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Biological Properties and Viral Surface Antigens of Burkitt Lymphoma- and Mononucleosis-Derived Strains of Epstein-Barr Virus Released from Transformed Marmoset Cells

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Three strains of Epstein-Barr virus (EBV), two from Burkitt lymphoma (BL) and one from infectious mononucleosis (IM) were used to transform separate cultures of the same batch of primary marmoset leukocytes, and the viruses released from the transformants were compared. The three viruses shared properties of the transforming biotype of EBV, namely, stimulation of DNA synthesis and immortalization of cord blood leukocytes, and failure to induce "early antigen" in lymphoblast lines. All viruses produced more virus in transformed marmoset cells than in transformed human cells, as measured by the number of EBV genomes detected by complementary RNA/DNA hybridization, by virus capsid antigen expression, or by released virions and biologically active virus. Reference human sera and sera from primary EBV infections were used to compare the three virus strains in a virus neutralization test based on inhibition of stimulation of DNA synthesis. Specimens taken late in convalescence from patients with mononucleosis and sera from marmosets experimentally infected with virus from a patient with mononucleosis neutralized the homologous virus, as well as the two virus strains isolated from patients with BL. This finding indicates that viral antigens that elicit neutralizing antibodies are shared among the strains. However, in certain sera the neutralizing-antibody titer against one strain was consistently higher than against another strain. Furthermore, sera taken early after onset of IM contained low levels of neutralizing antibody against IM-derived virus, but failed to neutralize BL-derived virus. These latter findings suggest the existence of heterogeneity among surface antigens of EBVs. The results emphasize the biological and antigenic similarity of EBV isolates from BL and IM and do not suggest major subtype variations. It remains to be determined whether antigenic diversity such as described or virus genome variation detectable by other means is epidemiologically significant.

A mystery surrounding the association of Epstein-Barr virus (EBV) with infectious mononucleosis (IM), Burkitt lymphoma (BL), and nasopharyngeal carcinoma is whether diversity in geographical distribution, age range, and histological characteristics of these diseases reflects different host responses to the same agent, or different pathogenetic properties of a family of closely related viruses. The experiments to be described represent attempts to analyze homogeneity and heterogeneity of biological properties and virus surface antigens of EBV strains derived from BL and IM.

Direct comparison of EBV strains from different sources has not previously been possible. The two laboratory strains available in high titer for study, P_3J-HR-1 from BL and B95-8 from IM, differ in many respects in addition to their disease origin. Currently available laboratory stocks of the P_3J-HR-1 BL strain do not cause transformation of primary lymphocytes into cell lines, and the B95-8 IM strain does not cause abortive infection and cell death of established EBV genome carrier lines (8). Furthermore, the two available laboratory viruses differ in the antigens that they induce in EBV genome-negative human lymphoid lines (6). Few other producer cell lines that regularly release EBV of transforming capacity are available; such producer lines as exist were each derived from different individuals. It was essential that direct comparison of biological behavior and of viral envelope antigens be made among viruses grown in the same or closely related cells. Finally, quantitative bioassays for transforming EBV have only recently become available (14).

The materials to study this problem were

developed by using three different EBV strains to transform peripheral blood leukocytes from one marmoset. Sufficient quantities of virus were released from these transformants for comparative study, and these viruses could be measured with precision by stimulation of DNA synthesis in cord blood lymphocytes (14). Inhibition of this reaction served as the basis of a serum neutralization test.

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MATERIALS AND METHODS

Derivation of the viruses. The Hawley strain was obtained by exposing cotton-top marmoset leukocytes to an extract of 10^8 cells of a lymphoblastoid line derived from the blood of a patient with mononucleosis following blood transfusion (9). The Olare and Nyevu viruses were obtained from lines derived by Burkitt tumors. These lines, a gift from George Klein, were received in our laboratory in 1972. Sufficient cell-free virus to transform marmoset leukocytes could not be extracted from the BL lines on repeated attempts; however, transformation of marmoset cells was achieved by co-cultivation of BL lines, lethally X-irradiated with 8,000 rads, with primary marmoset leukocytes by methods previously described (9). Virus stocks prepared from filtered (450 nm) supernatant fluids of the producer marmoset cells were used to transform cultured leukocytes obtained from a single bleeding of one marmoset. The resultant cell lines served as the source of virus in subsequent experiments.

