Persistent Infection of a Human Lymphoblastoid Cell Line with Equine Herpesvirus 1

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Infection of a human lymphoblastoid cell line (Jijoye line derived from a Burkitt lymphoma which contains Epstein-Barr virus) with equine herpesvirus 1, maintained and observed for 53 days, was characterized by the continuous production of infectious extracellular and intracellular virus. Maximum virus production correlated with active cell multiplication. Less than 15% of the cells possessed viral capsid antigen at any one time. Five percent of the cells in the Jijoye line possess Epstein-Barr viral capsid antigen; 80% of the Epstein-Barr viral caspid-containing cells also contained equine herpesvirus 1 antigen as detected by double staining. The interaction of equine herpesvirus 1 and lymphoblastoid cells provides a useful in vitro model which may help clarify the mechanism for herpesvirus latency.

Herpesviruses, within their group species, characteristically persist in host tissue even in the presence of specific circulating antibody. Bryans (2) showed that viremia in equine herpesvirus 1 (EHV-1)-infected horses persisted as long as 3 weeks and that the virus was associated with an element in the buffy coat layer of blood samples and coexisted with homologous neutralizing antibody.

Several herpesviruses, Epstein-Barr virus (EBV) (5), herpes simplex virus (13), and herpesvirus macaca (7) replicate and persist in vitro in human lymphoblastoid cell (LC) lines. Data obtained in our study demonstrate the ability of EHV-1 to infect, replicate, and maintain a persistent infection in human LCs.

MATERIALS AND METHODS

Cell cultures. The LC line used was Jijoye (11), derived from a Burkitt lymphoma and possessing B-lymphocyte antigenic characteristics. Jijoye cells are EBV-transformed human peripheral lymphocytes which proliferate indefinitely in vitro. This cell line contains the EBV genome and EBV nuclear antigens and produces demonstrable EB viral capsid antigen (VCA).

The LC line was grown in RPMI 1640 medium containing 2 mM glutamine, 100 U of penicillin per ml, and 50 mg of streptomycin per ml, with 20% fetal calf serum.

Rabbit kidney-13 (RK-13) cells were used for virus stock production and virus titrations. RK-13 cells for monolayers were propagated in Eagle minimum essential medium, containing 100 U of penicillin per ml and 50 mg of streptomycin per ml, with 10% fetal calf serum, and were maintained in minimum essential medium containing penicillin and streptomycin with 2% fetal calf serum.

Virus. EHV-1 (equine rhinopneumonitis virus), Kentucky-D strain, adapted to RK-13 cells was obtained from J. L. Blue (University of Georgia, Athens, Ga.) and from the American Type Culture Collection. The EHV-1 was passed twice in this laboratory in RK-13 cells; virus stock was prepared from the second passage. When 80% of the monolayer exhibited cytopathic effects, the cells were frozen and thawed three times without removing the culture fluid. The virus suspension was clarified by centrifugation at 8,700 × g for 30 min and concentrated by pelleting at 73,400 × g for 2 h. The virus was titrated in 16- by 125-mm screw-capped tubes containing 5-day-old RK-13 monolayers. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Reed-Muench (12) method.

Infected of the LC line. Jijoye cells were sedimented by centrifugation at 200 × g for 10 min, and the supernatant was discarded. Cells were counted in a hemacytometer, with 0.1% trypan blue exclusion stain used as an indicator of viability. The packed cells were adjusted to a volume of 2 × 10^6 viable cells per ml and were infected with EHV-1 at a multiplicity of infection of 0.3. The virus was allowed to adsorb at 37°C for 1 h. After adsorption, the cells were washed four times with RPMI medium to remove unattached virus and were then adjusted to a final concentration of 5 × 10^5 cells per ml. Uninoculated Jijoye cells maintained under identical conditions were used as a control.

Viable cells were counted at various times. Cell volumes were adjusted by adding fresh medium at 3-day intervals to obtain a cell volume of 5 × 10^5 cells per ml; or if the cell volume was lower than 5 × 10^5

cells per ml, the medium was discarded and replaced with an appropriate amount of fresh medium.

Samples of cultures were taken at different times to monitor virus production. The samples were centrifuged at $200 \times g$ for 10 min. The supernatant was frozen at $-70°C$ until titrated in RK-13 monolayers to determine the level of extracellular virus. The sedimented cells were washed three times in sterile phosphate-buffered saline to remove unattached virus. Half of the sedimented cells were reconstituted in RPMI 1640 medium, frozen and thawed three times, and titrated in RK-13 monolayers to determine the level of intracellular virus. The remainder of the sedimented cells were prepared for the direct and indirect fluorescent-antibody (FA) test).

