph node of a patient with lymphadenopathy in 1983 and called lymphadenopathy-associated virus (LAV) (9). Other isolates of similar viruses have been named human T-lymphotropic leukemia virus type III (HTLV-III) and AIDS associated retrovirus (ARV) (10,11). Numerous studies of their biological and molecular characteristics have confirmed that

they are all different isolates or strains of the same virus, for which the family name human immunodeficiency virus (HIV) has recently been proposed by an international committee (12).

HIV is characterized by a pronounced tropism for OKT4+ lymphocytes, magnesium-dependent reverse transcriptase, cytopathic effects on lymphocytes, and morphologically distinct budding from the host cell membrane (13,14). Like all replication-competent retroviruses, HIV contains three viral genes required for replication (Figure 1): gag genes, for the viral internal structural proteins; pol genes, for the reverse transcriptase, endonuclease and proteinase; and env genes, for the envelope proteins. All retroviral genomes, when integrated, are flanked on each end by sequence with powerful regulatory elements, called the long terminal repeat sequence (LTR). They not only regulate the expression of viral genes but also the expression of cellular genes nearby provirus integration. HIV also contains several genes that encode proteins with transcriptional modulating activity. One such gene (tat III) has been shown to be critical for virus replication and has also been implicated in the transforming capabilities of this virus (16,17,18,19).

Molecular analysis has revealed heterogeneity in restriction enzyme sites and nucleotide sequence between various viral isolates, and especially between isolates from different geographical regions (20,21,22,23). The greatest diver-

sity (17%) is present in the genomic region encoding the extracellular portion of the virion envelope glycoproteins. The various isolates are clearly highly related, however, since they show near complete antigenic cross-reactivity and hybridize under high-stringency conditions throughout the viral genome to subgenomic fragments of a full-length cloned viral probe (23,24,25).

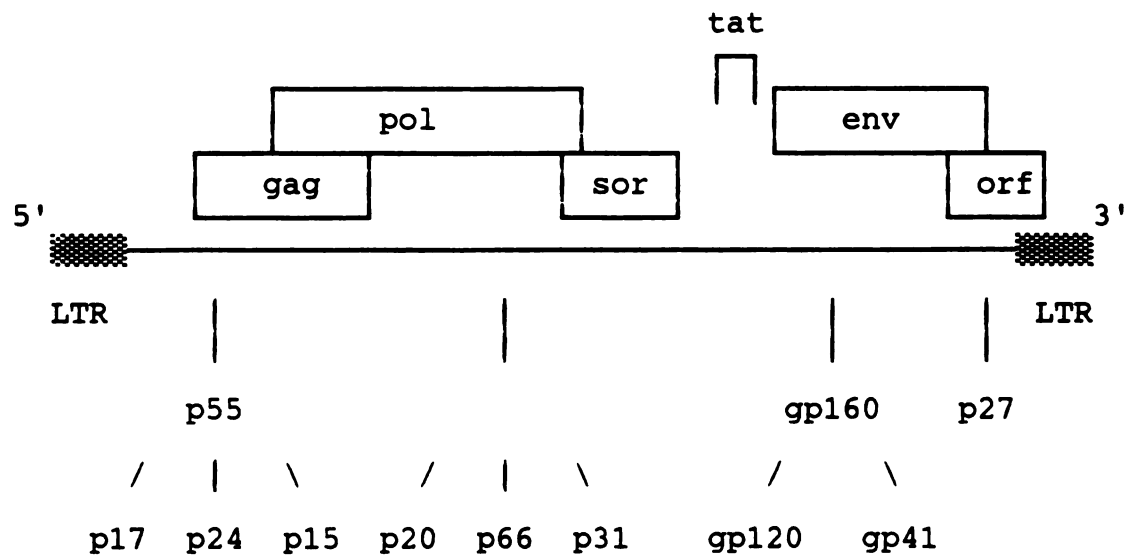


Figure 1. Schematic representation of the HIV genome and translational products.

C. Pathogenesis of AIDS

AIDS is associated with a variety of clinical disorders involving the lymphoreticular, respiratory, gastrointestinal, peripheral and central nervous systems. HIV particles have been successfully recovered from plasma, serum, saliva, sperm, tear, urine, cerebrospinal fluid, bone marrow, brain tissue and several other tissues of infected individuals (27,28,29,30). Of particular interest is the recent observation that the number of lymphocytes in the semen is elevated in individuals with a history of repeated sexually transmitted diseases (31).

Like most viruses, HIV has preferential target cells in which it replicates after entering the organism. HIV's tropism toward T4+ cells, with the associated cytopathic effects, is presumably responsible for the eventual loss of the immunologic functions that leads to evolution of the syndrome. However, it has subsequently been demonstrated that HIV can be present or even replicate in other cell types. For example, rare B lymphocytes and cells of monocyte-macrophage lineage (32,33,34,35) have been shown to contain viral particles. Furthermore, HIV has been shown to actually replicate in EBV-transformed B lymphoblastoid cell lines (36,37) and cell lines of monocytic origin (38,39).

The major biological property of HIV which distinguishes it from other previously described human retroviruses is its strong cytopathic effects (40). This cytolytic process is

not fully understood, but probably depends in part on the specific interaction between the viral envelope glycoprotein (gp110) expressed at the membrane of virus producing cells and T4 proteins expressed by other cells which are not necessarily infected (41,42,43,44). It has recently been reported that the expression of HIV envelope via transfection results in marked syncytium formation and concomitant cell death indistinguishable from the effects of infection with HIV itself (44,45).

The dynamics of HIV infection is complex, involving a balance between virus replication, impairment of the immune function and the host's immune response specific to the virus. For some cells, the infection results in productive HIV replication with prominent cytopathic effects, but in some other cells, infection results in proviral integration into the cellular DNA with maintained cell viability. This mode of viral persistence in the face of the antiviral immune response results in a long latency between antibody development and eventual disease outcome (46). Subsequent activation of latent provirus by immune activation (due to antigens, other viruses, etc.) leads to proviral gene expression, cytopathology, and progression to ARC/AIDS.

D. Laboratory Diagnosis of HIV Infection

D.1. Serologic Assays

At present, HIV appears to have genes that encode at

least six proteins or polyproteins (refer to Figure 1). At the 5' end of the genome is the gag gene, which encodes a polyprotein of approximately 55 kilodaltons (kd). This non-glycosylated 55-kd protein is subsequently cleaved to form p17, p24 and p15. Although p24 and p17 are detectable in both extracellular virion particles and disrupted virus-infected cells, the 55-kd precursor protein is not present in significant amounts in the virus. The env gene precursor protein, gp160, is cleaved to produce gp120, the most immunogenic protein in humans, and gp41. Both of these proteins are present in infectious virus particles and infected cells (47,48,49).

Several immunodiagnostic tests are currently being used to test patients and blood donors for exposure to HIV. The enzyme-linked immunosorbent assay (ELISA) (47,50); Western blotting test (WB) (47,50); radioimmunoprecipitation with sodium doldecylsulfate polyacrylamide gel electrophoresis (RIP-SDS/PAGE; RIPA) (49,51); indirect cytoplasmic immunofluorescence assay (IFA) (49,51,52) are used most frequently. The WB and RIPA tests can use either concentrated extracellular virus or homogenate from infected cells as the antigen source, whereas IFA employ infected cells. The ELISA tests currently available use concentrated virus as the antigen source, but it is likely that the proteins or small peptides made by expression vectors using recombinant DNA technology eventually could be substituted for whole virus,

resulting in improved test specificity.

All the serologic tests have advantages and disadvantages. Tests using only the proteins from purified extracellular virus, such as ELISA and WB tests, may lack sensitivity when the viral preparations contain little gp160, gp120, and, to some extent, gp41; these are readily lost during the purification process. Virus prepared in this manner is rich in p24 and p17, but these proteins are generally less immunogenic than the glycoproteins (49,51). These viral preparations are also contaminated with cellular products which were incorporated in the budding particles, and these cellular elements can result in false positive tests. Yet, the first generation ELISA tests have been most useful for both mass screening purpose and epidemiologic research (24,54). When the ELISA test is used for diagnosis, however, additional confirmatory tests, such as WB, RIPA and IFA are recommended to rule out the false positive results (55).

A confirmed antibody-positive result indicates that a person has been exposed to HIV and has mounted an immunologic response, but this does not definitively mean persistent infection. Furthermore, a few healthy people, apparently infected with HIV several months before culture, contain infectious virus in the absence of antibodies detected by any of the tests listed above (56). Therefore, these antibody tests do not indicate whether the person currently harbors the virus; carrier status can be firmly established only by

viral cultures.

D.2. Culture Monitoring Assay

Isolation of HIV from human peripheral blood mononuclear cells (PBMC) or body fluids of infected individuals is the definitive evidence of persistent infection and serves as a basis for assessing disease prognosis and monitoring the efficiency of therapy. In vitro cultivation of HIV in normal PBMCs or leukemic T4+ lymphocyte cell lines is also used for investigating the mechanism of viral replication, cytopathicity and persistence (57). The basis for HIV isolation includes lymphocyte activation, virus propagation, and virus detection (9,10,11). When mature cells are activated with antigens or mitogens, they turn on the gene for interleukin-2 (IL-2) receptors. Addition of IL-2 can then stimulate the growth of the cells. Proliferating lymphocytes are very susceptible to HIV, and virus amplifies to detectable levels.

Replication of HIV in cultures is monitored by the following methods:

D.2.i. Reverse Transcriptase Assay (RT)

All retroviruses including HIV have an outer lipid envelope and an inner protein nucleocapsid containing an RNA genome with an associated RNA-directed DNA polymerase also known as reverse transcriptase (RT) (58,59). A sensitive assay for detection of particle-associated RT activity in fluids from cultured PBMCs and other cells has been of major

importance in the isolation and propagation of HIV isolates. This enzyme synthesizes a double-stranded DNA copy of the genomic RNA. The DNA molecule can then integrate into the host genome and become a functional component of its genetic makeup (60). RT activity requires a 3'-OH-terminated primer complementary to a template. With natural endogenous heteropolymeric templates (e.g. viral RNA), all four deoxynucleotide triphosphates are required, but in vitro, RT efficiently copies synthetic homopolymer template-primer combinations (61,62,63). This enzyme always needs a divalent cation (Mg++ or Mn++) as a cofactor and is stabilized by the presence of nonionic detergents and sulfhydryl reducing agents (63).

The RT enzyme in HIV has been characterized, and the optimal conditions were established for a sensitive and specific RT assay. The RNA-dependent DNA polymerase of HIV gives highest activity with the exogenous synthetic primer-template, poly(rA)*oligo(dT)12-18 and prefers Mg++ over Mn++ as a divalent cation. Detection of HIV's RT in culture supernates is substantially increased with an optimal KCl concentration and a special combination of ethylene glycol-bis-(β-aminoethyl ether) N,N-tetraacetic acid (EGTA) (a calcium chelator) and reducing agents (63,64,65). We employed this optimized RT assay in our studies.

D.2.ii. Immunocytochemical Assay (IC)

HIV isolation and replication require prolonged human PBMC cultures, particularly with detection dependent on RT

activity. To reduce HIV culture time, various immunocytochemistry assays (IC) for detection of HIV antigens on lymphocytes were analyzed, including indirect immunofluorescence assay (IFA) (10,11), immunoperoxidase assay (IP) (67), alkaline phosphatase assay (AP) and alkaline phosphatase monoclonal anti-alkaline phosphatase assay (APAAP) (68). In addition, several different anti-HIV monoclonal and polyclonal antibodies were tested.

