

## Epstein-Barr Virus: Transformation, Cytopathic Changes, and Viral Antigens in Squirrel Monkey and Marmoset Leukocytes

(human placental fibroblasts/complement fixation/immunofluorescence/electron microscopy/herpesvirus)

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**ABSTRACT** Blood leukocytes of two species of new world primates, other than human, transform following exposure to Epstein-Barr virus. The transformed simian cells produce Epstein-Barr virus antigens and infectious (transforming) virus. The simian lymphoblastoid cells form multinucleate giant cells that appear to be selective sites for the production of Epstein-Barr virus. Multinucleate cells reveal intranuclear inclusions; in both species, a large proportion of giant cells contain Epstein-Barr virus antigen detectable by immunofluorescence.

Epstein-Barr virus (EBV) transforms human blood leukocytes into continuous lines of lymphoblastoid morphology in cell culture (1-4). An important question about these EBV-induced changes in human cell growth is "Do they represent induction of oncogenic potential in the blood leukocytes?" One experimental approach to the problem is to test the transformed cells for their capacity to cause lymphoproliferative disease in laboratory rodents, as has been done with leukocytes derived spontaneously from patients with leukemia, Burkitt lymphoma, or infectious mononucleosis. While certain human lymphoblastoid cell lines multiply *in vivo* in immature or immunosuppressed hosts, the proliferating cells retain human chromosomal and antigenic characteristics (5-7), and thus are heterotransplants. Another line of experimentation that might more closely approximate the situation in nature is the transformation of cells from a laboratory animal *in vitro* with EBV and, ultimately, the evaluation of the oncogenic potential of these cells in autologous hosts. As a preliminary step a method has been developed for establishment of lymphoblastoid cell lines from leukocytes of two species of South American primates: *Saimiri sciureus*, the squirrel monkey, and *Saimiri oedipus*, the cotton-top marmoset. Studies of the morphology of the simian lymphoblastoid cell lines (SLCL) and of the distribution of EB antigen in them have revealed some unique features of the SLCL, by comparison with EBV-transformed human leukocytes.

### METHODS

**Virus.** EBV was obtained from a human lymphoblastoid cell line (883L) derived from a patient with transfusion-induced infectious mononucleosis (8). The 883L cells were disrupted by three cycles of freezing and thawing ( $10^7$  cells/ml) in spent medium; the extract was centrifuged at  $400 \times g$  for 15 min; and the supernatant fluid was filtered through a 0.8- $\mu$ m Millipore filter. The factor in these filtrates that

is capable of transforming human leukocytes is neutralized by convalescent sera from patients with infectious mononucleosis, but is unaffected by pre-illness sera from the same patients (9).

**Cell Cultures and Media.** All lymphoblastoid cell lines were maintained on medium RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) with 10 or 20% fetal bovine serum; penicillin (100 units/ml), streptomycin, (100  $\mu$ g/ml); and amphotericin B (1.0  $\mu$ g/ml). Transformation assays were performed in the same medium. Cell strains of human placental fibroblasts established on Eagle's Basal medium plus 10% fetal bovine serum and antibiotics (as above) were used as "feeder layers" in the transformation assays (10). Monolayer cultures of a line of owl monkey kidney cells (OMK210), from Dr. L. V. Melendez, and a line of grivet monkey kidney cells (JR-1), from J. Riordan, were used to evaluate lymphoblastoid cell lines for the presence of cytopathic agents. The two monkey kidney cell lines were grown on Eagle's minimal essential medium plus 10% fetal bovine serum. Gas phase for all cultures was 5% CO<sub>2</sub> in air.

**Animals and Blood Leukocytes.** Squirrel monkeys were purchased from the Tarpon Zoo, Tarpon Springs, Fla. The primates in experiment B95 were bled at the New England Primate Research Center, Southboro, Mass. through the courtesy of Dr. L. V. Melendez. Peripheral blood was obtained from the femoral vein. Erythrocytes were sedimented in heparinized blood by addition of Dextran (molecular weight 240,000) at a final concentration of 1.5% in 0.85% saline. Leukocytes were washed three times with Puck's saline A and treated with phytohemagglutinin-M (Difco) (100  $\mu$ g/ml). They were cultured from 3 to 5 days before the addition of EBV (Table 1). Human leukocytes were handled as described (4).