Assay of the viruses and viral antigens. Morphological transformation of umbilical cord leukocytes and stimulation of DNA synthesis were detected by assays described (7, 14). Determination of the ability of the viruses to superinfect Raji cells was measured by induction of "early antigen" (4). Three milliliters of a virus stock was added to 3 ml of log-phase Raji cells at 2×10^6 cells per ml of fresh medium. After 3 days of incubation at 35 C, cell spreads were made, fixed, and stained by indirect immunofluorescence with a 1:10 dilution of a pool of human serum containing antibodies to early antigen and viral capsid antigen (VCA) (the gift of W. Henle and G. Henle). Human serum without anti-early antigen and Raji cells unexposed to virus served as controls. The number of viral particles in concentrated preparations was determined in counts of negatively stained hanging drops (10). VCAs were measured by indirect immunofluorescence (3).

Viral DNA. EBV DNA was detected by the method of complementary RNA (cRNA)/DNA hybridization on membrane filters; [^{3}H]thymidine ([^{3}H]TdR) cRNA was prepared with purified EBV DNA from the P_3J-HR-1 virus strain as template (11). The EBV cRNA had a specific activity of about 6×10^6 counts/min per μg. Hybridization was performed with 50 μg of total cellular DNA. Each determination was made in duplicate. The background of hybridization between cRNA and HEp2 cell DNA was subtracted. The number of EBV genome equivalents per cell was calculated on the basis of hybridization of the cRNA probe with Raji cell DNA as a standard. The Raji line has 60 genome equivalents per cell. Sixty "genome equivalents per cell" is equal to about 84.6 ng of EBV DNA per 50 μg of total cellular DNA. The values used for this calculation are 1.6×10^{-16} g for EBV DNA and 6×10^{-12} g for Raji cell DNA. The expression "genome equivalent per cell" is used as a convention, but it is recognized that in EBV producer lines the population consists of a mixture of producer cells with large numbers of copies of viral DNA per cell and nonproducer cells with fewer copies of EBV DNA in each cell.

Virus neutralization test based on inhibition of stimulation of DNA synthesis. The first step in this test was to determine equivalent inocula of biologically active virus of each strain. The titer of virus that caused stimulation of DNA synthesis was determined by serial twofold dilution. The DNA-stimulating titer was that dilution of virus which caused 0.3 log or more incorporation of [^{3}H]TdR counts/min above background; end points were read at 14 days. These preparations contained approximately equal numbers of viral particles (see Table 2). Approximately 4 "DNA-stimulating units" of virus was mixed with an equal volume of heated serum (56 C for 0.5 h), diluted in complete medium (RPMI 1640, 20% fetal calf serum, and antibiotics). The serum-virus mixture (0.1 ml) was added to four or more replicate cultures of leukocytes (0.4 ml at 2×10^6 cells/ml). Periodically, a tube was harvested for determination of the incorporation of [^{3}H]TdR during a 1-h isotope pulse performed as described previously (14). The time at which the results of the test could be read depended on the sensitivity of the individual batch of umbilical cord cells to transformation, and the extent of spontaneous DNA synthesis in the umbilical cord cells (a high level of spontaneous DNA synthesis delayed the time at which viral effects could be discerned). Final results were read 20 to 30 days after inoculation. In each test, the following controls were included: cord cells without virus, cord cells with the lowest dilution of each serum to test for serum toxicity, and cord cells inoculated with virus mixed with an equal volume of medium. The last mixture served as the "positive control." The background of uninfected cord cells was first subtracted to determine the net [^{3}H]TdR counts per minute stimulated by the virus preparation. The neutralizing-antibody titer was taken as that dilution of serum inhibiting incorporation of [^{3}H]TdR counts per minute by 0.3 log or more with reference to the positive control. Neutralization tests comparing antibody titers against the three virus strains were performed on the same batch of umbilical cord leukocytes.

RESULTS

Derivation and biological behavior of the EBV strains. The three strains were originally present in human lymphoblastoid lines with low levels of cells (1 to 3%) spontaneously producing VCA (Table 1). Minute amounts of bio-

TABLE 1. *Origin and expression of three EBV strains and their derivatives in cotton-top marmoset cells*

Strain from patient	Disease[c]	Original human line			Marmoset line				
					First passage[a]			Second passage[b]	
		Genomes[d]	VCA[e]	TD_{50}[f]	Genomes[d]	VCA[e]	TD_{50}[f]	VCA[e]	TD_{50}[f]
Hawley[g]	IM	63	<1	$10^{1.0}$	182	5	$10^{4.0}$	12	$10^{2.5}$
Nyevu[h]	BL	93	2	10^{0i}	261	5	$10^{2.5}$	10	$10^{3.0}$
Olare[h]	BL	198	3	10^{0i}	372	4	$10^{1.7}$	10	$10^{2.5}$

[a] The Hawley strain was passed to leukocytes of one marmoset; the Nyevu and Olare strains to leukocytes of another marmoset. The marmoset cells of first passage were transformed by a cell lysate of Hawley strain and by co-cultivation of X-irradiated Nyevu and Olare cells with marmoset leukocytes.

