Persistent Nonproductive Infection of Epstein-Barr Virus-Transformed Human B Lymphocytes by Human Immunodeficiency Virus Type 1

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We have studied the interaction of different strains of human immunodeficiency virus type 1 (HIV-1) with an Epstein-Barr virus-transformed human B-lymphocyte line, X50-7. Previously we found that some HIV-1 strains replicated rapidly and were exclusively cytopathic; others induced persistent noncytopathic infection associated with continued shedding of extracellular virus (K. Dahl, K. Martin, and G. Miller, *J. Virol.* 61:1602-1608, 1987). We now describe a third form of cell-virus relationship in which infection by strain IIIB is maintained in a highly cell-associated state in a small subpopulation (<2%) of X50-7 cells. Neither viral subcomponents nor infectious virus was detectable in culture supernatants; however, the carrier lines were fusogenic and HIV-1 could be recovered following prolonged cocultivation with susceptible cells. In these chronic carrier cultures, virions were not seen budding at the cell surface, but a few were found within cytoplasmic vesicles. HIV-1 infection of first- and second-generation cell subclones of the carrier cell line rapidly evolved from a productive to a cell-associated state. There were low levels of HIV DNA and RNA in the fusogenic secondary clones, but most clones lacked HIV-1 DNA, failed to express HIV-1 RNA, and exhibited no properties associated with HIV-1 infection. The experiments indicate that HIV-1 can be sequestered in human B lymphocytes. The cell cloning experiments introduce the possibility that the HIV-1 provirus may be lost from some lymphocytes.

Although human immunodeficiency virus type 1 (HIV-1) was initially characterized as a cytopathic T-cell lymphotropic virus, the spectrum of its biologic behavior and its host range have been found to be much broader than originally recognized. HIV-1 strains exhibit striking differences in cytotoxicity (4, 11, 25), replicative ability (4, 7, 25), and cell tropisms (7, 11, 19, 23, 25). Epitopes of the CD4 molecule, involved in binding the virus to cells (12, 22, 27, 28, 34, 37), are present not only on specific T cells but on a variety of other kinds of cells, including those of the monocyte-macrophage lineage (19, 25, 33), certain B lymphocytes (11, 25, 29, 30), and perhaps on some central nervous system cells (6, 8, 14, 47). Variations in structural and nonstructural HIV-1 genes may favor replication in each of these cell types.

In vitro studies suggest that, depending on the virus strain and type of cell infected, HIV-1 expression may range from high-level production of cell-free virus to viral latency with an unexpressed but potentially inducible provirus (18). Therefore, it is of interest to characterize the biologic behavior of individual strains of HIV-1 in distinct cell types in order to develop better understanding of the roles played by unique viral and cellular elements which mediate HIV-1 expression.

The behavior of HIV-1 in Epstein-Barr virus (EBV)-immortalized B cells is of interest for several reasons. EBV replication is often activated in patients infected with HIV-1 (1, 5, 10, 15, 40, 42). EBV-associated lymphoproliferative diseases, such as B-cell lymphomas and lymphocytic interstitial pneumonia, are common complications of acquired immunodeficiency syndrome (1, 24, 49). In vitro, HIV-1 is able to replicate in B cells immortalized by EBV as well as in B-cell lines without EBV (11, 29, 30, 36). Productive HIV-1

infection may be facilitated through EBV-encoded transactivators (21). It is also conceivable that HIV-1 replication may be down-regulated by putative EBV negative-regulatory elements. Furthermore, stimulatory effects of HIV-1 on EBV replication may be of importance.

We previously described two types of HIV-1-cell relationships in a human EBV-transformed B-lymphocyte cell line, X50-7 (11). Some HIV-1 strains (e.g., RF2 and YW) replicate and induce extensive cytopathic effects (CPE) which are ultimately lethal to the X50-7 culture. Other HIV-1 strains (e.g., IIIB and MN) replicate but induce only transient CPE; thereafter, the X50-7 cells recover and release large amounts of extracellular virus.

In this report, we describe a third type of interaction between EBV-transformed lymphoblastoid cells and HIV-1, in which the virus infection became highly cell associated in a small subpopulation of cells. This culture, designated LL58, consists of X50-7 cells infected with the IIIB strain of HIV-1. It was initially productive of high titers of virus which was released into supernatant fluids (11). However, over about 6 months the infection evolved from active HIV-1 maturation and release to one in which virus was expressed in very small to undetectable amounts and was tightly cell associated. The same evolution from productive to cell-associated behavior was reproduced in two successive clonal subpopulations of LL58 cells. This report describes experiments which characterize the biology of this nonproductive infection in B cells.

MATERIALS AND METHODS

Cells. X50-7 cells are human umbilical cord B lymphocytes immortalized in vitro by the B95-8 strain of EBV (48). These cells secrete immunoglobulin M. X50-7.8 and X50-7.30 are single-cell clones of the X50-7 line. Both X50-7 and its

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FIG. 1. Fluorescence-activated cell sorter analysis of X50-7 cells and LL58 cells with the anti-Leu 3a monoclonal antibody. (A) X50-7 cells. (B) LL58 cells 19 months after infection of X50-7 cells with the HTLV IIIB strain of HIV-1. Data are shown for a murine control antibody (—) and for anti-Leu 3a (---). Note that X50-7 cells express the CD4 antigen on their surfaces and LL58 cells do not.

subclones X50-7.8 and X50-7.30 express the Leu 3a epitope of the CD4 molecule (Fig. 1A and data not shown) and are susceptible to infection with diverse strains of HIV-1. X50-7 and its subclones express latent EBV nuclear antigens (EBNAs).

The cell line obtained by superinfecting X50-7 cells with the human T-lymphotropic virus (HTLV) IIIB strain of HIV-1 was designated LL58 (11). It did not react with the Leu 3a monoclonal antibody (Fig. 1B). A single-cell clone of LL58, selected for HIV-1 expression, was designated LL58-6. Four cellular subclones of LL58-6 were designated LL58-6.12, LL58-6.14, LL58-6.18, and LL58-6.20 (see Table 1).