Immunofluorescence detection of cytoplasmic HIV antigens in cultured PBMCs or in cells following in vitro infection has been conventionally employed as a confirmatory assay for viral replication (10,11). Immunoperoxidase labeling methods are widely used in pathology for the location of antigenic constituents in tissue sections and cells smears, but have not been used extensively in virology. This is partly because many cells contain endogenous peroxidase activity which may partially obscure specific IP reaction. These problems are improved by using AP or APAAP, which are particularly suitable for labeling cell smears (for both cytoplasmic and surface-membrane antigens) and for detecting small amounts of antigen-bearing cells in specimens (68).

D.2.iii. Antigen Capture Assay (AC)

The two most commonly used methods for monitoring virus production in culture, RT and cytoplasmic staining assays, are only semi-quantitative. In 1985, McDougal's group described a new enzyme-linked immunoassay, or antigen capture

assay (AC), that detects viral antigens in small volumes of culture supernates (66,82). This assay is a sandwich immunoassay in which test materials (culture supernates containing HIV) are added to plastic microtiter wells which have been coated with human anti-HIV IgG or monoclonal antibodies. After washing, the bound virus is detected with horseradish peroxidase-conjugated anti-HIV reagents. The major advantages of this assay are that it can be performed on 0.1 ml of culture fluids by an ELISA method and that it is quantitative. A similar assay has been developed by using monoclonal anti-HIV (see Materials and Methodology Section) for detection of HIV antigen(s).

D.2.iv. In Situ Hybridization Analysis (ISH)

In situ hybridization (ISH) is a new method to detect target DNA/RNA in a subpopulation of cells containing the target sequences. The ISH technique has been used to study viral infections, such as HBV and CMV (71). Although immunocytochemical methods have been widely used to localize gene products within cells and tissues, these techniques are limited because endogenous proteins cannot be distinguished from exogenous proteins that have entered the cells by receptor-mediated endocytosis. With the expanding field of recombinant DNA technology, cloned DNA encoding a wide variety of proteins have become available. These can be employed for localizing sites of gene transcription by ISH using radiolabeled or enzyme-labeled probes (69,70).

Identifying viral genes in a particular cell type by ISH can shed light on pathogenesis of viral infections and suggest the mechanistic role of viruses in chronic disease (71). However, different tissues or cellular preparations require individualized modification of ISH techniques in order to optimize detection of specific signals (72,73).

Molecular analysis of the HIV genome has recently been carried out following cloning of viral-specific DNA (22, 26, 74). It has been shown that the HIV genome is 9.5 kilobases (kb) in length and that it lacks any cross-hybridizing nucleic acid sequences with normal human DNA. In our laboratory, in situ hybridization experiments have been initiated to detect HIV viral RNA in primary tissue samples and cultured cells from AIDS or ARC patients (75,76).

D.2.v. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method for in vitro enzymatic amplication of specific segments of genomic DNA, thus allowing for augmented detection or direct cloning into vectors for sequence analysis. This in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension via Klenow fragment of DNA polymerase. The result is an exponential increase in copies of the region flanked by the primers. Regarding sequence isolation, this method greatly reduces the number of cloned DNA fragments to be screened, circumvents the need for generating full genomic libraries, and may allow

cloning from nanogram quantities of genomic DNA. In terms of signal detection, this method allows rapid amplification of even single copy DNA to detectable levels, and thus promises to greatly augment HIV detection.

E. Objectives of Thesis Work

My research goal was to improve the method of cultivation and detection of HIV. As isolation and propagation of retroviruses like HIV is fastidious, I focused initially on optimizing the culture conditions in order to permit high level HIV growth *in vitro*. I then explored the merits of various HIV detection assays, presuming the hypothesis that in situ hybridization (ISH) with cloned viral probes and immunocytochemistry (IC) with monoclonal antiviral antibodies could be adopted to monitor retrovirus cultures, affording early assessment of intracellular viral replication. I hoped that these methods would offer more information than the current standard RT or AC methods, which are dependent on extracellular virus production. The standard methods employing RT or AC activity to monitor cultures for HIV replication did not appear to be highly sensitive, and RT was also technically cumbersome and expensive. I thus attempted to detect HIV in cultured human PBMCs by monitoring intracellular viral RNA with ISH and proteins with IC rather than detecting the complete virion as assessed by RT or AC activity in the culture supernates. These methods were applied in a study

population consisting of healthy seronegative blood donors, virus-exposed (seropositive) healthy individuals and patients with AIDS/ARC. The new methods (ISH and IC) of monitoring cultures were critically compared with the standard RT or AC activity to determine which methods offer the most information about the level of cellular infection and the pattern of *in vitro* viral replication.

CHAPTER II

MATERIALS AND METHODOLOGY

A. Propagation of HIV

A.1. Human Peripheral Blood Mononuclear Cell Culture

A.1.i. Preparation of Donor Cells

One buffy coat derived from a fresh seronegative donor's packed cells was obtained from Irwin Memorial Blood Bank, San Francisco. The blood cells were diluted with an equal volume of Hank's solution, layered over Ficoll-hypaque (78), and centrifuged for 30 minutes (min) at 700g at room temperature (without brake-deceleration). The interface containing the lymphocyte-enriched PBMCs was removed and washed three times in Hank's solution. The cells were reconstituted to 1 X 10^6 cells/ml in PRMI-1640 media containing 10% (v/v) DMSO. The cells were divided into 4 ml aliquotes, which were placed in a styrofoam box for controlled freezing to -60°C overnight. The next day, tubes were removed from the styrofoam box and stored at -60°C until needed.

When donor cells were needed for cocultured, they were rapidly thawed (one aliquot per clinical culture specimen) in

a waterbath at 37°C and placed in an appropriate culture flask. An equal amount of CO_2-gassed RPMI-1640 media containing 0.09% Glutamine (Gibsco, Grand Island, NY), 250 µg/ml Fungizone, and 0.03% $NaHCO_3$ was added. The flask was incubated at 37°C upright for 2 to 4 hours, then the supernatant fluid was removed (without disturbing the cell pellet), and replaced with CO_2-gassed RPMI-1640 media containing 0.09% Glutamine, Gentamicin, Fungizone, 0.03% $NaHCO_3$, 20% (v/v) inactivated FCS, and 5 µg/ml phytohemagglutinin-P (PHA-P; Difco, Detroit, MI). The tube was capped tightly and incubated vertically at 37°C for three days.

A.1.ii. Isolation of HIV from Human PBMCs

Peripheral blood mononuclear cell (PBMC) cultures were established from ten normal seronegative blood donors, five healthy seropositive individuals and six AIDS/ARC patients. All these individuals were selected at random from the Irwin Memorial Blood Bank, San Francisco and the AIDS clinic, University of California, San Francisco.

Human blood was collected by venipuncture into heparinized tubes. The tubes were centrifuged for 10 min at 600 g at room temperature, and the plasma was removed and saved at -60°C. The mononuclear cells were resuspended in a minimum volume of RPMI-1640 media (approximately 0.5 ml) in round-bottom tissue culture glass tubes. At the same time, most of the supernatant fluid of the three-days PHA-stimulated donor cell culture was removed, without disturbing cell pellet.

The remaining cells were mixed slightly and distributed equally among the patient's lymphocytes. The round-bottom culture tubes were incubated upright at 37°C for 2 hours. After two hours, a complete medium consisting of 2 ml of CO_2-gassed RPMI-1640 media containing 0.09% Glutamine, 50 µg/ml Gentamicin, 250 µg/ml Fungizone, 0.03% $NaHCO_3$, 20% (v/v) inactivated FCS, 5% (v/v) interleukin-2 (IL-2; Cellular Products, Buffalo, NY), 2 µg/ml polybrene (Sigma, St. Louis, MO), and 0.1% anti-α interferon (Miles Scientific, Elkhart, IN) (10) was added and the culture was incubated vertically at 37°C overnight.

The next day, culture fluid was removed without disturbing the cell pellet and replaced with fresh culture media. After the initial four days in culture, supernatant fluids were routinely removed without disturbing cells and saved at -60°C for later assessment of RT and AC activity. The cultured PBMCs were resuspended carefully in fresh culture media, and an aliquot of cultured cells were harvested and spotted on pretreated microscopic slides for ISH and IC assays as described below. Once a week, the culture was fed with PHA-stimulated normal donor's PBMCs. Normal donor PBMC cell cultures were regularly checked for RT, AC, IFA, ISH and AP to ensure that no contamination by HIV had occurred.

A.2. Cultivation of Human T-Cell Lines

Three cell lines as well as the three-days-PHA-stimulated PBMC from seronegative donors were inoculated with HIV

in vitro. HUT-78, a human T-cell line, has been useful for the continuous propagation of HIV (11). H-9, which has also been used for the large-scale production of HIV, exhibits only modest cytopathic effects and no detectable cell death as a consequence of virus infection (40). A3.01, a variant of the CEM T-cell line, has been reported to be more than 95 percent susceptible to infection and undergoes all of the cytopathic changes associated with HIV, including cell death (79). These cells are maintained at a density of 1 to 3 x 10^6 cells/ml in culture fluids.

A.3. In Vitro Infection of Cultured Cells with HIV

Culture fluids from HIV-infected HUT-78 cells were filtered through a 0.45 μm membrane (Millipore, Bedford, Mass) and stored at -60°C as stock until used. The PHA-stimulated cells or HUT-78, H-9, and A3.01 cells were harvested by centrifugation (300 g for 5 min) and then resuspended to 2 x 10^6 cells/ml in the complete RPMI medium without PHA; no IL-2 was included in cell line cultures. Cells were inoculated with the stock HIV fluid (10,000 reverse transcriptase cpm per 2 x 10^6 cells) and incubated at 37°C for 18 hours. The cells were again pelleted by centrifugation and resuspended at 1 x 10^6 cells/ml in the complete media. Cultures were harvested every three days for RT, AC, ISH and IC assays. Uninfected cell lines and normal PBMC cultures were maintained and assayed in parallel as negative controls.

B. Reverse Transcriptase Assay

The presence of particle-associated RT activity in the culture fluids was generally assessed at intervals of three to four days after the first four days. These fluids were stored at 4°C if they were to be assayed within 5 days, or kept frozen in -60°C. In order to remove all cellular debris (which might contain polymerases), the supernates were either slow-spun (5,000 rpm for 20 min at 4°C in a Beckman-21 rotor; Beckman, Palo Alto, CA) or filtered through a 0.45 µm millipore filter. Virus was subsequently pelleted by one of three methods: (1) in an SW 41 rotor at 40,000 rpm for 45 min at 4°C in a Beckman L2-65B ultracentrifuge, (2) in an Eppendorf microfuge at 12,000 rpm for 2 hours at 4°C, or (3) in an airfuge at 150,000 rpm for 30 min at 4°C.

The virus pellet was then resuspended in 40 µl reagent mixture and mixed on ice. The reaction mixture included the following: 50mM Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), 5mM $MgCl_2$, 150 mM KCl, 0.05% Triton X-100, 0.3mM glutathione, 0.3mM EGTA. After 10 min on ice to solubilize the enzyme, 50 µg/ml poly(rA)*oligo(dT)12-18 (Pharmacia) and 20 µCi [^{3}H] thymidine triphosphate (100 Ci/mmole) (NEN, DuPont, DE) were added to the reaction mixture to a final volume of 50 µl. The tube was vortexed and placed in a water bath at 37°C for 60 min.

