COMPARISON OF THE YIELD OF INFECTIOUS VIRUS FROM
CLONES OF HUMAN AND SIMIAN LYMPHOBLASTOID
LINES TRANSFORMED BY EPSTEIN-BARR VIRUS*

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Two related questions led to the present experiments. What proportion of
cells in lymphoblastoid lines transformed by Epstein-Barr virus (EBV) contain
sufficient viral genetic information to code for infectious viral particles and,
by inference, contain the entire EB viral genome? Is the recently described en-
hanced production of infectious EBV by simian lymphoblastoid cell lines due to
differences in all cells or to a subpopulation of productive cells (1)? Both ques-
tions were approached by deriving single cell clones of human and monkey
lymphoblastoid lines known to be producers of infectious virus detectable by
the transformation assay.

All daughter clones of human lymphoblastoid cell lines express EB viral
capsid antigens, provided that the parent line also contains these antigens (2-4).
Therefore at least sufficient viral genetic information to code for capsid antigens
is present in all cells, even though these antigens are demonstrable in less than
10% of human cells at any given time. Furthermore, clones of nonproducer lines
exhibit EBV "early" antigens after treatment with 5'-bromodeoxyuridine
(BUdR) or 5-iodo-2'-deoxyuridine (IUdR) (5). Clones obtained in previous
studies have not been examined for infectious virus; consequently, the propor-
tion of cells containing the necessary information to code for infectious virions
is not known for any lymphoblastoid line. This question is difficult to study with
EBV-transformed human lines, which, if productive, release minute quantities
of virus. In fact, many human lymphoblastoid lines produce neither viral capsid
antigens nor infectious virus even though they contain DNA which is comple-
mentary to EB viral DNA (6, 7). In contrast, EBV-converted lymphoblastoid
lines derived from blood leukocytes of new world primates release 100-1,000
times more infectious virus than comparable human cells (1). Accordingly, the
simian lines provide the means to determine, by cloning, the proportion of cells
capable of producing mature virions.

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1 Abbreviations used in this paper: BUdR, 5'-bromodeoxyuridine; EBV, Epstein-Barr virus;
SW, 10%; human serum with an EBV neutralizing antibody titer of ≥ 1:128; VCA, viral cap-
sid antigen.

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Furthermore, cloning studies should allow a choice between several possible hypotheses to account for enhanced EB virus production by simian as contrasted with human cells. First, simian and human lines might differ in the proportion of producer and nonproducer cells, with more producer cells being present in the simian lines. Second, only a few cells in both human and nonhuman lines might possess the information to release infectious virus, but more virus is released by this subpopulation of simian cells. Third, human lines might contain a subpopulation of cells which are inhibitory to formation of infectious virus by potentially productive cells. Fourth, all cells in both human and nonhuman lines might potentially be producers, but the yield of virus from each activated cell would be greater for simian than human cells. Our results strongly support the last alternative.

Materials and Methods

Cell Lines and Clones.—The human line (883L) was derived spontaneously from peripheral blood leukocytes of a patient with mononucleosis; the nonhuman primate lines were established from blood leukocytes of a squirrel monkey (line B84-15) and a cotton-top marmoset (line B95-8) after exposure to the Hawley strain of EB virus in the form of a filtered extract of line 883L (8). The culture medium for maintenance of cell lines and for cloning experiments was RPMI 1640 with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 #g/ml), and amphotericin B (1 #g/ml).

To obtain clones logarithmic phase cells were resuspended at a concentration of 10^5/ml in complete medium. To eliminate the contribution of extracellular virus or virus adsorbed on the surface of cells 10% human serum with an EBV neutralizing antibody titer of > 1:128 (9) was added. This serum (SW) was obtained from a patient 6 mo after infectious mononucleosis. The cell-serum mixture was held at 37°C in a water bath for 1 h and then further diluted to 500-1,000 cells/ml. Clones were initiated by placing microdrops with single cells onto feeder layer of human placental cells (2). The same placental cell strain which is free of detectable EBV antigens was used for all experiments. Once growth of a clone was evident, subcultures thereafter were made in the absence of feeder cells. Clones were maintained by weekly replacement of one-half the volume of cell suspension with fresh medium.