X50-7.8 cells were productively infected with two other HIV-1 strains, MN and SB. These carrier lines were derived by incubating X50-7.8 with filtered supernatant fluids from H9/MN or by cocultivating X50-7.8 cells with fresh mononuclear cells from a seropositive individual, SB. The X50-7.8/MN and X50-7.8/SB cultures have produced infectious HIV-1 for at least 20 months. Both remain approximately 80 to 90% positive by immunofluorescence with HIV-1 polyvalent human antiserum.

H9 is a CD4-positive T-cell line (35). H9/RF2 and H9/MN are H9 cells productively infected with HIV-1 (38). BJAB and BL41 are Burkitt's lymphoma (B-cell) lines which are CD4 negative and also lack EBV. All cell lines were maintained in RPMI 1640 medium with 8% fetal bovine serum and antibiotics.

Cell cloning. X50-7 and LL58 cells were cloned by limiting dilution on feeder layers of human placental cells (20). LL58 cells were cloned in two stages. In the initial enrichment stage, LL58 cell subpopulations were derived from 50 cells. The cultures were then replica plated with a multichannel pipette onto indicator cultures of X50-7.8 cells and inspected for syncytia after 48 h. One cell population which formed syncytia was then subcloned at 1 cell per well by limiting dilution. A single fusogenic cell clone designated LL58-6 was identified. The LL58-6 cell population was subcloned at 0.1 cells per well. Thirteen random, unselected secondary subclones were studied.

Assay for CD4. From a logarithmic-growth-phase culture 5×10^5 cells were washed once in phosphate-buffered saline with 0.1% azide and suspended in either fluorescein isothiocyanate-conjugated murine monoclonal antibody anti-Leu 3a (Becton Dickinson and Co.) or isotype-matched control murine antibody for 30 min at room temperature. Cells were washed once in phosphate-buffered saline-azide, suspended in 0.5 ml of 0.5% Formalin in phosphate-buffered saline, and analyzed in a fluorescence-activated cell sorter (Becton Dickinson).

Assays for extracellular HIV-1. Samples (4 ml) of cell-free supernatant fluid were assayed for reverse transcriptase. The enzyme reaction mixture (100 μ l) contained 20 μ l of solubilized virus, 10 μ Ci of [3 H]TTP, 60 mM Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM dATP, 13 mM $MgCl_2$, and 1 μ g of primer, either poly(A-dT)₁₂₋₁₈ or, as a control, poly(dA-dT)₁₂₋₁₈ (Pharmacia, Inc.). The reaction was held at 37°C for 1 h and stopped with 10% cold trichloroacetic acid. Duplicate reactions were performed for each sample.

Supernatant fluids were assayed for p24 antigen with a commercial kit based on an enzyme-linked immunosorbent assay antigen capture assay (Abbott Laboratories). Standards and positive and negative controls were supplied by the manufacturer. A supernatant was considered antigen positive if the optical density of the enzyme-linked immunosorbent assay reading was twice that of diluent.

The content of infectious virus was measured by suspending X50-7.8 indicator cells in undiluted or serially diluted filtered supernatant fluids from LL58 cells or, as a positive control, filtered fluids from HIV-1 producer line H9/RF2 or X50-7.8/MN. The inoculated cells were held for 2.5 months and monitored periodically for CPE and viral antigens by using immunoblotting and immunofluorescence assays.

Bioassays for cell-associated HIV-1. LL58 and LL58-6 cells induced syncytia when cocultivated for 24 to 48 h with X50-7.8 cells. The number of fusogenic cells were estimated by cocultivating serial dilutions of LL58 cells with a fixed number of X50-7.8 cells. The assay was done in duplicate, and the number of foci in each well was estimated by using a grid and an inverted microscope. Incubation was in 5% CO₂-air at 37°C. In some experiments, the cocultures were examined for HIV-1 antigens by indirect immunofluorescence or immunoblotting after 48 to 72 h. In longer cocultivation experiments LL58, LL58-6, or LL58-6.20 cells, either intact or disrupted by repeated freezing and thawing, were mixed with equal numbers of intact X50-7.8 or X50-7.30 indicator cells. The cocultivated mixture was held for up to 2 weeks, at which time the filtered culture supernatants were passaged to fresh X50-7.8 or X50-7.30 cells. These cultures were monitored for CPE and for the appearance of viral polypeptides by immunoblotting 14 or 28 days later.

Immunologic assays for HIV-1. Cell-associated HIV-1 antigens were detected by indirect immunofluorescence. Cell-associated HIV-1 polypeptides were detected by immunoblotting, as described previously (11). Extracts of equal numbers of cells (1.6×10^6) were loaded in each lane of a polyacrylamide gel.

A syncytium inhibition assay was used to demonstrate the presence of HIV-1 antigens on the surface of LL58 cells. Samples of 2×10^5 LL58 cells were incubated for 1 h at 37°C with 0.5 ml of a serum dilution (1:20 to 1:360) in RPMI 1640 medium. Each serum dilution was tested in triplicate. After incubation with HIV-1-positive serum or, as controls, with medium or HIV-1-negative serum, the LL58 cells were added to 10^5 X50-7.8 cells. Incubation was continued for 48 h, at which time the syncytia were counted. The syncytium

inhibition titer was the reciprocal of the serum dilution which produced a 50% reduction in the number of syncytia observed when LL58 cells were preincubated with medium.

Antisera. BA, a human HIV-1-positive serum which recognizes many HIV-1 polypeptides, including the major structural proteins, was used in a 1:200 dilution for immunofluorescence and in a 1:1,000 dilution for immunoblotting. At this dilution BA also recognizes latent EBNA EBNA1 and EBNA4 (also called leader protein), which appear on immunoblots of both infected and uninfected cells. The control human antiserum S.C., containing very high antibody titers of EBV replicative antigens, low antibody titers to EBNA, and no reactivity to HIV-1, was used at similar dilutions. Human sera for the syncytium inhibition assay were obtained with informed consent from HIV-1-seropositive individuals with a spectrum of HIV-1-related clinical syndromes. Control sera were obtained from healthy HIV-1-seronegative workers in the laboratory. All human sera were heated at 56°C for 30 min prior to use. Rabbit monospecific antisera to HIV-1 *env* products gp160 and gp120 and guinea pig monospecific antiserum to gp41 were the gifts of Mark Cochran, Microgenesys, Inc., West Haven, Conn. Mouse monoclonal antibody to *gag* p55/24 was the gift of Paul Durda, Dupont, New England Research Products, Boston, Mass.

