Maintenance of Growth Transformation with Epstein-Barr Virus Is Mediated by Secretion of Autocrine Growth Factors in Two Serum-Free B-Cell Lines

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The characteristics of two tamarin (*Saguinus oedipus*) B-cell lines (sfBIT and sfBT) growth-transformed by Epstein-Barr virus (EBV) that proliferate continuously in serum-free medium are described. sfBIT was established by selecting cells for growth in RPMI 1640 supplemented with insulin, transferrin, and selenium (J. E. Shaw, R. G. Petit, and K. Leung, J. Virol. 61:4033-4037, 1987). sfBT, a subline of sfBIT cells reported here for the first time, required transferrin as the only protein supplement for continuous growth in RPMI 1640. Growth of sfBT cells was linear with human transferrin at 10^{-2} to $10 \mu\text{g/ml}$. Transferrin at $5 \mu\text{g/ml}$ yielded a culture density of 5×10^5 to 1×10^6 cells per ml, a cell doubling time of 2 to 3 days, and a culture viability greater than 95%. sfBIT and sfBT cells released transforming virus during continuous growth in serum-free culture medium without EBV-inducing agents. The spent medium of both serum-free lines supported cell growth at low culture density (1×10^4 to 5×10^4 cells per ml), but growth was arrested at low culture density with fresh serum-free medium. A procedure to measure growth-promoting activity (GPA) was established, and it revealed that the GPA of spent medium was greater than that of fresh medium for both serum-free cell lines. When fresh and spent media were dialyzed (molecular weight cutoff, 3,500) and subsequently concentrated by lyophilization, only the GPA of spent medium increased. We conclude that maintenance of growth transformation of tamarin cells latently infected with EBV is mediated by growth factors that are entirely autocrine in origin.

Human and nonhuman primate B lymphocytes infected with Epstein-Barr virus (EBV) become growth transformed and, unlike normal B cells, proliferate continuously in vitro (for reviews, see references 13 and 15). In most cell lines growth transformed by EBV, multiple copies of latent viral DNA are present in episomal (34, 38) and integrated (1, 35) forms in virus nonproducer cells. The number of copies of latent viral DNA per cell is constant for each cell line. The latent DNA is complexed with histones (51) and synthesized during the S phase by host DNA polymerases (8, 11, 54).

As long as latency is maintained, only a limited number of the 80 to 100 genes (2) that make up the EBV chromosome is expressed. When latency is disrupted by cryptic signals that occasionally activate viral genes (21) during the early S phase (22), a multitude of virus-induced proteins is expressed, including a DNA polymerase (11, 12, 17, 39) which amplifies the number of copies of circular EBV DNA (50). The circular genomes synthesized by the viral enzyme (50, 53) are presumably precursors of the linear DNA in virions.

Several EBV gene products of latently infected, virus nonproducer cells have been identified. Among these are the EBV nuclear antigens (EBNA-1 [27, 55], EBNA-2 [9, 28], EBNA-3 [26, 29, 46, 49], EBNA-4 [31, 46], and EBNA-5 [14]), a latent membrane protein (25), a leader protein (47, 61), and the EBV-encoded RNAs (33). EBNA-1 binds to DNA (42) in the *ori-P* region (43) of the plasmid and is required for plasmid replication (62). EBNA-2 and latent membrane protein may be essential for immortalization of cells (10, 44, 60). The functions of most of the latent viral gene products are not known.

Lymphokines are secreted by activated normal (36, 40, 41, 56-58) and EBV transformed (4, 18-20, 48, 56) human B cells, and some of these are autostimulatory in action in EBV-infected cells. It is possible that expression of one or more of the latent viral gene products is required for synthesis, secretion, and binding of mitogenic factors.

In the present study, two EBV-transformed tamarin cell lines were examined for secretion of autocrine growth factors to account for their capacity to proliferate continuously in serum-free medium. One of the lines (sfBIT) was selected for growth in serum-free RPMI 1640 supplemented with insulin, transferrin, and selenium (52). sfBIT cells expressed virus antigens, including the EBV DNA polymerase, and produced transforming virus when exposed simultaneously to phorbol 12-myristate 13-acetate and *n*-butyric acid (52).

The characteristics of sfBIT cells are extended in this report, and a subline, sfBT, which required transferrin as the only protein supplement for growth is described. Evidence is presented that maintenance of growth transformation in both cell lines is mediated by growth factors which are secreted by the cells during continuous proliferation in serum-free medium.