Assay for EBV Antigens in Clones.—Viral capsid antigens were measured by the indirect immunofluorescence technique (10). EBV complement-fixing (CF) antigens were detected by the standard microtiter CF test, or by the recently described anticomplement immunofluorescence test (11). Clones were examined for viral capsid and early antigens after treatment with 5'-bromodeoxyuridine (5, 12-14). Reference human sera with and without EBV antibody were used to measure all antigens. SW was the antibody-positive serum, and LH, from a healthy adult, was the antibody-negative serum. The human serum pool with high titer antibody to early antigen was a gift from W. Henle (15).

Preparation of Clones for Determination of Amount of Infectious EBV Released.—The cell concentration of each clone was adjusted to 3 × 10^5/ml in fresh medium. The clones were incubated at 35°C for 1 wk; they reached a maximum density of 1.0-1.5 × 10^6 cells/ml. Cells were sedimented at 500 g for 10 min, the pellets were discarded, and the supernatant fluids were rapidly frozen and thawed three times and passed through a 0.8 #m Millipore filter. In one experiment cell-associated infectious virus in clones of the human line was determined in an extract prepared from 10^9 cells/ml. Cells were resuspended in fresh medium, frozen and thawed, and filtered.

Transformation Assay for Infectious EBV Released by the Clones.—The content of infectious EBV in the filtered fluids was assayed by transformation of primary umbilical cord leukocytes. To prepare human cord leukocytes approximately 20 ml of blood obtained steriley from the
umbilical cord was placed in a tube with 0.2 ml of heparin (200 U). Red blood cells were sedimented by addition of sufficient 3% (wt/vol) dextran (mol wt 280,000) to bring the final concentration to 1%. The supernatant obtained after 45 min gravity sedimentation at room temperature was centrifuged at 500 g for 10 min, and the resultant cell pellet was washed three times by centrifugation in Puck's saline A. Cells were reconstituted at 10^6/ml in medium RPMI 1640 with 20% fetal calf serum and antibiotics. Gas phase was 5% CO2/air. After overnight incubation at 35°C, the cell concentration was readjusted to 10^6/ml and 0.8 ml of cell suspension was dispensed to individual 150 x 16 mm tubes. An end-point dilution assay was employed; four replicate cultures were inoculated with 0.2 ml of each 10-fold virus dilution. Each assay was examined at 2 to 3-day intervals and held for 60 days. End points, expressed as 50% transforming doses (TDs0) per 0.2 ml, were calculated by the Reed Muench formula (16). Transformation was marked by the appearance of large cell clumps, acid production, and rapid increase in the number of cells. These signs first appeared approximately 21 days after addition of virus. In each transformation assay there were four replicate cultures of cord leukocytes unexposed to EBV; these always failed to transform. To control for possible variations in different lots of cord leukocytes, a titration of a stock of EBV was performed in each test. Two reference virus stocks were prepared from frozen and thawed, filtered supernatant fluids of transformed marmoset leukocytes (line B95-8).

Preparation of Virus for Electron Microscopy.—From each clone supernatant fluids collected 1 wk after subculture were examined for viral particles. 60 ml of spent medium was centrifuged at 2000 rpm for 10 min in an International PR-2 centrifuge. The supernatant fluid was centrifuged at 28,000 rpm in a no. 30 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) for 2 h in a Beckman L-250 centrifuge. Each of the resulting pellets was reconstituted in 1 ml of buffer (pH 7.4) composed of 1 M NaCl-0.1 M Tris-HCl-10 mM EDTA. 3 ml of reconstituted pelleted material was placed on a discontinuous gradient of 4 ml 50% and 4 ml 10% potassium tartrate in the same buffer. After further centrifugation in an SW 41 rotor for 2 h at 25,000 rpm, the opalescent interface was collected by aspiration with a capillary pipette and reconstituted in 1 ml of buffer. The material examined by electron microscopy represented a 60-fold concentration of the original tissue culture fluid. Samples were placed on parlodion carbon-coated grids, stained with 2% phosphotungstic acid (pH 6.0), and examined in a Philips EM-200.

