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Release of Infectious Epstein–Barr Virus by Transformed Marmoset Leukocytes

(human leukocytes/extracellular virus)

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ABSTRACT **Marmoset blood leukocytes transformed *in vitro* by Epstein–Barr virus regularly release extracellular infectious Epstein–Barr virus with high titers of transforming activity. By comparison, human umbilical cord leukocytes and adult human leukocytes transformed by Epstein–Barr virus release either no extracellular infectious virus or small amounts, irregularly.**

Human lymphoblastoid cells that contain Epstein–Barr virus (EBV) restrict replication of the virus. In some human lymphoblastoid lines no viral nucleocapsids are found, yet the viral genome is detectable by nucleic acid hybridization (1, 2). In other lines nucleocapsids or viral antigens detected by immunofluorescence (immunofluorescence antigens) are produced in only a few cells, although cloning experiments demonstrate that all cells contain the viral genome (3–5). Furthermore, not all lines in which nucleocapsids or immunofluorescence antigens are found give rise to biologically active virus detectable by transformation or superinfection assays (6, 7). The mechanisms of restriction of EBV by the host cell are unknown and present important practical and theoretical problems. The lack of a cell type that yields large amounts of virus has hindered study of the virus. The means of restriction of virus by the cell deserves study as an important example of the control of gene expression.

We recently reported that leukocytes from two species of new-world primates transform into continuous cell lines after exposure to EBV (8). Certain features of these cells suggested that simian leukocytes might produce more EB virions than transformed human leukocytes. A higher fraction of monkey cells than human cells demonstrated intracellular immunofluorescence antigen. In simian cell lines syncitia evolved that were the site of accumulation of immunofluorescence antigen, and the multinucleate giant cells contained intranuclear inclusions. Accordingly, supernatant fluids of EBV-converted monkey cells were tested for the presence of cell-free virus, which was readily demonstrated. This report is concerned with identification of the extracellular virus as EBV, a description of the virus' morphology and growth characteristics, and evidence that production of extracellular EBV is due to factors inherent in the transformed cell type rather than to a permanent change in the virus resulting from passage through simian cells.

METHODS

Virus. Two EBV strains were derived from patients with infectious mononucleosis: one (Hawley) from peripheral blood leukocytes, the other (Lederer) from a throat washing. The Hawley strain was maintained as a line of the patient's blood leukocytes (883L) or as a line of marmoset blood leukocytes (B95-8), which was established after exposure to an extract of line 883L. A virus stock from the B95-8 line consisted of culture fluids that had been centrifuged (400 × *g*); the supernatant had been frozen and thawed and passed through a 0.8-μm filter and stored at −70°. Virus of the Lederer strain was obtained by having the patient gargle 100 ml of medium RPMI 1640. Fetal bovine serum (final concentration 2%) was added to gargle fluids, which were frozen and thawed three times, then centrifuged at 400 × *g* for 10 min; the supernatant was filtered and stored at −70°. Transforming activity in extracts of lines 883L and B95-8, and in the Lederer throat washing, was neutralized by human sera with EBV antibody and unaffected by human sera free of EBV antibody (refs. 7, 8, and Miller, G. & Niederman, J. C., *N. Engl. J. Med.* in press).

Infectivity Assay. Transformation of umbilical cord leukocytes was the measure of EBV infectivity. Transformation was recognized by clumping, acid production, increase in cell numbers, and acquisition by the culture of the ability to be repeatedly subdivided. The terminal dilution method was used, four cultures per dilution, and titers were calculated by the Reed Muench formula. Cord leukocytes were used at 10^6/ml, and one cord yielded from 50–150 cultures. As far as possible a single umbilical cord served as the source of assay cells in comparable titrations; the infectivity titers presented in Tables 1 and 3 and Fig. 2 were obtained with one cord each. Included in each experiment were cultures consisting of leukocytes not exposed to EBV; no transformation occurred in these controls.

EBV Antigens and Neutralization Tests. Four reference human sera, two antibody-positive from mononucleosis patients and two antibody-free sera from healthy adults, were used to identify EBV antigens. EBV antigens detectable by complement-fixation were measured in cell extracts prepared from 5×10^7 cells per ml, and also in frozen and thawed supernatant fluids (8). The indirect method of Henle and Henle was used to enumerate the proportion of cells with EBV immunofluorescence antigen (9).

Infectious transforming virus released from marmoset cell cultures was identified through neutralization tests with the same two antibody-positive and two antibody-negative reference human sera. About 30 infectious units were mixed with 2-fold dilutions of serum, beginning at 1:2, and incubated for

Abbreviation: EBV, Epstein–Barr virus.

