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## Epstein-Barr Virus-Positive and -Negative B-Cell Lines Can Be Infected with Human Immunodeficiency Virus Types 1 and 2

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Human immunodeficiency virus type 1 (HIV-1) can infect CD4<sup>+</sup> lymphocytes, monocytes-macrophages, and various other cell lines, including B-cell lines. To study the parameters of B-cell infections, we examined the susceptibility of 24 B-lymphoid cell lines to both HIV-1 and HIV-2 infections. These cell lines included a series of Epstein-Barr virus (EBV) genome-negative Burkitt's lymphoma cell lines and their EBV-converted counterparts. To infect these cells we used two HIV-1 isolates and one HIV-2 isolate. Infections were monitored with a cytoplasmic RNA dot-blot and a syncytium assay. HIV infection was also studied by a novel method based on electrophoresis of DNA liberated from cells that were lysed in situ in the well of an agarose gel. All human B-cell lines could be infected with HIV-1, regardless of the presence of EBV genomes; thus, EBV infection had no major effect on HIV susceptibility of B-cell lines. Integrated proviral HIV genomes could be detected by Southern blot analysis of DNA extracted from long-term, non-HIV-producing B-cell lines. This study suggests that B-lymphoid cells may serve as reservoirs for latent or persistent HIV infections in vivo, even in the absence of EBV infection.

While the human immunodeficiency viruses (HIV) preferentially infect and often deplete T4 lymphocytes (3, 7), a major unanswered question is why the latency period for the onset of acquired immunodeficiency syndrome (AIDS) can vary from a few months to more than 7 years. One explanation could be the ability of other viruses to act as cofactors in the stimulation of HIV expression and subsequent depletion of the T4 lymphocytes. It has been shown that HIV type 1 (HIV-1) can productively infect Epstein-Barr virus-positive (EBV<sup>+</sup>) lymphoblastoid cells (5, 12, 15, 18, 22). HIV-1 infection of EBV<sup>+</sup> B cells can result either in a persistently infected culture or in complete cytolysis of the culture, depending on the cell line and virus dilution (5). Several DNA viruses, including herpesviruses (cytomegalovirus, HSV-1, and EBV), can stimulate expression of HIV-1 by transactivation of the HIV-1 long terminal repeat (9, 10, 16).

This study was designed to examine the susceptibility of various B-lymphoid cell lines to HIV infection and to investigate the effect of prior EBV infection on the infectivity of these B cells by HIV. Most cell lines established from African and AIDS-associated Burkitt's lymphomas (BLs) are EBV<sup>+</sup>, whereas the majority of European and American non-AIDS-associated BLs are EBV<sup>-</sup>. Recently, one of us (A.C.) established a series of such EBV<sup>-</sup> cell lines from non-AIDS-associated European BL (4) and infected some of these cells with either the nonimmortalizing EBV strain P3HR-1 or the immortalizing EBV strain B95-8 to be able to study, in parallel, EBV<sup>-</sup> BL cells and their EBV-converted counterparts prior to extensive tissue culture passage. These cell lines (BL30, BL31, BL40, BL41, and BL70), another EBV<sup>-</sup> BL cell line (CA-46), an older series of EBV<sup>-</sup> and EBV-converted BL cells (RAMOS), and seven existing EBV<sup>+</sup> B-lymphoid cells of various origin (Table 1) were infected with HIV-1 and HIV-2. CEM, a human T4 leukemia cell line, was included as a positive for productive infection. B95-8, an EBV<sup>+</sup> lymphoblastoid cottontopped marmoset

B-cell line, was also infected with HIV-1 and HIV-2, since its progeny virus was used to convert several of the EBV<sup>-</sup> BL cells. It was observed that all human EBV<sup>-</sup> as well as EBV<sup>+</sup> cell lines could be infected with HIV.

The B-lymphoid cell lines used in this study are listed in Table 1, with their current EBV status. Cells were tested for the presence of EBV nuclear antigens by anticomplement immunofluorescence staining (17). EBV genomes were detected with Gardella gels (8), a simple gel electrophoretic technique utilizing intact cells. In addition, all EBV<sup>-</sup> cell lines were examined by Southern blot analysis (21) to confirm the absence of EBV genomes. These cell lines were also tested for the expression of CD4 antigen by indirect immunofluorescence using monoclonal antibodies OKT4a, Leu-3a, and MT151; CD4 is the HIV-1 and HIV-2 receptor (6, 11, 19a, 20).

Two types of HIV-1 were used to examine differences between various isolates. HTLV-IIIB (HIV-1 IIIB) is cytopathic for T4 lymphocytes, and HTLV-IIIRF (HIV-1 RF) is more highly cytopathic. In addition, we used one strain of HIV-2 (LAV-2 ROD, I-532) to study group-specific differences on B-cell infection. No data have previously been reported on HIV-2 infection of B-lymphoid cells. Each infection was repeated a minimum of three times.

