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Herpes Simplex Virus Type 1 Infection of Isogenic Epstein-Barr Virus Genome-Negative and -Positive Burkitt's Lymphoma-Derived Cell Lines

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The Epstein-Barr virus (EBV) genome-negative Burkitt's lymphoma-derived cell lines BJAB and Ramos and their in vitro EBV-converted sublines BJAB-B1, BJAB-A5, BJAB-B95-8, and AW-Ramos were infected with high multiplicities of herpes simplex virus type 1 (HSV-1; 10 to 70 PFU/cell). Cultures were monitored for cell growth and HSV-1 DNA synthesis. EBV-converted BJAB cultures were more permissive for HSV-1 infection than BJAB cultures. Significant cell killing and HSV-1 DNA synthesis were observed during the first 48 h of infection in the EBV-converted BJAB cultures but not in the BJAB cultures. The EBV-converted BJAB-B1 cell line contains an appreciable fraction of EBV-negative cells. Therefore, it was cloned. EBV-positive and -negative cells were identified by using EBV-determined nuclear antigen anti-complement immunofluorescence. Two types of subclones were identified: (i) those which contained both EBV-determined nuclear antigen-positive and -negative cells and (ii) those which contained only EBV-determined nuclear antigen-negative cells. When levels of HSV-1 DNA synthesis were measured in these subclones, it was found that the former were more permissive for HSV-1 infection than the latter. Thus, the presence of the EBV genome in BJAB cells correlates with increased permissiveness of these cells for HSV-1 during the first 48 h of infection. Nonetheless, persistent HSV-1 infections were established in both BJAB and EBV-converted BJAB-B1 cultures. No differences in extent of permissiveness for HSV-1 infection were found for Ramos and EBV-converted AW-Ramos cells.

Herpes simplex virus (HSV) causes productive infections in most cultured human cells; high levels of virus are produced, and the infected culture is rapidly destroyed. Such consequences are not observed when Epstein-Barr virus (EBV) genome-positive Burkitt's lymphoma-derived (BL) cell lines are infected with HSV (4, 7, 15). In fact, Robey et al. (15) reported the establishment of persistent HSV infections in such cell lines. These cultures continued to grow indefinitely, although a majority of the cells produced HSV antigens and a few produced low levels of virus. In related studies, Huang and Pagano (8) infected different nonisogenic EBV genome-positive and -negative human B-lymphoblastoid cell lines with another herpesvirus, cytomegalovirus, and examined the effects. No production of infectious virus was detected in either type of line. However, cytomegalovirus DNA synthesis was detected in EBV-positive lines and not in EBV-negative lines.

Because EBV-negative BL cell lines (containing less than 0.3 EBV genome per cell) and their isogenic in vitro EBV-converted sublines are now available (1, 2, 6, 9, 10), it was our goal to determine the effect of HSV type 1 (HSV-1) infection on these cell lines. The EBV-negative BL cell lines used were the BJAB and Ramos lines (9, 10). The EBV-converted BL cell lines used were the BJAB-B1, BJAB-A5, BJAB-B95-8, and AW-Ramos lines (5, 6, 9). The B1 and A5 lines are clones of cells obtained after infection of BJAB cells with the P3HR-1 strain of EBV. The BJAB-B95-8 line resulted from conversion of BJAB cells with the B95-8 strain of EBV. The AW-Ramos line arose from infection of Ramos cells with the P3HR-1 strain of EBV.

At least 90% of the cells in the EBV-converted BJAB-A5, BJAB-B95-8, and AW-Ramos cultures contain EBV genomes, as determined by their expression of EBV-determined nuclear antigen (EBNA) (5, 9). In contrast, BJAB-B1 cultures contain a variable fraction of EBNA-negative cells (5). When such a culture was cloned, two types of subclones resulted. The first type

contained both EBNA-positive and -negative cells. The second type contained only EBNA-negative cells. Furthermore, EBV genomes were detected in the former but not in the latter (5). Thus, in these studies, such subclones were infected with HSV-1.

After infection with high multiplicities of HSV-1, the various cell lines were monitored for growth and HSV-1 DNA synthesis. During the first 48 h of infection, no measurable cell killing and HSV-1 DNA synthesis were observed in BJAB cultures. In contrast, high levels of cell killing and HSV DNA synthesis were noted in EBV-converted BJAB cultures and in Ramos and EBV-converted Ramos cultures. The levels of HSV-1 DNA synthesized in B1 subclones containing only EBNA-negative cells and in B1 subclones containing both EBNA-positive and -negative cells were similar to the levels synthesized in BJAB and B1 cultures, respectively. HSV-1 infection of both BJAB and B1 cultures ultimately resulted in the establishment of persistent HSV-1 infections.