RESULTS

Reproducibility of the Assay for Infectious EBV.—The range of variability of the transformation assay performed on different batches of primary cord leukocytes was determined since the content of infectious virus released by all clones was not measured simultaneously. Table I lists 18 experiments, each with umbilical cord leukocytes from a different infant. The two virus stocks used were prepared from supernatant fluids of line B95-8, EBV-transformed marmoset leukocytes. In 14 consecutive titrations, started from 4 to 141 days after preparation of stock A, the median transformation titer was 3.7 log10 TDs0/0.2 ml (range 3.0-4.5 log10 TDs0/0.2 ml). There was thus no major change in titer of EBV with 4 mo storage at -70°C. Nearly identical results were obtained in four consecutive titrations of stock B which was prepared after the cell line had been carried 4 additional mo. The median value for the time to reach the end point was 36 days.

Efficacy of Neutralizing Antibody Used to Obtain Clones of Producer Lines.—Clones were derived in the presence of antiserum to assure that the results ob-
TABLE I
Repeated Titrations of Two Stock Preparations of EB Virus on Different Lots of Human Umbilical Cord Leukocytes

| Human umbilical cord leukocyte preparation | Virus stock | Time after virus stock prepared (days) | Time to reach transformation end point (days) | Infectivity titer (Log_{10} TD50/0.2 ml) |
|-------------------------------------------|-------------|----------------------------------------|---------------------------------------------|----------------------------------------|
| 1                                         | A*          | 4                                      | 42                                          | 4.3                                    |
| 2                                         | A           | 16                                     | 51                                          | 4.5                                    |
| 3                                         | A           | 30                                     | 47                                          | 3.8                                    |
| 4                                         | A           | 39                                     | 58                                          | 3.2                                    |
| 5                                         | A           | 43                                     | 49                                          | 3.5                                    |
| 6                                         | A           | 46                                     | 61                                          | 3.3                                    |
| 7                                         | A           | 78                                     | 28                                          | 3.5                                    |
| 8                                         | A           | 91                                     | 45                                          | 4.0                                    |
| 9                                         | A           | 92                                     | 43                                          | 4.5                                    |
| 10                                        | A           | 92                                     | 34                                          | 4.3                                    |
| 11                                        | A           | 128                                    | 41                                          | 3.2                                    |
| 12                                        | A           | 135                                    | 39                                          | 3.0                                    |
| 13                                        | A           | 137                                    | 38                                          | 3.5                                    |
| 14                                        | A           | 141                                    | 34                                          | 4.0                                    |
| Median stock A                            |             |                                        |                                              | 3.7                                    |
| 15                                        | B*          | 0                                      | 31                                          | 3.8                                    |
| 16                                        | B           | 41                                     | 25                                          | 3.7                                    |
| 17                                        | B           | 42                                     | 32                                          | 4.0                                    |
| 18                                        | B           | 47                                     | 27                                          | 3.7                                    |
| Median stock B                            |             |                                        |                                              | 3.8                                    |

* Stock A was prepared on 4/10/72; stock B on 8/11/72; both were stored at -70°C.

Source of virus was line B95-8, transformed marmoset leukocytes.

Gained reflected the presence of the viral genome within the cell, rather than viral particles adsorbed to the cell surface. Two types of experiments demonstrated that all extracellular virus had been neutralized in the cloning suspension. First, in each cloning experiment, after the suspension of 10^8 cells/ml had been used for selecting microdrops with single cells, the suspension was frozen and thawed and the extract was tested for transforming ability. In all instances, there was no residual transforming activity. Second, reconstruction experiments determined the capacity of the antiserum employed to neutralize cell-associated and extracellular virus (Table II). A suspension of 10^6 cells/ml of lines B84-15 and B95-8 was incubated at 37°C for 1 h in the presence of a 1:10 dilution of human serum containing EBV antibody or human serum which lacked EBV antibody. After incubation 10-fold dilutions to 10^8 and 10^9 cells/ml were made. Each cell concentration was then frozen and thawed three times and the suspension centrifuged at 1,000 rpm for 10 min. The supernatants were tested for residual infectivity by transformation in four replicate cultures. The results indi-
cated that the antiserum neutralized all cell-associated infectivity in line B84-15. In line B95-8 there was some residual infectivity in an extract of 10^4 cells/ml but none in the extract which had contained 10^6 cells/ml. Since suspensions containing 10^5 cells/ml were used for cloning, it was concluded that all extracellular infectious virus was neutralized at the time of cloning by the EBV antiserum present in the medium.