The reaction was stopped by adding 50 µl of cold 0.01 M sodium pyrophosphate (NaPP) in 1N HCl. The product was

allowed to precipitate on ice for 10 min, and subsequently vortexed and filtered through the dry Whatman No. 1 filter paper which had been pretreated in 0.1M NaPP. After spotting, the filter papers were completely dried under lamp, and then batch washed in 500 ml of 20% trichloracetic acid (TCA) in 0.01M NaPP for 10 min at 4°C (x2), followed by 10% TCA in 0.01M NaPP for 10 min (x2), 5% TCA for 10 min at the same temperature (x2), and then rinsed twice with cold 80% EtOH. Filters were dried, and then placed in 5 ml Aquasol-2 (NEN, Boston) scintillation cocktail and the radioactivity was counted for one minute. Level of the negative control supernatants on RT assays was never more than 5,000 cpm/ml (63, 64,65).

C. Antigen Capture Assay

The antigen capture assay was performed by Dr. K. S. Steimer of Chiron Corporation. Microtiter plates were coated with 10 µg/ml (100 µl/well) of an ammonium sulfate cut of ascites from anti-p25 monoclonal antibody. Triton X-100 (1% of final concentration) was added to the culture supernatants. The mixtures were added to duplicate wells of coated microtiter plates that had been washed in saline with 0.05% Triton (ST). The microtiter plates were incubated for 2 hours at 37°C, and then washed with ST. The plates were then incubated with rabbit antiserum to HIV (with a high titer of the anti-p25 antibody) diluted 1/1,000 in buffered saline

with 1% Triton and 0.1% Casein at 37°C for 1 hour. After incubation, the plates were washed and incubated with horseradish peroxidase conjugated goat antiserum to rabbit IgG (1/1,000 dilution) for 30 min at 37°C. The plates were washed and developed with diaminobenzidine.

D. Immunocytochemical Assay

D.1. Pretreatment of Glass Slides

All specimens (cellular preparations) to be analyzed by IC or ISH were placed on glass slides which had been pretreated to reduce background non-specific probe binding to glass and also to prevent the loss of cells or tissue sections from the slides during IC or ISH procedures. There were two ways to treat slides:

(1) Slides were incubated at 65°C for 3 hours in 450 mM NaCl/ 45 mM Na Citrate, pH 7.0, containing Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) (80). After the incubation period, slides were rinsed thoroughly with double distilled water and fixed for 20 min in ethanol: acetic acid (3:1) (73).

(2) Slides were placed in 1 N HCl for 15 min at room temperature, and then thoroughly rinsed with double distilled water. These slides were removed to 90% EtOH for 15 min and dry completely. Once dried, slides were then immersed in 0.01% poly-L-lysine (MW 1,500-8,000, Miles Scientific, Naperville, IL) in pH 7.8-8.0 phosphate buffer for 15 min at room

temperature. Slides were again allowed to dry in a dust-free oven at 65°C (81). Although we initially used Denhardt (above 1) solution, subsequently we have used poly-L-lysine (above 2) for pretreatment of glass slides.

D.2. Preparation of Cells for IC Assays

Cells were gently harvested and resuspended to 2 x 10^6 cells/ml in 0.1M phosphate-buffered saline (PBS). Between 2 and 4 x 10^4 cells were deposited per slide or per well of 10-well multispecimen slides (Polyscience Inc., Warrington, PA). Each multi-wells slide included HIV-infected HUT-78 cells as a positive control, uninfected cultured PBMC as a negative control, and eight test specimens. After the spots were dry, the slides were fixed for 15-30 min with fresh 2% paraformaldehyde, 0.075M lysine, and 0.01M periodate in 0.037M phosphate buffer (PLP fixation), dehydrated for 15 min in 90% EtOH, and stored at 4°C until tested by IFA and IC assays.

D.3. Immunofluorescence Assay

PLP-fixed slides were rehydrated with three 5-min changes of immunofluorescence assay buffer (FA buffer; Difco.) (PBS: 0.01M PO_4, 0.15M NaCl, pH 8.0). Slides were incubated with 1:500-1:300 diluted mouse monoclonal antibody against HIV core proteins (anti-p18 and anti-p25) (Genetic Systems, Seattle, Washington) for 30 min at 37°C in a humidified incubator. The slides were then washed with FA buffer (three changes, 5 min each). They were then incubated with fluorescein-isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-

mouse IgG (TAGO) for 30 min at 37°C in a humidified chamber. After incubation, the slides were washed with FA buffer (three changes, 5 min each) and mounted under a coverslip with 50% glycerol in PBS (10,11). The slides were then interpreted for cellular fluorescence with a fluorescent microscope.

D.4. Immunoperoxidase Assay

Immunoperoxidase assay (IP) was performed using avidin-biotin-horseradish peroxidase complex (ABC Kit, Vector Laboratories, Burlingame, CA) and 1:200 diluted mouse ascites fluid containing monoclonal antibody against HIV-p18 protein (Genetic Systems). The specific antigen-antibody binding was detected using H_2O_2 as substrate and diaminobenzidine (DAB) as chromogen. Non-immune mouse ascites fluid (Meloy Laboratories) was used on HIV-infected HUT-78 cells as another negative control.

Specifically, PLP-fixed slides were treated with 1% H_2O_2 in methanol to block endogenous peroxidase and then washed three times with PBS (0.05M PO_4, 0.15M NaCl, pH 7.45). Procedures were always carried out at room temperature and in humidified chambers. The cells were flooded with horse serum (ABC Kit) for 20 min so as to prevent non-specific protein binding to the cells in the later steps. Slides were then flooded with 1:200 diluted mouse monoclonal antibody to HIV p18 (Genetic Systems) for 1 hour. After the slides were washed three times, they were incubated with biotinylated

horse anti-mouse IgG as secondary antibody (ABC Kit) for 1 hour and then washed three times with PBS. The slides were incubated with ABC reagent (avidin-biotinized enzyme complex) for 1 hour, washed three times with PBS, and rinsed once in 0.1M Tris-HCl, pH 7.45. Then, the slides were immersed in 0.5 mg/ml DAB, 0.034% H_2O_2, 0.05M Tris-HCl, pH 7.45 for 7 min in dark condition and washed twice with water. The cells were counterstained by hematoxylin for 2 min, and immediately rinsed with lithium carbonate solution (pH 8-10) to turn the counterstain color blue. Then the slides were permanently mounted with Permount and coverslipped. Cells that showed typical brown color in the cytoplasm were considered positive (67).

D.5. Alkaline Phosphatase Assay

Alkaline phosphatase assay (AP) is an enzyme reaction whose principle is similar to IP as described above. The cells were first rehydrated in Tris buffered saline (TBS; 0.05M Tris-HCl, 0.15M NaCl, pH 7.4) and incubated with horse serum (VECTASTAIN ABC Kit. VECTOR) for 20 min. The procedures were also carried out in humidified condition and at room temperature. After the remaining serum was drained off, 1:300-1:500 diluted mouse monoclonal antibody against p18 was added for 40 min, and then slides were washed three times with TBS. The cells were incubated with secondary antibody (ABC Kit) as above for 30 min and washed with TBS. Then, the slides were incubated again with ABC Reagent for 30 min and

washed six times to clean the slides completely. 0.1M Levamisole was used to block endogenous enzyme. Fast red (Vector) was used to develope the visible red color indicating a positive reaction.

E. In Situ Hybridization Analysis

E.1. Preparation of Cells for ISH

Cells were prepared and fixed as described above for IC assay. PLP fixation satisfied two important requirements: morphological features of cells were retained at the level of the light microscope, and the nucleic acids were fixed in the cell under optimal conditions for hybridization.

E.2. Preparation of ^{35}S-labeled HIV Probe

^{35}S-labeled RNA probes specific for HIV were generated by nick translation of pARV-7 A/2 recombinant plasmids obtained from Chiron Corp. This plasmid contains a EcoRI permuted ARV-2 9.5 kb fragments, representing the entire HIV genome. The insert was labeled by nick translation with ^{35}S-dATP and ^{35}S-dCTP to specific activity of 2 x 10^8 dpm/µg. Purified probes were stored at -20°C until used.

E.3. Prehybridization

PLP-fixed slides were soaked in 0.2N HCl for 20 min at room temperature and then washed briefly. Slides were gently placed in 2XSSC (0.3M NaCl/ 0.03M Na Citrate) at 55-60°C for 30 min and rinsed in double distilled water. Then the cells were digested with 1µg/ml Proteinase K in 20 mM Tris-HCl, pH

7.4, 2mM $CaCl_2$ for 15 min at 37°C. The purpose of this treatment was to increase the accessibility of the RNA without altering structural morphology.

After digestion, the slides were briefly washed two times with water, incubated in 0.25% (v/v) acetic anhydride in 0.1M Triethanolamine (TEA) pH 8.0 for 10 min at room temperature, and rinse twice with double distilled water. A brief acetic anhydride treatment before hybridization serves to neutralize the positive charges on specimens and slides, thus reducing non-specific electrostatic binding of probes. As necessary, control slides were treated with RNase digestion: 100 µg/ml RNase A, 10 units/ml RNase T in 2XSSC at 37°C for 30 min; or DNase digestion: 200 µg/ml DNase I in 20 mM Tris-HCl pH 7.5, 10mM $MgCl_2$ at 37°C for 60 min. The slides were rinsed twice in 2XSSC.

E.4. Hybridization

The slides were post-fixed in 5% paraformaldehyde, 0.3N NaOH, in PBS pH 7.5 at room temperature for 1 hour in the dark and then washed twice with 2XSSC. This was followed by denaturation in 95% deionized formamide in 0.1XSSC at 65-70°C for 15 min. Then, slides were quenched in chilled 0.1XSSC for 3 minutes, rinse in cold double distilled water, and dehydrated in graded ethanol.

Hybridization mixture contains 10 mM HEPES, pH 7.4, 1mM EDTA, pH 8.0, 1X Denhardts' solution, 50 µg/ml polyadenylic acid (poly 'A' sodium salt; Sigma), 600nm NaCl, 10mM DTT, 50%

formamide, 1 µg/µl calf thymus DNA, 0.1 µg/µl Sigma baker's yeast RNA, and 0.3 ng/µl ^{35}S-labeled probe. The mixture was boiled at 90°C for 10-15 min to separate the DNA into single strand and then quickly quenched on ice. The appropriate volume of solution was placed on the specimen and covered with a glass coverslip which had been siliconized and heated to 180°C for 2 hours. The edge of the coverslip was sealed with rubber cement, and hybridization was carried out in the dark for 2-3 days at room temperature.

E.5. Washing Conditions

To reduce background level, proper execution of the washing step is essential for removing any non-specifically bound probes. After hybridization, the cover slips were gently removed, and the slides were rinsed with 2XSSC by squeeze bottle. Then the slides were transferred to 2XSSC at 42°C for 30 min. Slides were placed in 2,000 ml of hybridization washing media (HWM) consisting of 50% formamide, 100mM EDTA, 0.6M NaCl, 200mM Tris-HCl pH 7.4 for three days at room temperature. Scraps of nitrocellulose paper were included in the wash buffer to reabsorb free probes from solution. Slides were again rinsed with 2XSSC and dehydrated in graded EtOH containing 0.3M ammonium acetate to stabilize hybrids.

E.6. Autoradiography

Autoradiography should be carried out in absolute darkness. Nuclear track emulsion (Kodak NTB-2) was melted in a water bath at 42°C and diluted 1:1 with 600mM ammonium ace-

tate. Air bubbles of emulsion were removed by repeatedly dipping blank slides into the emulsion. The hybridized slides were dipped in the emulsion for a few seconds, and placed vertically in racks for drying. After drying for 3 to 4 hours at room temperature, the slides were placed in light-proof slide boxes containing a small amount of desiccant. The boxes were sealed with tape and put in a refrigerator (4°C) for 2 to 4 days.