MATERIALS AND METHODS

Cell lines. BJAB, Ramos, and AW-Ramos cells were provided by G. Klein. EBV-converted BJAB-B1, -A5, and -B95-8 cells were supplied by H. zur Hausen. These cells were grown in suspension in growth medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) at 37°C in a humidified 5% CO_2 atmosphere. Subculturing was performed twice a week by dilution so that cell densities did not exceed 10^6 cells per ml. All of the cell lines were assayed for EBNA expression by using anticomplement immunofluorescence (14). BJAB and Ramos cultures contained only EBNA-negative cells. At least 90% of the cells in BJAB-A5, BJAB-B95-8, and AW-Ramos cultures were EBNA positive. BJAB-B1 cultures contained between 15 and 80% EBNA-positive cells, depending on passage number.

Cloning of cells. B1 cultures containing approximately 15% EBNA-positive cells were counted and diluted in conditioned medium (growth medium in which BJAB cells were grown for 2 days and then filtered) to a density of 2.5 cells per ml. Samples (0.1 ml) were added to microtiter wells containing human placental feeder layers (provided by G. Miller). Clones were fed after 1 week with conditioned medium. After 2 weeks cells were transferred to flasks. Cloning efficiency was approximately 50%. Cultures were assayed for the presence of the EBV genome by EBNA anticomplement immunofluorescence (14); 45% of all clones obtained were EBNA negative.

Growth and preparation of virus. Stocks of HSV-1 strain Cl 101 (from S. Kit) were grown on Vero cells from plaque-purified virus by using multiplicities of 0.003 to 0.05 PFU/cell so as to minimize production of defective particles. Virus was prepared from infected cells by a freeze-thaw step and sonic treatment (3).

Infection of cells. Exponentially growing BL cells (8×10^6) were collected by centrifugation at $200 \times g$ for 5 min and resuspended in 1 ml of growth medium containing high multiplicities of HSV-1 (10 to 70 PFU/cell). Mock-infected cells were resuspended in 1 ml of growth medium or growth medium containing uninfected Vero cell extracts. No differences in cellular growth rates were observed for either type of mock infection. After 2 h at 37°C in a humidified 5% CO_2 atmosphere, the cultures were diluted to 40 ml with growth medium, and incubation was continued.

Analysis of DNA synthesis in cells. At 3 h after infection 10^6 BL cells were collected by centrifugation and suspended in 2 ml of growth medium containing 10 μCi of [^{3}H]thymidine (50 Ci/mmol; New England Nuclear Corp.) per ml. At 24 h after infection the cells were pelleted, washed with 2 ml of cold phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline, and stored at −70°C. DNA extractions were performed by using routine procedures; cells were lysed with sodium *n*-lauroyl-sarcosinate (Sarkosyl; ICN; final concentration, 0.5%), digested with autodigested Pronase (final concentration, 1 mg/ml) for 4 h at 37°C, and extracted twice with water-saturated phenol and then with ether. DNA solutions were mixed with 4 volumes of a saturated solution of CsCl to give a density of 1.712 g/ml and centrifuged in a fixed-angle 40 rotor at 33,000 rpm at 15°C for 60 h. The [^{3}H]thymidine-labeled material in the gradients was located by counting the gradient fractions with Aquasol (New England Nuclear Corp.).

Production of infectious virus. After adsorption of virus, cultures were washed three times with 1 ml of growth medium to remove as much of the input virus as possible. Then, at various times after infection, samples of cells were counted and assayed for virus as follows. Cell suspensions were subjected to a freeze-thaw step, sonically treated (1 min in a water-cooled Branson sonic oscillator), and plated on Vero cells. Plaques were counted after 2 days.

Infectious center assay. HSV-1-infected BL cells (10^6) were concentrated and suspended in 0.1 ml of rabbit anti-HSV-1 serum. After 1 h at room temperature, cells were washed once in RPMI 1640 medium and counted. Cells (1×10^4 to 9×10^4) were plated on confluent Vero cell monolayers in 25-cm^2 flasks. After a 30-min adsorption period, the monolayers were overlaid with 1% methyl cellulose in minimum essential medium plus 5% fetal calf serum. Incubation was continued at 37°C for 3 days, after which cells were stained with Giemsa stain and plaques were counted.