[b] Virus released into extracellular fluid of first-passage marmoset cells was used to transform blood leukocytes of one marmoset (CTM no. 677).

[c] IM, Line derived from blood leukocytes; BL, lines derived from tumors.

[d] Number of EBV genome equivalents per cell detected by hybridization between EBV [^{3}H]cRNA prepared in vitro with 50 μg of total-cell DNA.

[e] Percentage of cells with VCA.

[f] TD_{50} = 50% transforming doses per 0.1 ml of extracellular fluid; assayed on human umbilical cord leukocytes.

[g] The laboratory designations of this strain which have previously been published are: original human line, 883L; first-passage marmoset line, B95-8; the second-passage marmoset line, P94.

[h] The laboratory designation of the second-passage marmoset transformants are Nyevu, P69-26; Olare, P69-42.

[i] 10^0 = 1 of 4, or fewer, cultures transformed with undiluted virus. Virus present only in a crude lysate of 10^8 cells of the Nyevu line. Virus detected in undiluted supernatant fluid of Olare line.

logically active virus (10 transforming doses or less per ml) were released into the culture fluid. Nonetheless, all human lines contained multiple (63 to 198) copies of the viral genome per cell as detected by nucleic acid hybridization. The first passage of all three strains to marmoset cells resulted in an increase in the expression of EBV in the simian transformants, by comparison with its expression in the original human cell lines. There were two- to threefold increases in the amount of EBV DNA (now 182 to 372 genomes/cell) and in the proportion of cells activated to make capsid antigen. There was 50 to 1,000-fold more biologically active extracellular virus released by the marmoset cells than by the original human lines, as detected by morphological transformation of human umbilical cord leukocytes.

The second virus passage to a fresh preparation of leukocytes of one marmoset was now possible with filtered supernatant fluids from the first-passage marmoset cell transformants. Again, a high level of cells was activated to produce capsid antigen, and 300 to 1,000 50% transforming doses were present per 0.1 ml of extracellular fluid. Mature herpes-type virions were found in 1 to 3% of 100 cell profiles from each line studied by electron microscopy.

Concentrated supernatant fluids of each second marmoset passage producer cell line were examined for extracellular virions by negative staining (Table 2). Approximately the same number of virions was found in the fluids of each line, but the proportion of virions that was enveloped was always higher for the line derived from the patient with mononucleosis.

TABLE 2. *Extracellular virions of three EBV strains from concentrated extracellular fluids of marmoset transformants (second passage)*

Virus strain	Expt P-27[a]		Expt R-52[b]	
	Approx no. particles/ml[c]	% Enveloped	Approx no. particles/ml[c]	% Enveloped
Hawley (IM)	7×10^8	76	2×10^8	30
Nyevu (BL)	4×10^8	23	4×10^7	0
Olare (BL)	2×10^9	6	1×10^8	10

[a] Experiment P-27 used 200-fold concentrates prepared from supernatant fluids about 3 months after the lines were established. Virus was pelleted and then centrifuged on a 10 and 50% discontinuous potassium tartrate gradient for 2 h at 25,000 rpm.

[b] Experiment R-52 used 100-fold concentrates prepared 9 months after the two lines were established by method of precipitation with 10% polyethylene glycol (1).

[c] The estimate of Monroe and Brandt was used to calculate number of particles (8).

Early antigen induction. All three transforming viruses failed to induce early antigen upon addition to Raji cells. Both unconcentrated virus of known transforming titer and concentrated virus of known particle count were tested (Table 3). Only the nontransform-

TABLE 3. *Attempts to induce early antigen in Raji cells with four EBV strains*

Virus strain	Source	Expt U-2 (unconcentrated virus – known biologic activity)		Exp U-14 (concentrated virus)		
		No. of 50% transforming units added per 10^6 Raji cells	Fraction Raji cells with early antigen	Approx no. of viral particles per 10^6 Raji cells	Virus stock[a]	Fraction Raji cells with early antigen
Hawley[b]	IM	5×10^5	Nil[c]	5×10^6	P-27	Nil[c]
				7×10^7	R-52	Nil
Nyevu[b]	BL	3×10^3	Nil	4×10^7	P-27	Nil
				1×10^7	R-52	Nil
Olare[b]	BL	5×10^3	Nil	1×10^8	P-27	Nil
				3×10^7	R-52	Nil
Jijoye[d] (P_3J-HR-1)	BL	Nil	0.12	1×10^7		0.15

[a] See Table 2. Particles from stock P-27 were diluted approximately 1:30 in medium. Particles from stock R-27 were dialyzed against Tris (0.1 M), NaCl (1.0 M) (pH 7.2) and were diluted 1:2 in medium.
[b] Second-passage marmoset producer cell lines used.
[c] Nil, $<1:10^4$ Raji cells with early antigen.
[d] Human cell line.

ing P_3J-HR-1 virus induced early antigen in 10 to 20% of cells when added at a multiplicity of about 10 particles per cell.