Electron microscopy. A search for HIV-1 virions was made in four cell lines, X50-7, LL58, LL58-6, and X50-7/MN, as well as in a 3-day cocultivation of X50-7 and LL58 cells. Syncytia from the latter culture, enriched by low-speed centrifugation, were also examined. Cell pellets were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4°C and postfixed in 2% osmium tetroxide in the same buffer. Pellets were stained en bloc with 0.5% uranyl acetate, dehydrated with ethanol, and embedded in Epon 812. Plastic sections, 0.5 μ m thick, were stained with 1% toluidine blue in 1% sodium borate and were photographed under a light microscope. Thin sections, stained with lead citrate, were examined and photographed under a Philips 201 electron microscope operated at 60 kV.

Detection of HIV-1 DNA. Total cellular DNA was extracted from 2×10^7 cells by the method of Wahl et al. (46).

HIV-1 sequences in total cellular DNA were detected by the polymerase chain reaction (PCR). The reaction mixture of 100 μ l contained 1 μ g of total cellular DNA in 10 mM Tris (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 100 μ g of bovine serum albumin per ml, 200 μ M each of the four deoxyribonucleotide triphosphates, and 2.5 U of Taq polymerase (The Perkin-Elmer Corp. and Cetus Instrument Systems). The primers for the reaction, derived from HIV-1 long terminal repeat (LTR) sequences, were 5' GGGACTTTCGCTGGGG 3' (positions 5' 359 to 375 3') and 5' CAAGGCAAGCTTTATTGAGGC 3' (positions 5' 552 to 532 3'). The samples were subjected to 35 amplification cycles in a programmable cycle reactor (Ericomp, Inc.) set to denature the DNA for 1 min at 94°C, to anneal the primers for 2 min at 37°C, and to extend the annealed primers for 3 min at 72°C in each cycle. The extension step in the final cycle was performed for 10 min at 72°C.

The amplified HIV-1 DNA was detected by Southern analysis (13). A 30- μ l volume of each amplified sample was resolved in a 5% acrylamide gel and electrotransferred to a Nytran membrane (Schleicher & Schuell, Inc.) (9). After the membrane was dried for 1 h at 80°C it was probed with an end-labeled oligonucleotide, 5' TGTACTGGGTCTCTCT 3', from positions 5' 458 to 473 3' in the LTR.

Detection of HIV-1 RNA. Cytoplasmic RNA was extracted

from 5×10^6 cells. The cells were washed with phosphate-buffered saline (pH 7.2) and suspended in 200 μ l of lysis buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 1.0% Nonidet P-40, 0.5 U of RNasin per ml [Promega Biotec]). Lysis proceeded on ice for 10 min. The nuclei were pelleted by centrifugation in a Microfuge for 1 min at 4°C. The supernatant fluid, containing the cytoplasmic extract, was added to 200 μ l of urea mix (10 mM Tris [pH 7.5], 350 mM NaCl, 7 M urea, 20 mM EDTA, 1% sodium dodecyl sulfate) and incubated on ice for 5 min. The suspension was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol. The RNA was precipitated at -20°C from 300 μ l of the aqueous phase with 2 volumes of 95% ethanol containing 300 mM sodium acetate. The RNA was pelleted by centrifugation in a Microfuge for 15 min at 4°C, dried under vacuum, and suspended in 20 μ l of MOPS buffer (20 mM 3-*n*-morpholinepropanesulfonic acid [pH 7.0], 5 mM sodium acetate, 5 mM EDTA) containing 6% formaldehyde and 50% deionized formamide.

HIV-1 RNA was detected by Northern (RNA) hybridization. The entire cytoplasmic RNA sample was resolved on a MOPS-agarose gel in the presence of 6% formaldehyde and transferred to a Nytran membrane. The membrane was dried at 80°C for 1 h. Three probes were used for the detection of HIV-1 RNA. Probe 1 was the entire BH10 clone derived from the HTLV IIIB strain of HIV-1 and excised from its vector with *Sac*I. Probe 2 was the 1.8-kilobase-pair (kbp) *Kpn*I-*Hind*III subfragment of BH10 from positions 5706 to 7496. Probe 3 was a mixture of the 2.1-kbp *Hind*III-*Kpn*I fragment (positions 1034 to 3188) and the 2.2-kbp *Kpn*I-*Kpn*I fragment (positions 3516 to 5706). The probes were radiolabeled by the random primer method (16). The membrane was stained with 0.5% methylene blue after hybridization to assay the condition of the RNA and the positions of the rRNAs.

RESULTS

Decrease over time in HIV-1 expression by EBV-transformed cells infected with the HTLV IIIB strain. The LL58 culture was established in July 1985 by inoculation of X50-7 cells with a 10^{-4} dilution of supernatant fluids from the H9/HTLV IIIB cell line. After a transitory period of CPE the LL58 culture recovered, and 3 and 4 months later supernatants contained infectious HIV-1, to a dilution of 10^{-4} when tested on fresh X50-7 cells (11). In December 1985, about 5 months after the original isolation of LL58 cells, the titer of extracellular infectious HIV-1 was noted to have fallen to 10^{-1} when the same indicator cell line was used; by March 1986, infectious virus was no longer detectable in the supernatants of the LL58 line.

This fall in titer of infectious virus was associated with a marked decrease in polypeptide expression (Fig. 2). Immunoblots of LL58 cell extracts, 4 months after the initial infection, revealed numerous HIV-1 polypeptides, including prominent ones of 17, 19, 24, 35, 41, 55, and 66 kilodaltons (kDa) (Fig. 2, lane 3). However, by 8 months the number and abundance of HIV-1 polypeptides had declined dramatically and remained low thereafter. An extract prepared 26 months after isolation showed only a few HIV-1-specific polypeptides of about 24, 55, and 66 kDa (Fig. 2, lane 4). By contrast, X50-7 and X50-7.8 cells chronically infected with either the MN or the SB strain of HIV-1 continued to show the full spectrum of HIV-1 polypeptides since they were originally isolated (see Table 1 and Fig. 8).

