Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 4006–4010, October 1974

Differences Between Laboratory Strains of Epstein–Barr Virus Based on Immortalization, Abortive Infection, and Interference

(human lymphocytes/viral transformation/viral interference/extra-cellular virus)

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Communicated by John F. Enders, July 19, 1974

ABSTRACT **Biologic activities of extracellular Epstein–Barr virus (EB virus) from two laboratory strains, namely, P_3J-HR-1 (P-H) from Burkitt lymphoma and B95-8 (B95) from infectious mononucleosis, were compared. Virus stocks from both sources contained approximately the same number of virions. Virus from the P-H line induced "early antigen" in six nonproducer EB virus genome carrier cell lines; virus from B95 did not induce "early antigen." Extracellular virus from B95 regularly caused lymphocytes from human umbilical cords to form continuous lines (immortalization); P-H virus did not cause primary cultures of human lymphocytes to grow continuously. B95 virus stimulated DNA synthesis as determined by rate of incorporation of [^{3}H]thymidine into acid-insoluble material; P-H virus did not stimulate DNA synthesis. Pretreatment of lymphocytes with undiluted P-H virus inhibited immortalization and stimulation of DNA synthesis by B95 virus. The inhibitory properties of the P-H virus were sedimented at 100,000 × *g* and inactivated by heat and UV irradiation; interference by the P-H virus was neutralized by human serum with antibody to EB virus and not by antibody-negative human serum.**

The hypothesis most consistent with these results is that the P-H virus is defective in gene(s) needed for initiation of immortalization. We speculate that the absence of this gene allows early antigen to be expressed upon superinfection of nonproducer cell lines. The availability of two laboratory strains of EB virus which differ in biologic behavior provides starting material for analysis of the mechanism of lymphocyte immortalization by EB virus and of virus structural differences which affect immortalization.

Two biologic properties of Epstein–Barr virus (EB virus) can be studied in cell culture systems. The first is transformation or, as we prefer, immortalization (1–4). The virus causes normal human lymphocytes with a limited life span *in vitro* to form continuous cell lines. The second activity is abortive replication. When EB virus is added to certain continuous human lymphoblastoid cell lines, an EB virus associated antigen complex termed "early antigen" (EA) appears (5). The term "early antigen" is used because superinfected cells do not produce mature virus and because production of "early antigen" occurs in the presence of levels of cytosine arabinoside which inhibit DNA synthesis (6). However, abortive infection and the appearance of early antigen are accompanied by inhibition of cell DNA synthesis (7) and by impaired ability of the superinfected cells to form colonies in soft agar (8).

Abbreviations: EB virus, Epstein–Barr virus; EA, early antigen; P-H, a clone of cells (P_3J-HR-1) from Burkitt-lymphoma line; B95, B95-8 virus strain originally derived from a case of transfusion-induced mononucleosis.

A major obstacle to comparative study of EB virus strains has been that most producer cell lines release minute quantities of extra-cellular virus, and there is yet no completely permissive culture system. For most studies heretofore, purified extracellular EB virions and EB viral DNA have been prepared from the P_3J-HR-1 line of Burkitt lymphoma cells (9, 10). Recently it was demonstrated that high titers of EB virus, biologically active by the immortalization assay, were found in the extracellular fluid of marmoset cells converted into lines following exposure to EB virus (11). Therefore, it was possible to determine whether the two biologic properties of EB virus which have been described were shared by virus stocks of two laboratory strains of different origin but with approximately equal numbers of virions.

METHODS

Viruses. A summary of the properties of the two cell lines and the virus released therefrom are listed in Table 1. P_3J-HR-1 (abbreviated P-H) is a clone obtained in soft agar by Hinuma *et al.* from the "Jijoye" Burkitt lymphoma line (12). Our laboratory strain was obtained from Dr. W. Henle (University of Pennsylvania). The B95-8 virus strain (abbreviated B95) was originally derived from a case of transfusion-induced mononucleosis. The mononucleosis virus was used to immortalize marmoset blood leukocytes which are the source of extracellular EB virus (13). There was close antigenic similarity in four, cell-associated, EB virus-related antigens found in the two producer cell lines. The viral capsid antigen, the soluble complement-fixing antigen, a soluble antigen detectable by immunodiffusion, and the EB nuclear antigen of each cell line cross-reacted when tested with reference positive human sera and failed to react with antibody-negative sera.

