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Clonal Transformation of Adult Human Leukocytes by Epstein-Barr Virus

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We have developed a clonal transformation assay for Epstein-Barr virus which uses adult human leukocytes as target cells. The target cells were isolated from Epstein-Barr seronegative donors, and the same donor's cells could be studied repeatedly over long periods of time. When these cells were transformed by Epstein-Barr virus and had proliferated sufficiently to be studied, they had an average cloning efficiency of 3%. Assuming this average cloning efficiency obtains at the onset of transformation, we calculate that transformation by Epstein-Barr virus leads to immortalization maximally of about 1 in 30 of the adult peripheral leukocytes exposed to the virus. Studying the number of colonies transformed as a function of the amount of virus to which the cells are exposed indicates that a single DNA-containing virus particle is sufficient to transform a cell. All of the transformed clones studied harbored viral DNA. This technique will now permit, for the first time, our studying clonal variations in adult peripheral leukocytes transformed by Epstein-Barr virus as a function of input multiplicity of the virus and of the donor's immune status.

The capacity to transform human lymphocytes in tissue culture is a distinctive biological property of Epstein-Barr virus (EBV). Transformation by EBV means that the transformed cells will proliferate indefinitely in tissue culture. This property has been used in an end point dilution assay to quantify EBV (4, 10). Yamamoto and Hinuma (16) have introduced a clonal transformation technique in which colonies of umbilical cord leukocytes are transformed by EBV and cloned directly in agarose. Their technique, however, is not readily adapted to studying the clonal transformation of adult human leukocytes, because the frequency of the event under their conditions is too low for easy study. We have developed a clonal transformation technique for adult human leukocytes which uses a human fibroblast feeder layer covered with agarose and over which the infected leukocytes are suspended in a second layer of agarose. This technique can be used to assay EBV, and it allows us to study the variety of cell types EBV transforms early after infection, to analyze the numerical balance between viral and host genomes, and to monitor the alterations that arise in clones of transformed cells as they are passaged in tissue culture. In particular, this method permits the study of the in vitro transformation of cells derived from donors who are susceptible to infectious mononucleosis, a disease in which some peripheral lymphocytes are found to harbor EBV antigens and from which EBV-transformed cells can be rescued (7).

MATERIALS AND METHODS

Cells. B95-8 cells (9) were freshly cloned by end point dilution on gamma-irradiated human fibroblast monolayers. Clones that had 5% of their cells containing viral capsid antigen were grown in RPMI 1640 medium containing 5% calf serum and antibiotics (200 U of penicillin per ml plus 200 μg of streptomycin per ml). They were adapted to grow in suspension cultures, where they remained virus producers for different lengths of time (usually 3 to 9 months). They were found negative for pleuropneumonia-like organism (PPLO) contamination when tested for uridine phosphorylase activity (8).

Two human fibroblast strains, WI38 and 356 (a gift of Robert DeMars), were grown in Dulbecco medium containing 10% fetal bovine serum and antibiotics.

Adult human mononuclear leukocytes were isolated from four EBV-seronegative donors (aged 21 to 25) by centrifugation of EDTA-chelated blood through a cushion of Ficoll plus Renografin. This is essentially the method of Böyum (2) except that we have substituted Renografin for Hypaque.

Karyotypic analysis was performed as described by Hsu and Pomerat (5).

Virus. EBV was harvested from the supernatant of B95-8 cell cultures which had grown to saturation. The cells were removed by low-speed centrifugation, and the supernatant was passed through a 0.22-μm membrane filter (Millipore Corp.) to insure removal of viable cells. These filtered virus pools were stored at 4°C and generally used without further

concentration or purification. When we wished to determine virion DNA concentrations, we layered 20 ml of a virus pool over 15 ml of 20% sucrose in phosphate-buffered saline (3) and centrifuged the solutions at 25,000 rpm for 60 min at 4°C in an SW27 rotor. The DNA was then extracted from the viral pellet by digesting it in 100 μl of 0.01 M EDTA, 0.01 M Tris-hydrochloride (pH 9.0), self-digested Pronase (1 mg/ml), and 1% sodium Sarkosyl at 37°C for 2 h. This DNA was assayed without further purification.

Labeling viral DNA. EBV DNA was purified as described (14). It was labeled in vitro by nick translation in a manner similar to that of Kawai et al. (6).