**Transformation Assay.** Primary cultures of human and other primate leukocytes at a concentration of  $(0.3-2.0) \times 10^6$  cells/ml, after treatment with phytohemagglutinin, were exposed to EBV or to culture medium and cultivated in combination with human placental fibroblasts "feeder layers" on the bottom of 15  $\times$  150-mm glass test tubes. Transformation was recognized with the aid of an inverted microscope by the appearance of clusters of rapidly growing enlarged leukocytes. The suspended cells were subcultured first in the presence and then in the absence of "feeder layers" until sufficient numbers were available for examination of

Abbreviations: EBV, Epstein-Barr virus; SLCL, simian lymphoblastoid cell lines; TD<sub>50</sub>, median transforming dose.

karyotype and morphology, and evaluation for the presence of EBV antigens and infectious units.

**Cytogenetic and Cytologic Studies.** Chromosome studies were made on metaphase cells of cultures prepared by conventional methods as described (4). 50 Cells of each line were studied. The morphology of SLCL cells was studied in the following manner: Cells were centrifuged from the culture medium RPMI 1640 plus 10% fetal bovine serum and resuspended at a density of  $5 \times 10^5$  cells/ml in Eagle's minimal essential medium plus 5% fetal bovine serum. In Eagle's medium, many cells adhered firmly to glass and were fixed with Bouin's fixative, embedded in collodion, and stained with hematoxylin and eosin (11). Suspended cells were processed for electron microscopy by standard methods (23).

**Tests for EBV Antigens and Antibody to EBV.** Simian lymphoblastoid cell lines (SLCL) were tested for EBV antigens by complement fixation and by the indirect immunofluorescent technique of Henle and Henle (12). Complement fixing antigens were prepared by washing SLCL three times in veronal buffered saline with 0.1% bovine serum albumin, and freezing and thawing of  $10^8$  cells/ml in veronal buffered saline plus 0.1% bovine serum albumin (13). This

TABLE 1. Transformation of human and other primate leukocytes by Epstein-Barr Virus

Expt. no.	Species	No. transformed/ no. attempted		Time to transformation (days)
		exposed to EBV	control	
B-84*	Squirrel monkey—Fang	1/5	0/5	40
	Man—G.M.	4/4	0/4	25
B-95†	Cotton-top marmoset	0/3	0/3	
	Cotton-top marmoset	1/3	0/3	40
	Owl monkey	0/3	0/3	
	Owl monkey	0/3	0/3	
	Cebus albifrons monkey	0/3	0/3	
	Cebus albifrons monkey	0/3	0/3	
	Squirrel monkey	0/3	0/3	
	Squirrel monkey	0/3	0/3	
C-42‡	Man—G.M.	3/3	0/3	35
	Squirrel monkey—596	2/5	0/5	45
	—597	1/5	0/5	39
	—598	1/5	0/5	45

\* Pretreated leukocytes with phytohemagglutinin for 3 days; added 0.5 ml of leukocytes ( $3 \times 10^6$  cells) to human placental fibroblasts "feeder layers"; added 0.5 ml of virus filtrate (transforming titer in human cells =  $10^{1.5}$  TD<sub>50</sub> per 125  $\mu$ l) or medium.

† Added 1.0 ml of leukocytes ( $4 \times 10^6$ – $2 \times 10^6$  cells) plus phytohemagglutinin to feeder layers (as in \*); after 3 days, added 0.5 ml of virus filtrate and 0.5 ml of medium containing phytohemagglutinin.

‡ Pretreated leukocytes with phytohemagglutinin for 5 days; 0.5 ml of leukocytes ( $3$ – $7 \times 10^6$  cells) were mixed with 0.5 ml of virus. After 10 min at 5°C and 60 min at 35°C, the cell-virus mixture was added to human placental fibroblast feeder layers.