Virus stocks were prepared and assayed for the number of viral particles by electron microscopy as described (11). The assay for infectious units capable of immortalization of human leukocytes from umbilical cord blood was performed as described (11). To assay virus by superinfection of nonproducer cell lines, cells in log phase were sedimented and resuspended in 3 ml of fresh medium and 3 ml of the superinfecting virus stock or, as a control, extracellular fluid prepared from the nonproducer Raji cell line. The cultures were incubated at 36° for 3 days when cell spreads were examined for "early antigen." The antibody to "early antigen" D type, a pool of sera from patients with chronic lymphocytic leukemia, was a gift from Dr. Henle. Since superinfection does not result in production of progeny virus, the number of EA positive cells is a reflection of the number of EA inducing units

TABLE 1. *Properties of two high-titered EB virus laboratory strains*

	Strain P_3J-HR-1	Strain B95-8
Properties of cell line which carries the virus		
Disease origin	Burkitt lymphoma	Infectious mononucleosis
Tissue origin	Tumor	Blood
Species	Human	Marmoset
% of cells with capsid antigen	5–10	10–15
% of cells with nuclear antigen	100	100
Properties of the released virus		
Approximate no. particles released/ml	3×10^6	6×10^6
% of particles enveloped	~60	~60
Titer—immortalization of lymphocytes from human umbilical cord blood	Nil	10^5 ID_{50}/ml
Titer—induction of early antigen in EB virus genome-positive nonproducer lines	$\sim 10^{4.6}$*	Nil

* EA inducing units/ml.

added initially. The number of EA inducing units per ml can be estimated as follows:

$$\frac{\text{Fraction cells showing EA}}{\text{Volume of inoculum}} \times \text{Total number of cells in the culture.}$$

Assay for Stimulation of Incorporation of [^{3}H]Thymidine. The rate of incorporation of [^{3}H]dT in virus-treated cultures was compared with uninoculated cord blood lymphocytes (14). [^{3}H]dT incorporation may be used as a rapid quantitative assay for EB virus-induced immortalization. (J. Robinson and G. Miller, manuscript in preparation).

RESULTS

Virions and biologically active virus in extracellular fluids of the two producer lines: (Table 1)

To estimate the number of particles present in virus stocks, four lots of 250 ml of extracellular fluid from each line were concentrated 60-fold and examined by electron microscopy. We used the estimate of Monroe *et al.* that 1 EB virion per grid square equals approximately 3.4×10^7 particles per ml (15). Concentrated fluids from the P-H and B95 lines contained 10^8 and 2×10^8 particles per ml, respectively. Assuming quantitative recovery of virus particles, there were 3 to 6 $\times 10^6$ EB virions per ml of extracellular fluid of the two cell lines after 1 week incubation. Both naked and enveloped herpes virus particles were seen in these preparations and no other morphologic type of virus particle was identified. The median diameter of 33 B95 capsids was 108.6 nm and of 32 P-H capsids was 110.7 nm.

The P-H virus regularly induced "early antigen" in Raji cells. At a concentration of 10^6/ml, 5–20% of the cells developed "early antigen" after exposure to 3 ml of extracellular virus from various stocks; when calculated as shown in *Methods*, there were 1.6 to 6.6 $\times 10^4$ EA inducing units per ml of supernatant fluid of the P-H line. In contrast B95 extracellular fluid consistently failed to induce EA in Raji cells.