Direct, indirect, and double-staining FA technique. Samples of infected and uninfected Jijoye cells were washed three times in phosphate-buffered saline, pH 7.5, and adjusted to 3×10^6 cells per ml. A volume (0.05 ml) of the cell suspension was placed in wells of epoxy-surface microscope slides. The cells were air dried, fixed in acetone for 10 min at room temperature, and stored at $-20°C$ until used.

For direct FA determination of EHV-1 antigen, 0.05 ml of fluorescein isothiocyanate-conjugated EHV-1 horse antiserum. (U.S. Department of Agriculture, Veterinary Services Laboratories, Ames, Iowa) was placed in each well, incubated in a moist chamber for 30 min at 37°C, and then washed twice with phosphate-buffered saline, pH 7.4. The slides were dried, and cover slips were mounted on a drop of buffered glycerin, pH 7.5.

Indirect FA testing of EB-VCA was done by the method of Dye and Feorino (4). A double-staining FA test was performed on the sample slides to observe if a single cell was producing both EHV-1 and EB-VCA. For the double-staining test, 0.05 ml of a known EBV-positive and -negative serum was placed in wells containing acetone-fixed samples of Jijoye cells. The slides were incubated in a moist chamber for 1 h at 37°C and washed with phosphate-buffered saline. After the slides dried, 0.05 ml of an equal-part mixture of fluorescein isothiocyanate-conjugated EHV-1 horse antiserum and rhodamine-conjugated goat anti-human immunoglobulin G (Bionetics, Kensington, Md.) was placed in each well, incubated in a moist chamber for 30 min at 37°C, and then washed with phosphate-buffered saline. The slides were dried, and cover slips were mounted. Slides were read for the presence of EB-VCA using the rhodamine system. When a rhodamine fluorescent cell was observed, the filter system was switched to observe if fluorescein isothiocyanate fluorescence occurred within the same cell. Slides were read with a Leitz Orthoplan fluorescence microscope equipped with a 100-W mercury lamp.

Filter combinations used with fluorescein isothiocyanate conjugate were KG 1, BG 38, KP 500, K 480, TK 510, and K 515; and KG 1, BG 38, KP 560, TK 580, K 590 were used with the rhodamine conjugate.

Neutralization index. A constant serum-varying virus titration was used in determining the neutralization index. Pooled normal horse sera and EHV-1 antisera (obtained from National Veterinary Services Laboratories, Ames, Iowa) were inactivated at 56°C for 30 min. Stock virus used for LC infection and a 45-day postinfection virus sample were prepared in serial 10-fold dilutions. Each virus dilution was added to equal parts of a 1:25 dilution of normal horse serum and a 1:25 dilution of EHV-1 antisera. The mixtures were incubated at 4°C for 3 h before 0.3 ml of each was inoculated into two 16- by 125-mm screw-capped tubes containing 5-day-old RK-13 monolayers. Inoculated tubes were checked daily for 14 days, and the 50% endpoints and neutralization indexes were calculated for the original input virus stock and for the 45-day persistent virus sample.

RESULTS

Growth rate of lymphoblastic cells. Growth rates of viable cells in both infected and uninfected cultures are presented in Fig. 1A. From day 21 on, the cell count of the infected cells was lower than that of controls, but relative growth rates were roughly parallel. Lymphoblastic cell lines normally had growth cycles 21 days long—moving from slow growth to rapid growth as shown in the rates for uninfected control cells in Fig. 1A. The cytotoxic effect of EHV-1 on Jijoye cells as measured by percentage of viable cells at various times is shown in Fig. 2. For the first 4 weeks the percentage of viable persistently infected cells varied greatly compared with that of control cells. From day 30 on, the percentage of viable persistently infected cells stabilized at about 70 to 80% of the number of viable control cells.

Virus production. The $TCID_{50}$ of extracellular virus per 10^5 cells per ml at various times is shown in Fig. 1B. Extracellular virus production peaked on days 11 and 30, with titers of $10^{4.3}$ and $10^{6.2}$ $TCID_{50}$, respectively. The $TCID_{50}$ of intracellular virus per 10^5 cells per ml is presented in Fig. 1B. Intracellular virus production yielded a maximum titer of $10^{3.4}$ $TCID_{50}$ on day 30.