Immunofluorescence assays of lines stably productive of

FIG. 2. Loss of HIV-1 polypeptide expression in LL58 cells over time. Shown is an immunoblot reacted with human antiserum BA, which has antibody to EBV latent products found in X50-7 cells (EBNA1 and EBNA4) and to many HIV-1 polypeptides. HTLV III B (HTLV3B) extract contains partially purified HIV-1 virions. LL58(B) cells were propagated *in vitro* for 26 months (until December 1987) after LL58(A) cells (October 1985). The HIV-1 polypeptides detected in extract B include p66, p55, and p24.

HIV-1, such as X50-7/MN, X50-7.8/MN, and X50-7.8/SB, showed that 90 to 95% of the cells were brightly reactive with polyvalent human HIV-1-positive serum and nonreactive with HIV-1-negative human serum which had high titers of antibodies to EBV replicative antigens. By contrast, less than 0.01% of cells in late LL58 cultures reacted with HIV-1-positive human antiserum. Thus, by available immunoassays, LL58 cells had evolved to an exceedingly low or nonproductive state.

Absence of HIV-1 in supernatant fluids of LL58 cells. Three different assays for extracellular HIV-1 were performed. Supernatants from LL58 cells were compared with those from three productively infected cell lines, H9/RF2, X50-7/MN, and X50-7.8/SB, in a reverse transcriptase assay (Fig. 3A). When the control template poly(dA-dT) was used, the background activity varied between 600 and 2,600 cpm/ml of supernatant. With the poly(AdT) template, the virus producer lines H9/RF2, X50-7/MN, and X50-7.8/SB showed reverse transcriptase activity of 17,000 to 261,000 cpm, or 7 to 100 times the control level. The activity from LL58 supernatants did not differ for control or experimental templates (Fig. 3A).

Similarly, high levels of p24 core antigen could be detected in H9/RF2 supernatants (428 pg/ml or 33 times the diluent control) while LL58 supernatants contained no detectable antigen (1.08 times the diluent control) (Fig. 3B).

Infectivity assays for the presence of HIV-1 in LL58 culture supernatants were also negative. In three separate trials, beginning at month 8 of the LL58 culture, LL58 supernatants, undiluted or diluted 10^{-1} , were unable to induce HIV-1 polypeptides detectable by immunoblotting in X50-7 or X50-7.8 cells (data not shown). X50-7.8 cells failed to show morphologic changes or to express HIV-1 antigens

FIG. 3. Lack of extracellular HIV-1 in supernatant fluids of LL58 cells. (A) Reverse transcriptase assay. Reverse transcriptase activity of LL58 supernatant fluids was compared with those of supernatant fluids of two HIV-1-uninfected cell lines (H9 and X50-7.8) and fluids from three cell lines chronically infected with three different strains of HIV-1 (H9/RF2, X50-7/MN, and X50-7.8/SB). Activity with poly(AdT) (■) and with control template poly(dA-dT) (□) is shown. (B) HIV-1 core antigen (p24) assay. p24 activity was measured by using the Abbott enzyme-linked immunosorbent assay kit. The curve was generated by using standards provided in the kit. Symbols: △, p24 antigen in supernatant fluid of productively infected H9/RF2 cells; ○, assay diluent; ▲, LL58 supernatant.

for 2.5 months after exposure to LL58 supernatants. In contrast, X50-7.8 cells which had been incubated in supernatant from the X50-7.8/MN cells were 90% antigen positive by 2 months after inoculation (data not shown).

LL58 cells induce syncytia upon cocultivation with receptor-positive cells. Despite the lack of evidence for production of extracellular HIV-1 by LL58 cells and the limited evidence for synthesis of cell-associated HIV-1 polypeptides, cocultivation of LL58 cells with CD4-positive cell lines such as X50-7, X50-7.8, and H9 induced the typical HIV-1-associated CPE of multinucleate giant cell formation (Fig. 4A).

FIG. 4. Syncytium formation 48 h after cocultivation of LL58 and X50-7 cells. (A) Thin section (stained with toluidine blue) viewed by light microscopy. (B) Indirect immunofluorescence using BA antiserum. The large syncytium is HIV-1 antigen positive (see text).

These syncytia were usually evident by 24 h and reached a maximum number at 48 to 72 h. However, cocultivation did not initiate progressive CPE in the remainder of the culture. When mixtures of LL58 and X50-7.8 cells were analyzed by immunofluorescence 48 and 72 h after cocultivation, the giant cells were positive for HIV-1 antigens (Fig. 4B). The giant cells were nonreactive with EBV-positive HIV-1-negative serum.

Experiments were performed to evaluate the conditions required for syncytium formation. Syncytium formation could be seen after cocultivation with either viable or frozen and thawed LL58 cells; however, intact X50-7.8 cells were required. Supernatant fluids from neither cell line induced fusion. These experiments indicated that LL58 cells induced the fusion event and that X50-7.8 cells were the responder cells. That LL58 cells did not fuse with themselves was probably explained by their lack of CD4 (Fig. 1). Syncytium formation did not occur when LL58 cells were cocultivated with CD4-negative B-cell lymphoma lines, such as BJAB or BL41. Thus, one requirement for syncytium formation seemed to be the presence of CD4 on the indicator cells (12, 22).

The number of LL58 cells capable of giving rise to a fusion event was estimated by quantitative cocultivation experiments. Cell fusion was rarely observed when fewer than 1,000 LL58 cells were used. In cocultivations of 1,000 LL58 cells with 10^5 X50-7.8 cells, about 20 giant cells were

observed. If a single LL58 cell expressing *env* on its surface was sufficient to recruit uninfected CD4-positive cells and to induce giant cell formation, this indicates that about 2% of LL58 cells were fusogenic. The 2% level of fusogenic activity was a stable property of the LL58 population cultured continuously for at least 3 years.

HIV-1-positive human sera inhibit syncytia induced by cocultivation of LL58 with X50-7.8 cells. To show that syncytium formation was due to expression of an HIV-1-related product (presumably an *env* product) on the surface of LL58 cells, a group of HIV-1 antibody-positive and HIV-1 antibody-negative human sera were studied in a syncytium inhibition assay. A total of 67% (29 of 43) of sera positive for HIV-1 antibodies by Western (immuno-) blotting showed 50% or more inhibition of syncytium formation at a titer of $>1:20$. None of 16 sera which were HIV-1 negative by Western blot inhibited syncytium formation induced by LL58 cells. Preincubation of X50-7.8 cells with HIV-1-positive sera did not lead to inhibition of syncytium formation.