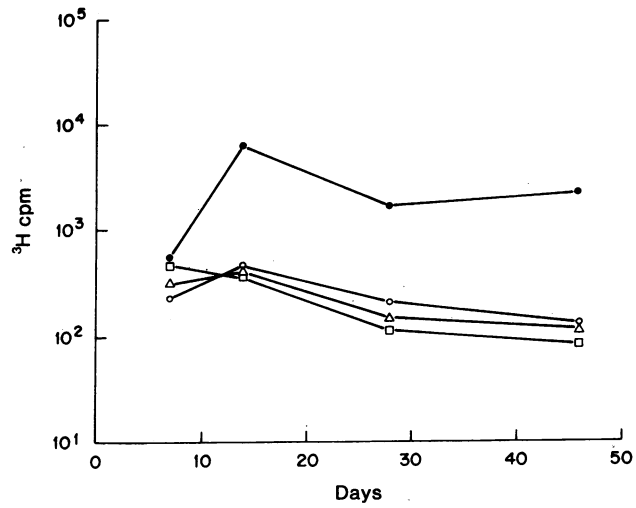

FIG. 1. Rate of DNA synthesis in leukocytes from umbilical cord after addition of two EB virus strains. Undiluted virus from the P-H or B95 line were added to leukocytes on day 0. As a control, extracellular fluid from the Raji (nonproducer) cell line and a cell strain of human placental fibroblasts was prepared and inoculated in parallel. Incorporation of [^{3}H]dT was measured on days 7, 14, 28, and 46. ●, B94; ○, P-H; △, medium from Raji cells; □, medium from placental cells.

Extracellular fluids from the B95 line contained approximately 10^5 50% immortalizing units per ml on repeated titration, but virus with immortalizing properties in the supernatant fluid of the P-H line was never found despite numerous attempts. Single cell clones of the two lines have been obtained. All clones from both lines exhibit viral capsid antigens in from 0.1–22.2% of cells. None of 14 P-H clones released extracellular virus detectable by immortalization; all six B95 clones released virus with immortalizing properties (16).

Stimulation of the incorporation of [^{3}H]dT

Fig. 1 shows the rate of incorporation of [^{3}H]dT into acid insoluble material in umbilical cord leukocytes following exposure to the two virus strains. Stimulation of incorporation of isotope is evident by 14 days after inoculation of approximately 10^4 ID_{50} of B95 virus. The rate of incorporation in umbilical cord cells exposed to undiluted P-H virus was the same as in cells exposed to either supernatant fluid from the Raji cell line, which contains no extracellular EB virus, or to culture medium.

We examined the possibility that undiluted preparations of P-H failed to stimulate DNA synthesis but that with serial dilution, a stimulatory effect would be observed. Such a result might be obtained if the P-H virus inoculum contained relatively few "interfering" particles and a larger number of immortalizing particles. Cultures of human umbilical cord leukocytes were observed for 35 days after exposure to dilutions of P-H from 10^{-1} to 10^{-4}. There was no difference in the incorporation of the isotope in the presence of P-H virus as compared to cultures exposed only to medium, nor were morphologic signs of immortalization observed in cells exposed to serial dilutions of P-H virus. In parallel titrations, dilutions of B95 from 10^{-1} to 10^{-5} all caused increased incorporation of [^{3}H]dT and morphologic conversion of the cells (data not shown).

TABLE 2. *Comparison of induction of "early antigen" in human lymphoblastoid cells after addition of two strains of EB virus*

Cells	Origin	EB virus carrier (EB nuclear antigen present)	Percent cells showing "early antigen" after addition of virus	
			P_3J-HR-1	B95-8
Raji	Burkitt lymphoma	+	20	Nil
NC37	Normal	+	14	Nil
WIL2A	Normal	+	4	Nil
G90-5	Cord†	+	10	Nil
E94	Cord†	+	1	Nil
K63-5	Cord†	+	0.5	Nil
CCRF-CEM	Leukemia	−	Nil	Nil
MOLT-4	Leukemia	−	Nil	Nil
Primary	Umbilical cord	−	Nil	Nil

Procedure: Virus stocks from P-H and B95 were prepared. P-H contained $10^{4.8}$ EA inducing units/ml. B95 contained $10^{5.0}$ ID_{50}/ml. Three ml of each virus or, as control, fluid from the Raji line, were added to log phase cultures of various continuous lymphoid lines or to primary cord cells. Three milliliters of fresh medium plus 10% fetal bovine serum were added and cultures incubated for 3 days at 35°. On day 3, cell spreads were fixed in acetone and replicate spreads were stained by indirect immunofluorescence with a serum pool containing antibody to early antigen. Controls in this test included human sera free of EB virus antibody and a human serum containing antibody to EB viral capsid antigen but not early antigen.