1 hr in a 37° water bath. The mixture was assayed for infectivity on umbilical cord leukocytes.

Ether and Chloroform Sensitivity of EBV. The effect of lipid solvents on the transforming capacity of virus released from marmoset cells was determined as follows: 2 ml of B95-8 virus stock was mixed with 1 ml of anhydrous ethyl ether and placed at 4° for 4 hr. For chloroform treatment, 2.7 ml of B95-8 virus stock was mixed with 0.3 ml of chloroform and placed at 4° for 1 hr. The organic layers were discarded, and air was bubbled through both mixtures for 2 min. Titrations of infectivity were done on untreated B95-8 stock virus and on the aqueous phase of chloroform-treated and ether-treated virus.

Examination of EBV Particles by Electronmicroscopy. Cells were sedimented from 200 ml of culture fluid at 400 × *g* for 20 min. The supernatant was centrifuged at 20,000 rpm in an SW25.2 rotor at 5°, and the resulting pellets were suspended in about 1/30 volume of Tris·HCl-buffered saline plus 1% bovine-serum albumin. This material was placed on top of a discontinuous gradient of 10 and 50% potassium tartrate in buffer (pH 7.4) composed of 1 M NaCl–0.1 M Tris·HCl–10 mM EDTA. An interphase with an opalescent band, which appeared after centrifugation at 25,000 rpm in an SW41 rotor at 5° for 2 hr, was collected. This interphase was then centrifuged in a linear 20–30% potassium tartrate gradient for 17 hr at 25,000 rpm in an SW41 rotor. An opalescent band in the bottom one-third of the tube was collected for electronmicroscopic examination and for density determination by weighing. Aliquots from the cell culture fluids after initial clarification, from the pellet after the first high-speed centrifugation, and from the interphase after centrifugation in the discontinuous gradient were also examined by electronmicroscopy. Samples were placed on Formvar carbon-coated grids, stained with 2% phosphotungstic acid (pH 6.0), and examined in a Phillips EM-200.

RESULTS

Transforming activity of extracellular virus from line B95-8 (marmoset cells) was neutralized by two human EBV-positive reference sera, and was unaffected by two EBV-negative human sera. Titrations of the antibody-positive sera demonstrate neutralizing activity at a 1:128 dilution or greater. When about 30,000 transforming units of extracellular virus from line B95-8 were mixed with ether or chloroform, all transforming activity was abolished.

Herpes virus nucleocapsids were seen in clarified supernatant fluid obtained 6 days after subculture of B95-8 cells. When the fluid was centrifuged at 48,000 × *g*, virions were found in the pellet. Similar viral particles were seen in the band between 10 and 50% potassium tartrate and in the band collected after overnight centrifugation. Debris was still present in the preparation after this step. The virions seen (Fig. 1) consisted of enveloped particles as well as naked nucleocapsids. The nucleocapsids were hexagonal and of uniform size (about 100 nm). A core could be seen in many of the particles. The density of the virus-containing band after centrifugation in the 20–30% potassium tartrate linear gradient was 1.186 g/ml. When line 883L was treated in an identical manner, only a rare naked nucleocapsid was identified in the banded material.

Time course of production of extracellular EBV

Extracellular virus increased in parallel with the exponential phase of cell growth (Fig. 2). The amount of infectious EBV