LL58 cells contain low levels of cell-associated HIV-1. To learn whether syncytium formation by LL58 cells was associated with the synthesis and release of mature HIV-1 particles or only with expression of subviral components, both the LL58 culture and the X50-7 culture were examined by electron microscopy (see Fig. 6A; data not shown). The X50-7 cells showed no retroviral particles. Similarly, more

than 99% of LL58 cells were morphologically indistinguishable from uninfected X50-7 cells and showed no evidence of retrovirus. A rare LL58 cell (less than 1 in 2,000) was noted to contain intravesicular structures resembling HIV-1 virions (Fig. 5A). By contrast, at least 75% of X50-7 cells productively infected with the MN strain contained numerous virions, with typical rod-shaped cores, distributed in a polar fashion in close proximity to the extracellular surface of the cell membrane (Fig. 5B). Such mature virions were never seen budding from LL58 cells or in the extracellular space. However, 3 days after cocultivation of LL58 with X50-7 cells, populations enriched for multinucleated giant cells showed small numbers of extracellular virions which were closely associated with plasma membrane processes and appeared to have typical HIV-1 morphology (Fig. 5C).

Thus LL58 cells can encode mature virions which appear after cocultivation. To learn whether these virions were infectious, intact or disrupted LL58 cells were cocultivated with indicator H9 or X50-7.8 cells for 2 weeks, at which time cell-free extracellular fluids were passaged to fresh indicator cultures. Cocultivations of intact or disrupted LL58 cells with X50-7.8 cells gave rise to virus which could infect fresh X50-7.8 cells (Fig. 6). The virus recovered was cytopathic, as evidenced by CPE seen by light microscopy and by the disappearance of EBNA proteins from immunoblots of infected cells (Fig. 6). EBNA expression in these cells was correlated with their viability. No virus was recovered after cocultivation of whole or disrupted LL58 cells with H9 cells, despite early syncytium formation. This finding might be explained by the observation that in earlier experiments X50-7 cells and their derivatives were at least 10-fold more sensitive to infection by HIV-1 IIB than were H9 cells (11).

Recovery of a fusogenic subclone of LL58 cells. Immunofluorescence assays, the quantitation of syncytium formation, and electron microscopy all indicated that only a small subpopulation of LL58 cells expressed HIV-1 products. Cellular clones of LL58 were derived in order to learn whether one could be isolated which would behave as a virus producer line and express viral products in a large proportion of cells.

None of an initial set of 70 single-cell clones produced HIV-1 polypeptides or induced fusion upon cocultivation with X50-7.8 cells. This result indicated that the LL58 line contained at least two different kinds of cells: those which were fusogenic, presumably as the result of synthesis of HIV-1 *env* products, and those which were not fusogenic. We then attempted to isolate and characterize the fusogenic population.

Since the fusogenic cells were rare, cloning was carried out in two stages. Populations enriched in the fusogenic cells were derived by seeding 50 LL58 cells per well in 96-well plates. In this phase of cell cloning, 10 of 288 wells (containing a total of 14,400 cells) gave rise to fusogenic cultures which were identified by replica plating on indicator X50-7.8 cells. The remainder of the enriched cultures were not fusogenic. Two populations which were most enriched for fusogenic cells (designated LL58-E7 and LL58-E8) both

failed to release extracellular infectious HIV-1 (data not shown).

One of the enriched populations was cloned by limiting dilution at 1 cell per well. From 2,304 wells, 280 (12%) produced single foci; therefore, these foci were thought to be true clones. They were expanded; upon replica plating and cocultivation with X50-7.8 cells, one fusogenic clone (LL58-6) was identified.

Evolution of the LL58-6 subclone from a virus-releasing state to a nonproductive state. The LL58-6 subclone evolved from a productive to a nonproductive state over a period of about 3 months in culture (Table 1). When the LL58-6 culture was first examined by electron microscopy (3 weeks after it was identified as a fusogenic line), about 25% of the cells contained extracellular virus (Fig. 5D) and a similar fraction of cells stained brightly and specifically for HIV-1 by indirect immunofluorescence. At 6 weeks 5% of the cells reacted positively by indirect immunofluorescence, and by 9 weeks only 1 to 2% of the cells expressed HIV-1 antigens.

By immunoblotting, the first extract showed numerous HIV-1-related polypeptides with estimated molecular masses of 19, 24, 35, 41, 55, 66, and 120 kDa. After 6 weeks of culture, however, only a few faint polypeptide bands remained. Their molecular masses were approximately 24, 55, and 41 kDa (Fig. 7).

The identities of some of the HIV-1 polypeptides expressed in early passages of the LL58-6 clone were determined by reactivity with monoclonal and monospecific antibodies to HIV-1 gene products (Fig. 8). Definite reactions were evident with antibodies to *env* gp160 and gp120 and *gag* p55/24. The reaction with the monospecific guinea pig antiserum to *env* gp41 was unusual. This antiserum recognized many polypeptides in extracts of productively infected X50-7/MN cells and in partially purified strain IIB virions (Fig. 8, lanes 2 and 4) but only a single polypeptide (about 60 kDa) in LL58-6 cells (Fig. 8, lanes 3). The LL58-6 extracts also demonstrated EBV replicative polypeptides (as shown by the reaction with the S.C. antiserum).

There was a rapid fall-off in release of infectious HIV-1 by the LL58-6 clone. LL58-6 supernatants were initially infectious when tested undiluted, but 6 weeks later and thereafter no infectivity was found in the supernatant fluids (Fig. 9A). However, at this time frozen and thawed cell extracts from clone LL58-6 still contained infectious virus at a 10^{-1} dilution (Fig. 9B).

Thus by all criteria the LL58-6 cell subclone evolved, over a period of 3 months, to resemble the parental LL58 cells. This state of low-level cell-associated infection was persisted for at least 18 months.