The increase in virus production paralleled with the rapid cell production rate noted between days 7 and 11 and days 21 and 30. After 53 days of continuous growth, the infected LCs still maintained the ability to produce EHV-1.

Direct, indirect, and double-staining FA test. The percentages of EHV-1-infected and uninfected cells with EHV-1-specific fluorescence in the direct FA test are shown in Fig. 1C. About 2,000 cells were examined for each sample. EHV-1-positive LCs showed diffuse specific fluorescence and were enlarged and rounded, contrasting sharply with the negative cells in the same preparation (Fig. 3). EHV-1-specific fluorescence was not detected until day 7 post-EHV-1 infection. A peak of 15% of the cells demonstrated EHV-1 antigen on day 16. After day 21, the proportion of positive cells remained constant at 2%. The control cells of each sample
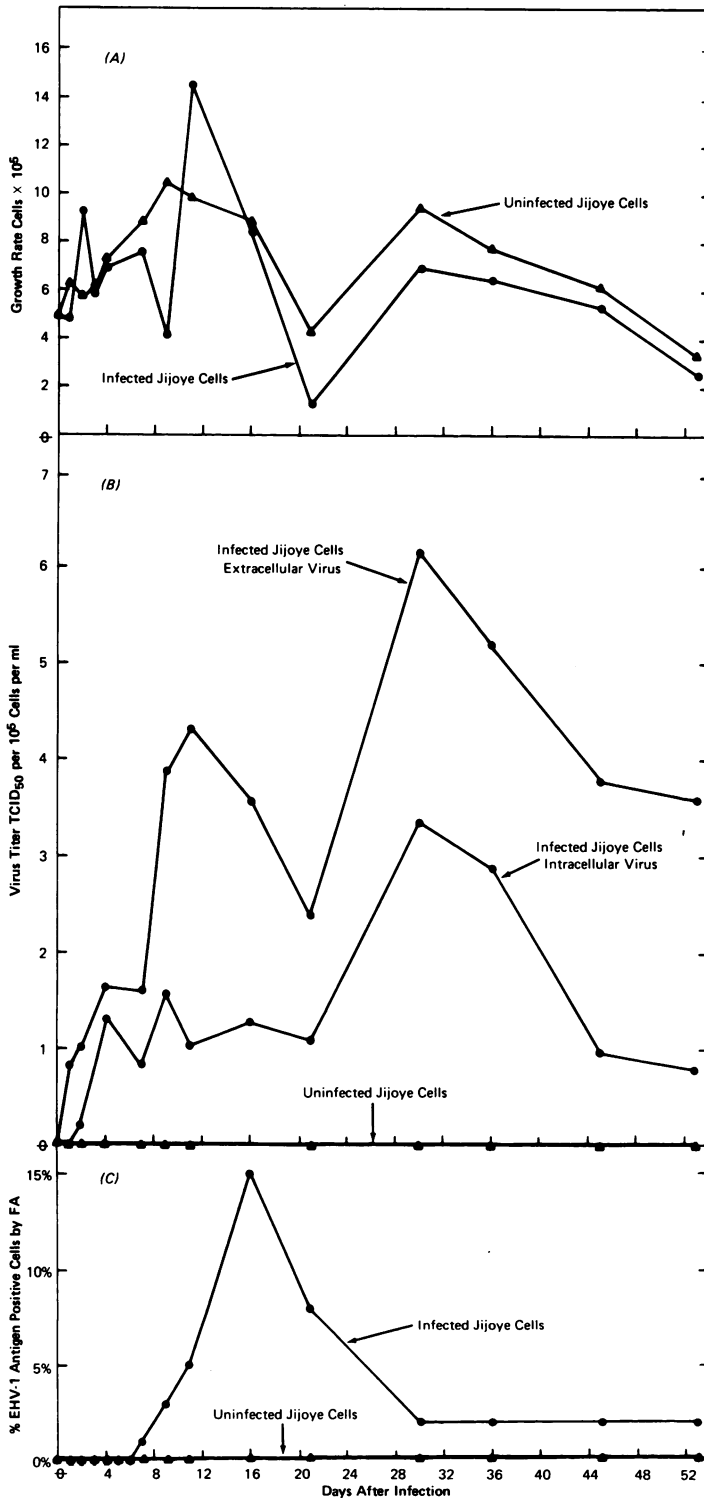
FIG. 1. (A) Growth rates of EHV-1-infected and uninfected Jijoye LCs. (B) Virus production in EHV-1-infected Jijoye LCs. Intracellular and extracellular virus was measured in $TCID_{50}$ per 10^5 cells per milliliter. (C) Percentage of infected Jijoye LCs showing EHV-1-specific immunofluorescence.