In all, 3 ml of cells (at 10<sup>6</sup>/ml) was infected with 0.1 ml of virus stock (stored at -70°C) and incubated at 37°C in 5% CO<sub>2</sub> for 24 h. An equal volume of fresh medium (RPMI 1640-10% fetal calf serum) was added on day 2. BL cell lines were fed with RPMI 1640-20% FCS. Cells were sampled and fed with the same medium twice weekly, starting on day 4. HIV infection was monitored by using the cytoplasmic RNA dot-blot assay for HIV-1 (14) along with the C-8166 syncytium assay for HIV-1 and HIV-2. The syncytium assay (23) was performed by mixing equal volumes (0.5 ml) of C-8166 indicator cells (19) (5 × 10<sup>5</sup> cells) and cell-free supernatant from the infected cultures in 24-well tissue culture plates. The cultures were monitored for syncytium formation at 24, 48, and 72 h. For each virus stock used, titers were deter-

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TABLE 1. HIV infection of 24 EBV<sup>+</sup> and EBV<sup>-</sup> B-cell lines<sup>a</sup>

Cell line	Origin <sup>b</sup>	EBNA <sup>c</sup>	EBV genome	CD4 antigen	HIV infectivity <sup>d</sup>				
					IIIB DB	IIIB SYN	RF DB	RF SYN	HIV-2 SYN
Jijoye	Afr BL	+	+	-	++	++	+++	+++	+/-
P3HR-1	Jijoye	+	+	-	+/-	+	++	+++	+
Raji	Afr BL	+	+	-	-	+	+	+++	-
8392	LCL	+	+	-	+++	+++	+++	+++	++
IB4/B95-8	LCL	+	+	ND	+++	+++	+++	+++	-
QIMR-WIL	LCL	+	+	ND	-	+	+	++	-
CA-46	BL	-	-	ND	++	+++	++	+++	++
BL30	Eur BL	-	-	-	-	+	+/-	++	-
BL30E1/P3HR-1	BL30	+	+	++	+++	+++	+++	+++	++
BL30E2/P3HR-1	BL30	+	+	-	+	++	+++	+++	+++
BL31	Eur BL	-	-	-	+	++	++	+++	-
BL31E/B95-8	BL31	+	+	+	+++	+++	+++	+++	+++
BL40	Eur BL	-	-	-	-	+	-	++	+
BL40E/B95-8	BL40	+	+	+	+/-	+	+	+++	++
BL41	Eur BL	-	-	+	-	+	+	+++	+
BL41E/B95-8	BL41	+	+	++	+++	+++	+++	+++	+++
BL70E1/P3HR-1	BL70 (Eur BL)	+	+	-	-	+	+/-	++	++
BL70E2/P3HR-1	BL70	+	+	-	+	+	++	++	+++
BL70E3/B95-8	BL70	+	+	++	+++	+++	+++	+++	+++
RAMOS	Amer BL	-	-	+/-	++	++	++	+++	+
RAMOS2/P3HR-1	RAMOS	+	+	+	+++	+++	+++	+++	++
RAMOS3/P3HR-1	RAMOS	+	+	-	+	++	++	+++	++
RAMOS4/P3HR-1	RAMOS	+	+	+	+++	+++	+++	+++	++
RAMOS5/B95-8	RAMOS	+	+	+/-	+++	+++	+++	+++	++
B95-8	CTM-LCL	+	+	-	-	-	-	-	-
CEM	TCL	-	-	++	+++	+++	+++	+++	+++

<sup>a</sup> Cell lines were tested for EBV nuclear antigens, EBV genomes, and CD4 antigen as described in the text. HIV infection with similar titers was also performed as described in text.

<sup>b</sup> LCL, Lymphoblastoid cell line; CTM-LCL, cottontopped marmoset lymphoblastoid cell line; TCL, T-cell leukemia; Afr BL, African Burkitt's lymphoma; Eur BL, European Burkitt's lymphoma; Amer BL, North American Burkitt's lymphoma.

<sup>c</sup> EBNA, EBV nuclear antigen.

<sup>d</sup> IIIB, HIV-1 IIIB; RF, HIV-1 RF; DB, Dot-blot assay; SYN, C-8166 syncytium assay; ND, not done; -, never observed; +/-, occasionally observed; +, weak; ++, strong; +++, very strong.

mined on C-8166 cells and appropriate dilutions were made so that each virus inoculum had a similar titer.