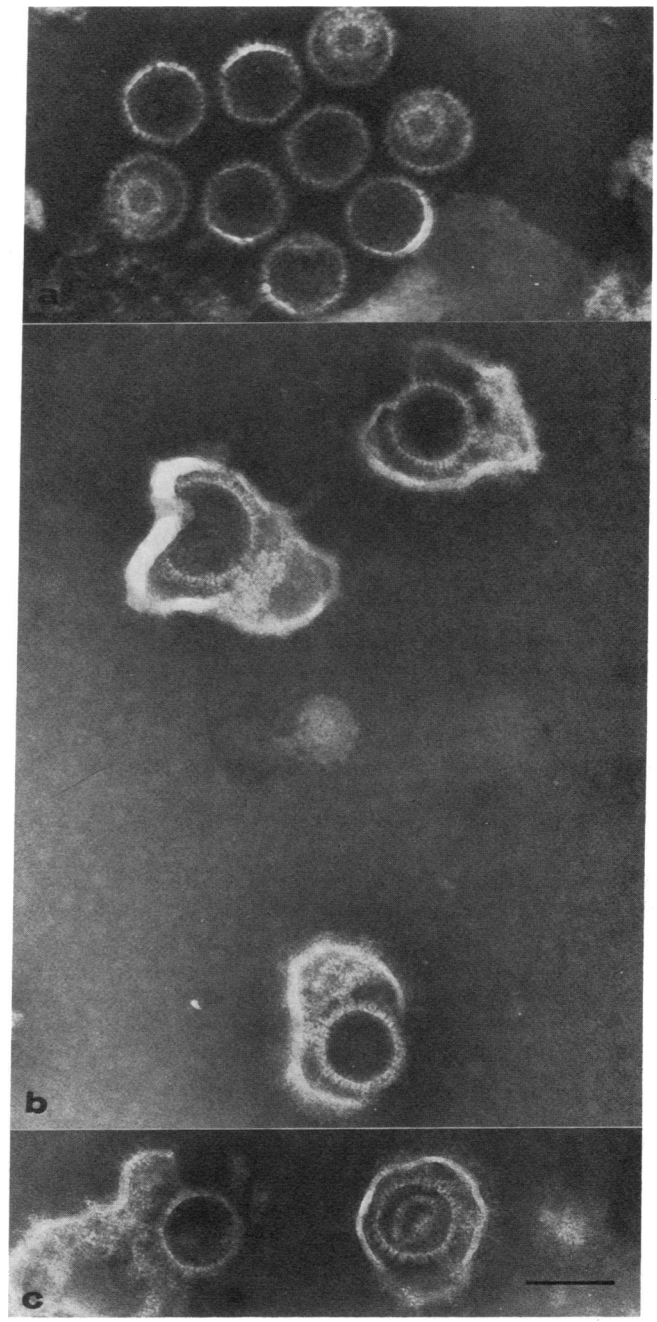

FIG. 1. Epstein–Barr virus (EBV) from infected marmoset leukocyte cell line B95-8. Tissue culture fluid was pelleted, resuspended in Tris·HCl-buffered saline, and centrifuged on a 10 and 50% potassium tartrate, noncontinuous gradient for 2 hr at 25,000 rpm. The band between the layers was collected, stained with 2% phosphotungstic acid, and examined by electron-microscopy (*a*). Group of nucleocapsids, three of which contain cores. Capsomeres are arranged in an orderly fashion and suggest the 5:3:2 axial symmetry of the herpes group of viruses. (*b*) Three enveloped particles were found in one field. Two have disrupted envelopes and the third a disintegrating nucleocapsid. (*c*) An intact enveloped EBV, which also contains a core, is accompanied by a nucleocapsid without an envelope. All micrographs were taken at the same magnification. *Bar* represents 100 nm.

TABLE 1. *Expression of EBV in a human and in two simian lines and in human cord cells transformed by virus derived from them*

Designation	Species	Method of transformation	Expression of EBV: CF antigen*	Expression of EBV: IF antigen†	Expression of EBV: Infectivity‡	Calc. yield§ of infectious EBV per 10^6 IF-positive cells
883L	Human (adult)	Spontaneous	4	2.8	0.3	36
B95-8	Marmoset	883L virus	16	12.5	3.8	25,000
B84-15	Squirrel monkey	883L virus	8	8.8	2.6	2,300
E46	Human (newborn)	883L virus	8	0	nil	0
E49-32	Human (newborn)	B95-8 virus	16	0	nil	0
E14	Human (newborn)	B84-15 virus	8	0	nil	0

Lines of transformed squirrel monkey and marmoset leukocytes were derived by exposure to EBV from line 883L (Hawley strain). EBV obtained from cell-free supernatant fluids of these three lines was used to transform cells from three umbilical cords. All six lines were carried in parallel for several months by subdividing 2:1 every 5–7 days. Cells and fluids were harvested for determination of antigen content and infectivity 1 week after last subdivision. At the time of harvest there were 10^6 viable cells per ml of each line.

* CF antigen, no. of complement fixing units per 25 μl of antigen prepared from an extract of 5×10^7 cells per ml.

† IF antigen, % cells demonstrating immunofluorescence in the indirect test, based on counts of 1000 cells.

‡ Infectivity, $\log_{10}$ 50% transforming units per 0.2 ml of supernatant fluid.