DNA:DNA renaturation. DNA:DNA renaturation studies were performed as described (14).

Cloning of EBV-infected lymphocytes. Tissue culture dishes (15 to 60 mm in diameter) were seeded with human fibroblasts (10^5 cells for 15-mm wells and proportionately more for larger surface areas). The cells were allowed to settle for 24 h or longer and then overlayed with a solution (0.5 ml for 15-mm wells) of RPMI 1640 plus 10% fetal bovine serum plus antibiotics in 0.5% (wt/vol) agarose (Seaplaque, Main Colloid Corp., Rockland, Maine). The agarose solution was gelled rapidly by placing the plates at 4°C for 5 to 10 min. If transformed clones were to be picked from these plates, the feeder layers were irradiated (4,000 rads) at this stage with a gamma-emitting source. They could be kept at 37°C for up to 2 weeks before being used.

Purified mononuclear leukocytes in RPMI 1640 plus 10% fetal bovine serum plus antibiotics (complete medium) were incubated with dilutions of EBV and rocked on a reciprocating platform for 4 to 8 h at room temperature. To 9 volumes of these infected cell suspensions at 37°C was added 1 volume of 3 or 3.5% (wt/vol) agarose in phosphate-buffered saline (3), which had been equilibrated to 42°C. The cells and agarose were mixed thoroughly, and samples (0.5 ml for 15-mm wells) were added to the feeder layers already covered with agarose. This second layer of agarose plus cells was also gelled by placing the plates at 4°C for several minutes. The plates were incubated in a humidified CO_2 incubator at 37°C for 2 days, sealed with adhesive tape to minimize dehydration, and placed back in the incubator. Between 12 and 14 days after plating, the clones were fed with an additional volume (0.5 ml for 15-mm wells) of complete medium in 0.35% agarose. Clones were counted approximately on days 21 and 28 after plating.

Harvesting clones. Clones were identified with a microscope by marking the plate immediately beneath the clone, and only those that were well separated from one another were then picked with a Pasteur pipette tip. The agarose plug was suspended in 1 ml of complete medium, blended in a Vortex mixer, and plated directly on gamma-irradiated feeder layers of fibroblasts without an intervening layer of agarose. The progeny of the clones were subsequently passaged on gamma-irradiated fibroblast feeder layers, and at each passage a sample of cells was added to tubes with complete medium but no feeder layer. When clonal progeny grew successfully in tube cultures without feeder layers, they were further maintained only in tubes or glass bottles.

RESULTS

EBV transforms human mononuclear leukocytes in tissue culture. When we have plated 10^4, 10^5, or 10^6 mononuclear leukocytes per ml from four EBV-seronegative donors, we have consistently failed to observe clones of proliferating cells under our assay conditions. At the higher cell concentrations used, clumps of cells can be found at the beginning of the experiment which persist as nondividing cells for several weeks. When these clumps are picked and passed on fibroblast feeder layers, they do not proliferate. The presence of these clumps can be avoided by plating fewer than 10^5 cells per ml of agarose. The results in Table 1 indicate that EBV first treated with serum not containing antibodies to EBV-associated antigens successfully transforms human leukocytes. The results of Table 1 further support the notion that EBV is the agent responsible for the transformation, because treatment of the virus pool with human sera containing antibodies to EBV-associated antigens prevents the transformation events.

The transformed colonies scored by this assay largely are proliferating clones. In five separate experiments we have picked 86 clones from which we have rescued 28 permanent lines. We believe that the reason only 25 to 45% of the clones in the separate experiments are found to

TABLE 1. *Colonies counted on day 24 after infection with EBV pretreated with human sera negative or positive for antibodies to EBV viral capsid antigens*[a]

Well no.	EBV-positive serum 1	EBV-positive serum 2	EBV-negative serum
1	0	0	4
2	0	0	0
3	0	0	7
4	0	0	4
5	0	0	2
6	0	0	3
7	0	0	3
8	0	0	6
9	0	0	6
10	0	0	4
11	0	0	3
12	0	0	2

[a] Samples of EBV were incubated with equal volumes of 1:4 dilutions of human sera for 90 min at 37°C. Cells from a single seronegative donor were exposed to the treated virus samples for 8 h with rocking, and 5×10^3 cells were plated in each well. Colonies were scored 24 days later. Approximately 1 cell per 1,100 exposed to the virus treated with the EBV-negative serum formed a colony.

proliferate is that our method of mechanically picking the clones probably misses many, which therefore never get transferred to the feeder layer. In addition, not all picked clones escape from their agarose plugs.