were negative. EHV-1 infection had no effect on the expression of EB-VCA as detected with the indirect FA test. In all samples containing control and infected cells, positive EB-VCA was always present in 5% of the cells. In the double-staining experiment 5% of the Jijoye cells were positive for EB-VCA using rhodamine stain and 15% of the cells were positive for EHV using the fluorescein label. Of those cells positive for EBV, 80% were also positive for EHV. This strongly suggests an association between the production of EHV and EB-VCA in this cell system. The 20% nonassociation between EHV and EB-VCA production may be real or may be due to the limitation of the FA test.

Virus neutralization test. To verify the persisting virus as EHV-1, a virus neutralization
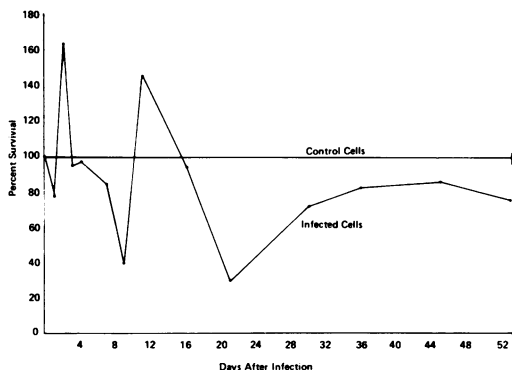

FIG. 2. *Cytotoxic effect of EHV-1 on Jijoye cells. Percent survival was calculated by dividing viable cell count of EHV-1-infected Jijoye cells by viable cell count of uninfected Jijoye cells on samples from the same day.*
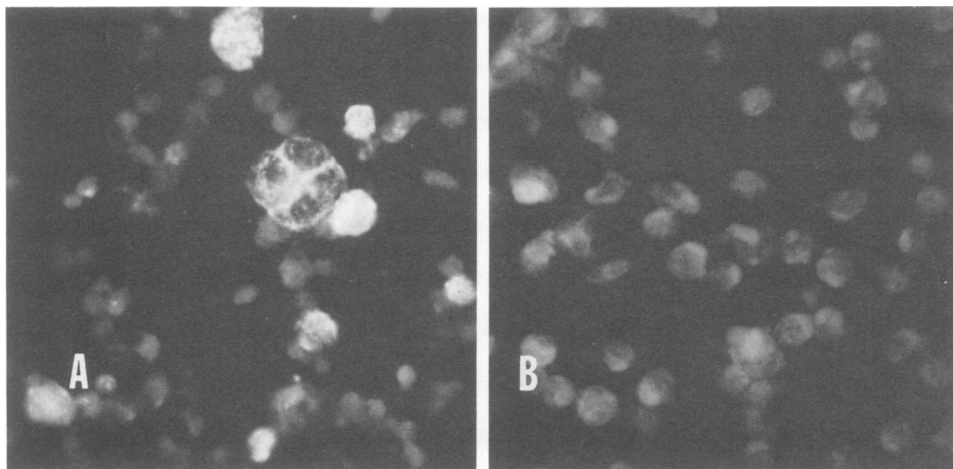
test was performed with virus obtained from the persistently infected Jijoye cells. Specific EHV-1 antisera neutralized the 45-day virus sample from infected LCs and the parental virus sample equally well (neutralization index, 4); thus, the virus obtained from infected cells still appeared to be antigenically intact EHV-1.

DISCUSSION

The ability of EHV-1 to replicate and persist in human LCs has not previously been reported. The Jijoye LC line supported the replication of EHV-1 and was persistently infected by the virus. The characteristic total cytopathic destruction of cells in EHV-1-infected LC cultures was not observed.