Morphology of transformed marmoset cells. The appearance of the marmoset leukocytes transformed by the three EBV strains was similar. All lines grew as a mixture of cells which attached to glass or plastic and free-floating cells (see Fig. 1). Scattered multinucleated cells and occasional cells with intranuclear inclusions were seen. The line transformed by the Olare virus consisted of larger rounder cells; in preparations of surface adherent cells from this line, only rare spindle shaped cells were noted. This mixture of adherent and floating cells was also seen in sublines obtained by limiting dilutions and in single-cell clones. Human umbilical cord lymphocytes transformed by the three viruses did not demonstrate the property of adherence to glass or plastic. Other cytological differences between human and marmoset cells transformed by the EBVs were noted and will be described separately (J. Robinson, W. A. Andiman, G. Miller, manuscript in preparation).

Expression of the three viruses in human umbilical cord lymphocytes. Cellular controls governing the expression of the viral genome were similar for the three viruses. Transformed marmoset cells were productive of mature virions (Table 1); by contrast, human umbilical cord lymphocytes, derived from three different individuals and transformed by the three strains, did not spontaneously exhibit capsid antigen or early antigen synthesis (Table 4). All transformed cord cells contained the EB nuclear antigen. Fewer copies of EBV DNA (from 14 to 61 genomes per cell) were found in the nonproductive transformed cord cells than in the virus producer marmoset cells, or in the original human producer cell lines. No extracellular virus was found in supernatant fluids of human cord leukocytes transformed by the three viruses.

All three viruses stimulated DNA synthesis in umbilical cord lymphocytes (Fig. 2), and the incorporation of [^{3}H]TdR was proportional to the amount of virus added. For each virus stock, a dilution could be determined which caused stimulation of incorporation of approximately 10^3 counts/min above the background on a pulse performed 14 days after inoculation. Thus, equivalent amounts of biologically active virus could be compared in neutralization tests.

Comparative neutralization by selected reference human sera. Sera that lacked antibody to EBV VCA invariably failed to neutralize any of the viruses, whereas sera containing antibody to VCA always showed neutralizing activity against two or three strains (Table 4). However, one reference serum, RM, obtained from an individual many years after mononucleosis, consistently showed a higher neutralizing antibody titer to the mononucleosis EBV strain and to the Nyevu BL strain than to the Olare BL strain (Table 5). The results obtained with serial dilutions of this serum in two experiments are illustrated in Fig. 3, which demonstrates poor neutralization of the Olare BL strain and efficient neutralization of the other strains. Although the same virus stocks were used in both experiments, the time of appearance of stimulation of DNA synthesis differed by about 1 week with the two sets of primary umbilical cord

cells. Within each experiment, comparable results were obtained when results were "read" on different days. In experiment Q78, low dilutions of serum mixed with the Hawley and Nyevu viruses were not as inhibitory as higher dilutions. This phenomenon, for which we have no ready explanation, was not seen in experiment R2. Stimulation of DNA synthesis by the three virus "positive controls" (virus mixed with antibody-negative serum or with medium)