The transformation of adult human mononuclear leukocytes under our conditions is not general to all DNA-transforming viruses. Infecting 10^6 mononuclear leukocytes with 10 or 50 PFU of simian virus 40 per cell (four replicate cultures at each dose) led to no transformed colonies, whereas infection under similar conditions with 1 ml of an EBV virus pool led to more transformed colonies than could be counted.

EBV transforms adult human mononuclear leukocytes in a dose-dependent fashion. The results shown in Fig. 1 indicate that diluting a virus stock by 10 and infecting with this diluted stock results in approximately one-tenth as many transformed colonies. The slope of the graph in Fig. 1 is therefore consistent with one particle of EBV being sufficient to transform one target cell, as has been found by Yamamoto and Hinuma (16) and Henderson et al. (4).

Parameters that might affect colony formation by EBV-transformed mononuclear leukocytes. We tested several obvious parameters of our cloning conditions to ascertain which of them particularly affected the efficiency of colony formation. Two parameters were found to be influential in determining the efficiency of colony formation: the brand of agarose used and the presence of a fibroblast feeder layer. Several brands of agarose did not form stable gels at a concentration of 0.3% (wt/vol) and therefore could not be used. The elimination of a fibroblast feeder layer reduced the efficiency of colony formation at maximum virus input from 1 per 1,500 cells plated to 1 per 10^5 to 2×10^5 cells plated. This decreased frequency of colony formation by EBV-infected adult human mononuclear leukocytes would preclude many interesting transformation experiments, and it is therefore desirable to include a fibroblast feeder layer in the assay. We found that neither the addition of 2-mercaptoethanol (25 to 75 μM) nor the removal of glass-adherent cells affected the efficiency of colony formation of this assay.

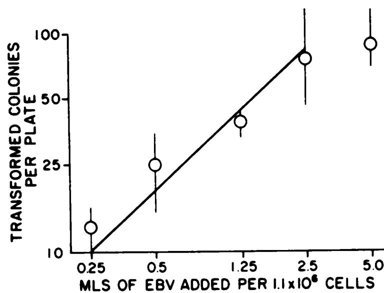

FIG. 1. *Adult leukocytes exposed to EBV were plated in 60-mm dishes to facilitate the counting of high numbers of colonies. Five dishes were each plated with 1.5×10^5 cells for each concentration of virus. The vertical bars for each point represent ± the standard deviation of the mean of each point. The multiplicity of infection ranges from about 0.5 to 20 EBV DNA-containing particles per cell. The data for the highest ratio of virus to cell are, within our error, identical to those for the second highest ratio, indicating that all of the cells that can be transformed (about 1 per 1,500 cells exposed) are transformed with a multiplicity of infection of about 10 DNA-containing particles per cell. No colonies were found when uninfected leukocytes were plated; no colonies were found when a concentrated virus solution was plated, indicating that the virus stocks are free of viable cells.*

Properties of EBV-transformed clones. The cloning efficiency of the isolated EBV-transformed adult human lymphocytes is low relative to that of established lymphoblastoid cell lines. The results of several recloning experiments are presented in Table 2. The cloning efficiency of Raji is about 80%, whereas that of the newly transformed colonies is on the order of 3%. A second result shown in Table 2 is that given our cloning conditions, the cloning efficiency of the EBV clones is independent of the number of transformed cells seeded.

We have determined the chromosome number of four clones. Each is diploid. We have made these determinations at about generations 30, 40, 50, and 60 for each of the clones, and so far have found a modal number of 46 for each determination.