Before the slides were developed, the boxes were warmed to room temperature for 1 hour to prevent moisture condensation on the slides. The slides were developed in the dark for 3 min in Kodak D-19 developer at room temperature, briefly dipped in tap water, transferred for 3 min in Kodak Rapid Fixer, and then briefly rinsed in tap water. The slide were washed two final times in water and then stained with Hematoxylin for 2 min. They were coverslipped with permount. Quantitation of the positive cells was achieved by examining six fields at 450X magnification, and expressed as a percentage of all cells (72,73,75,76).

CHAPTER III

RESULTS

A. Specificity of In Situ Hybridization for HIV RNA

Specificity of the ISH method was shown by use of a T-cell line, HUT-78. This is a neoplastic cell line which is susceptible to HIV, but is relatively resistant to the cytopathic effects (e.g. cell lysis) of HIV infection. Thus, HIV infected HUT-78 cells chronically produce HIV. Cells from uninfected HUT-78 cells exhibited essentially no grains when hybridized with HIV probes, as shown in Figure 2. In contrast, Figure 3 and 4 depict the highly significant labeling obtained over HUT-78/HIV cells by ISH. By microscopy, approximately 50-200 grains per cell were observed on individual cells, while 200-500 grains were observed on some large, multinucleated cells (a characteristic cell type observed after HIV infection).

The specificity of ISH was also shown by nuclease digestion and control probe experiments. When infected cells were treated with RNase prior to HIV hybridization, no labeling of cells was observed. This indicates that the predominant

signal detected was RNA. In addition, only the background signal was observed when cells were hybridized with the ^{35}S-labeled HBV DNA control probe or the plasmid-specific control probe. Thus, observation of grains following hybridization with ^{35}S-labeled HIV probe correlated conclusively with the presence of HIV, demonstrating that ISH according to this method exhibited specificity for viral sequences.

B. Specificity of Immunocytochemical Assays

Specificity of IC is inherent in the use of monoclonal antibodies which are against specific HIV proteins. Two enzymatic procedures were used, using either horseradish peroxidase or alkaline phosphatase. The specific antigen-antibody binding was detected by use of DAB or fast red as respective chromogens, and cells that showed dark brown by IP or bright red by AP in the cytoplasm were considered positive. Although both procedures gave comparable results, the result from AP was much more easily discernible than IP. Uninfected cells were used as a negative control. Non-immune mouse ascites fluids were also used on HIV infected cell preparations as a second negative control. Figure 5 shows the typical result on infected HUT-78/HIV cells by IC.

C. Specificity of RT and AC assays

The RNA-dependent DNA polymerase of HIV gave high specificity with a characteristic preference for Mg++ over Mn++ as

a divalent cation and rAdT as a template. This fact distinguished HIV'S polymerase from the other human retroviruses. RT activity was eventually elevated by using the new culture techniques and optimal RT assay procedures.

When we compared the RT activity from pellets obtained by ultracentrifuge, top-speed microfuge, and airfuge, the result indicates that no substantial difference between ultracentrifuge and microfuge which could handle 1.0 ml volume of culture supernates. However, the airfuge at speed of 150,000 rpm appeared to be the most efficient method for pelleting virus particles. Because ultracentrifuge took shorter time than microfuge and also because airfuge could not handle 1.0 ml volume for pelleting HIV particles, we chose the ultracentrifugation as the method of choice for virus concentration.

The specificity of antigen capture assay was given by use of monoclonal antibody against the p25 gag protein. Figure 6 shows the relative sensitivity of diluted inoculum of the HIV culture supernatant tested by RT and AC. In conclusion, AC activity appeared to be relatively parallel to RT.

D. Patterns of <u>In Vitro</u> Infection with HIV

The time course of acute HIV infection in normal PHA-stimulated PBMCs and three selected cell lines, HUT-78, H-9, and A3.01, was characterized. Virus replication was readily

detected in cultured cells by ISH detection of intracellular viral RNA and IP staining of cytoplasmic viral proteins, and by release into the culture supernatants of mature viruses as detected by particulate reverse transcriptase activity and antigen activity.

Figure 7 depicts the kinetics of in vitro inoculation of HUT-78 cells with HIV. Figure 8 showed the kinetics obtained by in vitro infection of H-9 cells with HIV. In both HUT-78 and H-9 infected cell cultures, ISH signal was observed in a small proportion, less than 0.01% of cell populations, within 24 hours of HIV inoculation. By day 4 post-inoculation, up to 90 percent of cells showed strong silver grains by ISH in both infected cultures. Numerous multinucleated giant cells which are characteristics of acute infection were observed at the same time. Subsequently, HUT-78 cells maintained 80-90% of HIV positive cells and later declined to 20% of the cells. The positive IP reaction was first found in rare HUT-78 and H-9 cells on day 3. Over the following days, an increasing proportion of cells expressed viral antigens until approximately 90% of HUT-78 cells and 4% of H-9 cells were IP positive. RT and AC activity were first detected in the culture fluids of infected HUT-78 cells on day 9, and of infected H-9 cells on day 12. None of these assays were positive on control uninfected cultures, which were cultured and assayed in parallel.

The kinetics of HIV infection in normal PHA-stimulated

PBMC is illustrated in Figure 9. Intracellular viral RNA was detected in 1% of cells by ISH within 24 hours of inoculation and generally peaked at day 5 post-inoculation with as many as 15% of cells showing ISH signal. By day 10 following inoculation, the number of ISH positive cells diminished to about 1% of the viable cell population, and the infection persisted at that level until the termination of culture. Simultaneously, the expression of viral antigens was detected by IP in 1 of 1,000 cells on day 3, increased to 7% by day 12 and then declined to 1% by day 18. As with the infected cell lines, cytopathic effects, including the presence of cell fusion, cytoplasmic ballooning, multinucleated giant cells and degenerative cellular forms, were observed in the positive PBMCs during the several days coinciding with peak ISH and IP reactivity. The RT and AC assays on this culture were first positive on day 12, peaked at day 15 and subsequently declined to background level.

In another group of experiments, the sensitivity of A3.01 cells to HIV infection was compared to those of HUT-78, H-9, and PHA-stimulated normal PBMCs by detection of RT activity (see Table 1). The results showed that the A3.01 cells were definitely more resistant to HIV than HUT-78, H-9, or PBMCs; the inoculum employed was unable to cause a persistent infection; viral RNA and antigens expression was much lower and delayed. Thus, A3.01 cells were not as susceptible to HIV infection as originally reported (79) as well as the

other cells.

E. Preliminary Studies of HIV in an Antibody-Positive Healthy Gay Man

PBMCs purified from an anti-HIV positive, healthy gay man were PHA-stimulated and allowed to proliferate for 6 days, following which they were cocultured by adding PHA-stimulated normal PBMCs every three to five days. Hybridization of HIV probe to the uncultured PBMCs resulted in labeling of very rare cells, only 1 in 10,000 cells, which represented in vivo HIV-expressing cells. Three days after activation by PHA, 10% of cells displayed definitive ISH signals localized to the cytoplasm. Viral antigen, p18 and p25, were identified by IP beginning on day 9 and reached 8% of cells on day 15. RT activity was found transiently in the culture supernates 15 days after activation, and AC assay was evidently positive on day 20. After this period, the culture exhibited abundant cell lysis and the cell viability declined to less than 30% of cells. When the culture was carried until day 30, RT and AC activity had returned to baseline level, but about 1% of the residual cells remained positive by ISH and IP. Figure 10 depicts the kinetics of HIV replication in the PHA-stimulated PBMCs from this seropositive man. Similar pattern was repeatably observed on two subsequent cultures during a six month period.

I next compared whether PBMCs or T cell lines were more

susceptible to HIV infection in the setting of cocultivation. I established several different cocultures by adding to a primary PBMC culture from this seropositive person either: (1) PHA-stimulated normal PBMCs, (2) H9 cells, or (3) A3.01 cells. The viral expression was monitored by measuring RT activity. All three cell types could readily propagate HIV from the primary PBMC cultures. Cell to cell transmission appeared to be particularly efficient, given the low level of cell-free HIV in the culture. Results indicated that the infectivity of HIV was higher with PHA-stimulated homologous PBMCs (see Table 2).

F. Studies of Healthy Blood Donors Found to be HIV Antibody Positive

Based on the above preliminary experiments, I improved the HIV culture method so as to yield a much greater percentage of positive cultures from seropositive persons. Our laboratory adopted this technique, including the following modifications: (1) the test PBMCs are cocultured with PHA-stimulated normal PBMCs at the initiation of culture (2) the cellular aggregates are not disturbed, allowing the compacted cells to transmit HIV through cell to cell contact (3) the media are acidified with CO_2 to pH 6.8. This method increased the rate of recovery of virus from clinical patients and anti-HIV positive individuals. It also increased the level of replication of HIV as determined by RT activity in

culture fluids. The RT detection was substantially increased with optimized reaction conditions and a modified washing procidure.

Five anti-HIV positive and five anti-HIV negative healthy individuals were chosen randomly from IMBB, and their PBMCs were cultured. Recovery of HIV in the five seropositive PBMC cultures was 60% (3 out of 5). RT activity form the three positive cultures was detected within 9-18 days after initiating cultures of PBMCs. Viral antigens were first identified by both AP and IFA assays on day 4 in very rare cells, and subsequently increasing positive signals in these positive cultures. However, ISH signals were found later than antigen expression. In these three positive cultures, ISH detection almost paralleled the RT activity. Figure 11 and 12 depict the viral expression detected by ISH, AP, IFA and RT in these three positive cultures. HIV was not isolated from any of five individuals which were anti-HIV negative healthy donors.

G. Studies of AIDS/ARC Patients

PBMCs from six AIDS/ARC patients were also cultured for HIV. By using the modified culture protocol, HIV was recovered from AIDS/ARC patients at a frequency of 100% (6 out of 6). RT activity was first detected within day 6-10, usually with a rapid increase to millions of cpm. When compared with the antibody-positive healthy individuals' cultures, RT

activity form the AIDS/ARC patients' cultures were positive at 3 to 10 days earlier and had three times higher RT activity. This success presumably reflects the presence of high viral activity in the infected cells of these patients. Three to five days after the initiation of cultures, the antigens were detected by AP and IFA. By these methods, antigen signals were increasing to 5-10% of cells the following days. As mentioned above, ISH signals were also found to be positive in parallel with the RT activity. Cytopathic effects including multinucleated giant cells and cell death were also characteristics of these cultures. Figure 13, 14 and 15 show the viral propagation in the six AIDS/ARC patients by detection of ISH, AP, IFA and RT.

*	DAY 3	DAY 6	DAY 9	DAY 12	DAY 15
PBMCs	3.2	**10.8**	**40.3**	**80.1**	**46.3**
HUT-78	4.0	4.2	**568.0**	**532.8**	**517.2**
H-9	2.8	4.1	4.6	**76.6**	**236.9**
A3.01	2.5	5.2	4.1	8.7	**105.8**

* : unit of RT activity : cpm (x 10^3)

positive control : 560 x 10^3 cpm

negative control : 3.0 x 10^3 cpm

Table 1. Comparison of PHA-stimulated normal PBMCs, HUT-78, H-9 and A3.01 cells <u>in vitro</u> infected with HIV by detection of RT activity.

*	DAY 4	DAY 6	DAY 9	DAY 12	DAY 15	DAY 18
PBMCs	4.4	3.1	**9.7**	**12.5**	**27.2**	**85.6**
H-9	2.8	2.5	2.8	**10.6**	**36.3**	**14.3**
A3.01	3.2	3.0	3.7	6.5	**14.5**	**13.7**

* : unit of RT activity : cpm (x 10^3)

positive control : 560 x 10^3 cpm

negative control : 3.0 x 10^3 cpm

Table 2. Comparison of coculture of primary PBMCs with PHA-stimulated normal PBMCs, H-9 and A3.01 cells by detection of RT activity.