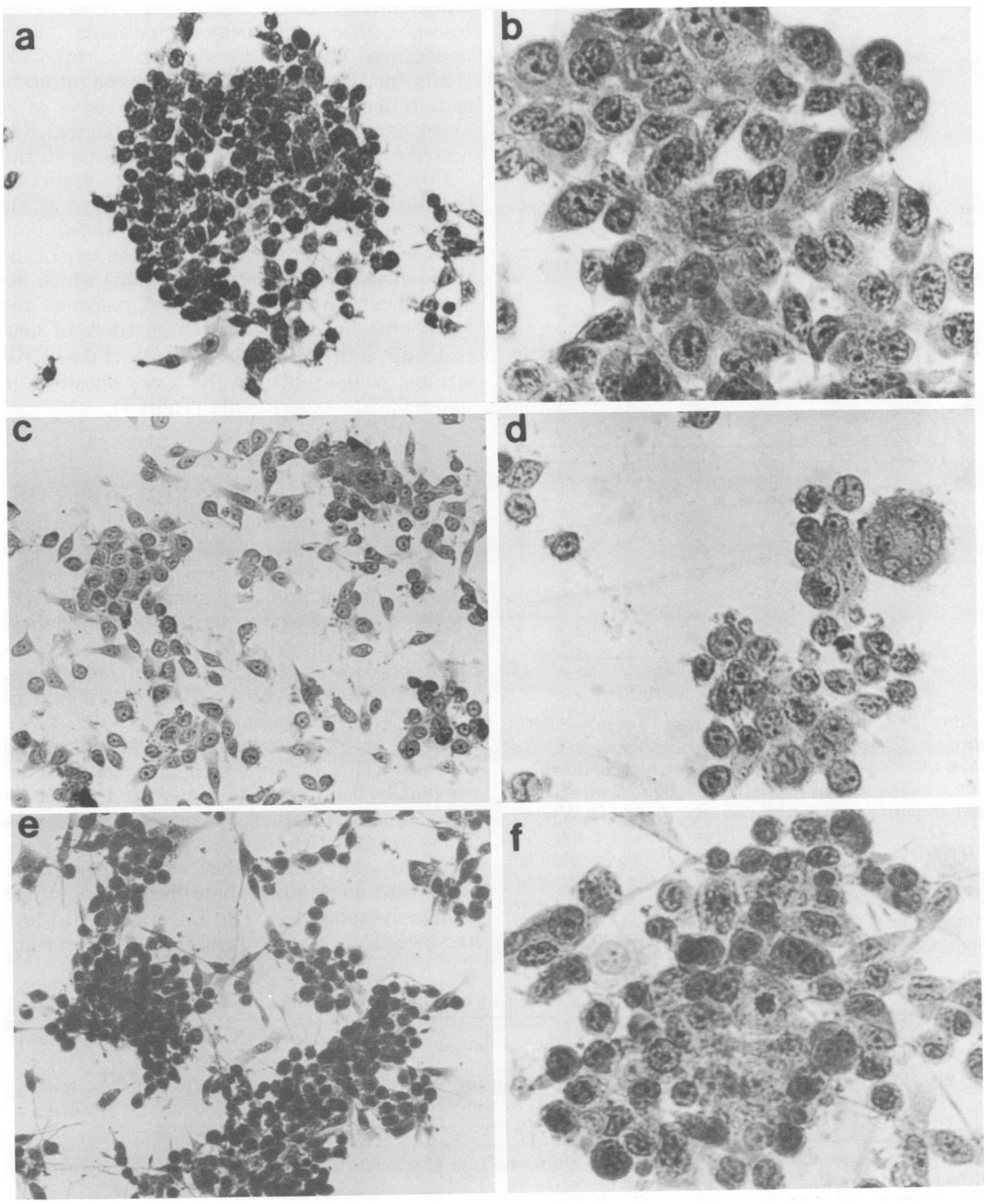

FIG. 1. *Hematoxylin and eosin-stained preparations of glass-adherent marmoset blood leukocytes transformed by three different EBV strains: (a, b) transformed by Nyevu (BL) virus; (c, d) by Olare (BL) virus; (e, f) by Hawley IM virus. Magnification: a, c, e ×320; b, d, f ×800.*

was equivalent for each experiment. The Olare virus strain, which was poorly neutralized by the RM serum, was inhibited by other antibody-positive sera (Table 4), a finding which indicates that this virus strain is not intrinsically difficult to neutralize.

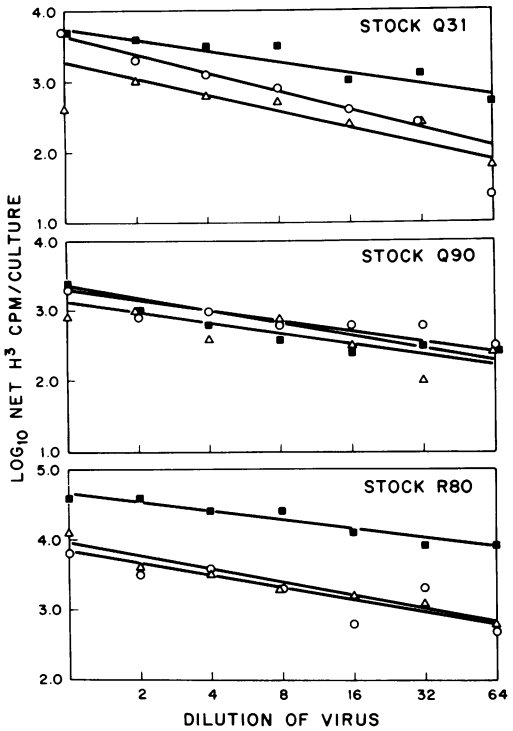

FIG. 2. *DNA-stimulating activity of three EBV strains. Three different virus stocks of each strain were prepared, and assayed by serial twofold dilution for their ability to stimulate [^{3}H]TdR incorporation in cord blood leukocytes. Symbols: (○) Nyevu, (△) Olare, (■) Hawley. Results shown are for [^{3}H]TdR pulses on day 14 after addition of virus. The ^{3}H counts per minute subtracted for the background of uninfected cells include stock Q-31, 527 counts/min; stock Q-90, 310 counts/min; stock R-80, 550 counts/min.*