† Immortalized *in vitro* by B95-8 virus.

Comparison of induction of early antigen after addition of two strains of EB virus: (Table 2)

P-H virus caused the appearance of "early antigen" in all six lymphoblastoid cell lines which contained the EB viral genome as evidenced by the presence of EB nuclear antigen. The B95 virus did not induce "early antigen" in any of these cell lines which included lines which were originally derived by exposure to the B95 virus. The P-H virus did not induce "early antigen" in two continuous lymphoblastoid cell lines, MOLT-4 and CCRF-CEM, which lack the EB nuclear antigen and which have been shown by other investigators to have characteristics of T-lymphocytes (thymus-derived lymphocytes) (17, 18). In primary cultures of umbilical cord leukocytes, P-H virus induced no "early antigen."

Interference by P-H virus with stimulation of DNA synthesis by B95 virus

When P-H virus was added 1 day before B95 virus, there was transitory inhibition of stimulation of DNA synthesis, detectable 7–11 days later. (Fig. 2). In five experiments, pretreatment of cord leukocytes with P-H virus resulted in 48–94% (median 82%) inhibition of incorporation of [^{3}H]dT induced by B95 virus. This inhibitor in the P-H virus preparation was characterized by treating the stock in various ways and then adding the treated stock to umbilical cord leukocytes. (Table 3). Twenty-four hours later, the cord leukocytes were exposed to B95 virus and the rate of incorporation of [^{3}H]dT was measured after 7 days. The interfering properties of the P-H virus were diminished by heating at 56° for $^1/_2$ hr.

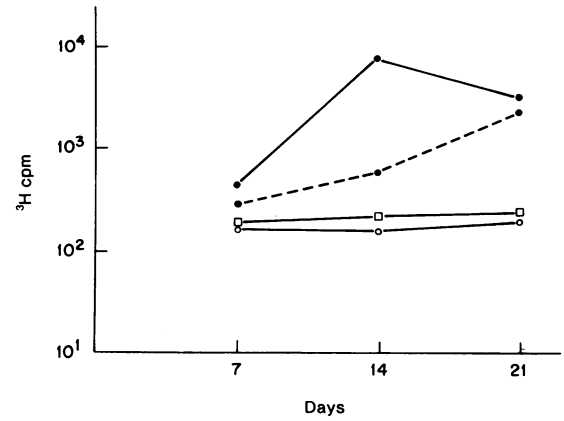

FIG. 2. Interference by P-H virus with stimulation of DNA synthesis by B95 virus. On day 0, two cultures each of cord leukocytes were exposed to medium or P-H virus. On day 1, B95 virus was added to one culture of each group; the other culture received medium. [^{3}H]dT incorporation was measured on day 7, 14, 21. ●——● medium day 0, B95 day 1; ●- - -● P-H day 0, B95 day 1; ○——○ P-H day 0, medium day 1; □——□ medium day 0 and day 1.

They were also decreased by UV irradiation for 16 min at a dose of 10 ergs/mm^2 per sec. After centrifugation at 100,000 × *g*, the interfering properties were found in the pellet of the preparation and removed from the supernatant fluid. These findings were compatible with interference being due to the virus *per se* rather than some soluble component found in the extracellular fluid such as interferon.