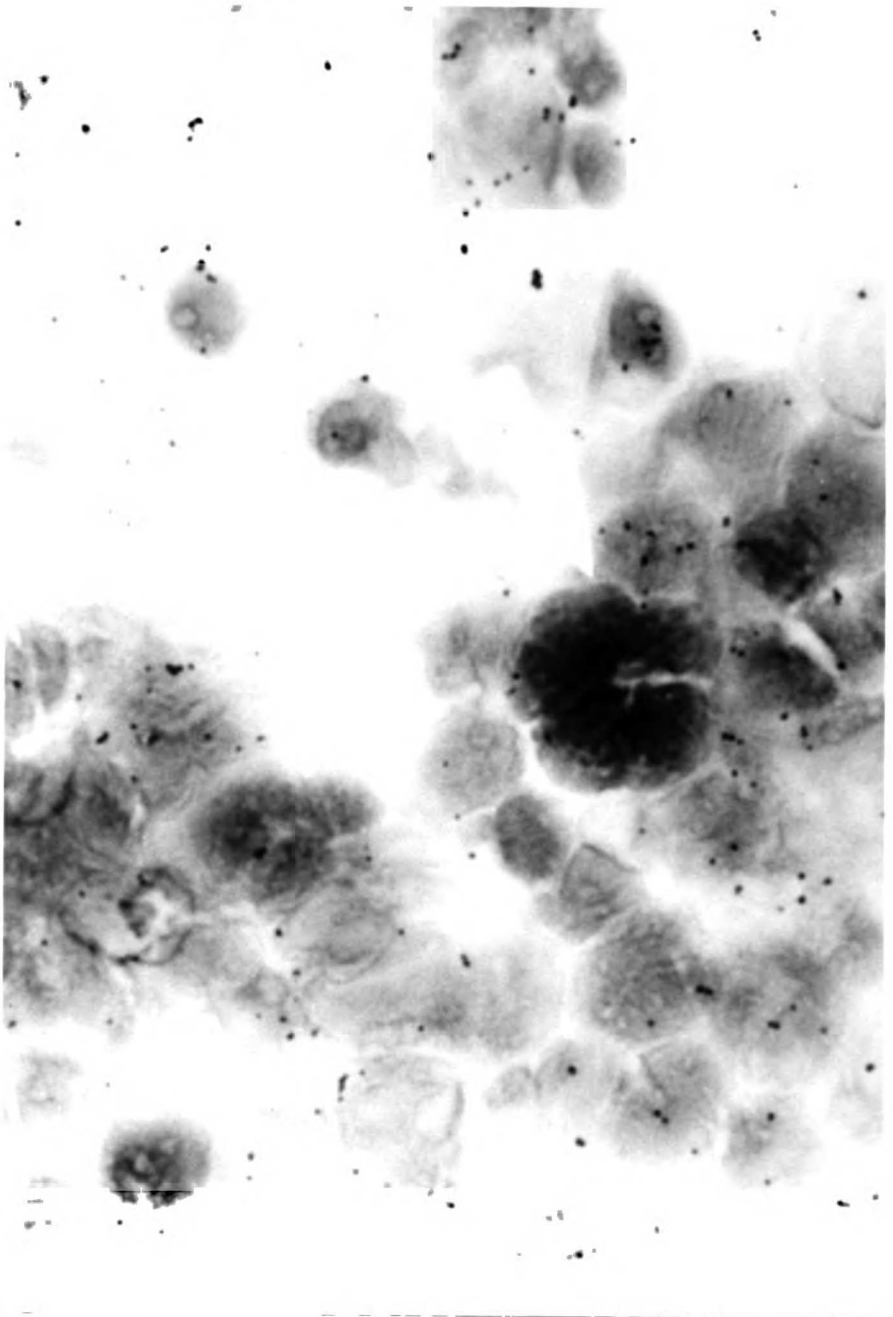


Figure 2. Photomicrograph of ISH on uninfected HUT-78 cells.

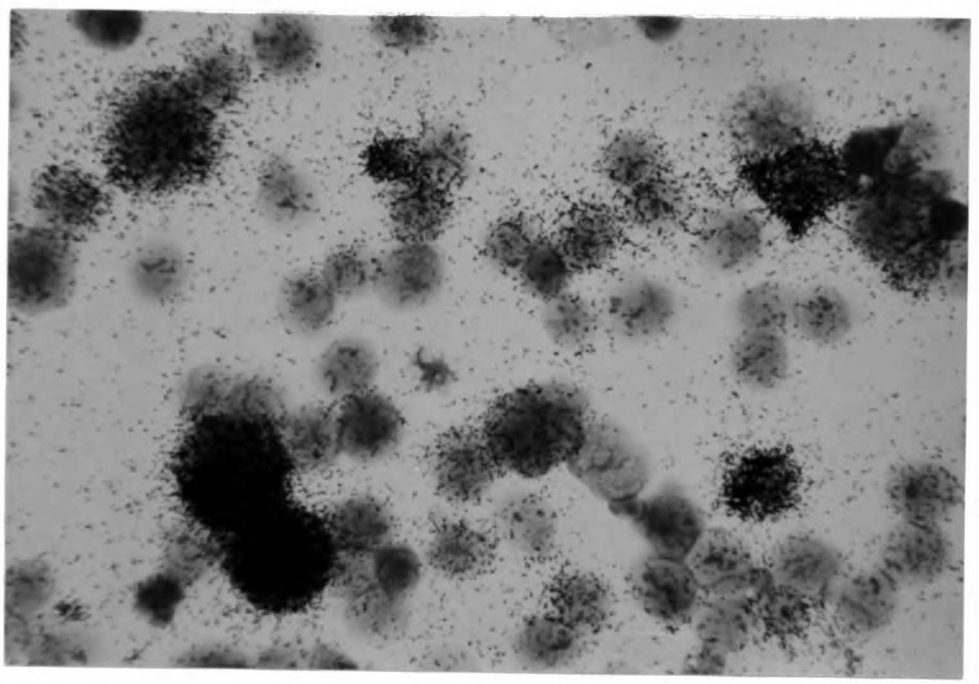


Figure 3. Photomicrograph of ISH on HIV-infected HUT-78 cells.

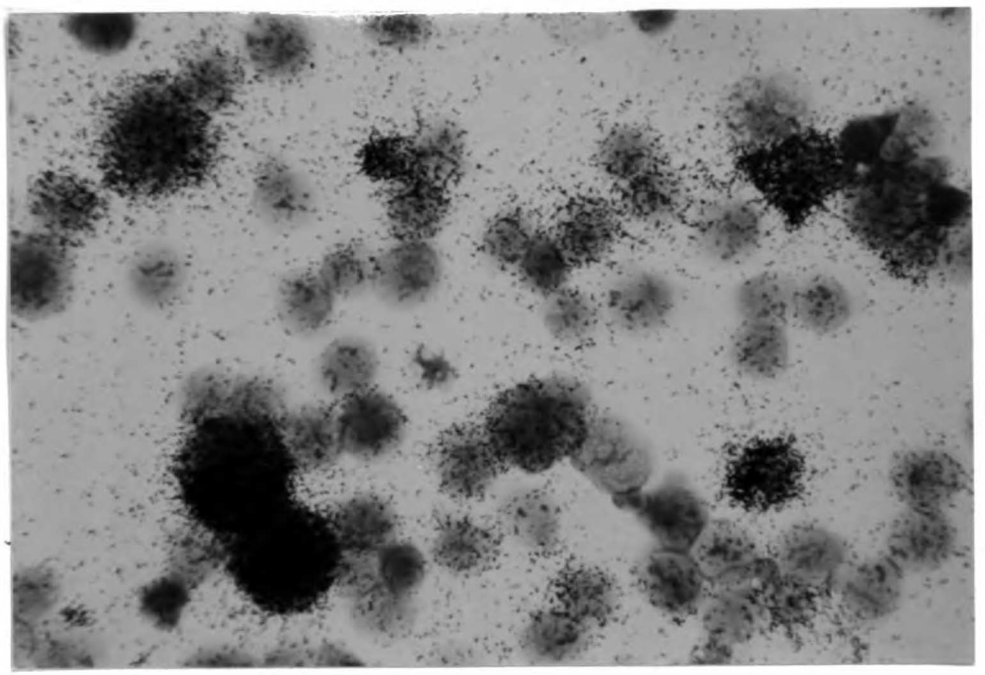


Figure 4. Photomicrograph of ISH on HIV-infected HUT-78 cells.

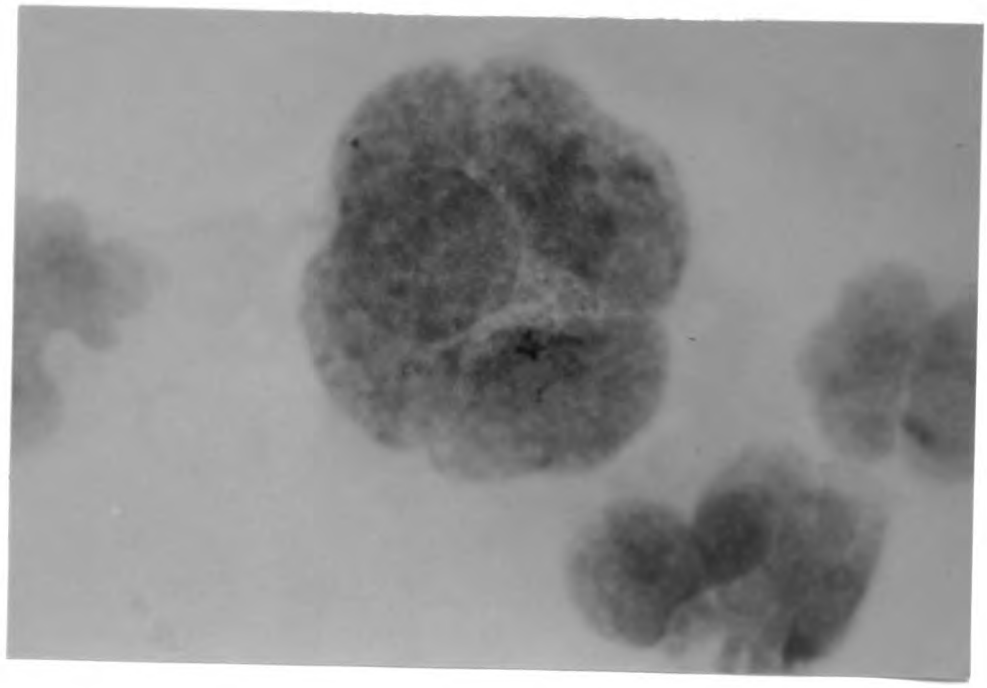


Figure 5. Photomicrograph of IC on HIV-infected HUT-78 cells.