Comparative neutralization by human and marmoset sera following primary EBV infections. The development of neutralizing activity in matched sera obtained prospectively from six patients with mononucleosis was studied (Table 6). All pre-mononucleosis sera failed to neutralize any virus. Sera obtained late in convalescence after mononucleosis (patients 1 to 3) neutralized each of the strains. In three patients (no. 4 to 6, Table 6), early convalescent sera collected between 11 and 69 days after onset demonstrated low levels of neutralizing activity only against the mononucleosis strain.

Preinoculation sera from two marmosets that received the mononucleosis (Hawley) strain lacked antibody. One year after inoculation, the serum of animal 654 neutralized two of the three viruses. Another animal (625) which developed a high-titered anti-VCA response and lymphoma, also developed high titers of neutralizing antibodies against all three virus strains; comparable results were obtained in two replicate experiments (Table 7).

DISCUSSION

Biological properties of EBV strains. The three strains, one from IM and the two new high-titered laboratory strains from BL, share several biological attributes. They are transforming viruses and they stimulate DNA synthesis in lymphocytes with high efficiency. They are all more productive in marmoset blood leukocytes than in human leukocytes, whether tested at the level of the amount of viral DNA, the fraction of cells with capsid antigen, or the amount of extracellular virus released. This difference in host control had previously been described only for IM-derived virus (7). Furthermore, marmoset leukocytes transformed by the three viruses are surface adherent and share other cytological differences that distinguish them from transformed human lymphocytes. The IM strain, Hawley, has been shown to be tumorigenic in marmo-

TABLE 4. *Expression of EBV in transformed human cord cells*

Original virus strain (source)	Genomes/cell[a]	Nuclear antigen	Capsid antigen[b]	Early antigen[b]	Released virus
Hawley (IM)	31, 40, 61	Positive	Nil[c]	Nil[c]	Nil[d]
Nyevu (BL)	14[e]	Positive	Nil	Nil	Nil
Olare (BL)	19[e], 41	Positive	Nil	Nil	Nil

[a] If more than one value is shown, more than one line of transformed umbilical cord leukocytes from different infants was examined.
[b] Determined on three different cord cell lines transformed by each virus.
[c] <1/10,000 cells.
[d] None of four cultures exposed to undiluted supernatant fluids was transformed.
[e] Derived from the same umbilical cord.

sets; the other strains have not yet been studied (15).

It is important to emphasize that the two BL strains are unlike the widely studied P_3J-HR-1 BL virus which, at its present state, is not transforming, but, instead, undergoes abortive replication, as indicated by appearance of early antigen, when used to infect cells that contain the EBV receptor (4). Thus the biological behavior of the P_3J virus is not representative of EBV strains originating from BL, but in fact, is exceptional. The finding that none of the transforming viruses induced early antigen supports our earlier hypothesis (8) that the transforming and abortive-infecting biotypes of EBV are mutually exclusive.

There are few clues yet to biological heterogeneity among the transforming class of EBV strains that might be expected to vary in their transforming efficiency, in their ability to replicate, or perhaps in their host range or tumorigenicity. However, two biological variations observed in these experiments require further analysis. The marmoset cell line which releases the Olare virus strain consists of larger cells that grow to a lower cell density than the other two lines, even though all lines were obtained from one bleeding of the same animal. This observation raises the possibility that some morphological variations among EBV-transformed cells might be virus specified. Hu-

TABLE 5. *Neutralizing-antibody titers of selected human sera against three EBV strains*

Serum	Donor status	Anti-VCA titer	Neutralizing-antibody titer against indicated virus strain		
			Nyevu (BL)	Olare (BL)	Hawley (IM)
LH	Healthy	<10	<10[a]	<10	<10
DN	Healthy	<10	<10	<10	<10
AF	Healthy	<10	<10	<10	<10
WAA	Healthy	80	80	80	≥160
RM	Healthy	80	≥160	10	80
TW	Burkitt	160	≥320	≥320	≥320
Nalwoga	Burkitt	160	40	<20	40

[a] Reciprocal of dilution of the human serum causing inhibition of 0.3 log or more of stimulation of [^{3}H]TdR incorporation by the indicated virus. [^{3}H]TdR incorporation by uninfected cultures is first subtracted (background). [^{3}H]TdR incorporation is compared for virus mixed with antibody-negative serum (positive control) and virus mixed with antibody-positive serum.