The EBV-transformed clones contain viral

TABLE 2. *Cloning efficiencies of EBV-transformed leukocytes*

Clone	No. of cells seeded per well	No. of wells counted	Mean no. of colonies per well ± standard deviation of the mean	Cloning efficiency (%)
A	10^3	18	14 ± 4	1
A	5×10^3	12	74 ± 10	2
B	5×10^2	29	16 ± 5	3
C	10^3	17	63 ± 18	6
D	5×10^2	5	6 ± 3	1
D	10^3	22	14 ± 9	1
D	5×10^3	17	53 ± 13	1
D	10^4	11	92 ± 22	1
E	5×10^2	15	20 ± 6	4
E	10^3	17	45 ± 10	5
Raji	15	22	12 ± 5	80
Raji	30	12	23 ± 4	80

DNA sequences. The results of experiments measuring the increased rate of renaturation of purified, in vitro labeled EBV DNA by total cell DNA isolated from several EBV-transformed clones indicate that these transformed clones contain between 2 and 80 genome equivalents of EBV DNA. In addition, experiments similar to those of Botchan et al. (1) indicate that the fragments generated by *Eco*RI endonuclease digestion of isolated viral DNA are each represented in four of the freshly transformed clones (Sugden, manuscript in preparation). Therefore, the clones of adult human leukocytes freshly transformed by EBV harbor viral DNA of the same or nearly the same sequence complexity as the DNA found in the virus stocks used to transform them.

Absolute efficiency of transformation of adult human leukocytes by EBV. From the data presented in Table 1 and Fig. 1, we can conclude that when adult human leukocytes are infected with excess EBV, between 1 in 1,000 and 1 in 1,500 cells forms a colony under the conditions of our assay. We have found this maximum number of colonies to be formed with leukocytes from each of the four donors tested. From the experiments presented in Table 2, we know that the cloning efficiency of newly transformed lymphoblasts is on the order of 3%. If we assume that the cloning efficiency of cells immediately after transformation by EBV is similar to that which we determine in our cloning experiments, which are conducted about 30 to 40 generations after transformation, then about 1 in 30 to 1 in 50 of the adult peripheral leukocytes we isolate is capable of being transformed by EBV. (Our assumption, which is practically untestable, is partially supported by our finding that the cloning efficiencies of four clones did not change significantly between cell generations 30 and 60.)

Absolute infectivity of EBV. We have measured the number of viral DNA-containing particles in our virus stocks by first pelleting the virus through a sucrose cushion, extracting DNA, and determining its concentration by a reassociation kinetics assay. The results of one such assay are shown in Fig. 2. They indicate that one stock of virus has approximately 5×10^6 DNA-containing particles per ml, assuming that each particle can contain only one molecule of DNA. We repeated this measurement but first treated the virus stock with 20 μg of DNase I per ml in 0.01 M $Mg(O_2C_2H_3)_2$ at 37°C for 60 min before pelleting it. In this second determination we found the stock to contain 2×10^6 DNA-containing particles per ml (data not shown). Our method is not sufficiently accurate to permit us to conclude whether this difference is a result of the DNase digestion or the errors inherent in our method. A total of 1.8×10^6 adult human leukocytes were exposed to 50 μl of this stock of virus, and 95 transformed colonies grew out. Given the assumed initial average cloning efficiency of 3%, we calculate that about 2,900 cells were transformed by exposure to 10^5 to 2.5×10^5 DNA-containing EBV particles, which indicates that at least between 1 in 30 and 1 in 100 of the EBV particles is infectious.

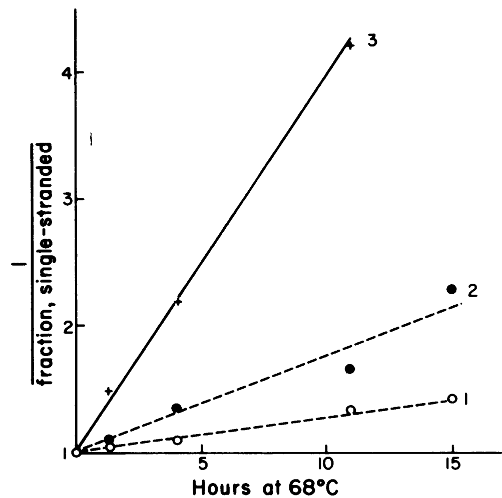

FIG. 2. *DNA extracted from pelleted EBV particles from a stock of virus used for our transformation experiments was assayed for its content of viral DNA by reassociation kinetics. The dashed lines represent renaturation of the labeled viral DNA in the presence of known amounts of purified viral DNA plus carrier calf thymus DNA: no. 1 = 4 ng of EBV DNA; no. 2 = 13 ng of EBV DNA. The solid line represents the renaturation of DNA extracted from a virus stock and equals 40 to 50 ng of viral DNA, which was extracted from particles pelleted from 45 ml of the stock. This finding indicates that the stock contained about 5 × 10⁶ DNA-containing particles per ml as determined by this assay, assuming that each particle contains no more than one molecule of viral DNA.*