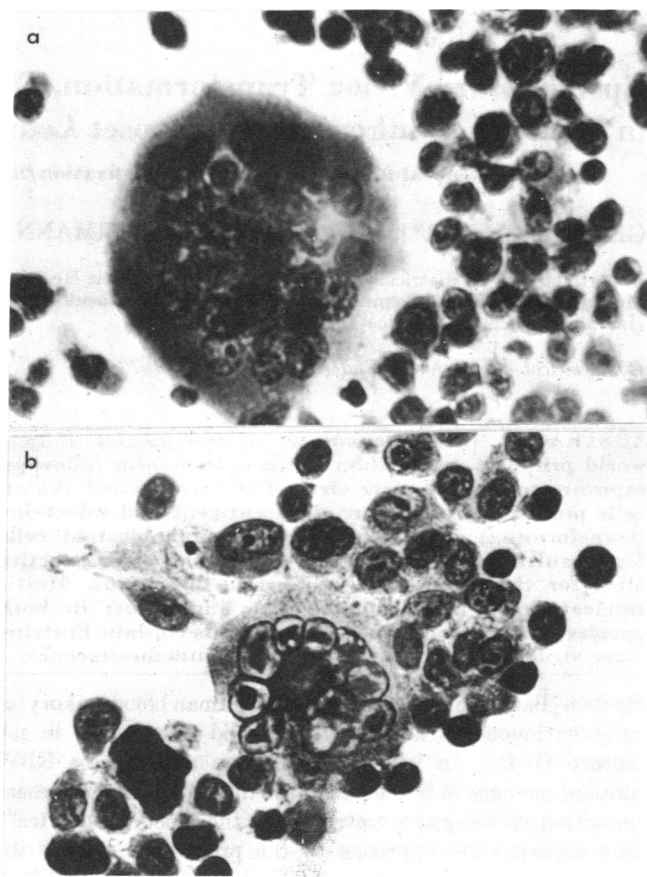


FIG. 1. Simian lymphoblastoid cell lines stained with hematoxylin and eosin. (a) Shows single cells and a multinucleate giant cell of the B84-15 (squirrel monkey) line. (b) Shows single cells and a multinucleate cell of the B95-8 (marmoset) line. 10 of the B95-8 giant cell nuclei contain intranuclear inclusions, and an occasional single cell contains an intranuclear inclusion. Original magnification  $\times 400$ .

extract was centrifuged at  $400 \times g$  and the supernatant fluid was used as an antigen in a microtiter complement fixation test; 1.1–1.4 units of guinea pig complement were used (14). The standard antibody to EBV used in the complement fixation test was in adult human serum with a complement fixing titer of 1:64; EBV antigen that was prepared in human cells was used. Simian sera were tested for EBV antibody by the complement fixation test. Anticomplementary activity in some primate sera, other than human, was reduced by overnight incubation of sera with complement (15) or heating of sera at 65°C for 20 min (16).

The immunofluorescent method for detection of EBV antigen was used; thin films from a pellet of  $3 \times 10^6$  suspended cells and cells attached to glass cover slips in Leighton tubes were stained. The reference antibody EBV in the immunofluorescent tests was a human serum (immunofluorescent titer  $> 1:160$ ) from a patient convalescing from infectious mononucleosis.

In immunofluorescent and complement fixation tests, human sera, without antibody to EV virus, and buffered saline were included as controls.

**Infectious EBV and Neutralization.** The presence of infectious EBV in filtrates prepared from  $10^7$  SLCL cells/ml was detected by transformation of human leukocytic cells.

Human sera with and without EBV antibody were used to neutralize infectivity.

## RESULTS

### *Transformation of Squirrel Monkey and Marmoset Leukocytes.*

In three experiments, SLCL were established in leukocyte cultures that were exposed to EBV from four adult squirrel monkeys and from one cotton-top marmoset; no cell lines were derived in cultures without added EBV (Table 1). Only 5 of 26 squirrel monkey cultures (from six monkeys) and one of six marmoset cultures (from two monkeys) were transformed, in comparison to transformation of all seven human cultures that were exposed to EBV (from one subject). Transformation appeared later in the monkey cells than in the human cells. In these preliminary experiments, SLCL were not formed after exposure of owl monkey or cebus monkey leukocytes to EBV.

*Chromosome Studies of the Transformed Lines.* Karyotypes of four lines derived from squirrel monkey leukocytes and of the single marmoset line showed chromosome numbers and morphology characteristic of each species (17, 18). The squirrel monkeys had a chromosome number of  $2n = 44$ . The karyotype of a normal cell from line B84-15 was identical with that of cells obtained from a primary culture of peripheral leukocytes of the same animal. Aneuploidy ranged from 6 to 42% in the four lines; it was mainly due to loss of chromosomes. Dicentric chromosomes were noted in all four lines, the incidence ranging from 2 to 12%; a few chromatid breaks were also noted. The marmoset line (B95-8) showed a chromosome number of  $2n = 46$ , with an aneuploidy of 16%. There were eight chromatid breaks, but no dicentrics or other chromosome type aberrations were found.