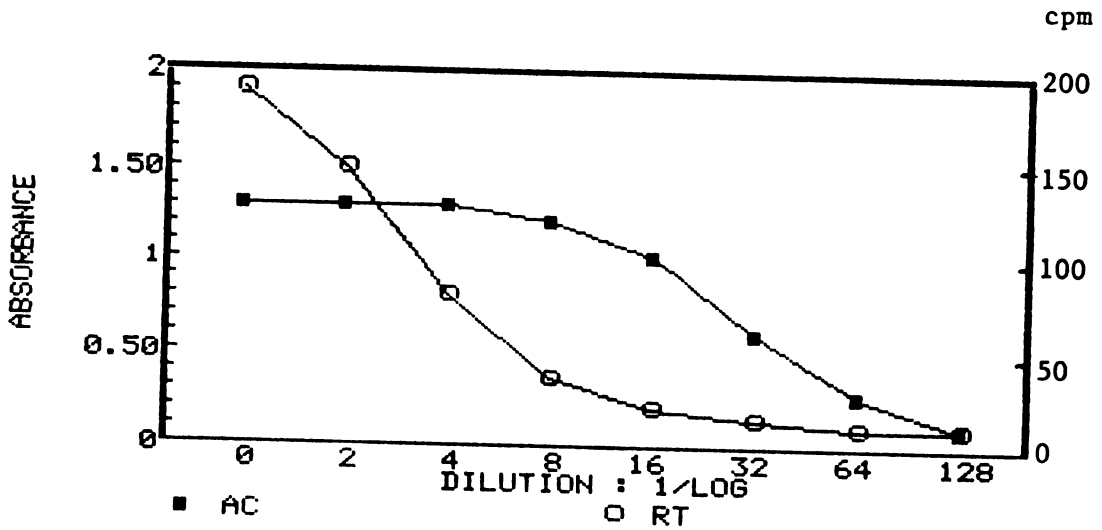
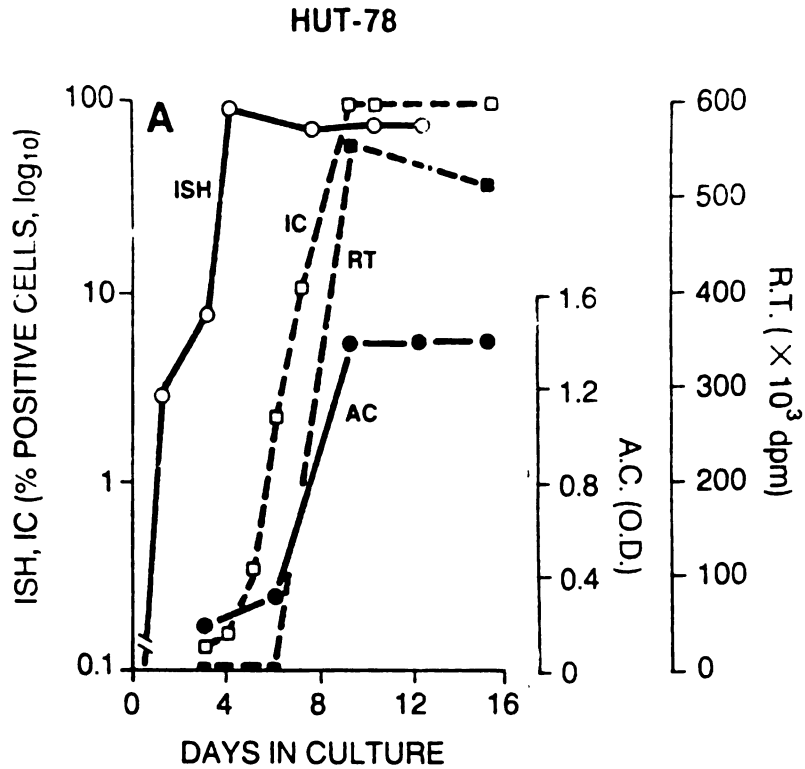


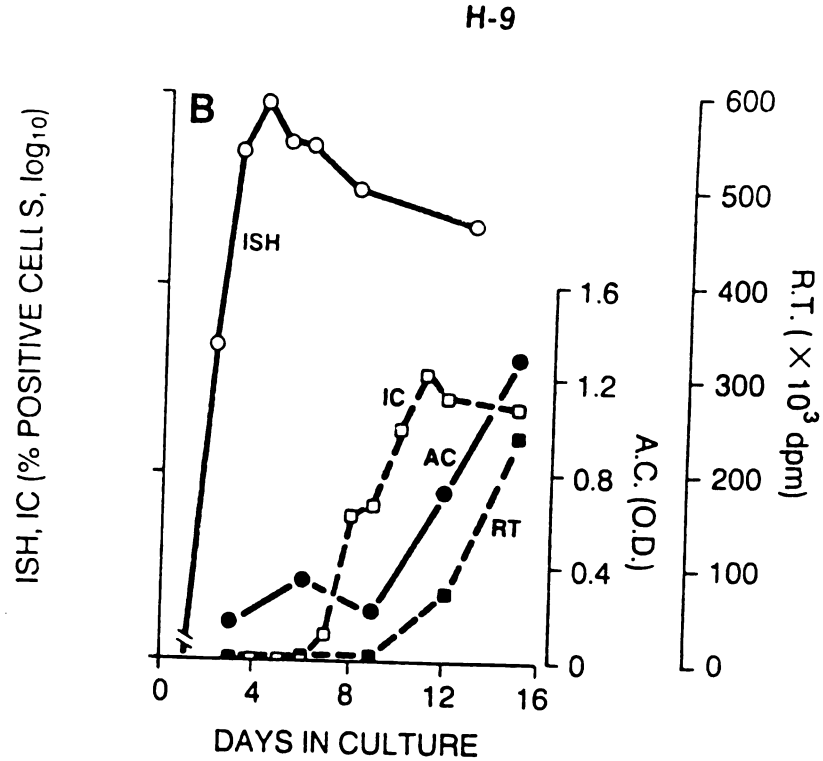
Figure 6. Relative sensitivity of diluted inoculum of the HIV culture fluids tested by RT and AC.



ISH : —○— IC : ----□----

RT : ----■---- AC : —●—

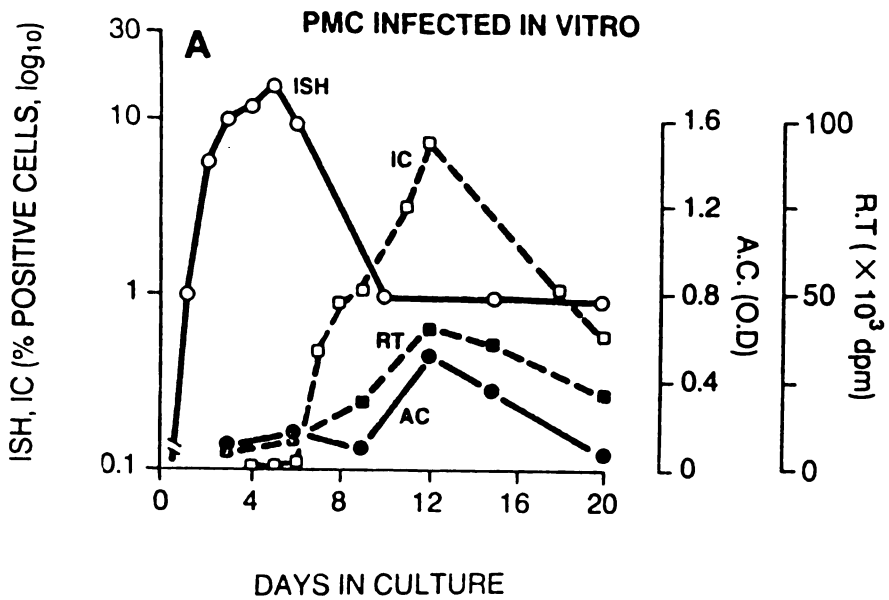
Figure 7. The kinetics of <u>in vitro</u> inoculation of HUT-78 cells with HIV.



ISH : —○— IC : ----□----

RT : ----■---- AC : —●—

Figure 8. The kinetics of <u>in vitro</u> inoculation of H-9 cells with HIV.



ISH : —○—

RT : ----■----

IC : ----□----

AC : —●—

Figure 9. The kinetics of <u>in vitro</u> inoculation of PHA-stimulated normal PBMCs with HIV.

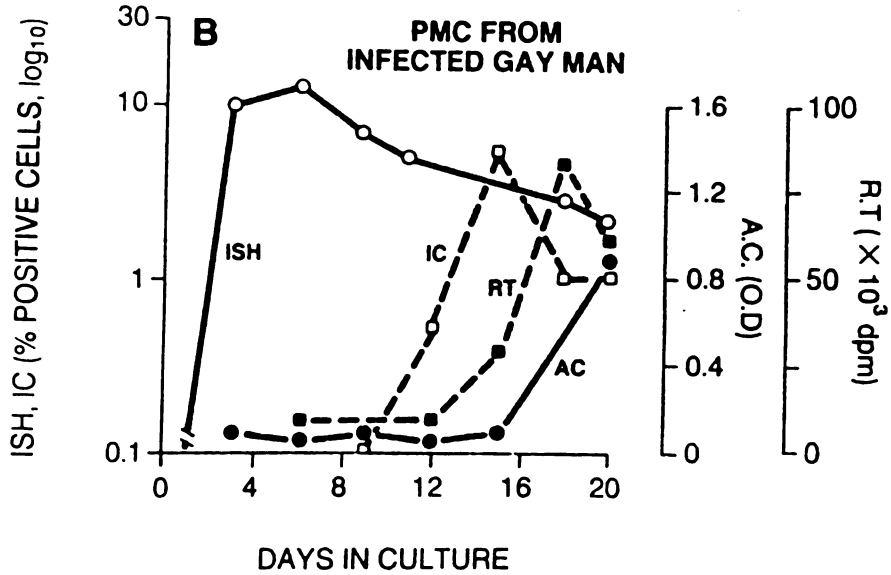


Figure 10. The kinetics of HIV replication in the PHA-stimulated PBMCs from a seropositive man.

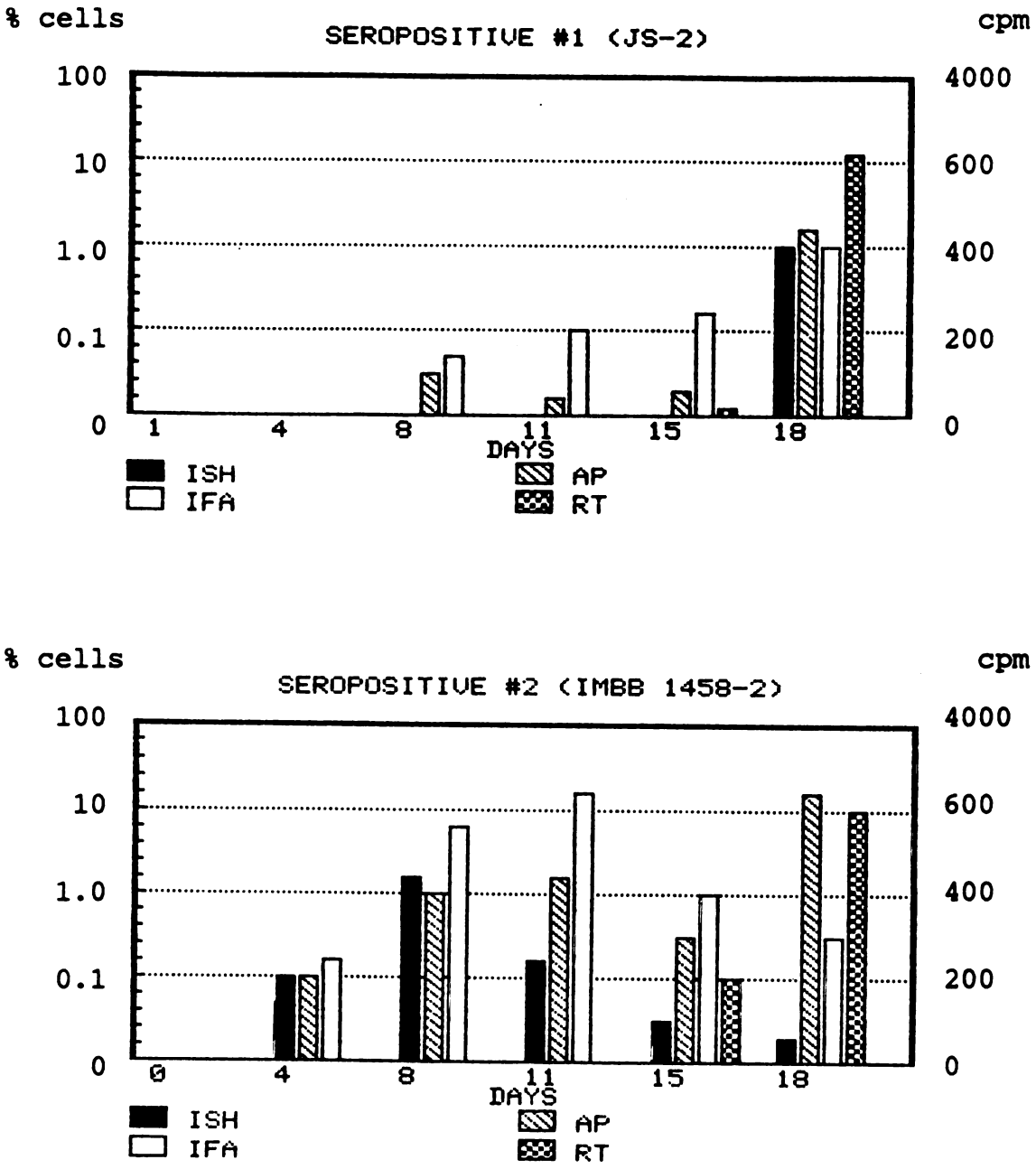


Figure 11. Viral expression detected by ISH, AP, IFA and RT in three positive PBMC cultures (seropositive individuals) .