HIV-1 DNA in second-generation subclones. The LL58-6 clone was subcloned 3 months after its isolation, at a time when less than 0.1% of the population was expressing antigen detectable by indirect immunofluorescence and approximately 2% of the cells were fusogenic. Thirteen second-generation clones chosen at random were examined for HIV-1 DNA by PCR, using probes for the LTR. Two clones, LL58-6.12 (Fig. 10) and LL58-6.20 (results not shown) were

FIG. 5. Electron micrographs of HIV-1 particles seen in LL58 cells (A), X50-7/MN cells (B), LL58 cells cocultivated with X50-7 cells (C), and LL58-6 cells (D). (A) In LL58 cells, only one of several thousand cells contained retroviral particles contained within or budding from vesicular compartments in the cytoplasm (arrows). Bar, 1 μ m. Maturation at the plasma membrane was not seen. (B) X50-7 cells infected for 18 months with HIV-1 strain MN showed budding retroviral particles at the cell surfaces of the majority of cells. (C) At 48 h after cocultivation of LL58 with X50-7 cells, occasional HIV-1 particles were seen outside the cells (arrow and inset). (D) LL58-6 cells studied 3 weeks after isolation showed small numbers of mature virions at the cell surface. The respective magnifications of panels A to D are $\times 94,500$, $\times 87,000$, $\times 18,900$ (inset, $\times 63,000$), and $\times 27,400$.

FIG. 6. Recovery of cell-free HIV-1 from LL58 cells after prolonged cocultivation with X50-7.8 cells. Intact LL58 cells or LL58 cells disrupted by freezing and thawing were cocultivated with either H9 or X50-7.8 cells. Controls were LL58, H9, or X50-7.8 cells which were not cocultivated. After 13 days, supernatant fluids were passaged to uninfected X50-7.8 cells. Extracts shown in this immunoblot were prepared 28 days after supernatant passage. Lanes: 1, HIV-1 virions; 2 to 8, X50-7.8 cells plus undiluted supernatant from H9 (lane 2), X50-7.8 (lane 3), LL58 (lane 4), LL58 plus H9 (lane 5), LL58 (freeze-thawed) plus H9 (lane 6), LL58 plus X50-7.8 (lane 7), and LL58 (freeze-thawed) plus X50-7.8 (lane 8). Recovery of HIV-1 (lanes 7 and 8) was associated with loss of EBNA expression due to HIV-1-induced cytolysis.

positive; the remaining clones (represented by LL58-6.14 and LL58-6.18 in Fig. 10) were negative for HIV-1 DNA in multiple assays using PCR. The PCR technique demonstrated HIV-1 DNA, as expected, in producer cell lines and in the parental LL58 and LL58-6 cells; uninfected H9 and X50-7 cells were negative (Fig. 10).

The sensitivity of the PCR technique was estimated by comparing serial 10-fold dilutions of cellular DNA from H9/RF2 with similar dilutions of DNA from LL58 and LL58-6.12 cells. A signal could be readily detected from 100 pg of DNA from H9/RF2 cells (equivalent to 20 cells) and from 10 ng of DNA from LL58 and LL58-6.12 cells (2,000 cells). However, no signal was detected with 1 μ g of DNA, equivalent to 200,000 cells, from clones such as LL58-6.14 or LL58-6.18. Therefore, the LL58 and LL58-6.12 cells contained about 1% as much HIV-1 DNA as did the producer

FIG. 7. Loss of expression of HIV-1 polypeptides in parental LL58 cells and in the LL58-6 clone. Shown is an immunoblot containing extracts from early (A) and late (B) passages of the LL58 line and its LL58-6 subclone. The LL58 extracts, which are the same as those shown in Fig. 1, were prepared about 26 months apart. The LL58-6 extracts from the same number of cells were prepared 6 weeks apart. The only HIV-1 polypeptides seen in LL58-6-(B) are p55 and p24.

line H9/RF2, while lines such as LL58-6.14 and LL58-6.18 harbored less than 0.01%. From these titrations we concluded that among 200,000 cells of a PCR-negative clone, there were fewer than 20 cells with HIV-1 DNA. It is likely, therefore, that these clones lack a HIV-1 provirus.

Expression of HIV-1 RNA in second-generation subclones. EBV-transformed cell lines which were productively infected with HIV-1, such as X50-7/MN and X50-7.8/SB, contained large amounts of HIV-1 RNA, equivalent to the amount detected in H9/RF2 cells. In each of these lines, the three prominent HIV-1-specific RNAs of 9.5, 4.1, and 1.9 kilobases (kb) (2, 3, 31) could be detected (Fig. 11A). In

TABLE 1. Description of EBV-immortalized cell lines and HIV-infected counterparts used in these studies

Cell line designation	Description	Date isolated	Expression of HIV		
			Extracellular ^a	Intracellular ^b	% IF+ ^c
X50-7	EBV-immortalized umbilical cord cells		—	—	
X50-7.8	Subclone of X50-7	Jan. 1986	—	—	
X50-7/MN	Infected with MN	Nov. 1985	+	+	>90
X50-7.8/MN	Infected with MN	June 1987	+	+	>90
X50-7.8/SB	Infected with SB	June 1987	+	+	>90
LL58	X50-7 infected with HTLV IIIB	July 1985	— ^d	+	<0.1
LL58-6	Subclone of LL58	Feb. 1988	— ^e	+	<0.1
LL58-6.12	Subclone of LL58-6	May 1988	—	+	<0.1
LL58-6.20	Subclone of LL58-6	May 1988	—	+	<0.1
LL58-6.14	Subclone of LL58-6	May 1988	—	—	
LL58-6.18	Subcloned of LL58-6	May 1988	—	—	

^a As assayed by infectivity of supernatant fluids.

^b As assayed by syncytium formation.

^c IF+, Positive by immunofluorescence.

^d As of March 1986.

^e As of May 1988.