*Morphology of the Transformed Simian Cells.* B84-15 (squirrel monkey) and B95-8 (marmoset) lines were used in the remaining studies. The two SLCL were subcultured as suspended cells and cell clumps in medium RPMI 1640. However, when cells were transferred to Eagle's minimal essential medium with Earle's salts and 5% fetal bovine serum, a proportion of cells in the SLCL attached to glass. After 3-12 days, about 10% of the nuclei of adherent cells had formed multinucleated giant cells in both lines (Fig. 1). Giant cells of the B84-15 (squirrel monkey) line often contained 50-100 nuclei and those of the B95-8 (marmoset) line contained 10-20 nuclei. Nearly all the nuclei in the B95-8 giant cells contained intranuclear inclusions. The intranuclear inclusions were also seen in an occasional binucleate or mononuclear cell of the B95-8 line. Intranuclear inclusions were rarely found in the giant cells of B84-15 line. Two human lymphoblastoid cell lines, HR1K from Burkitt lymphoma and 883L, handled in parallel in the same experiments did not attach to glass or form multinucleate giant cells.

*EBV Antigens, Particles, and Infectious Virus in SLCL.* EBV antigens were detected in SLCL by the complement fixation and immunofluorescent tests (Table 2). Differences were found in the distribution of immunofluorescent antigens between single cells and multinucleate cells. About 2% of single cells and from 10-30% of multinucleate cells of the B84-15 line contained immunofluorescent antigens. In the B95-8 line, 7% of single cells and all multinucleate giant cells contained immunofluorescent antigens (Fig. 2).

Viral particles resembling herpesvirus were seen in the nucleus of 9 of 174 cells (5%) of the B95-8 line by electron microscopy (Fig. 3). All intranuclear particles were unenveloped and approximately one-half of the particles lacked electron-dense cores. A rare enveloped particle was seen in the cytoplasm. No EBV particles were found in a preliminary screening of 40 cells from the B84-15 line. Neither line contained identifiable "C" type particles.

Infectious EBV with a titer of  $10^{2.0}$ - $10^{2.5}$  median transformation dose ( $TD_{50}$ ) per  $125 \mu\text{l}$  was present in both simian lines; it was neutralized by a 1:4 dilution of human antibody to EBV but was unaffected by a 1:4 dilution of antibody-negative human serum.

*Tests for Cytopathic Agents in the SLCL.* For determination of whether indigenous simian viruses were associated with the SLCL, monolayer cultures of green monkey kidney line JR-1, owl monkey kidney line 210, and a human placental cell strain were exposed to intact cells and cell extracts of the

