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# Epstein-Barr Viral Antigen in Single Cell Clones of Two Human Leukocytic Lines

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A method was devised for obtaining single cell clones of human leukocytic cell lines in the presence of a human placental cell feeder layer. Clones of two lines, LS-B and $EB_3$, which contain Epstein-Barr viral (EBV) antigen in approximately 1% of cells were tested for EBV antigen. Since all $EB_3$ clones and LS-B clones contained EBV antigen, it is concluded that in vitro EBV genome is associated with all of the cells.

Many established cell culture lines derived from Burkitt tumors, blood leukocytes of patients with infectious mononucleosis, leukemia, and other diseases, and from blood of normal persons harbor the Epstein-Barr virus (EBV). EBV is seen in less than 10% of cells whether the cells are examined with an electron microscope or by immunofluorescent assay on acetone-fixed cells. Studies by Henle and Henle (4) and by Epstein and Achong (1) suggested that the same infected cells are identified by electron microscopy and immunofluorescence. Since one major question about the presence of EBV in these cultures is whether the virus is responsible for the continued growth of the leukocytes in vitro, it seemed important to define the virus-cell interaction precisely.

Several models for the EBV-cell relationship were considered. First, leukocytic lines may carry EBV in a small number of infected cells that produce cell-free virus able to infect other cells in the culture. Second, the lines may consist of two distinct populations of cells, infected cells that do not release virus and noninfected cells. Third, all cells in infected lines may contain viral genome, but only a fraction of cells produce complete particles. To choose among these possibilities, clones were derived from single cells of two human leukocytic lines that contain EBV antigen, and these clones were examined for the presence of the antigen.

The use of clones from single cells to study the EBV-cell relationship has been considered in other laboratories as well. Abstracts of such experiments have appeared (B. A. Maurer and S. M. Wilbert, Bacteriol. Proc., p. 195, 1970; G. Kohn and B. A. Zajac, Fed. Proc., **29**:636, 1970) while our work was in progress.

**Cell lines.** The LS-B line was established in our laboratory from the peripheral blood of a child with acute leukemia. LS-B was maintained in culture for 14 months before the clones were derived. It demonstrated EBV antigen in 0.1 to 2.0% of cells on various examinations. The $EB_3$ line, from a Burkitt tumor (2), was obtained from A. S. Evans. When $EB_3$ was examined at random intervals without preincubation either at low temperature or in arginine-depleted medium, approximately 1.0% of the cells stained in the indirect immunofluorescence test (FA test). Both leukocytic lines were maintained on medium RPMI 1640 with 20% fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B. Every week the suspension cultures were divided 2:1 by adding fresh medium. Monolayers of human placental cells (HPC) growing on the bottoms of upright glass tubes (100 by 13 mm) were employed as "feeder layers" (3). HPC were established with Eagle basal medium plus 10% FBS and antibiotics. On the day of cloning, this medium was replaced with 0.5 ml of RPMI 1640 containing 20% FBS. No EBV antigen was detected in HPC. HPC did not grow in suspension and were examined by the indirect FA procedure by using several different human sera with a high titer of EBV antibody.

**Method of cloning.** To obtain single cells, the parent line was diluted to contain approximately 500 to 1,000 intact cells/ml. By means of a capillary pipette, one droplet of cell suspension was placed in a 35-mm plastic petri dish (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.). Individual droplets were examined with an inverted microscope (100× magnification), and microdrops with only one cell were aspirated into a capillary pipette partially filled

with medium. The droplet and medium were then expelled into a tube containing an HPC monolayer, 1 to 2 weeks of age. The tubes, filled with 5% $CO_2$ in air, were incubated upright at 35 C. Spent medium was partially removed without disturbing the cells in the bottom of the tube, and fresh medium was added weekly. When sufficient growth of the clone had occurred, usually after 3 to 4 weeks, the leukocytic cells were cultivated thereafter as suspension cultures without HPC.

Subclones of clone 16 of $EB_3$ were also obtained in 5% human serum containing antibody to EBV (FA antibody titer = 1:160 dilution of serum). In experiment A59, droplets were made by using a cell suspension without antiserum, and then 5% antiserum was included in the medium of individual tubes for 1 week. In experiment A66, cells were preincubated with medium containing 5% antiserum for 1 hr before the droplets were made, and this concentration of antiserum was maintained in the medium during the first week of cultivation.

**Test for EBV.** To test for EBV antigen, the indirect immunofluorescence procedure (1) was employed with acetone-fixed cell spreads. Fluorescein-conjugated rabbit antihuman gamma globulin was purchased from Antibodies, Inc. As a source of EBV antibody, the serum of a healthy adult was used diluted 1:10. Clones of LS-B were also examined with a 1:10 dilution of a serum from a patient with nasopharyngeal tumor. This serum was a gift from W. Henle. Duplicate cover slips were treated with phosphate-buffered saline followed by conjugate, and all slides were evaluated in a coded, double blind fashion.

**Effect of HPC feeder layer on number of cells required to establish a growing culture.** After several unsuccessful attempts to obtain clones of LS-B in microdrops without a feeder layer, it was determined that approximately $10^3$ LS-B cells were required to initiate a growing culture. The initial size of the inoculum was not markedly reduced by adding LS-B cells to feeder layers of human lymphoblastic lines that had received approximately 4,500 r of X-irradiation. Because a growth-promoting effect of HPC was noted in earlier studies of the transformation of normal human leukoyctes into established lines (5), the number of cells in the LS-B line required to initiate a culture was determined in the presence and absence of an unirradiated HPC monolayer (Table 1). In the absence of HPC, 800 or fewer cells failed to multiply. In contrast, a small number of LS cells grew in the presence of HPC. Similarly, 100 or fewer $EB_3$ cells did not start a subculture in the absence of HPC.