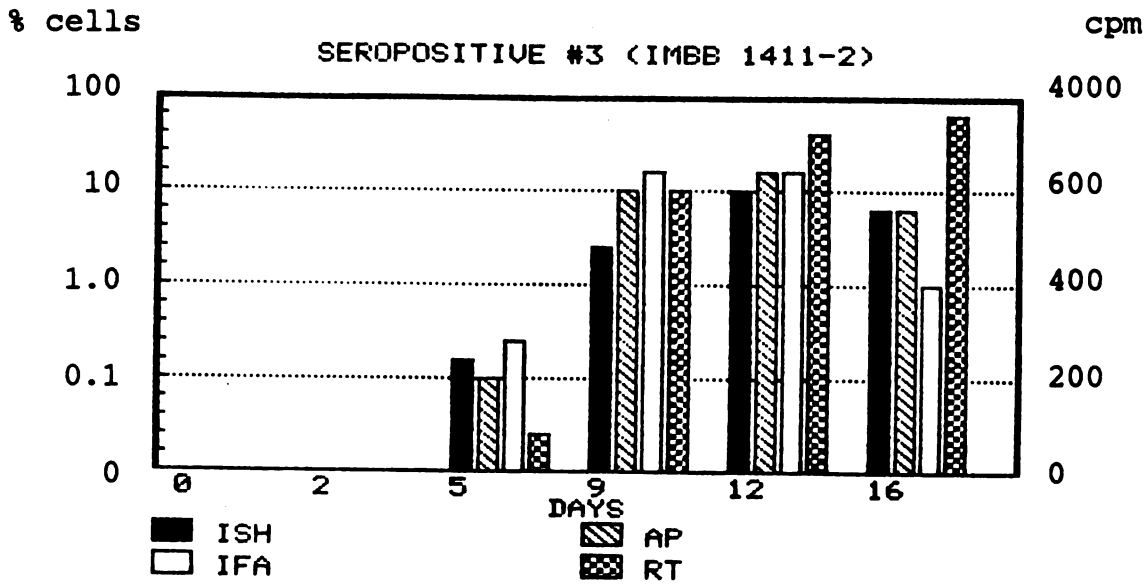


Figure 12. Viral expression detected by ISH, AP, IFA and RT in three positive PBMC cultures (seropositive individuals). (continued)

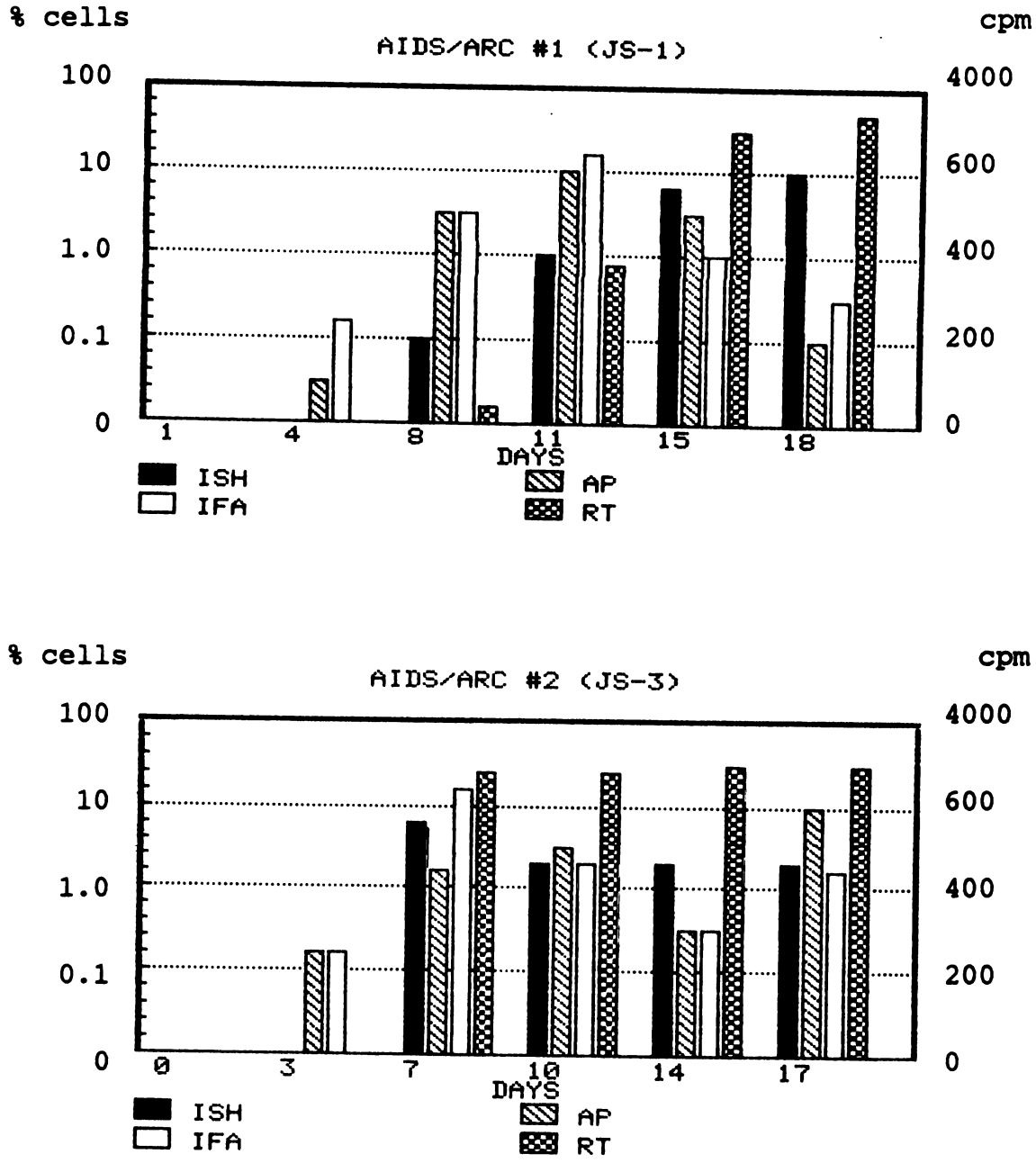
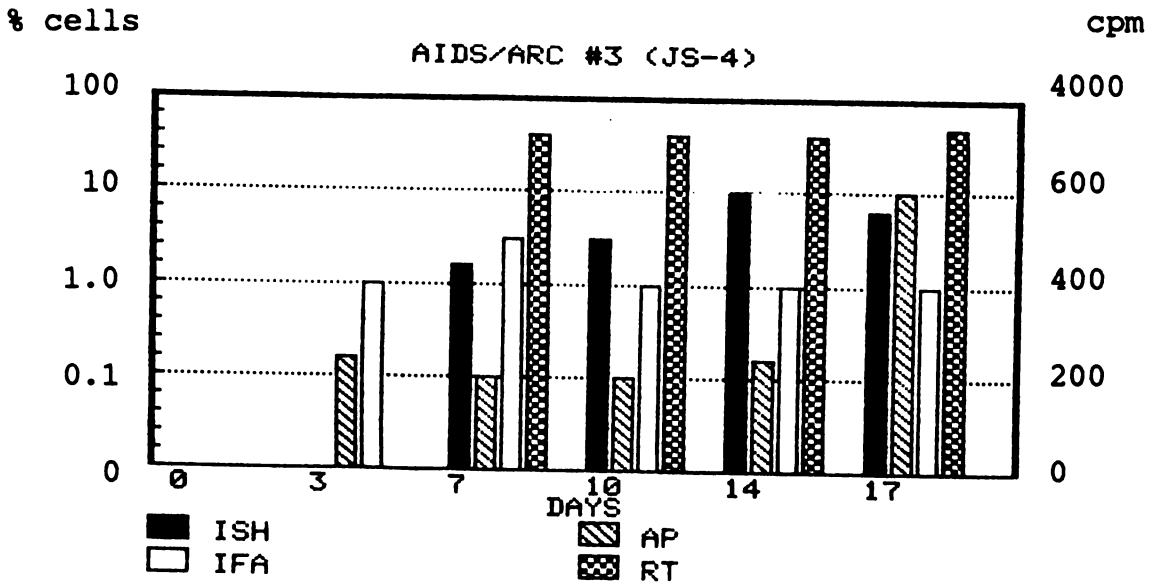


Figure 13. Viral propagation in six AIDS/ARC patients by detection of ISH, AP, IFA and RT.



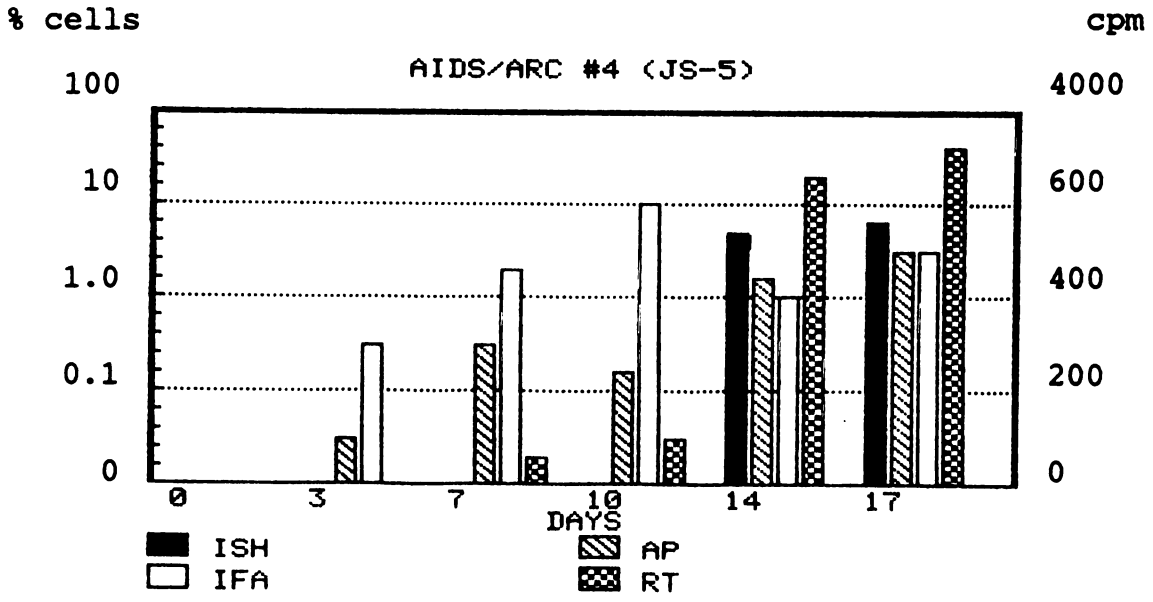


Figure 14. Viral propagation in six AIDS/ARC patients by detection of ISH, AP, IFA and RT. (continued).