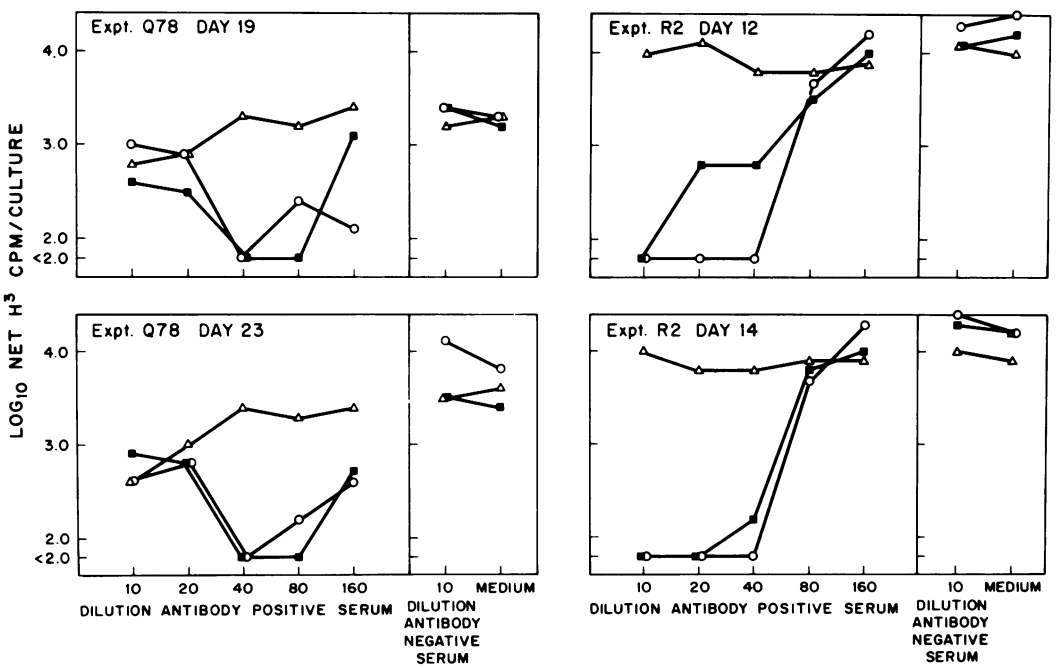

FIG. 3. *Neutralization of three EBV strains by reference sera. The figure illustrates two replicate experiments in which serial dilutions of an antibody-positive serum were added to four DNA-stimulating units of the Nyevu (○), Olare (△), and Hawley (■) EBV strains. For controls, the viruses were mixed with antibody-negative human serum or with medium. Each experiment was "read" by [^{3}H]TdR pulses on two different days. Note poor or absent neutralization of the Olare virus in comparison with the other two viruses.*

TABLE 6. *Neutralizing-antibody titers of sera from acute IM patients tested against EBV strains from mononucleosis and BL*

Mononucleosis patient	Serum	Time after onset	Anti-VCA titer	Neutralizing-antibody titer against virus strain (source)		
				Nyevu (BL)	Olare (BL)	Hawley (LM)
(1) L.D.	Preillness		<1	<20[a]	<20	<20
	Postillness	13 months	40	20	20	40
(2) F.M.	Preillness		<10	<10	<10	<10
	Postillness	32 days	10	<10	<10	<10
	Postillness	380 days	20	80	20	40
(3) A.D.	Preillness		<10	<10	<10	<10
	Postillness	240 days	40	40	160	20
(4) Sullivan	Preillness		<10	<20	<20	<20
	Postillness	48 days	40	<20	<20	20
	Postillness	71 days	40	<10	<10	20
(5) W.B.	Postillness	6 days	10	<10	<10	20
	Postillness	24 days	80	<10	<10	20
	Postillness	51 days	40	40	10	40
(6) A.H.	Preillness		<1	<10	<10	<10
	Postillness	11 days	160	<10	<10	10

[a] See footnote Table 5.

TABLE 7. *Neutralizing-antibody titers of sera from marmosets experimentally infected with Hawley EBV strain tested against three EBV strains*

Marmoset	Pathological result	Serum	Day	Anti-VCA titer	Titer against virus strain		
					Nyevu	Olare	Hawley
654	Inapparent infection	Preinoculation		<10[a]	<20	<20	<20
		Postinoculation	361	40	<20	40	40
625[b]	Reticulum cell sarcoma	Preinoculation		<5	<10	<10	<10
		Postinoculation	224	320	≥160	≥160	≥160
625[c]	Reticulum cell sarcoma	Preinoculation		<5	ND[d]	<20	<20
		Postinoculation	224	320	≥320	160	160

[a] See footnote Table 5.
[b] Experiment R-100.
[c] Experiment S-19.
[d] ND, Not done.

man umbilical cord cells transformed by the B95-8 virus are said to differ from those transformed by the QIMR-WIL strain; the former virus induces spherical colonies; the latter induces discoid ones (5). The process of viral envelopment, as judged by electron microscopy examination of negatively stained preparations, occurred more often with the mononucleosis-derived virus than with the two BL viruses.