FIG. 8. Detection of HIV-1 proteins in very early passage of the LL58-6 subclone. Shown are immunoblots reacted with monospecific polyclonal antibodies to *env* gp160, gp120, and gp41 and a monoclonal antibody to *gag* p55/24. Two polyclonal human antisera are also included. BA recognizes EBV latent proteins and HIV-1. SC recognizes EBV replicative products and is HIV-1 negative. The panel of antigens are: lanes 1, X50-7; lanes 2, X50-7/MN; lanes 3, LL58-6 (April 1988); and lanes 4, HTLV IIIB virions.

contrast, the amounts of HIV-1 RNA found in LL58, LL58-6, and the two second-generation subclones (LL58-6.12 and LL58-6.20) which contained HIV-1 DNA were considerably smaller than those present in producer cell lines (Fig. 11B). Nonetheless, LL58-6 and its two HIV-1-positive subclones, LL58-6.12 and LL58-6.20, expressed all three major RNA species of 9.5, 4.1, and 1.9 kb (Fig. 11B). Clones such as LL58-6.14 and LL58-6.18 which were negative by PCR were likewise negative for HIV-1 RNA by Northern analysis. Thus, those clones which contained HIV-1 DNA expressed low levels of the appropriate HIV-1 RNAs. We did not detect clones which contained HIV-1 DNA but failed to express RNA. The second-generation clones LL58-6.12 and LL58-6.20, which contained HIV-1 DNA and RNA, were fusogenic. The remainder of these clones, which lacked detectable provirus, were not fusogenic.

DISCUSSION

Evolution of the cultures. We report novel biologic observations about the behavior of the IIIB strain of HIV-1 in an EBV-transformed B-lymphocyte cell line. The original LL58 culture and its first-generation clone, LL58-6, evolved over several months from a state in which HIV-1 was produced and released extracellularly in significant amounts to one in which there was no detectable extracellular virus. Two fusogenic second-generation clones, when examined 2 months after expansion, expressed similarly low levels of cell-associated virus. A minor subpopulation of the cultures from all three generations continued to express highly cell-

associated HIV-1 products for long periods of time. HIV-1 has persisted in the original LL58 culture for 3 years, in the LL58-6 subclone for 1.5 years, and in the LL58-6.12 and LL58-6.20 clones for 1 year. The majority of cells in the cultures appeared not to express any HIV-1 replicative products. The cultures thus eventually consist of two populations of cells: a fusogenic one which contains the HIV-1 genome and expresses HIV-1 RNA and a population which is HIV-1 genome negative.

The evolutionary process consists of two distinct events: the infection evolves from one in which extracellular virus is released to one in which virus is cell associated, and the fraction of cells which express HIV-1 products eventually reaches a very low level which is maintained over time. These two events may be interrelated.

A viral factor is likely to be involved in the evolution to the cell-associated state. This evolution from HIV-1 production to low-level cell-associated behavior appeared to be specific to the IIIB strain; other HIV-1 strains which replicate in the X50-7 and X50-7.8 cells show either rapid viral multiplication associated with extensive CPE and release of virus (e.g., RF2) or persistent noncytopathic productive infection in which at least 70 to 95% of the cells continue to express replicative products and to shed virus (e.g., MN and SB). The IIIB strain is known to be genetically and biologically heterogeneous. In LL58 and its clonal subpopulations there may be predominance of one or more variants with high levels of expression of the *nef* gene product, which seems to down-regulate synthesis of structural components of the virus (17, 26, 44, 45). There may also be selection for variants which have a mutation in the *vpu* gene which seems to facilitate extracellular virus release (41).

One or more cell components are also likely to favor the cell-associated state. The need for a cell factor is suggested by the cocultivation experiments which rescue virus from the LL58 cells. These cells evolved to a CD4-negative state and therefore probably can not be reinfected by any virus which is produced in the culture. The uninfected X50-7.8 indicator cultures supply something needed for extracellular viral maturation, since extracellular viral particles (Fig. 5) and infectious virus (Fig. 6) can be recovered after cocultivation. A component of this cell factor is probably CD4 and the remaining elements of the virus receptor.

Replicative subpopulation of cells. Using a number of different assays, we found that in the steady state, between 0.01 and 2% of cells express HIV-1 genes encoding structural components of the virus. The capacity for fusion and its inhibition by HIV-1 antibody-positive sera indicated that cells from the replicative subpopulation expressed the *env* gene product on their surfaces. It is not yet clear, however, whether *env* products were expressed only in cells which produced intracellular viral particles or whether some cells expressed *env* at the surface but did not undergo viral maturation. The *env* polypeptides gp160, gp120, and gp41 were detected with monospecific antisera on immunoblots of early passages of the LL58-6 subclone (Fig. 8). Furthermore, the characteristic *env* RNA of approximately 4.1 kb (2, 31) was observed in cultures of LL58-6, LL58-6.12, and LL58-6.20 (Fig. 11B). Further studies of LL58 cells using a panel of anti-*env* antibodies and immune electron microscopy should help localize *env* peptides within these cells.

The analysis of the expression of the remainder of HIV-1 genome in the replicative subpopulation is still incomplete. This analysis is difficult because such a small subpopulation of cells expresses these replicative products. Nonetheless, immunoblotting (Fig. 8) and Northern analysis (Fig. 11B) do

FIG. 9. Comparison of extracellular (A) and cell-associated (B) infectious HIV-1 in the LL58-6 clone. The materials for the infectivity assay were prepared 3 weeks after the LL58-6 extracts shown in Fig. 7 were made. These were filtered supernatant fluids (A) or frozen and thawed cell extracts (B) which were inoculated onto X50-7.8 indicator cells in serial dilutions. Extracts for Western immunoblotting were prepared 14 days after inoculation. Lanes: 1, X50-7.8 cells; 2, HTLV IIIB virions; 3, LL58-6 cells; 4 to 6, X50-7.8 cells incubated with 10^0 , 10^{-1} , or 10^{-2} dilutions, respectively, of supernatant fluid (A) or frozen and thawed cell extract (B). No infectious HIV-1 was found in the supernatant fluid of the LL58-6 clone, but the cell extract contained infectious HIV-1 at 10^0 and 10^{-1} dilutions. Two HIV-1 polypeptides (p55 and p24) are seen in the extract of clone LL58-6 itself (lanes 3).

not indicate a gross abnormality in gene expression. The core polypeptides (p55 and p24) were made, and the three expected RNAs were detected. The relatively low levels of HIV-1 RNAs in LL58 cells and their fusogenic derivatives are most certainly the result of studying a mixed population in which only a few cells in the culture are producing the RNAs.