§ Yield of extracellular EBV = (no. of infectious units per ml/10^6 cells per ml × % IF-positive cells) × 10^5.

released exceeded the increase in cell numbers; the cell population increased 6-fold (from $10^{5.5}$/ml to $10^{6.3}$/ml), and the content of infectious EBV increased more than 300-fold (from $10^{3.2}$/ml to $10^{5.7}$/ml). A maximum yield of about 0.3 infectious units per cell was obtained 8 days after subculture. A rise in complement-fixing antigen present in culture supernatant fluids occurred in parallel with the rise in cell number and infectious virus. Complement-fixing activity was removed from supernatant fluids and recovered in the pellet when fluids from day 5 were centrifuged at 88,000 × *g* for 1 hr.

Comparison of expression of EBV in transformed human and monkey cells

A series of experiments were undertaken to determine whether the release of extracellular EBV by marmoset cells was due to a permanent change in the virus resulting from passage in marmoset cells ("adaptation") or to differences in the capacity of leukocytes from different sources to permit expression of infectivity of EBV.

First, a comparison was made of the content of EBV-specific antigens and of extracellular infectious virus in simian lines from two species and in the human line (883L) that was the original source of EBV used to transform the monkey cells (top half of Table 1). Simian lines contained higher titers of complement fixation antigen (2- to 4-fold) and immunofluorescence antigen (2- to 4-fold), and they released considerably more infectious virus (200- to 3000-fold) than the human line. A calculation related the yield of infectious virus

TABLE 2. *Expression of EBV in marmoset and human cells transformed by one stock of EBV derived from marmoset cells*

Species and designation	Expression of EBV*: CF antigen	IF antigen	Infectivity
Marmoset			
D39-32	16	5.7	3.7
D39-44	16	4.5	4.5
D39-14	16	12.5	4.5
D39-16	32	6.0	4.5
Human (newborn)			
E74	4	0	nil
E82	4	0	nil
E94	8	0.1	nil
E81	4	0	nil
Human (adult)			
F22-1	8	0.1	nil
F22-13	8	1.5	0.2
F22-25	16	1.2	nil
F22-37	8	0	nil

All cell lines were derived after exposure to about $10^{3.5}$ transforming doses of virus from line B95-8. Transformed cells were subdivided 2:1 every 5–7 days before assay for EBV antigens and infectivity.

* See footnotes for Table 1.

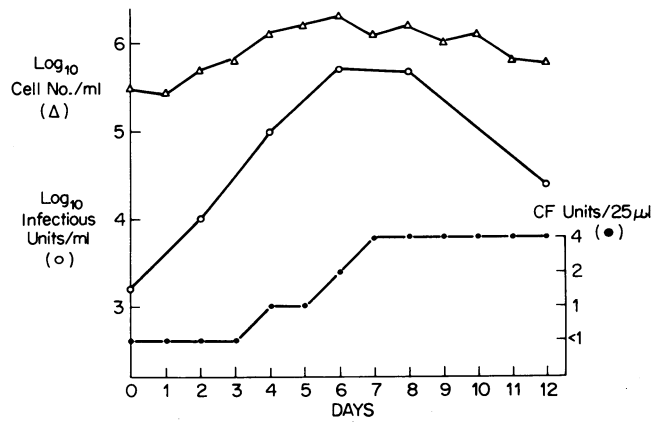

FIG. 2. EBV-transformed marmoset leukocytes (line B95-8) were diluted 1:4 in 50 ml of fresh medium to a concentration of 3×10^5 viable (trypan-blue excluding) cells per ml. An aliquot of 2 ml was removed daily. Daily cell counts were performed (Δ). Cells were sedimented by centrifugation (400 × *g* for 10 min); the supernatant fluids were frozen and thawed three times. Aliquots of supernatant fluids were assayed for infectivity by the capacity to transform human umbilical cord leukocytes into continuous cell lines (O). All the infectivity assays were performed in leukocytes from a single cord. The titer of complement-fixing (CF) antigen in the supernatant fluid was determined in a microtiter assay (●).

to the number of cells producing immunofluorescence antigen, on the assumption that the cells producing immunofluorescence antigens were the cells ultimately responsible for production of infectious virus. The results suggested that the greater amounts of infectious virus released by the monkey cells was due to an increased yield of infectious virus per virogenic cell. EBV from each of the three lines was used to transform leukocytes from three different human umbilical cord bloods. The transformed cord leukocytes were tested for expression of EBV antigens and infectious virus in parallel with the parent lines. The results (bottom half of Table 1) showed that cord cells transformed by the Hawley strain from three different cell types expressed only EBV complement fixation antigens and not immunofluorescence antigens. No transforming virus was detected in the supernatant fluids from the transformed human cord cells.