RESULTS

Growth kinetics of BL cells infected with HSV-1. Cultures of EBV genome-negative and EBV-converted BL cells were mock infected or infected with high multiplicities of HSV-1. Viable cell counts were made at various times after infection. The resulting growth curves are shown in Fig. 1. The growth rate of EBV genome-negative BJAB cultures was not significantly altered by HSV-1 infection (Fig. 1A). The growth rate of EBV-converted BJAB-B1 cultures was significantly altered by HSV-1 infection, how-

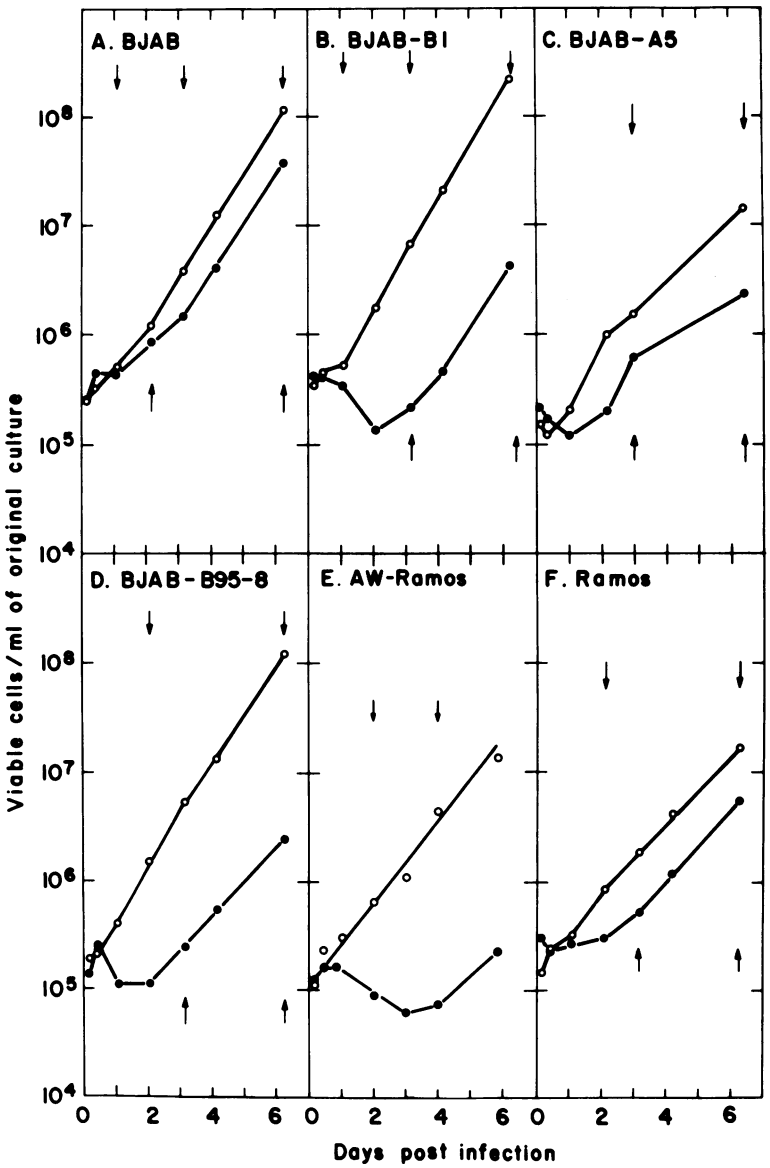

FIG. 1. *Growth kinetics of BL cell lines after HSV-1 infection (●) or mock infection (○). Cultures were infected at a multiplicity of 40 to 70 PFU/cell. Viable cell counts were made by using trypan blue and a hemacytometer. Cell concentrations are plotted in terms of concentration of the original culture, although dilutions were made where indicated (↑, infected cultures; ↓, mock-infected cultures) so that cell densities did not exceed 10^6 cells per ml.*

ever. During the first 48 h after infection, cell densities decreased, indicating that large numbers of cells were killed. Then cell densities increased at rates similar to those for mock-infected cultures. Thus, a small percentage of the cells of the original B1 culture survived the infection. The number of B1 cells which survived was estimated by extrapolation of the positive component of the growth curve to zero time. Approximately 5% of the original B1 culture survived. Similar killing and outgrowth of a small population of cells were also observed for EBV-converted BJAB-A5 and -B95-8 cultures (Fig. 1C and D). HSV-1 infection of EBV-negative Ramos cultures and EBV-converted AW-Ramos cultures also resulted in a decrease in the growth rate of these cells which, after 2 to 4 days, returned to that of the mock-infected cultures (Fig. 1E and F).