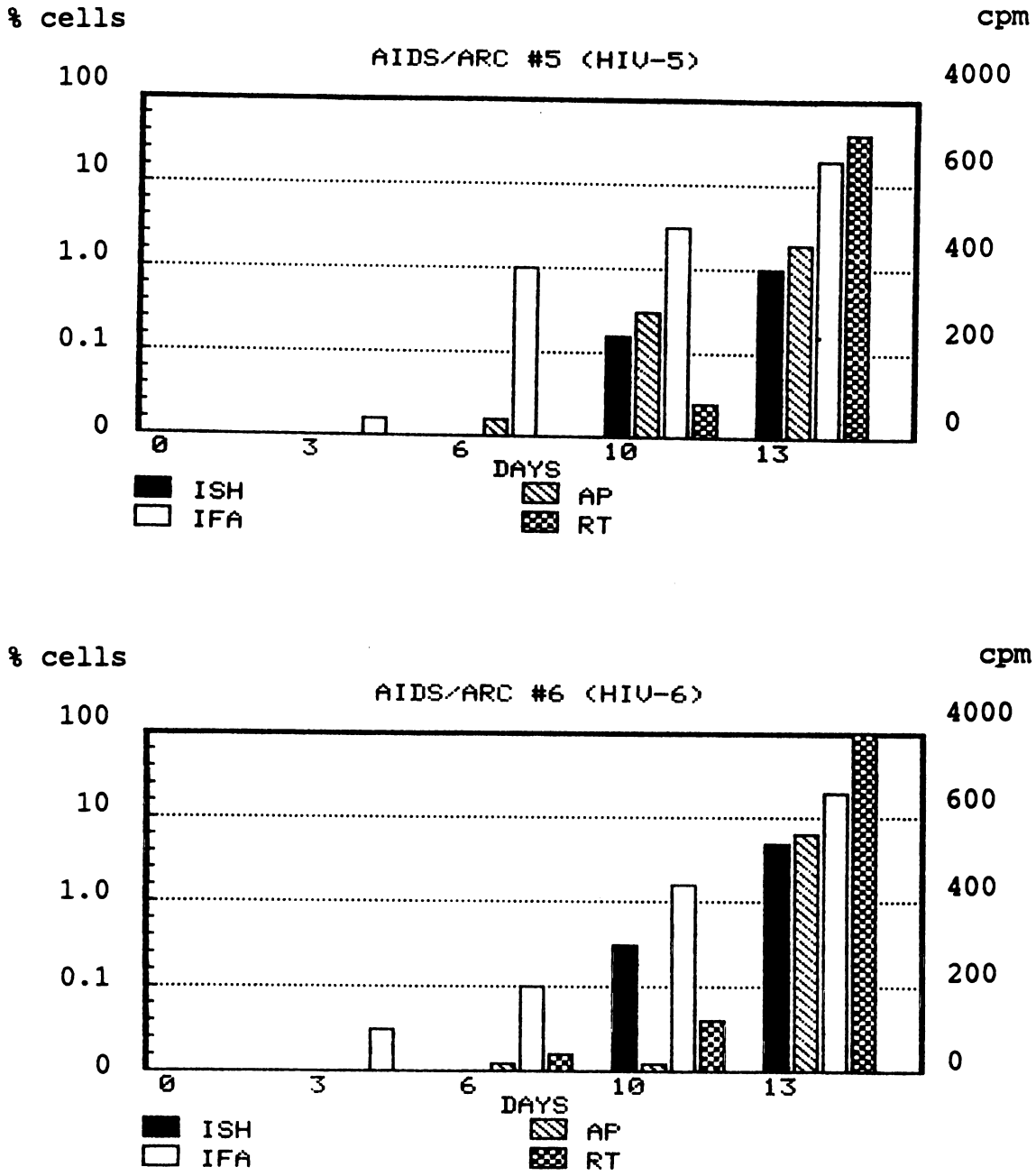


Figure 15. Viral propagation in six AIDS/ARC patients by detection of ISH, AP, IFA and RT. (continued).

CHAPTER IV

DISCUSSION

To establish the presence of persistent HIV infection, human lymphocytes must be cultured and monitored for HIV replication. The detection of HIV in PBMC cultures has been greatly aided by several recent improvements in cell culture techniques. In addition, new molecular methods for detecting the presence of HIV in the cultures have become available. My thesis work was directed at critically comparing various culture and detection methods, in order to develop an optimally sensitive, specific and cost-effective HIV culture protocol.

When I began my thesis work, I adopted a standard HIV culture protocol (11). Once this method was established, I assessed several modifications of the method, focusing particularly on the role of coculture cells. The relative yield of HIV was compared in patients' PBMCs cultured without addition of any feeder cells, and cocultured with normal PBMCs versus with different cell lines as feeder cells. The

HIV replication level and recovery rate in cultures without addition of feeder cells were found to be much lower than the yields with feeder cells in cocultures. These results most likely reflect the fact that low levels of HIV in the primary culture need to be amplified in the coculture procedure. In my preliminary studies, HIV replicated significantly more efficiently in mitogen-stimulated normal human lymphocytes than in continuous cell lines (79). This apparent replication tropism of HIV for crude PBMC preparations may possibly reflect intracellular events between normal helper T4 cells and other cell phenotypes (namely, macrophages, T8 cells, monocytes, null cells, etc.), which contribute to the spread of HIV within the feeder cells. Thus, maximally efficient replication of HIV may require a mixed population of fresh mononuclear cells, as opposed to the homogeneous phenotype of cell lines. However, it is alternatively possible that purified T4 cells used as in feeder cells may provide enhanced virus expression in the coculture procedure.

Eventually, our laboratory adopted a new culture protocol (from the California State Department of Health Services, Viral and Rickettsial Diseease Laboratory, Berkeley, CA) which was found to be clearly superior to independent PBMC culture or coculture with cell lines. This protocol includes several key modifications: (1) the test PBMCs are not directly stimulated with any mitogen, (2) the test culture is mixed with PHA-stimulated normal PBMCs at the initiation of the

culture and then maintained with addition of PHA-stimulated feeder cells once a week, (3) once in culture, the cellular sediments are not disturbed, thus allowing the compacted cells to transmit HIV effectively via cell-to-cell contact, (4) an optimal concentration of IL-2 (5%) in the RPMI medium, (5) the RPMI medium is acidified to pH 6.8 by gassed with CO_2 (facilitating acid-dependent virus penetration). These modifications have not only increased our rate of recovery of HIV from clinical cultures, but it also has greatly increased the level of HIV replication as determined by all assay methods. However, the optimized coculture method has two possible disadvantages: (1) PBMCs used as feeder cells are derived from various donors (of different HIV susceptable) and (2) the rapid expression of HIV detection apppers to be irrespective of the clinical status of HIV-positive individuals. Thus, this method is apparently not highly quantitative.

I detected HIV in cultured human lymphocytes by monitoring intracellular viral RNA with ISH and proteins with IC as well as by detecting the complete virion as assessed by RT and AC activity in the culture supernatants. The first two methods (ISH and IC) take advantage of the high specificity of molecular DNA probes and monoclonal antibodies as well as the sensitivity afforded by microscopic localization of the target signal to a subpopulation of virus-infected cells. In addition, they identify early intracellular events of viral

replication rather than relying on detectable levels of progeny viral particles appearing in the supernatant fluid. Since these techniques allow for more quantitative and early detection of the virus, they should facilitate further study of the biology of early HIV replication in vitro. Furthermore, ISH and IC can be performed concurrently on the same cytologic preparation, thus allowing for detailed correlation of morphologic and antigenic changes coinciding with viral replication at the cellular level (75).

In the studies of in vitro infection of various cell lines and normal human PBMCs, ISH detected cytoplasmic viral RNA within 24 to 48 hours of inoculation and the signal reached a maximun by day 4 or 5. IC-detectable core proteins (p18 and p25) increased over the subsequent 2 to 10 days of inoculation. But, RT and AC activity are detected much later (as shown in Figure 7, 8 and 9). This is a clear demonstration that ISH and IC are highly sensitive means for detecting viral RNA and protein expression, particularly in the early stage of the replication cycle.

In clinical studies, I used an immunofluorescence assay in addition to the above assays as a confirmatory assay for each culture. IFA is employed in many laboratories as a confirmatory assay on the culture cells when RT activity is repeatedly detected for HIV replication. My results showed that IFA functioned in parallel with IC detection by AP. However, my results with these cultures showed that ISH

signal appeared later than antigen expression detected by both AP and IFA. ISH signal was in fact detected almost coincident with RT activity. Thus, the temporal patterns of ISH signal in the PBMCs inoculated in vitro were divergent from those observed in cultures of HIV-infected patients' PBMCs.

We considered the posibility that large amounts of antigens present on the culture cells in the early stage of viral replication (pre ISH singal) might reflect virion particles bound to T4 receptors but not yet into the HIV replication cycle. This was ruled out by a subsequent experiment of in vitro inoculation which showed that the T4 cell saturated with HIV were antigen-negative. Following culture, the progression of signal (ISH->IC->RT) observed in our preliminary studies of in vitro inoculation were additionally validated. Therefore, the results with clinical cultures probably reflect a lack of consistent sensitivity of the ISH method. ISH is technically more tedious than the other assays. Hence, although ISH is a powerful tool, it remains a research assay because of its technical complexities and inconsistent performance with clinical samples.

By comparing Figure 11 to 15, we noted that the yield of HIV from the cultures of AIDS/ARC patients was significantly earlier and higher than from the cultures of anti-HIV positive healthy individuals. Even among the AIDS/ARC patients' cultures, some give high RT production on day 7, whereas the

others give RT activity only on day 13-17. Variations between HIV isolates may be explained by previous reports (53), which suggest a relation between the clinical severity of HIV infection and the in vitro replication potential of the virus. Further clinical studies defining the prognostic relevance of HIV culture as monitored by these various assays need to be carried out in the near future. Such studies should yield insight into the spectrum of persistent viral infections (latent, chronic low-grade, or chronic high-grade), and will allow us to correlate the value of culture in prognosis and therapeutic monitoring of HIV-infected patients .

In summary, HIV infection is associated with a wide spectrum of clinical presentation, ranging from the asymptomatic carrier state to ARC and AIDS. Currently the clinical laboratory diagnosis of HIV infection is based on the serological detection of anti-HIV antibody. These antibody tests do not indicate whether the person currently harbors HIV; carrier status can be firmly established only by viral cultures. The current standard methods for monitoring PBMC cultures for the presence of HIV depend principally on the detection of RT or AC activity in the culture supernates. We found two newly developed techniques (ISH and IC) allow for more quantitative and earlier detection of HIV. Continued use of the ISH technique in conjunction with the IC assay will allow for correlation of specific viral and cellular RNA

and antigen expression. This should help us to further define and characterize cells which are infected with HIV, including reservoir cells which may harbor HIV during the long incubation phase of the infection. These latently infected cells may be induced to active HIV expression by cofactor viruses, antigenic stimulation, stress, etc. This activation may precipitate progression of the disease. In vitro study employing ISH and IC may help to elucidate the mechanism of HIV persistence and the role of cofactor activation. Certainly, these techniques will open up new vistas for imaginative investigations.

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