DISCUSSION

EBV transforms or immortalizes adult human leukocytes in tissue culture. We have shown that EBV is the transforming agent in our clonal assay by the sum of several observations: first, transformation is prevented when virus is treated with EBV-neutralizing antisera; second, the number of cells transformed is proportional to the number of EBV particles to which the cells are exposed; and finally, all proliferating clones harbor EBV DNA. Our as-

say conditions do not permit transformation of adult human leukocytes by all transforming DNA viruses. In particular, simian virus 40, which transforms human fibroblasts, does not transform human leukocytes under the assay conditions we use.

This transformation assay requires a feeder layer but is not affected by the presence of macrophages or 2-mercaptoethanol. Yamamoto and Hinuma (16) did not use a feeder layer in their transformation assay, which used human umbilical cord cells as target cells. They found that under the conditions of their assay adult human leukocytes were transformed 100 to 1,000 times less efficiently than were umbilical cord cells. This frequency was too low to be useful. Our including a feeder layer permits up to 1 per 1,000 adult leukocytes to form a transformed colony, which is particularly desirable because now the leukocytes of a single donor may be studied in many subsequent experiments. Pope et al. (12) found that macrophages promoted their end point transformation assay for EBV, whereas we have found that removing all glass-adherent cells does not affect our transformation assay. This observation is consistent with the feeder layer's providing the same functions as the macrophages in the end point assay, which does not use a feeder layer. The efficiency of the clonal transformation technique is not affected by 2-mercaptoethanol in the range of 0 to 75 μM. This observation demonstrates a difference in the growth requirements of transformed primary human and murine leukocytes. Efficient transformation of murine leukocytes by Abelson virus requires 2-mercaptoethanol (13).

The transformed clones have different numbers of copies of viral DNA per cell. We do not know if these numbers reflect the multiplicity of infection for those clones we have studied to date. However, we have now established nine clones that are the progeny of a transformation experiment with a multiplicity of infection of 0.1 DNA-containing particle per cell. Determining the number of viral DNA copies per cell in these and similar clones should aid in determining whether transformation by EBV is associated with disproportionate replication of the viral genome.

We have calculated that about 2 to 3% of the peripheral leukocytes of adult donors are capable of being transformed by EBV. This conclusion is a result of our having found that 1 cell per 1,000 forms a colony after exposure to excess virus and that transformed clones, once they have divided sufficiently to be studied, have a cloning efficiency of about 3%. Our calculation is based on our assumption that the cloning efficiency of the transformed cells does not change from the onset of transformation until the time we can test it. Both the transformation and cloning efficiencies that we find for adult human leukocytes are similar to those found for human umbilical cord leukocytes by Henderson et al. (4) using a transformed-center assay. These authors, however, found the transformation and cloning efficiencies of adult leukocytes to be significantly lower than that of umbilical cord leukocytes in their assay. Henderson et al. (4) have shown that depleting an umbilical cord leukocyte population of T-cells increased its efficiency of transformation. Pattengale et al. (11) have shown that EBV-transformed cells carry surface immunoglobulin molecules. These two findings indicate that the B-lymphocyte is probably the target cell for transformation by EBV. Winchester et al. (15) have shown that approximately 10% of human peripheral leukocytes bear immunoglobulin molecules, that is, are B-lymphocytes. Our finding that only 3% of peripheral leukocytes is capable of being transformed by EBV is indirect; we cannot now conclude whether all or only a subset of all adult B-lymphocytes are susceptible to being transformed by EBV.

EBV has a high absolute infectivity. Given our assumption of the constancy of cloning efficiencies with cell passage, we find at least between 1 in 30 and 1 in 100 DNA-containing particles is infectious. This number is similar to that found by Henderson et al. (4). In addition, we have found that virus stocks stored in 10% fetal bovine serum plus medium maintain this level of infectivity at 4°C for up to 6 months.

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