The differential cell killing noted after HSV-1

infection of BJAB and its EBV-converted sublines was not due to differences in adsorption or nuclear penetration of HSV-1. The extent of adsorption of ^{32}P-labeled HSV-1 and transport of ^{32}P-labeled HSV-1 DNA to the nucleus was the same for BJAB and EBV-converted B1 cultures (data not shown).

Synthesis of HSV-1 DNA in BL cells. HSV-1 DNA synthesis was also measured in infected BL cultures. Cells were pulse-labeled with [^{3}H]thymidine for 3 to 24 h after infection, and total DNA was extracted and fractionated by CsCl equilibrium gradient centrifugation. For HSV-1-infected and mock-infected BJAB cultures, one peak of [^{3}H]thymidine-labeled material was found at the density of cell DNA (Fig. 2A and B). No [^{3}H]thymidine was incorporated into material which banded at the density of the HSV-1 marker DNA. For HSV-1-infected B1 cultures, two peaks of [^{3}H]thymidine-labeled DNA were found (Fig. 2C). One banded at the density of HSV-1 DNA, and the other banded at the density of cell DNA (Fig. 2D). From such data it was determined that 32, 16, and 37% of the total DNA synthesized in BJAB-B1, -A5, and -B95-8 cultures, respectively, was HSV-1 DNA (Table 1). No more than 2% of the total DNA synthesized in BJAB cultures was HSV-1 DNA. HSV-1 infection of both Ramos and AW-Ramos cultures resulted in measurable HSV-1 DNA synthesis; of the total DNA synthesized, 28 and 37%, respectively, was HSV-1 DNA (Table 1).

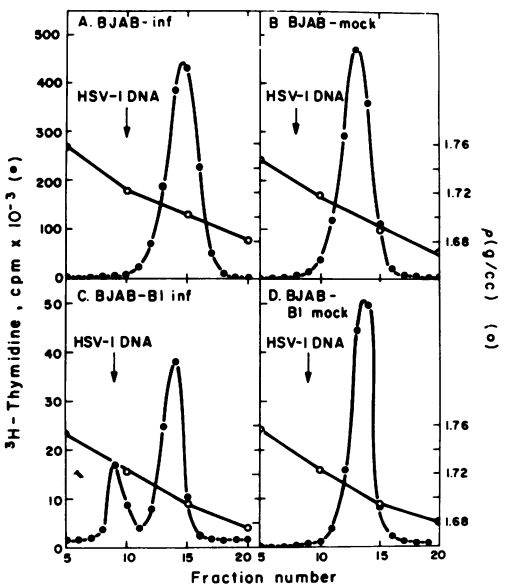

FIG. 2. *Cesium chloride density gradient equilibrium centrifugation of [^{3}H]thymidine-labeled DNA extracted from HSV-1-infected and mock-infected BJAB and B1 cells. Cells were infected at multiplicities of 70 PFU/cell. Labeling was from 3 to 24 h after infection. Marker ^{32}P-labeled HSV-1 DNA banded where indicated (↓). Densities (O) were measured with a refractometer.*

TABLE 1. *HSV-1 DNA synthesis in BL cell lines*[a]

Cell line	EBV genome present	% HSV-1 DNA synthesized[b]
BJAB	–	2[c, d]
BJAB-B1	+	32[d]
BJAB-A5	+	16
BJAB-B95-8	+	37
Ramos	–	28[d]
AW-Ramos	+	37[d]

[a] Cells were infected with HSV-1 (30 to 70 PFU/cell), labeled with [^{3}H]thymidine for 3 to 24 h after infection, and analyzed for HSV-1 DNA synthesis as described in the text and in the legend to Fig. 2.

[b] Percent HSV-1 DNA synthesized = (counts per minute of [^{3}H]thymidine-labeled material cobanding with ^{32}P-labeled HSV-1 DNA)/(total counts per minute of [^{3}H]thymidine-labeled material) × 100.

[c] The lower limit of detection (no distinct peak of [^{3}H]thymidine-labeled DNA banded at the density of HSV-1 DNA).