Comparison of EB viral envelope antigens by neutralization. Previous work with herpes simplex viruses emphasizes the complexity of the problem of antigenic speciation in the herpesvirus group by means of cross-neutralization tests. There are likely to be several neutralization sites on the virion, some of which are held in common among strains and some of which are distinct (17). There may be neutralization sites both on the envelope and on the core. Laboratory passage appears to alter the antigenic sites. The results are also influenced by the nature of the sera used: sera from primary infections differ from those from reactivated infections, and hyperimmune sera differ from those that are obtained after natural infection.

Two principal points emerged from comparative neutralization tests with the EBV strains. First, antigenic sites that elicit neutralizing antibodies are shared among EBVs derived

from BL and IM because sera obtained late after primary infection, either from mononucleosis patients or from experimentally infected marmosets, neutralized all three strains. Second, certain evidence suggests that there is heterogeneity among these antigenic sites. One reference serum consistently showed a higher neutralizing-antibody titer to two of the viruses (one from BL and one from IM) than to a third. Furthermore, during IM, antibodies to the IM-derived virus appeared earlier in illness than antibodies to the BL viruses. Although this finding could be interpreted as evidence for a surface antigen unique to the IM-derived virus or an antigen present in excess on it, another interpretation is that neutralizing antibodies that appear at a low level early in IM inhibit the Hawley (B95-8) strain because a greater proportion of the particles are enveloped (Table 2). Chang (2) has reported that some sera taken early in mononucleosis have neutralizing activity against the B95-8 virus, but fail to neutralize the leukocyte-transforming activity present in some throat washings; both B95-8 and throat washings are neutralized by late IM sera. Further study of serial bleedings and comparison with a larger battery of viruses is needed to determine whether sera taken early in IM are invariably able to discriminate between IM-derived and BL-derived agents.

Some EBV-associated antigens on the viral envelope responsible for eliciting neutralizing antibodies are also thought to be present on the cytoplasmic membrane of producer cell lines (16). It is known that the membrane-antigen complex consists of several different components, on the basis of blocking experiments (18). Such heterogeneity in virus neutralization as has been described here may be a reflection of this mosaic character to the viral surface antigens.

Comparative study of EBV genomes. Two other investigators have supplied evidence for heterogeneity of the EBV genome, based on nucleic acid hybridization studies with the technique of DNA reassociation kinetics. Pritchett et al. (13) found that nucleotide sequences, amounting to approximately 15% of the genome, were present in the nontransforming P_3J BL virus and absent in the transforming B95-IM virus. These investigators suggested that transforming viruses are defective. Alternatively, the P_3J-HR-1 virus may have acquired additional genetic information. We have also found that cellular DNA from B95-8 cultures lacks approximately 30% of the sequences found in P_3J virus DNA (J. Pagano, unpublished data). It will now be of considerable interest to compare the genetic complexicity of the new BL laboratory strains described herein with the nontransforming variant. Such studies will help to determine whether the amount of information in the viral genome correlates with the transforming property of the virus or with other properties such as its disease or geographical origin. Using labeled P_3J virus DNA as a probe, additionally we have found various degrees of homologous DNA ranging from 100% to as little as 60%, represented in different nasopharyngeal carcinoma biopsy specimens (12). These results may imply that there are EBV-genomic sequences common to the tumors and that there is also another region of the EB genome that is variable or missing. The availability of additional laboratory virus strains should help to control geographical or host-specific variations that might arise, for homology studies can now be performed with a variety of different EBV DNAs (and fragments thereof) and tumor cell DNA.

Certain caveats must be raised about the technique of adapting EBV to high-producer status in marmoset cells in order to obtain comparative measurements of virus strains. One possible objection to this technique is that the viruses obtained by co-cultivation of X-irradiated BL lines with marmoset leukocytes may have mutagenized genomes. This seems unlikely in view of the small target size of the viral genome, but has not been excluded. In the present state of the art, with EBV there are systems for isolation of transforming virus, but none are yet available for the primary isolation of viruses that are lytic (if such exist). Hence, upon primary isolation a viral subpopulation may be selectively amplified by the producer marmoset cell transformants. The virus recovered from a BL cell line may not necessarily represent the virus that transformed the cell; further modification of the viral genome may occur while the host cell is carried in vitro. Nonetheless, such laboratory strains of EBV now available do not indicate the existence of BL and IM subtypes at the level of analysis that has thus far been carried out.

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