The results of both the cytoplasmic RNA dot-blot and the C-8166 syncytium assays are listed in Table 1. Since HIV-2 has very low genomic homology to HIV-1 and since HIV-2 clones were not yet available to us for hybridization, only the syncytium assay was used to detect HIV-2 infection.

All 24 human B-cell lines tested could support HIV production of at least the two HIV-1 isolates used; 19 of these could also be productively infected with HIV-2. HIV-1 RF consistently gave the highest yield of HIV-1 RNA and the strongest syncytium production in the majority of the cell lines studied. About half of the EBV<sup>+</sup> cell lines showed high HIV production (Table 1). High levels of both cytoplasmic RNA and extracellular virus could be detected in these cells well past 30 days after infection. No production of HIV could ever be detected in the cottontopped marmoset B95-8 cell line, although cottontopped marmoset T4 lymphocytes are infectible by HIV-1 and HIV-2 (13; our unpublished results).

All of the six EBV<sup>-</sup> Burkitt's lymphoma cell lines examined could be productively infected with HIV-1. Earlier reports (14, 17) that EBV<sup>-</sup> B cells could not be productively infected with HIV-1 are clearly at odds with our results. Those results could be due to the use of a less virulent virus strain, lower virus titers, less sensitive assays used by Salahuddin et al., or a combination of these three phenomena (18). Thus, this is the first report of an EBV<sup>-</sup> B-cell line productively infected by HIV. Production of HIV-1 IIIB in three of the six EBV<sup>-</sup> cell lines (BL30, BL40, and BL41)

was very weak and was detectable only with the syncytium assay. This was also the situation with some of the EBV<sup>+</sup> cell lines for HIV-1 IIIB infection.

To confirm that these cell lines with very low virus production were truly infected with HIV-1, cell-free supernatants from cultures at 10 days postinfection were used to infect CEM cells. These CEM cells were then tested for HIV-1 production by the dot-blot assay, starting on day 7 postinfection. Most of the HIV-1 infections that had been either negative or weakly positive with the dot-blot assay (Table 1) were tested in this manner. Of the 10 such infections examined, only 1 remained negative by dot-blot assay after CEM infection (data not shown): the HIV-1 IIIB infection of the BL-40 cell line. Since the B95-8 cell line was always negative for HIV infection, supernatants from B95-8 cultures were included in the CEM infections as a negative control to ensure the absence of residual virus from the original inoculum. CEM cells infected with the supernatants of the HIV-infected B95-8 cells remained negative.

While HIV production could be detected in the EBV<sup>-</sup> cells (Table 2), we observed that, unlike infection of the EBV<sup>+</sup> cells, HIV infection of several of the cell lines resulted in cytolysis by day 12 after infection. This phenomenon occurred with the BL30, BL40, and BL41 cell lines and occasionally with the BL31 cell line. Most of the uninfected EBV<sup>-</sup> BL cell lines were very fastidious when compared with their EBV-converted counterparts. This could explain the different types of biological behavior after HIV infection. However, HIV infection of the CA-46 and RAMOS EBV<sup>-</sup> cell lines always yielded a persistent infection similar to that

TABLE 2. HIV infection of EBV<sup>-</sup> B-cell lines<sup>a</sup>

Cell line	HIV infectivity <sup>b</sup>	Cytolysis <sup>c</sup>	Persistently infected cells <sup>d</sup>
BL30	+	+	-
BL31	++	+/-	+/-
BL40	+/-	+	-
BL41	+	+	-
CA-46	+++	-	+
RAMOS	+++	-	+

<sup>a</sup> -, Never observed; +/-, occasionally observed; +, weak; ++, strong; ++++, very strong.

<sup>b</sup> HIV infectivity of each cell line was scored by compiling the data in Table 1.

<sup>c</sup> Cell destruction occurring between days 10 and 14 postinfection.

<sup>d</sup> Cell cultures that survived past 30 days postinfection.

of the HIV-infected EBV<sup>+</sup> cell lines. BL31 infections usually resulted in persistent HIV infections, but with lower HIV yields.

A majority of cell lines were tested for the expression of CD4 antigen by indirect immunofluorescence with three monoclonal antibodies against CD4. These results are listed in Table 1. Several HIV-infectible cell lines expressed no detectable CD4 surface markers. Similar results had been reported earlier (18). Although these results could be interpreted to indicate the existence of alternative HIV receptors besides CD4 (18), other possible explanations appear more attractive. Several nonlymphoma cell lines without detectable CD4 surface expression, but infectible by HIV-1, were subsequently found to contain CD4 mRNA (2; J. Weber, personal communication). Similarly, HIV-1 infection of Raji cells could be blocked by MT151, an anti-CD4 monoclonal antibody (C.M. and P. R. Clapham, unpublished observation). Both of these observations indicate CD4 expressions, albeit at a low level, and the use of CD4 as the HIV receptor. Therefore, we are currently testing several of the B-cell lines for the presence of CD4 mRNA and for the ability to block infection with antibodies against CD4 epitopes.