TABLE 3. *Effects of various treatments on interfering properties of P-H virus suspension*

Treatment of P-H virus	[^{3}H]dT pulse day 7		
	cpm	Net cpm*	% Stimulation†
None	382	93	6
56° for 1/2 hr	1266	977	64
Pellet 28,000 rpm for 2 hr reconstituted in orig. vol	818	529	35
Pellet 28,000 rpm for 2 hr reconstituted in 1/10 vol	347	58	4
Supernatant fluid after centrifugation at 28,000 rpm	1828	1539	100
UV‡ 2 min	697	408	27
4 min	537	248	16
8 min	465	176	12
16 min	1298	1009	66
B95 virus control	1818	1529	100
Cord cell control	289		

Procedure: On day 0, P-H virus was treated as indicated and placed on cultures of leukocytes from cord blood (0.1 ml of virus plus 0.4 ml of cells). Two groups of cultures, the cell control and B95 virus control, were not inoculated with P-H virus. On day 1, all cultures except the "cord cell control" were inoculated with 0.1 ml of undiluted stock B95 virus. The incorporation of radioactivity into acid insoluble material was determined on day 7, after a 0.5 hr pulse at 37° with [^{3}H]dT (5 μCi/ml).

* Cord cell control subtracted.

† (Net cpm/1529) × 100.

‡ Delivered at 10 ergs/mm^2 per sec.

TABLE 4. *Summary of three experiments demonstrating neutralization of interference by P-H virus with EB virus antibody*

Material added day 0	Material added day 1	Exp. no. 1* Net cpm§	Exp. no. 1* % Stimulation¶	Exp. no. 2† Net cpm§	Exp. no. 2† % Stimulation¶	Exp. no. 3‡ Net cpm§	Exp. no. 3‡ % Stimulation¶
1. P-H + EB virus antibody-positive serum	B95	2132	127	937	100	1225	91
2. P-H + EB virus antibody-negative serum	B95	562	33	336	36	575	43
3. P-H + medium	B95	597	36	172	18	697	52
4. P-H + medium	nil	Not done		Not done		1	0
5. Medium + EB virus antibody-positive serum	B95	Not done		1032	110	1419	105
6. Medium + EB virus antibody-negative serum	B95	Not done		909	97	941	70
7. Medium	B95	1684	100	940	100	1348	100

Procedure: On day 0, an aliquot of 0.3 ml of 10-fold concentrated P-H virus stock was mixed with 0.3 ml of a 1:10 dilution of antibody-positive or antibody-negative human sera or no serum. Similar mixtures were made with serum and medium. The mixtures were incubated at 37° for 1 hr and then 0.1 ml of the mixture was added to 0.4 ml of leukocytes from umbilical cord blood. After 24-hr incubation, all tubes were washed twice and B95 virus was added to all except the cord cell controls or the P-H virus control. On the indicated day, one tube from each group was incubated with [^{3}H]dT (5 μCi/ml) for 0.5 hr and the incorporation of the isotope into acid insoluble material was measured.

* [^{3}H]dT pulse, day 11.

† [^{3}H]dT pulse, day 7.

‡ [^{3}H]dT pulse, day 11.

§ Net cpm, value obtained for cells exposed to culture medium was subtracted from each experimental group; Exp. 1, 1949 cpm; Exp. 2, 437 cpm; Exp. 3, 613 cpm.

¶ $\%\ \text{Stimulation} = \dfrac{\text{net cpm in experimental culture}}{\text{net cpm in culture no. 7 (B95 control)}} \times 100.$

Table 4 demonstrates three separate experiments in which EB virus antibody-positive serum neutralized the capacity of the P-H virus to interfere with the B95 virus. Interference occurred in cultures inoculated with P-H virus preincubated with antibody-negative human serum or with culture medium. There was no inhibition of stimulation of DNA synthesis by the antibody-positive human serum alone because the sera were removed from cultures by washing before challenge with the B95 virus.