MATERIALS AND METHODS

Cell lines and growth conditions. sfBIT and sfBT are EBV-transformed tamarin (*Saguinus oedipus*) B-cell lines that grow continuously in serum-free medium. sfBIT was derived from B95-8 cells (37) as previously described (52). sfBT, a subline of sfBIT cells, is discussed in the present report for the first time. Both cell lines were cultured with serum-free medium which consisted of RPMI 1640 (Flow Laboratories, Inc.) supplemented with $5 \mu\text{g}$ each of bovine insulin and human transferrin (Sigma Chemical Co.) per ml

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(sfBIT) or 5 μ g of human transferrin only per ml (sfBT). All serum-free media contained 3×10^{-8} M selenium dioxide (52), penicillin G (100 IU), and streptomycin sulfate (100 μ g/ml). Serum-free cultures were fed twice weekly with 0.5 to 1 volume of fresh medium.

Measurement of cell growth. Culture viabilities were determined with trypan blue. Growth was measured by direct cell counts, by estimating the concentration of DNA with diphenylamine (5), or by measuring incorporation of [3 H]dTMP into trichloroacetic acid (TCA)-insoluble material. [3 H]dTMP-labeled cells were collected on glass fiber filters (1.2- μ m retention) and washed with 5 ml of fresh medium. The filters were covered for 5 min with 1 ml of 20% TCA containing 20 mM sodium PP_i and then washed three times with 5 ml of 5% TCA–20 mM sodium PP_i and twice with 5 ml of 95% ethanol. TCA and ethanol were precooled to 0°C. The filters were dried and counted by scintillation spectrometry.

Removal of salt and concentration of culture media. Cells were removed from culture media by centrifugation ($10^3 \times g$, 5 min, 22°C), and then spent and fresh culture media were dialyzed (molecular weight cutoff, 3,500) against 10 volumes of water (>10 M Ω -cm) for 3 to 4 h at 4°C. Dialysis was repeated four times with fresh water. The material remaining after dialysis was frozen below -40°C and lyophilized. The freeze-dried residue from 600 ml of medium was dissolved in 3 ml of 50 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.7, containing 135 mM NaCl, 1.2 mM each MgSO₄ and KCl, and 200 μ g of bovine serum albumin per ml. Insoluble material was removed by Microfuge centrifugation (5 min, 22°C), and then the concentrates were filtered (0.2- μ m pore size) and frozen as aliquots at -20°C .

Assay for growth factor activity. Growth-promoting activity (GPA) was determined by measuring incorporation of [3 H]dTMP with cells at low culture density (1×10^4 to 5×10^4 cells per ml). The sensitivity of the assay varied with culture density, the concentrations of spent media, the specific activity of the labeled precursor, and the period during incubation in which the cells were labeled. The details of the assay will be reported elsewhere.

In this report, spent medium, with the exception of that described in Fig. 4, was diluted with fresh serum-free medium, and then 1 ml of each dilution was mixed with 1 ml of cells (2×10^4) in fresh serum-free medium. After 72 h at 37°C (6% CO₂), 0.1 ml of fresh medium containing [3 H]thymidine (0.5 μ Ci; 5 Ci/mmol) was added and incubation was continued for 18 to 24 h. The quantity of [3 H]dTMP recovered in TCA-insoluble material was determined as described for measurement of cell growth.

Assay for transforming virus. Spent media were assayed for transforming virus with human umbilical cord blood cells (24). Cells were removed from culture media by centrifugation ($10^3 \times g$, 10 min, 4°C), and then heat-inactivated (56°C, 30 min) fetal bovine serum was added (15% vol/vol) and the mixture was filtered (0.2 μ m pore size). Tenfold dilutions of the filtrate were prepared with RPMI 1640 containing heat-inactivated fetal bovine serum (15% vol/vol), and then 1 ml of each dilution was mixed with an equal volume of identical medium containing 2×10^6 human umbilical cord blood cells. The cells were fed twice weekly by replacing one-half of the culture medium with fresh medium. After 2 to 4 weeks of incubation, the cultures were examined for outgrowth.

FIG. 1. Growth of sfBIT cells in serum-free medium with different supplements. At day zero, sfBIT cells were washed three times with RPMI 1640 without supplements and then suspended to 1×10^5 to 2×10^5 cells per ml with RPMI 1640 supplemented with insulin and transferrin (Δ), insulin ($\circ$), or transferrin ($\bullet$). At the times indicated, 5 ml of cells was collected by centrifugation in triplicate and frozen at -20°C . The quantity of DNA in each sample was determined as described in the text.