DNA extracted from several long-term-HIV-infected cell lines (BL31E, RAMOS 2, Jijoye, P3HR-1, and CEM) were positive for HIV genomes by Southern blot analysis (data not shown). At the time of extraction, these cells were producing only very low levels of or no detectable HIV-1 RNA as measured by the dot-blot assay. This finding suggests that these cells had been latently infected with HIV.

To monitor HIV-1 genome production in the initial stages of infection, we subjected infected B cells to a modified Gardella gel analysis (8). A suspension of 10<sup>6</sup> B cells, at various days postinfection, was pipetted into the wells of a vertical agarose gel (1.4%) and overlaid with sodium dodecyl sulfate and pronase. Electrophoresis of sodium dodecyl sulfate into the cell layer, at low voltage, induces a very gentle lysis of the cell and nucleus. As a result, the cellular DNA remains too large to enter the gel, and only small DNA such as unintegrated proviral DNA enters the gel and can then be detected by hybridization as two or three bands, a covalently closed circular DNA, and a linear DNA that migrates below a diffuse band of broken cellular DNA (100 to 500 kilobases); this diffuse band is probably derived from dead cells (8). The hybridization frequently detected at the top of the gel could be due to integrated proviral DNA or unintegrated proviral DNA, or both, trapped in the network of high-molecular-weight cellular DNA. This simple gel technique requires only a small number of cells (5 × 10<sup>5</sup> to 1 × 10<sup>6</sup>) and does not require DNA extraction and purification.

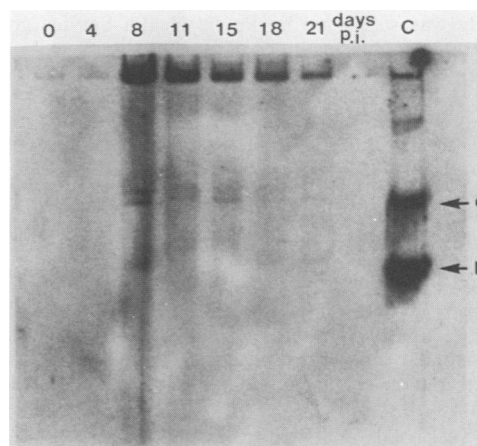


FIG. 1. Detection of circular and linear HIV-1 DNAs in an HIV-1 RF-infected EBV<sup>+</sup> lymphoid cell line (BL41E). A suspension of 10<sup>6</sup> cells from an HIV-1 RF-infected culture was sampled on days 0, 4, 8, 11, 15, 18, and 21 postinfection (p.i.) as described in the text. The control lane (C) contains both the circular (c) and linearized (l) forms of pH6B5.0, a 10-kilobase plasmid with pBR322 sequences which cross-hybridize to pJM(HIV-1)8.9, the HIV-1 clone (12) used for hybridization. The band between the origin of the gel and the circular band in the control lane represents a small portion of nicked circular DNA that was present in the original plasmid preparation.

A higher-percentage agarose gel (1.4%) instead of the normal 0.75% gel used for larger episomal DNA (8) was used for this study to achieve better separation of the circular and linear forms of the HIV genome. In the 0.75% gel, the circular and linear forms of 10-kilobase plasmid migrate very close together, whereas in a 1.4% gel the linear form migrates much faster than the circular band. The relative position of each band was detected by running each form in separate lanes (data not shown). The relative positions of linear and covalently closed circular molecules depend on size and agarose concentration (1).

Using this method, we consistently detected high levels of both circular and linear unintegrated HIV-1 genomes 8 days postinfection (Fig. 1). The number of unintegrated genomes diminished thereafter and, by day 21 postinfection, became barely detectable (Fig. 1). The double band visible at the circular region of the HIV-1 RF-infected cells could possibly represent a mixture of circular genomes with one and two copies of the long terminal repeat region.

While most EBV<sup>+</sup> B cells produced higher amounts of HIV and HIV RNA than their EBV<sup>-</sup> counterparts, it was clearly seen that they can all be productively infected with HIV. It was also observed that one isolate of HIV (HIV-1 RF) is more virulent for these B cells than other isolates (HIV-1 IIIB, and HIV-2 ROD). This study indicates that most B-lymphoid cell lines (both EBV<sup>-</sup> and EBV<sup>+</sup>) can be persistently infected with HIV. Therefore, we have to consider the hypothesis that in vivo B cells serve as reservoirs for HIV infection. These results should also act as a warning against the use of spontaneous or EBV-induced B-cell lines established from individuals with unknown HIV status.

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