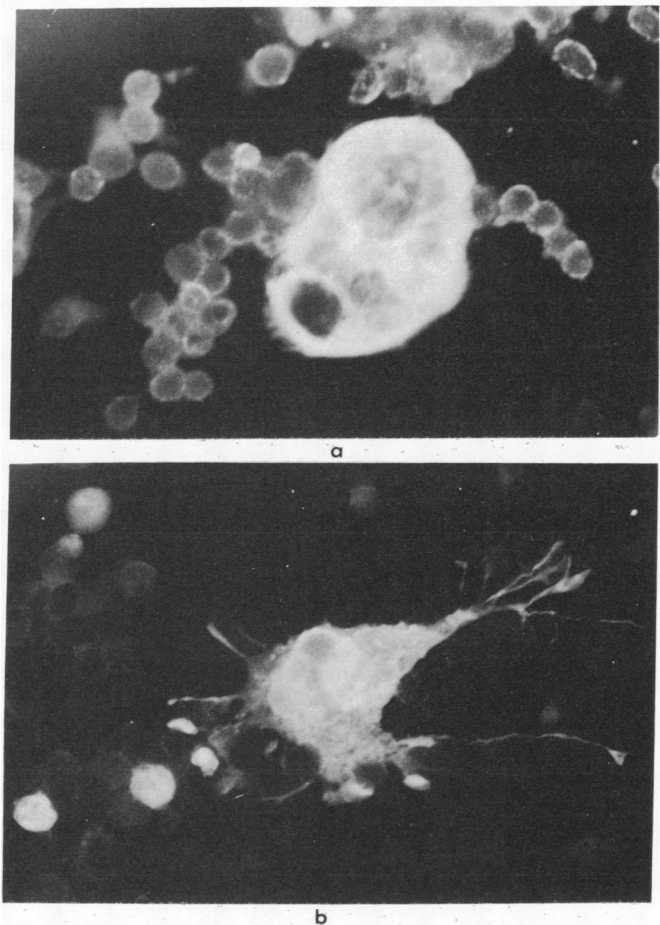


FIG. 2. Indirect immunofluorescent staining of acetone-fixed SLCL with human antibody to EBV. (a) Shows a multinucleated giant cell of the B84-15 (squirrel monkey) line with EBV antigen in the cytoplasm. Some of the nuclei of this giant cell are devoid of antigen. The ring of fluorescence around some individual cells is not considered to be EBV-specific. (b) Shows EBV antigen in single cells and in a multinucleated giant cell of the B95-8 (marmoset) line. The giant cell appears to contain viral antigen in nuclei and cytoplasm. The majority of single cells in both lines do not contain detectable antigen. Original magnification  $\times 250$ .

TABLE 2. Characteristics of two lines of EBV-transformed primate (other than human) leukocytes

Line	Source	Karyotype	Morphology			EBV antigens			
			Giant cells	Inclusions	EBV particles	CF	IF		Infectious EBV
							Single cells (%)	Giant cells (%)	
B84-15	SM	Simian-44	Present	Rare	0/40*	1:2†	2	10-30	10 <sup>2.0</sup> ‡
B95-8	CTM	Simian-46	Present	Frequent	9/174*	1:4†	7	100	10 <sup>2.5</sup> ‡

Abbreviations: SM, squirrel monkey; CTM, cotton-top marmoset; CF, complement fixation; IF, immunofluorescence.

\* No. of cells with EBV particles/no. of cells examined.

† Titer of CF antigen prepared from 10<sup>8</sup> cells/ml.

‡ Titer of transforming virus per 125  $\mu$ l of filtrate prepared from 10<sup>7</sup> cells/ml.

SLCL. No cytopathic effects were noted in the 4-week observation period.

*Survey of Simian Sera with Antigens Prepared from Human Lymphoblastoid Cell Line and SLCL.* For determination of whether the simian lymphoblastoid cell lines contained other antigens, and perhaps virus, besides those of EBV, complement fixing antigens were prepared from the SLCL and reacted with simian and human sera. Both SLCL contained antigens that reacted with a known EBV-positive human

serum and not with an EBV-negative human serum. None of the monkeys whose leukocytes were transformed contained EBV antibodies as measured by the complement fixation test with antigen prepared from the HR1K Burkitt lymphoma line. The monkey sera also did not contain measurable complement fixing antibodies when antigens derived from the transformed simian leukocytes were used. These results suggested that the SLCL did not contain indigenous viruses to which the original simian leukocyte donor possessed complement fixing antibodies.

## DISCUSSION

*Evidence That EBV is Responsible for Transformation of Simian Leukocytes.* In previous studies, by use of adult human leukocytes as the assay system, the transforming virus has been shown to be neutralized by human sera with EBV antibody detectable by immunofluorescence, but unaffected by human sera lacking EBV antibody (2-4, 9). Since transformation of simian leukocytes is an irregular event, by comparison to transformation of human leukocytes, neutralization tests appeared to be an impractical way of providing evidence that EBV was responsible for transformation of the monkey cells. Nonetheless, a body of indirect evidence taken together supports the hypothesis that EBV caused the continuous growth of the simian leukocyte. First, EBV antigens were detected by immunofluorescent and complement fixation methods in the transformed cells, EBV particles were seen in one cell line by electron microscopy, and infectious EBV was present in both cell lines. Second, the monkeys that were the source of the transformed leukocytes did not possess EBV antibodies detectable by the complement fixation test, and thus were presumably not carriers of EBV. Third, SLCL formed only in cultures exposed to EBV and not in unexposed cultures. Fourth, the cytopathology seen regularly in the B95-8 line of transformed marmoset leukocytes and occasionally in the B84-15 line of squirrel monkey leukocytes, consisting of multinucleate cells with intranuclear inclusions, is compatible with cytopathic effects described for the herpesvirus group, of which EBV is a member. Fifth, the SLCL did not yield cytopathic agents when cocultivated as intact cells or cell extracts with monolayer cultures known to be sensitive to simian herpesviruses such as Herpes Saimiri (19). Finally, there was no evidence in the SLCL for the presence of other antigens that reacted with the sera of animals whose leukocytes were transformed.