Cloning Efficiency, Derivation of Clones, and Presence of EB Viral Capsid Antigens.—Preliminary experiments demonstrated that the plating efficiency of the three lines from different primate species was less than 0.1% in the absence of a feeder layer, but increased to 16-24% in the presence of human placental feeder cells (Table III). Individual clones were examined for viral capsid antigen (VCA) by immunofluorescence as soon as sufficient cells were available to do the test (usually 2-4 mo after cloning) and at intervals thereafter. 16 of 17 clones demonstrated VCA on the initial test; the single clone without capsid antigen originated from EBV-transformed squirrel monkey cells. This clone has remained free of detectable VCA in the 15 mo since its derivation;

| TABLE II | EBV Neutralizing Capacity of Human Serum Used in Cloning of EBV-Transformed Primate Cells |
|----------|------------------------------------------------------------------------------------------------|
| No. cells/ml used to prepare extract | Line B84-15 (squirrel monkey) | Line B95-8 (marmoset) |
| | Antibody-positive human serum | Antibody-negative human serum | Antibody-positive human serum | Antibody-negative human serum |
| 10^5 | 0/4* | 4/4 | 1/4 | 4/4 |
| 10^4 | 0/4 | 4/4 | 0/4 | 4/4 |
| 10^3 | 0/4 | 4/4 | 0/4 | 0/4 |

* Number of cultures of human cord leukocytes demonstrating transformation per number inoculated with 0.2 ml of cell extract.

| TABLE III | Cloning Efficiency and EB Viral Capsid Antigens in Single Cell Clones of a Human and Two Nonhuman Primate Lymphoblastoid Cell Lines |
|------------|------------------------------------------------------------------------------------------------|
| Line | Source | Method of transformation | Cloning Efficiency | No. clones with capsid antigen per no. tested | No. clones releasing transforming virus |
| 883 L | Mononucleosis patient | Spontaneous | 6/25* 24% | 6/6 | 5 |
| B84-15 | Normal squirrel monkey | EBV from 883L | 6/32 19% | 5/6 | 5 |
| B95-8 | Normal marmoset | EBV from 883L | 6/38† 16% | 5/5‡ | 5 |

* Number of clones per number of single cells plated.
† One clone in this series was lost before it could be examined.
however, it contained EBV complement-fixing antigens detectable both by the standard CF test and the anticomplement immunofluorescence test. Thus, in confirmation of previously published results with human cells, all simian clones contain the EBV genome.

Serial determinations of the content of viral capsid antigen in each clone showed a downward drift in the fraction of cells with VCA over the observation period of 12–26 mo (Table IV); in 14 of the 17 clones the highest proportion of cells with antigen was seen on the first examination. The fall in content of viral capsid antigen was most apparent in the human clones and least so in the marmoset clones. The median level of VCA in the human clones after initiation was

| TABLE IV |
| Serial Determination of Content of Viral Capsid Antigen in Clones of EBV-Transformed Human, Squirrel Monkey, and Marmoset Cells |
|---|---|---|---|---|---|---|---|
| 2 mo* | 5 mo | 7 mo | 9 mo | 12 mo | 20 mo | 26 mo |
| Human line 883 L |
| Clone 34 | 9.6 | 0.9 | 0.1 | <0.1 | 0.7 | 3.2 | 0.5 |
| 35 | 3.3 | 1.5 | 0.5 | 0.6 | 1.5 | 2.8 | 1.4 |
| 42 | 3.5 | 0.4 | 0.2 | <0.1 | 0.5 | 0.6 | <0.1 |
| 44 | 3.4 | 1.0 | 0.6 | 0.4 | 0.8 | <0.1 | <0.1 |
| 50 | 1.8 | 0.9 | 1.5 | 0.4 | 1.1 | 0.8 | <0.1 |
| 52 | 2.2 | 0.3 | <0.1 | <0.1 | <0.1 | nil | nil |
| Squirrel monkey line B84-15 |
| Clone 9 | 4.0 | 0.9 | 0.8 | 1.7 |
| 13 | 6.2 | 2.3 | nil | nil |
| 15 | nil | nil | nil |
| 21 | 1.8 | 0.5 | 1.2 |
| 30 | 5.2 | 1.9 | 1.1 |
| 32 | 1.0 | 0.8 | 0.4 |
| Cotton-top marmoset line B95-8 |
| Clone 18 | 17.0 | 12.0 | 6.2 |
| 20 | 12.7 | 10.0 | 5.8 |
| 21 | 22.2 | 23.0 | 4.0 |
| 22 | 14.4 | 7.3 | 5.9 |
| 29 | 8.0 | 10.0 | 7.9 |