RESULTS

Growth of sfBIT cells and development of subline sfBT. sfBIT cells were selected for growth in serum-free RPMI 1640 supplemented with insulin and transferrin (52). It was not determined whether both supplements were required for continuous growth once the cell line was established. To determine this, three cultures with 1×10^5 to 2×10^5 sfBIT cells per ml were prepared in RPMI 1640 which contained insulin or transferrin or both supplements. The cultures were examined for increases in DNA during 10 days of incubation (Fig. 1).

An increase in DNA above that at day zero was detected after 5 days in the culture containing insulin and transferrin and after 7 days in the culture with transferrin as the only protein supplement. The quantity of DNA did not increase in the culture with insulin as the only protein supplement. Cell doubling times were 2.5 days in the culture containing both supplements and 6.2 days in the culture with transferrin as the only protein supplement. These values were determined from the slopes of the lines connecting the datum points from days 7 to 10.

Incubation of the cultures supplemented with insulin or transferrin (Fig. 1) was continued for several weeks. Cells with transferrin continued to proliferate as determined by increases in culture density and medium acidity. This subline of sfBIT cells, designated sfBT, continues to grow after 1 year in medium with transferrin as the only protein supplement. The culture viability was greater than 95% once the cells had adapted to the new growth medium. There was no evidence of growth in the culture with insulin during the extended incubation period. The viability of the cells with insulin alone decreased with time, and the culture ultimately had no viable cells.

Growth of sfBT cells with human transferrin. Conditions for optimal growth of sfBT cells in medium with human

FIG. 2. Growth of sfBT cells with human transferrin. sfBT cells were washed as described in the legend to Fig. 1 and then suspended in triplicate to 1×10^5 to 2×10^5 cells per ml with 2 ml of fresh RPMI 1640 containing human transferrin. The quantity of [^3H]dTMP incorporated into acid-insoluble material during the last 24 h of a 96-h incubation period at 37°C was determined as described in the text. The logarithmic scale denotes the concentration (micrograms per milliliter) of transferrin.

transferrin was determined by measuring incorporation of [^3H]dTMP (Fig. 2). Growth was linear in medium with human transferrin at 10^{-2} to $10 \mu\text{g/ml}$. Although [^3H]dTMP was incorporated when transferrin was below $10^{-2} \mu\text{g/ml}$, growth of the cells was not optimal. Transferrin inhibited growth above $10 \mu\text{g/ml}$, with significant inhibition occurring at 10^2 to $10^3 \mu\text{g/ml}$. Incorporation of [^3H]dTMP in medium with $5 \mu\text{g}$ of transferrin per ml was 92% of that observed when transferrin was present at $10 \mu\text{g/ml}$.

Growth of sfBT cells in serum-free medium. Growth of sfBT cells was measured at two culture densities 1 year after they had been passaged in medium without serum and insulin (Fig. 3). At day zero, the cells in an actively growing culture were diluted to 1×10^4 and 1×10^5 cells per ml with fresh RPMI 1640 containing $5 \mu\text{g}$ of transferrin per ml.

The most significant feature of Fig. 3 is that the culture with 1×10^4 cell per ml was growth arrested for the first 7 days of incubation. The inability of cells to grow optimally at low culture density made it possible to develop a sensitive assay to measure GPA (see Materials and Methods).

A second feature of Fig. 3 is that growth of sfBT cells was linear immediately after they were diluted with fresh serum-free medium to 10^5 cells per ml. The linear increase in cell number continued until the culture density approached 10^6 cells per ml, which occurred approximately 10 to 12 days after dilution. After 12 days without refeeding, the number of cells in the culture decreased.

A third feature of Fig. 3 is that sfBT cells exhibited a greater rate of growth after long-term passage (Fig. 3) than they did when they were initially removed from the sfBIT medium with insulin and transferrin (Fig. 1). The cell doubling times of the cultures, compared at 7 to 10 days of growth, were 2.8 days (Fig. 3) and 6.2 days (Fig. 1). The greater rate of growth exhibited in Fig. 3 indicated that the cells removed from the sfBIT culture had adapted to the new growth medium. After adaptation, the growth rate of sfBT cells (Fig. 3) was essentially identical to that of parental cells grown with both protein supplements (Fig. 1).

FIG. 3. Culture density versus growth of sfBT cells with fresh serum-free medium. At day zero, sfBT cells were diluted with fresh medium containing $5 \mu\text{g}$ of transferrin per ml to 10^4 (●) and to 10^5 (○) cells per ml. At the times indicated, 5 ml of cells was removed from each culture in triplicate and the quantity of DNA was determined as described in the text. The insert shows the ordinate expanded for the culture at day zero with 10^4 cells per ml.