There is, nonetheless, a defect in viral maturation and/or viral transport in the cells which express HIV-1 RNA: infectious virus could not be recovered in supernatant fluids, and mature virus was not seen budding at the cell surface. Instead, virions were observed within cytoplasmic vesicles (Fig. 5A) which were similar in morphology to those described in HIV-1-infected monocyte lines (19, 32). To our

knowledge, this is the first description of HIV-1 sequestration in vesicular compartments within lymphocytes. Although vesicles with HIV-1 were less abundant in the EBV-transformed cells than in monocytes, and although the number of viruses sequestered in each vesicle was lower, the virions contained therein were enveloped, as has been described for infected monocytes. The maturation defect therefore seems to consist of an impairment of the normal pathway of egress of virus from infected cells. The maturation defect may be analogous to some other retrovirus systems, e.g., mammalian cells infected with avian sarcoma viruses, in which nonproductive infection is associated with defective virus maturation but virus can be recovered by cocultivation (39, 43).

FIG. 10. Detection of HIV-1 DNA in second-generation subclones of the LL58 line by using PCR. The amplified DNA was from the HIV-1 LTR. The LL58-6 clone and one of its subclones, LL58-6.12, contained HIV-1 DNA. (When LL58-6.20 was included in other experiments under identical conditions, it also contained HIV-1 DNA). Two LL58-6 subclones (LL58-6.14 and LL58-6.18) and uninfected H9 and X50-7 cells lacked detectable HIV-1 DNA. Size markers (in base pairs) are indicated.

FIG. 11. Detection of HIV-1 RNA in EBV-transformed cell lines chronically infected with HIV-1. (A) HIV-1 RNAs found in X50-7 cells and X50-7.8 cells productively infected with HIV-1 strains MN and SB, respectively. The probe was the entire BH10 clone of HIV-1, which detects the characteristic HIV-1 RNAs of 9.5, 4.1, and 1.9 kb. (B) HIV-1 RNAs found in second-generation clones of LL58 cells. Using the 2.1-kbp *Hind*III-*Kpn*I fragment and the 2.2-kbp *Kpn*I-*Kpn*I fragment of BH10 in combination as a probe reveals the three characteristic HIV-1 RNAs in LL58-6 and LL58-6.20; on a longer exposure, they were present in LL58-6.12 as well. Two subclones, LL58-6.14 and LL58-6.18, were negative.

Recovery of cell-associated virus. Three mechanisms were considered to explain HIV rescue by cocultivation with uninfected cells which express a virus receptors. (i) Cocultivation may induce a silent HIV-1 provirus. However, we did not obtain experimental support for this mechanism. There was no difference in the relative abundance of HIV-1 polypeptide expression in LL58 cells alone compared with that in cocultures of LL58 and X50-7 cells (data not shown). Numerous experiments were performed in an effort to activate a quiescent provirus by using chemicals and cytokines.

Among the agents tested in several concentrations were 5-azacytidine, 5-iododeoxyuridine, tetradecanoylphorbol acetate, *n*-butyrate, phytohemagglutinin (with or without interleukin-2), and human interleukin-6. In no instance was there an increase in the number of HIV-1 antigen-positive cells following the addition of the inducing agent. (ii) Cocultivation, which is accompanied by HIV-1-induced cell fusion, conceivably facilitates entry of cell-associated virions into susceptible target cells. (iii) The process of cocultivation not only permits virus entry but also temporarily restores normal virion maturation, transport, and egress at the cell surface. Therefore, the indicator cells may provide one or more factors which are necessary for HIV-1 maturation but which are not provided by the LL58 population.

Cell subpopulation which does not express replicative products. More than 99% of the cells in each fusogenic clone did not express HIV-1 replicative products. Most clones were negative for HIV antigens and were nonfusogenic. There were initially three hypotheses to account for these nonproducer cells: one was that they contained an intact but latent HIV-1 provirus whose expression was repressed; another possibility was that they contained a deleted or otherwise defective provirus; finally, they might lack HIV-1 DNA entirely. All the available evidence favors the last alternative.

If HIV-1 was latent in the nonproducer cultures, we should be able to detect HIV-1 DNA in them. We would also expect to be able to activate HIV-1 expression by using inducing agents; all these experiments were negative. Initially we were unable to find HIV-1 DNA in LL58, LL58-6, LL58-6.12, and LL58-6.20 cells by a Southern hybridization procedure which could detect between 1 and 10 pg of DNA. These cell lines were expected to contain an HIV-1 genome because a subpopulation of each was fusogenic. When PCR became available, we could readily detect HIV-1 DNA in these fusogenic cultures; however, on the basis of titrations, the amount of DNA was at least 100-fold less than in cell lines which express HIV-1 in all the cells. Had a significant number of cells harbored an intact but latent provirus, the PCR DNA amplifications would have demonstrated a stronger signal. Furthermore, many secondary clones lacked HIV-1 DNA by PCR.

That some nonfusogenic clones contain a deleted provirus cannot be excluded by our data. These clones will need to be examined further by PCR with a large panel of probes spanning the entire HIV-1 genome. Nonetheless, the more likely explanation is that those clones which lack HIV-1 DNA detectable by PCR using the LTR probes do in fact lack the HIV-1 genome.

What is the origin of these HIV-1 genome-negative cells in the culture? The possibility that they represent cells which initially escaped infection when HIV-1 IIIB was originally added to X50-7 cells is feasible for the original LL58 cell population, but this scenario seems less likely for the succeeding generations of fusogenic cell subclones, which seemed to arise from single cells. Even if we assume that the LL58-6, LL58-6.12, and LL58-6.20 subclones were each initially contaminated with a few HIV-1 genome-negative cells, the contaminating population could not account for the very low frequency of HIV-1 replication-positive cells (<1%) unless there was a marked selective overgrowth of cells which did not contain the HIV-1 provirus. In this case, the number of fusogenic or HIV-1 antigen-positive cells would be expected to fall to 0 after a period of time in culture. Rather, the number of such cells has persisted at roughly the same level for 1 to 3 years (Table 1).

Alternatively, some clones may have originally been HIV-1 genome positive but subsequently lost the provirus. If the provirus is being lost from a state in which it is integrated into the cell genome, there may be some special feature of the integration site which permits the provirus to excise. Loss of an integrated provirus may be accompanied by loss of the cell chromosomal integration site. This possibility could be explored experimentally. Another possibility is that the provirus was not integrated in the majority of LL58 or LL58-6 cells. The absence of a plasmid partitioning system may explain why HIV would not persist in a nonintegrated state.

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