In fewer than 15% of the LCs was antigen production detected with the direct immunofluorescence technique. Because of the low percentage of cells which immunofluoresced and the low titer of intracellular virus, we concluded that at any one time even less than 15% of the cells were actively producing either EH-VCA or infectious virus. The low percentage of cells producing virus has been documented by Robey et al. (13) and Graze and Royston (7) with other virus-LC systems. As measured by cell death, EHV-1 was cytotoxic to the lymphoblastoid culture. Cytotoxicity was as high as 70% on day 21, but this level did not correlate with a high release of virus as would be expected with a normal EHV-1 lytic cycle. Only $10^{2.4}$ TCID$_{50}$ of extracellular virus per 10^5 cells per ml were detected in the 21-day sample.

The percentage of cells possessing EHV-1 antigen by FA does not correlate with the production of infectious virus. Infected cells which

FIG. 3. *(A) Enlarged, rounded EHV-1-infected Jijoye cells; (B) uninfected Jijoye cells stained by using the FA technique.*

lysed, releasing large amounts of infectious virus, can no longer be detected by FA, whereas the released infectious virus can still be detected. Samples for virus isolation and FA testing after day 11 were taken at periods 5 days or greater apart. Because of the length of the time between samples and the fact that samples for virus isolation and FA were taken on the same day, it is not possible to ascertain an accurate picture of the relationship between infectious virus and EHV-1 antigen within the cell population as detected by FA.

EB viral capsid-specific antigen in the Jijoye cell line was not altered by EHV-1 infection as detected with immunofluorescence. Similar observations were noted by Henle et al. (8), Zajac et al. (15), Robey et al. (13), and Floyd et al. (6) in LC lines infected with other viruses.

The EHV-1 persistently infected Jijoye cells were maintained for 53 days. During this interval, maximum virus titers correlated with active cell metabolism as indicated by cell growth. Robey et al. (13) concluded in their study of herpes simplex virus-infected LCs that the virus replication in LC lines may be dependent on cell growth.

Apparently, only a small proportion of LCs are susceptible to EHV-1 at any given time. After day 28, the viable cell counts of the persistently infected Jijoye cells remained 20 to 30% lower than the uninfected cells, as shown in Fig. 2. The decrease in viable cells in the persistently infected culture is possibly due to an EHV-1 lytic cycle which occurs in 20 to 30% of the cell population. Based on the number of infected LCs that died and the fact that normal permissive EHV-1 infections are lytic, we concluded that infected EHV-1 LCs lyse after a viral replicative cycle is completed. In EBV-transformed lymphoid cell lines that are EBV producers, only a small proportion of the cells actively produce detectable virus at any one time (B. A. Maurer and S. M. Wilbert, Bacteriol. Proc., p. 195, 1970). Studies by Zajac and Kohn (14) and Miller et al. (9) on cloning indicate that one EBV genome-containing cell gives rise to a cell population of identical parental characteristics, with the same percentage of cells possessing VCA. These studies on EBV raise the question of whether the reason for low cell infectivity is that most of the EHV-1 cells are initially infected and contain the EHV-1 genome. The initiation of a rapid cell metabolism followed by cell division would then activate the latent virus in the cell genome, and the virus would undergo a lytic cycle.

EHV-1 persistently infected Raji and HSB LC lines are currently being investigated in this laboratory. We were unable to persistently infect the BJAB cell line. Raji (10) and BJAB (3) cell lines are derived from Burkitt's lymphomas and possess B-lymphocyte antigenic characteristics. The HSB cell line (1) is derived from an acute lymphocytic leukemia and possesses T-lymphocyte characteristics. BJAB and HSB cell lines do not contain detectable EBV genomes, EBV nuclear antigens, or EB-VCAs. The Raji cell line contains EBV genome and EBV nuclear antigen; but unlike the Jijoye cell line, it does not produce demonstrable EB-VCA. These results indicate that neither active EB-VCA production nor EBV genomes were necessary for EHV-1 production.

Attempts to culture infected cells in the presence of specific herpesvirus antiserum resulted in clumping and cell death to antigen-producing cells. When the immune sera were washed off the remaining viable non-FA-positive cells, the culture continued to again produce herpesvirus as detected by FA and isolation tests. This observation, as well as the fact that herpesvirus-producing cells undergo a lytic cycle, strengthens the proposal that the Jijoye-EHV-1 in vitro model represents a latent viral infection with periodic activation.

To date, there is no record of EHV-1 infections of humans. However, as this report shows, EHV-1 has the potential to infect human lymphoblastic cells in vitro. The potential of EHV-1 to be transmitted naturally to humans must be fully assessed. The interaction of EHV-1 and LCs provides a useful in vitro model which may help clarify the mechanism of herpesvirus latency.

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