**Clones of the LS-B line.** Ten clones were derived from single cells of LS-B and were examined for EBV antigen. Viral antigen was not consistently present and varied from one examination to another. When the test was performed with a healthy adult serum as the source of antibody, four clones contained antigen on more than one test. In two clones antigen was not detected in several assays (Table 2). When the clones were studied with the "Mwangi" serum, all but one clone revealed antigen. When present, the proportion of fluorescent cells was low, usually not exceeding 0.1 to 0.2%.

**Clones of the $EB_3$ line.** All clones of $EB_3$ contained viral antigen (Table 3). The parent line demonstrated about 1.0% cells with antigen, and similarly, each clone showed a low level of fluorescence. All subclones, derived from clone 16 in the presence of human serum containing

TABLE 1. *Effect of a human placental cell feeder layer on number of LS-B cells needed to initiate a culture*

| No. of LS-B cells[a] | No. of growing cultures per no. of tubes inoculated[b] | |
|---|---|---|
| | HPC present | HPC absent |
| $8 \times 10^3$ | 4/4 | 4/4 |
| $8 \times 10^2$ | 4/4 | 1/4 |
| $8 \times 10^1$ | 3/4 | 0/4 |
| $8 \times 10^0$ | 2/4 | 0/4 |
| $8 \times 10^{-1}$ | 1/4 | 0/4 |

[a] Serial 10-fold dilutions.
[b] Period of observation 3 months, with medium changes weekly.

TABLE 2 *Tests for EBV antigen in single cell clones of LS-B line*

| Clone no. | No. positive tests/no. tests performed | |
|---|---|---|
| | Serum 1[a] | Serum 2[b] |
| 7 | 0/5 | 2/2 |
| 36 | 0/7 | 1/2 |
| 9 | 1/6 | 1/2 |
| 14 | 1/6 | 2/2 |
| 24 | 1/1 | NT |
| 40 | 1/6 | 1/2 |
| 23 | 4/5 | 2/2 |
| 25 | 2/6 | 1/2 |
| 42 | 2/7 | 0/2 |
| 49 | 6/6 | 2/2 |

[a] From a healthy adult without a past history of infectious mononucleosis.
[b] From a patient with nasopharyngeal tumor. Both sera used diluted 1:10. NT, not tested. Clone 24 lost after one test for EBV.

TABLE 3. *Summary of studies with single cell clones of two human leukocytic lines*

| Expt | Parent | Anti-serum[a] | No. of single cells cultured | No. of clones | EBV antigen | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | No. tested | No. positive | Percentage with antigen | |
| | | | | | | | Parent | Clones |
| 931L | LS-B | − | 33 | 10 | 10 | 10 | 0.5 | <0.1–1.0 |
| A35 | $EB_3$ | − | 55 | 28 | 25 | 25 | 1.2 | <0.1–1.5 |
| A59 | $EB_3$CL-16 | +[b] | 20 | 13 | 6 | 6 | 0.4 | 0.5–1.5 |
| A66 | $EB_3$CL-16 | +[c] | 16 | 10 | 6 | 6 | 0.7 | <0.1–3.5 |

[a] A 5% concentration of healthy adult serum with EBV titer 1:160.
[b] Present after clones picked.
[c] Present before and after clones picked.

EBV antibody, likewise contained EBV antigen in a small proportion of cells. No qualitative differences were noted in the nature of the fluorescent reaction in any of the $EB_3$ clones, although minor quantitative differences were detected. Giemsa-stained smears of the $EB_3$ clones showed cells that varied in size and some multinucleate giant cells.

These studies of single cell clones suggest that, in the $EB_3$ and LS-B lines, the EBV genome is associated with many more cells than demonstrate EBV antigen. EBV antigen was seen in approximately 1% of each parental line, yet EBV antigen was observed in all LS-B clones on at least one occasion and regularly in 100% of $EB_3$ clones. Since the cloning efficiency was 30 to 60%, it is probable that cells initially devoid of EBV antigen gave rise to clones capable of producing EBV antigen.

Antiserum was used to obtain subclones of $EB_3$ to exclude the possibility that the presence of EBV infection in the clones resulted from viral particles present at the cell membrane. The human serum used in the subcloning experiments contained a high titer of antibody measured in the FA test and also, in preliminary unpublished experiments, inhibited the capacity of EBV to induce continuous growth of normal leukocytes. To determine whether this serum is capable of neutralizing 100% of infectious EBV virions, a more sensitive assay for infectious virus than is presently available is required. Single cell clones of LS-B were not propagated in the presence of antibody, but in an earlier preliminary experiment cultures of LS-B were not cured of EBV infection when maintained in 10% antiserum for 3 months. With these reservations in mind, it seems more likely that infection is maintained in lines LS-B and $EB_3$, not by transmission of virus from infected to uninfected cells, but by passage of viral genetic material from parent cell to progeny.

If the EBV genome is present in all the cells of an infected line, as our results indicate, the low level of viral antigen production in parent lines and in their clones implies a precise mechanism of regulation of the synthesis of viral products and suggests that some viral proteins are not made in most cells. These regulatory mechanisms may differ in the two lines studied, because the level of viral antigen was lower in LS-B clones than in $EB_3$ clones, and on some examinations several LS-B clones failed to demonstrate viral antigen. In future experiments it would be of interest to attempt to induce virus and to demonstrate viral-specific nucleic acid in the LS-B clones.

The findings with cloned tissue culture lines cannot be arbitrarily extrapolated to virus-cell relationships existing in vivo. It may, however, be possible to determine whether all cells of Burkitt's tumor are infected with EBV by obtaining single-cell clones directly from tumor biopsies.

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