To verify that the phenomenon represented a general difference between transformed marmoset and human cells, we used a single stock prepared from extracellular EBV from line B95-8 to transform leukocytes from four marmosets, four additional human umbilical cords, and four EBV antibody-free adult humans. The transformants were studied for their content of EBV antigens and infectious EBV (Table 2). All lines of transformed marmoset cells contained both EBV antigens and also released infectious virus. By contrast, only one of four lines derived from human cells of newborns demonstrated a very low titer of immunofluorescence antigen, and none of the newborn lines released detectable infectious EBV. In adult human cells transformed by the same virus stock, complement fixation antigen was detected in all lines, low titers of immunofluorescence antigens were found in three of four lines, and extremely low titers of transforming activity were detected in culture fluids from one of four lines.

To establish whether these differences were peculiar to the Hawley strain of virus, which had undergone many passages in transformed human and marmoset cells, we performed further transformation studies with the Lederer strain, which was stored as a throat gargle from a patient (Table 3). Marmoset leukocytes transformed by the Lederer strain contained more than $10^{3.5}$ transforming units per 0.2 ml of both cell-associated and extracellular infectious EBV; human cord leukocytes transformed by the same strain demonstrated comparable titers of complement fixation antigen, but barely detectable levels of immunofluorescence antigen and infectious EBV.

TABLE 3. *Expression of EBV in human cord and marmoset cells transformed by a strain of EBV obtained from a throat washing from a patient with infectious mononucleosis*

Designation	Species	Expression of EBV*			
		CF antigen	IF antigen	Infectivity	
				Cells	Fluids
E49-3	Human (newborn)	4	<0.1	nil	<0.0
F26-11	Marmoset	4	3.6	3.5	4.3

Lines of human umbilical cord leukocytes and marmoset leukocytes were obtained after exposure to a throat washing. The transforming capacity of the throat washing was neutralized by EBV antibody and unaffected by EBV-free human sera. The cell lines were subdivided 2:1 every 3–5 days and tested for antigens and infectious EBV in supernatant fluids and extracts of 10^7 cells/ml.

* See footnotes of Table 1.

DISCUSSION

These experimental results indicate notable differences in the expression of EBV in transformants derived from different sources. Marmoset leukocytes yield considerable quantities of biologically active virus, adult human leukocytes yield minute quantities of virus, and human cord leukocytes rarely release biologically active EBV detectable by the transformation assay. At least two distinct processes are responsible for the increased yield of extracellular EBV by marmoset cells. First, a higher proportion of marmoset cells than human cells produce immunofluorescence antigens, i.e., a greater proportion of cells are activated to produce nucleocapsids. Second, on the basis of calculations made in Table 1 and similar calculations (not shown) for the data in Tables 2 and 3, those transformed marmoset cells that have been activated to produce immunofluorescence antigen yield much more infectious virus than comparable adult human cells. The yield is from 0.25–2.6 infectious units per marmoset cell with immunofluorescence antigen, while for those transformed adult human cells that yield infectious EBV one infectious unit is released per 200–3000 cells with immunofluorescence antigen.

The factors responsible for the higher yields of transforming virus per activated marmoset cell deserve further study. The most likely explanation is that envelopment of the virus proceeds more efficiently in marmoset cells. In keeping with this hypothesis, infectivity of virus from the B95-8 line was destroyed by ether and chloroform, and enveloped particles were seen by electronmicroscopy. By contrast, only a rare nucleocapsid, and no enveloped particles, were found in fluids of line 883L, which has a low titer of extracellular EBV. It is conceivable, therefore, that EB viral release from cells, in the absence of cellular degeneration, is facilitated by envelopment.

Activation of the viral genome of the transformed marmoset cells leading to release of infectious virus apparently occurs during the exponential phase of cell growth (Fig. 2), for there was no increase in infectious virus production once the cells reached stationary phase. The availability of a line that releases biologically active virus should permit close analysis of the events in the cell cycle in which viral activation proceeds. However, it is apparent that exponential cell growth *per se* is insufficient to activate the genome, for biologically active virus is not released at all by transformed cord cells. Some of the transformed cord cells have been tested for cell-associated virus; barely detectable transforming activity was found in an extract of one cord cell line and none in several others.

Finally, several biochemical, biophysical, and immunological experiments should be facilitated by the availability of a system that produces reasonably large quantities of cell-free virus that can be partially purified. One objective, now approachable, is to determine whether EBV associated with Burkitt lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis is the same agent.

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