[d] Average of at least three determinations.

Subclones of B1 cultures were also analyzed for HSV-1 DNA synthesis (Table 2). For subclones containing both EBNA-positive and -negative cells, 27 to 56% of the total DNA synthesized was HSV-1 DNA. For subclones containing only EBNA-negative cells, 1 to 8% of the total [^{3}H]thymidine-labeled material banded at the density of HSV-1 DNA in CsCl gradients.

Differences in the various cell lines were noted not only for HSV-1 DNA synthesis but also for HSV-1 protein synthesis. During the first 24 h of infection, the levels of HSV-1 proteins synthesized in Ramos and EBV-converted BJAB cultures were dramatically higher than in BJAB cultures, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and indirect immunofluorescence (data not shown).

Virus production in HSV-1-infected BL cells. When BJAB cultures were infected with high multiplicities of HSV-1, virus titers decreased from 2 to 0.4 PFU/cell during the first 12 h of infection (Fig. 3A). In contrast, in EBV-converted B1 cultures, HSV-1 titers increased from 1.5 to 9.5 PFU/cell (Fig. 3B). Thereafter, virus production continued for over 45 days after infection in both cultures. Thus, persistent HSV-1 infections were established in both cultures.

At 24 h after infection, about 1 and 15% of the cells in BJAB and B1 cultures, respectively, produced HSV-1, as determined by the infectious center assay. Approximately 1 to 2% of the cells in the persistently infected cultures produced HSV-1.

TABLE 2. *HSV-1 DNA synthesis in B1 subclones*

Subclone	% EBNA-positive cells	% HSV-1 DNA synthesized[a]
103	65	34
106	60	51
114	80	30
120	65	56
121	75	27
122	95	53
101	0	1
107	0	1
108	0	2
110	0	1
112	0	8[b]
113	0	3
125	0	4[b]

[a] Determinations were made as described in the footnotes to Table 1.

[b] Average of at least two determinations.

When persistently infected B1 cultures were superinfected with HSV-1, the same events occurred as in the initial infection. About 95% of the cells were killed, and about 22% of the total DNA synthesized was HSV-1 DNA. These results suggest that a subpopulation of B1 cells was not selected by the initial HSV-1 infection.

DISCUSSION

HSV-1 infection of EBV genome-negative BJAB and EBV-converted B1 cultures (10 to 70 PFU/cell) results in persistent infections, as has been noted previously for EBV-positive BL cell lines (15). However, during the first 24 to 48 h of infection, BJAB and B1 cultures respond differently to HSV-1. In BJAB cultures few, if any, cells are killed. No measurable HSV-1 DNA synthesis occurs. Only 1% of the cells produce infectious HSV-1. In B1 cultures, about 95% of the cells are killed. HSV-1 DNA synthesis comprises 32% of the total DNA synthesized. About 15% of the cells produce infectious virus. Effects similar to those observed for B1 cells were also observed for the EBV-converted BJAB-A5 and -B95-8 cells after HSV-1 infection. Thus, it appears that the EBV genome increases the permissiveness of BJAB cells for HSV-1 during the first 48 h of infection.

An alternate explanation is possible, however. It might be that initial conversion of BJAB cells by EBV resulted in selection for a variant cell which is more permissive per se for virus growth. In this case, the EBV genome would not function to increase the permissiveness of BJAB cells for HSV-1 infection. To distinguish between these two possibilities, the BJAB-B1 cell line was used because Fresen et al. (5) found that subclones of this line are of two types with respect to EBNA expression. The first type contained both EBNA-positive and -negative cells. The second type contained only EBNA-negative cells, and no EBV DNA was detectable by reassociation kinetics. Thus, if permissiveness for HSV-1 resulted from selection of a cell type and is unrelated to EBV function, then the subclones containing only EBNA-negative cells should be as permissive for HSV-1 replication as the subclones containing both EBNA-positive and -negative cells. If permissiveness for HSV-1 is related to EBV function, then the subclones containing only EBNA-negative cells should be less permissive for HSV-1 replication than the subclones containing both EBNA-positive and -negative cells.