DISCUSSION

Relationship Between Abortive Infection, Immortalization, and Interference. The data provide a clear demonstration of heterogeneity in the EB virus group although discussion of mechanisms underlying these differences must be speculative. The results indicate that P-H virus which is capable of abortive infection is incompetent in immortalization and stimulation of DNA synthesis. Conversely B95 virus, with high titers of immortalizing activity, fails to effect abortive infection, as indicated by the appearance of early antigen in nonproducer cell lines. In the two strains studied, abortive infection and immortalization thus appear mutually exclusive, although this may not always be the case. However, it is tempting to relate these two functions since B95 virus contains information for making complete mature virions, as well as the entire complex of EB virus-associated cellular antigen, including early antigen. Therefore, it seems reasonable to propose that in lymphocytes from umbilical cord blood, the B95 viral genes responsible for immortalization in some way inhibit expression of genes responsible for early antigen and the structural components of the virus. This may occur as a result of viral-specified or cellular regulatory mechanisms.

At this point it is not known whether P-H virus fails to immortalize cord lymphocytes because it lacks relevant genetic information or because, in this virus, expression of genes related to abortive infection is predominant. If abortive infection and inhibition of host cell DNA synthesis occurs in cord cells exposed to P-H virus, immortalization obviously cannot occur. Although the P-H virus induces no detectable "early antigen" in these cells, it interferes with subsequent immortalization by B95 virus. This result implies some early gene function by P-H in the cord lymphocytes, but the nature of the gene function which induces interference is purely speculative. It may be related to inhibition of cellular DNA synthesis, to induction of interferon, to intrinsic interference, or possibly to alteration of receptor sites for B95. Since interference occurs with relatively small amounts of biologically active virus, some P-H particles which appear biologically "inert" may be participating in the interference phenomenon.

Origins of Different EB Virus Strains. There are three general hypotheses which might account for the origin of immortalizing and nonimmortalizing EB virus strains. The first, and to us the most likely, hypothesis is that "wild type" EB virus is an immortalizing virus, such as B95, and that nonimmortalizing virus, such as P-H, is a mutant which is defective in functions needed to stimulate cellular DNA synthesis and to effect immortalization. In favor of this view is the finding that virus with immortalizing properties can be regularly found in the throats of patients with mononucleosis and less frequently in the oropharyngeal secretions of normal individuals and patients with diverse disease states (19, 20). It is not known whether superinfecting EB virus can also be detected in the throat and low concentrations of EA inducing virus may be difficult to detect. Thus far, only P-H virus has been shown to be capable of superinfection and induction of early antigen, whereas a wide variety of EB virus strains have demonstrated immortalizing properties. If a parent of the P-H virus originally initiated immortalization of the line which produces P-H

virus, then the defect in the P-H virus arose after the line was immortalized. Functions necessary for initiation of immortalization may be defective, but functions necessary for maintenance of immortalization may still be present in the P-H virus genome.

The second hypothesis which cannot be excluded on the basis of available data is that outside the laboratory, both immortalizing and nonimmortalizing viruses exist, perhaps sometimes as a virus mixture. The parent of the P-H clone, the Jijoye line may, at one time in its history, have released such a mixture of particles. Virus with immortalizing properties have been detected in small amounts in the cells and fluids of this line (1, 2).

The third hypothesis, least likely to us, is that immortalizing virus particles are defective or damaged versions of the lytic superinfecting virus which is the wild type. Damage induced by UV light or photodynamic inactivation appears to be requisite for transformation by herpes simplex and cytomegalovirus (21, 22). However, UV light inactivates the immortalizing capacity of EB virus.

The strain differences observed do not reflect a general difference between EB viruses from Burkitt lymphoma and mononucleosis. However, description of major biologic differences between the two laboratory strains studied might serve as a stimulus to search for variations among EB viruses arising from diverse diseases. Furthermore, in the absence of classical methods for selection of conditional lethal mutants, the availability of these two "spontaneous" variants should facilitate analysis of the virus-cell interaction and definition of viral gene products which affect lymphocytic immortalization.

We are grateful to G. LeBouvier for advice and to V. Thompson for assistance. This work was supported by grants from U.S. Public Health Service (CA-12055, HD-00177, and AI-11-611); American Cancer Society (VC-107); Damon Runyon Memorial Fund (DRG-1127). G.M. is an Investigator of the Howard Hughes Medical Institute.

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