Cell morphology and adherence to culture plasticware. sfBIT and sfBT cell lines differed in their capacities to adhere to culture plasticware. sfBIT cells adhered firmly to the bottom of the culture flask and gave rise to boluses of cells once the cells became confluent (52). sfBT cells were weakly adherent and readily detached by gentle shaking. Most of the cells in sfBIT cultures were fibroblastic in morphology, whereas most of those in sfBT cultures were spherical.

Is exposure to extrinsic virus-inducing agents required for production of transforming EBV in serum-free cultures? It was shown previously that sfBIT cells produced transforming virus during serum-free growth if they were exposed simultaneously to two EBV-inducing agents (52). This finding established that the virus lytic-cycle-inducing factors in serum (3) were not necessary for induction and full expression of the EBV replicative phase.

It was not established in the earlier study that noninduced cultures of serum-free cells would produce EBV. The findings in this study establish that noninduced cultures of sfBIT and sfBT cells produced EBV with titers of 10^3 to 10^5 transforming U/ml. These findings are included in Table 1, which summarizes the characteristics of both serum-free cell lines.

Detection of growth-promoting factors in serum-free media. A procedure that measures GPA was established (see Materials and Methods) and then used to compare fresh and spent media (Fig. 4). At all concentrations of spent media (5 to 100%), incorporation of [^3H]dTMP by sfBIT cells was greater than with fresh medium (0%). Twenty percent spent medium yielded maximum incorporation of [^3H]dTMP. Repeated experiments of this type showed that the GPA varied with the serum-free culture. Maximum GPAs were generally obtained with spent medium adjusted to 5 to 40% with fresh serum-free medium.

Detection of GPA in a salt-free concentrate of spent medium. The data in Fig. 4 illustrate that the GPA of spent

TABLE 1. Characteristics of sfBIT and sfBT cell lines^a

Cell line	Origin (cell line)	Plasticware adherence	General cell morphology	Mean doubling time (days)	Maximum density (cells/ml)	RPMI 1640 supplements ^b	Transforming, U/ml of culture
sfBIT	B95-8	Strong	Fibroblastic	2.5	1×10^6	I, T, S	10^4 – 10^5
sfBT	sfBIT	Weak	Spherical	2.8	5×10^5 – 1×10^6	T, S	10^3 – 10^4

^a The viability of both cell lines was 95%, both contained autocrine growth factors, and both had no serum requirements for growth.

^b I, Insulin; T, transferrin; S, selenium.

medium increased when it was diluted with fresh medium. This finding is consistent with depletion of essential growth substances or accumulation of inhibitory metabolites during proliferation of the cells. The GPA of spent medium increased without dilution when it was dialyzed and subsequently concentrated by lyophilization (Fig. 5). Moreover, the data illustrate that a direct relationship exists between the quantity of [³H]dTMP incorporated and the concentration of spent medium used in growth factor assays, from 0.01 to approximately 10 times the original medium concentration.

Does EBV affect GPA? Spent media used for growth factor assays in this study contained transforming EBV. To determine whether the virus affected the GPA measurements, the concentration of EBV was reduced in a culture of sfBT cells by centrifugation and then the GPA of the conditioned medium was determined. The GPA was not affected when EBV was removed from spent medium or when it was returned to spent medium at a concentration which exceeded that of the original culture (Table 2).

DISCUSSION

A mechanism to account for maintenance of growth transformation which is associated with EBV infection was explored with two serum-free B-cell lines (sfBIT and sfBT). The cell lines are unique among the continuous cultures of EBV-transformed tamarin cells because they proliferate

without serum in RPMI 1640 that contains insulin and transferrin (sfBIT) or transferrin alone (sfBT) as a protein supplement. Thus, the media used for growth of both cell lines were chemically defined. sfBIT and sfBT are the only cell lines that produce transforming EBV and proliferate continuously in serum-free medium.

It was suggested in earlier work by others that EBV-transformed human B cells secrete autostimulatory factors when incubated for a short term (3 days) in medium without serum (19, 58). The present study extends the earlier work with continuous serum-free tamarin lines that were derived from cells (B95-8) which produce the prototype strain of EBV. We show that during growth in serum-free medium autostimulatory factors with a molecular weight of 3,500 or greater are secreted and required at a threshold concentration by the cells for continuous proliferation.