Accordingly, we isolated these two types of B1 subclones. They were infected with HSV-1, and the amount of HSV-1 DNA synthesized during the first 24 h of infection was used as an assay for permissiveness for HSV-1. Six subclones containing both EBNA-positive and -negative cells

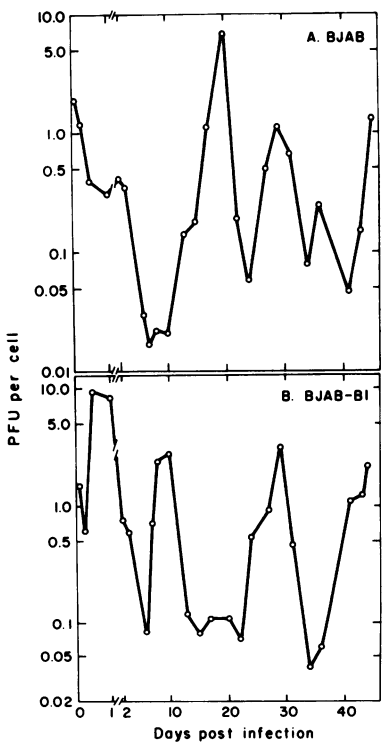

FIG. 3. *Production of infectious virus in HSV-1-infected BJAB (A) and B1 (B) cells. Cells were infected at a multiplicity of 50 PFU/cell. After 2 h of adsorption, cells were washed. At the indicated times samples of cells were assayed for virus production (PFU/cell) as described in the text. Cultures were diluted every 4 to 5 days. The cycles of virus production do not correspond to the cycles of cell density resulting from subculturing.*

and seven subclones containing only EBNA-negative cells were examined. For the former, 27 to 56% of the total DNA synthesized was HSV-1 DNA. For the latter, no more than 8% of the total DNA synthesized was HSV-1 DNA. Therefore, increased permissiveness of BJAB cells for HSV-1 infection correlates with the presence of the EBV genome in these cells.

If this correlation holds, then the levels of HSV-1 DNA synthesized in BJAB cultures and in EBNA-negative B1 subclones should be the same. This was the case for BJAB and six of the seven EBNA-negative B1 subclones. On the average, 2% of the total [^{3}H]thymidine-labeled DNA banded at the density of HSV-1 DNA. Whether this material was HSV-1 DNA could not be determined due to the limit of sensitivity of this analysis. However, because 1% of the cells in BJAB cultures produce infectious virus as determined by the infectious center assay, low levels of HSV-1 DNA must be synthesized. This fact could explain why HSV-1 DNA synthesis was detected in EBNA-negative subclone 112, where 8% of the total DNA synthesized was HSV-1 DNA. Subclone 112 may have originated from one of these permissive BJAB cells. Thus, we conclude that the EBV genome functions in some way in BJAB cells to increase permissiveness for HSV-1. It plays a role other than initial selection because its loss results in return to the parental BJAB phenotype.

A number of investigators have compared the properties of the BJAB and Ramos cell lines with their EBV-converted sublines to determine the effect of the EBV genome on these cells (2, 11–13, 16–20). The EBV genome appears to change their growth requirements (16, 17) and membrane properties (11, 12, 18, 19). Another effect of the EBV genome on BJAB cells has been identified in this study. Whether the increased permissiveness of EBV-converted BJAB cells for HSV-1 infection is related to the EBV function associated with these altered growth requirements or to a different EBV function remains to be determined. The growth rates of the BJAB and EBV-converted BJAB cultures and the B1 subclones did not differ significantly, however. Moreover, since no differences in adsorption and nuclear penetration of HSV-1 were observed for the BJAB and EBV-converted B1 cells, it is unlikely that the altered membrane properties of the EBV-converted BJAB cells are involved.

Whereas BJAB cells were essentially nonpermissive for HSV-1 during the first 48 h of infection, Ramos cells were permissive. Significant amounts of cell killing and HSV-1 DNA synthesis were observed after HSV-1 infection of Ramos cells. Thus, even though both BJAB and Ramos cells do not contain EBV genomes (1), they differ in at least one property. In fact, they have different histories. The BJAB cell line was derived from biopsy material of an African Burkitt lymphoma patient (10). The Ramos cell line was derived from biopsy material of an American Burkitt lymphoma patient and was subsequently passed in nude mice (9).

The effect of the EBV genome on Ramos cells also differed from that on BJAB cells. For EBV-converted AW-Ramos and Ramos cultures, there were no significant differences in the levels of HSV-1-induced cell killing and HSV-1 DNA synthesis. These results suggest that the degree of permissiveness of BL cells for HSV-1 infection is limited. Even though Ramos cells are inherently permissive for HSV-1 infection, the EBV genome does not increase this permissiveness.

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