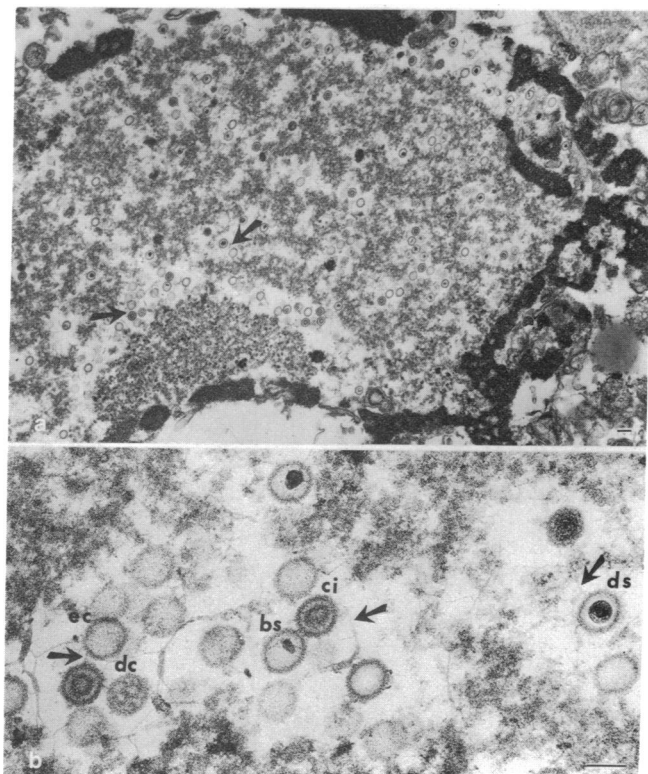


FIG. 3. A section of EBV-infected B95-8 cell line stained with uranyl acetate and lead citrate. (a) Shows a portion of a nucleus outlined with dense chromatin and filled with virus capsids, almost half of which are devoid of a core. The arrows designate the area of the nucleus shown in b. Higher magnification allows visualization of various states of core development such as empty capsid (ec), diffuse conformation (dc), circular (ci), bar-shaped (bs), and dense core (ds). The thread-like matrix attached to the capsids is indicated by arrows. Bar represents 100 nm.

*Pathogenesis and Significance of the Multinucleated Cells.*

The unique feature of the transformed simian leukocytes, by comparison to human lymphoblastoid cells, is the formation of multinucleate cells in the SLCL. These cells contain a high proportion of nuclei bearing inclusions in the B95-8 line; inclusions are rarely seen in giant cells of the B84-15 line. The multinucleate cells appear to be a preferential site for synthesis of EBV antigens, since all the multinucleate cells of B95-8 and 10-30% of similar cells of the B84-15 line contain viral antigens, while only 2-7% of single cells in these lines contain immunofluorescent antigen. Preliminary electron micrographic studies suggest that viral particles are present in multiple nuclei of the giant cells.

It will be important in future experiments to study the mechanisms whereby formation of multinucleate giant cells favors EBV replication. At least three hypotheses may be considered. First, fusion may specifically involve those cells that possess changed cell membranes as the result of a commitment to produce viral antigen; that is, the giant cells form from single cells already producing or about to produce viral antigens. Second, fusion may result from events not related to EBV, but following fusion, the EBV genome is induced to replicate complete virus. This theory is based on analogy with the induction of SV40 virus in transformed cells following Sendai virus-mediated cell fusion (20). It is of note that giant cells made up from cells that possess and cells that do not possess T-antigen of SV40 are T-antigen positive (21). Third, a small amount of infectious virus released by a few cells in the culture may be selectively taken up by "spontaneous" cell-fusion and replicate in the fused cells, but not in the unfused cells that may have a barrier to viral entry. This theory is based on the experimental observations of Tegtmeyer and Enders (22), who found that feline herpesvirus replicated to the state of induction of intranuclear inclusions (but not to the point of production of infectious viral particles) in human cells when infection by the cat herpesvirus was accompanied by cell fusion that was affected by Sendai virus.

Transformation of new world primate leukocytes by EBV appears to hold promise as an experimental tool for the study of the "tumorigenicity" of EBV as well as for the study of the EBV-cell relationship. Experiments are in progress for the determination of the oncogenic potential of the transformed cells in autologous hosts.

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