The identities of the growth-promoting factors are currently being established. There is certainty with sfBIT and sfBT cell lines that the growth factors in culture media were not derived from serum or one of its metabolites. Moreover, serum components internalized by and attached to the surfaces of cells cannot be eliminated by extensive washing and therefore continue to influence metabolism during the period that immediately follows the transfer of cells to serum-free medium. Whereas this may be a problem in assays which use cells grown with serum, it was not a

FIG. 4. Response of sfBIT cells at low culture density to spent culture medium. sfBIT cells were collected by centrifugation and suspended in 5 ml in triplicate at low culture density (5×10^4 cells per ml) with fresh serum-free medium (0%), fresh serum-free medium containing spent medium (5 to 80%), or spent medium (100%). The quantity of dTMP incorporated during the last 4 h of a 52-h incubation period at 37°C was determined as described in the text.

FIG. 5. Response of sfBT cells at low culture density to concentrated spent medium. sfBT spent (○) and fresh (●) culture media were dialyzed to remove salt and then concentrated by lyophilization as described in the text. The concentrates were diluted with fresh medium and then assayed for GPA by using triplicate 2-ml cultures at low culture density (10^4 cells per ml). The quantity of [³H]dTMP incorporated during the last 24 h of a 96-h incubation period at 37°C was determined as described in the text. Zero on the logarithmic scale indicates the dilution that yielded the original medium concentration.

TABLE 2. GPA of virus-free spent medium^a

% Spent medium	EBV content	[³ H]dTMP incorporation/2 × 10 ⁴ cells (cpm)
0	Not present	1,178
25	Removed	6,575
25	0.4-fold	6,965
25	2-fold	6,664
100	Removed	876

^a The concentration of EBV was reduced in an sfBT culture by centrifugation (22,000 × g, 90 min, 4°C), and then the medium was diluted with fresh serum-free medium (25%) or not diluted (100%). For assay of GPA, sfBT cells were washed three times with fresh medium and then suspended (2 × 10⁴ cells) in 2 ml in triplicate with fresh medium (0%), spent medium without EBV (100%), or a mixture of fresh and spent media (25%) without virus or with EBV at 0.4- and 2-fold the original culture concentration. Assays for GPA were performed as described in the text.

problem in the present study because sfBIT and sfBT cells were cultured for 1 year in serum-free medium before use in growth factor assays.

Secretion of lymphokines, such as interleukin-1 (IL-1) (36, 48, 58), IL-4 (40, 41), and IL-6 (57), by either EBV-transformed B cells or activated normal cells affects the growth of cells latently infected with EBV. EBV-transformed human epithelial cells passaged in nude mice produce IL-1α (6). The B-cell activation antigen BLAST-2, which is induced by EBNA-2 (59), is an autocrine growth factor when it is shed from normal and EBV-transformed B cells (56).

The earlier findings and those in the present study indicate to us that cells latently infected with EBV exhibit a stage-specific growth factor association. This is consistent with the capacity of EBV to arrest developing hematopoietic cells at a particular point during their progression from pre-B cells to immunoglobulin-secreting plasma cells (7, 16, 23, 32, 45, 63).

The findings reported here and in our previous study (52) establish that neither serum components (3) nor extrinsic virus-inducing agents, such as phorbol 12-myristate 13-acetate and *n*-butyrate, are required to convert cells latently infected with EBV to those which produce a transforming virus. Thus, the components of serum that induce EBV early antigens (3) are probably not essential for expression of EBV genes, such as ZEBRA (21), whose functions include disruption of latency.

EBV was recovered in a virus concentrate after exposure of serum-free sfBIT cells to virus-inducing agents (52). Our initial attempts to recover EBV directly from serum-free culture media without exposure of cells to virus-inducing agents were not successful. We attribute the detection of transforming EBV in this study to bovine serum albumin, which was added to stabilize the virus when spent medium was filtered before dilution and inoculation of umbilical cord blood cells.

Synergistic activation of normal cells with UV-inactivated EBV and B-cell growth factor was reported recently (30). In the present study, we removed EBV from a culture by centrifugation and demonstrated that the GPA of spent media with or without EBV were essentially the same. These findings are consistent with different mechanisms for maintenance and initiation of growth transformation in EBV-transformed B cells.

A provisional assignment is that EBV induces a growth factor-mediated malignant response in tumor B cells in vivo. Accordingly, tumor cells with EBV DNA would constitutively express autocrine growth factors which would free them from factors in serum that regulate the mitogenic

response. This hypothesis can be tested in vitro with cells derived directly from B-cell tumors now that the conditions for serum-free growth of EBV-transformed B cells have been established.

ACKNOWLEDGMENTS

This work was supported by a grant from the American Cancer Society, Ohio Division, and by Ohio State University core grant CA-16058.

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