* Months after seeding clones.
† Percent cells with viral capsid antigen. 500–1,000 cells counted after indirect immunofluorescence test using SW (convalescent IM) serum and LH (EBV-antibody negative) serum, as control.
23% of the median value at the first examination; the comparable value for VCA in the marmoset clones was 40% of the initial level. One clone of human origin completely lost evidence of nucleocapsid production.

**Biologically Active Virus Produced by Clones.**—15 of the 17 primary clones released infectious EBV. Those derived from the human line, 883L, have been examined at three different times using cell extracts 9 mo after cloning and supernatant fluids 12 and 20 mo after cloning. Five of the six human clones released infectious EBV on either two or three of the examinations (Table V), while the sixth clone, in which viral capsid antigen was at a very low level or not present, failed to release infectious virus on any examination. Five of the six clones of EBV-transformed squirrel monkey cells also released EBV; the sixth was negative for viral capsid antigen and also failed to release virus on repeated tests. All five clones of transformed marmoset leukocytes released infectious virus.

**Table V**

| Clone | 9 mo | 12 mo | 20 mo |
|-------|------|------|------|
| 34    | 0.0† | 1.5  | 1.5  |
| 35    | 0.5  | 1.5  | 1.7  |
| 42    | <0.0 | nil§ | 0.3  |
| 44    | 0.3  | 0.5  | nil  |
| 50    | nil  | 1.5  | 0.0  |
| 52    | nil  | nil  | nil  |
| Parent| 1.3  | 1.3  | <0.0 |

* Log_{10} 50% transforming doses per 0.2 ml as tested on human umbilical cord leukocytes.
† 0.0, < 0.0 = 2 of 4 or 1 of 4 cultures transformed after inoculation of undiluted virus.
§ No transformation.

Titers of infectious virus released by sister clones were similar and paralleled the amount of EBV released by the parent line (Table VI). To illustrate, the titers of virus released by the human, squirrel monkey, and marmoset lines were, respectively, 10^{1.3}, 10^{3.7}, and 10^{4.0} TD_{50}/0.2 ml; the median titers of virus released by their respective daughter clones were 10^{1.5}, 10^{3.0}, 10^{4.3} TD_{50}/0.2 ml.

The amount of virus released estimated from electron micrographic examination correlated well with measurements made by the transformation assay. Viral particles were not seen in 60-fold concentrates prepared from clones of the human line, with a low level infectivity; herpes virus particles were found in fluid concentrated from two of the squirrel monkey clones and from all of the marmoset ones. Approximately 66% of the virus particles from squirrel monkey and 84% of the particles from marmoset clones were enveloped (Fig. 1). These
results indicate that differences among clones are not due to the production of biologically inactive viral particles by clones which yield low levels of infectious EBV.

From the data presented in Table VI, the amount of infectious virus released per activated cell can be estimated for the producer clones. In these calculations

| TABLE VI | Infectious Virus and Viral Particles in Supernatant Fluids from Individual Clones* |
|-----------|---------------------------------------------------------------|
| **Line** | **Clone** | **Viral capsid antigen** | **Infectivity§** | **Calc. Yield¶** | **No. grid squares examined** | **Detected** | **Enveloped** | **Naked** |
|-----------|-----------|--------------------------|-----------------|-----------------|-----------------------------|--------------|--------------|-----------|
| 883L¶ (human) | Parent 0.017 | 100 0.006 | 5 | -- |
| 34 0.032 | 158 0.005 | 5 | -- |
| 35 0.028 | 251 0.009 | 5 | -- |
| 42 0.006 | 10 0.002 | 5 | -- |
| 44 <0.001 | nil nil | 5 | -- |
| 50 0.008 | 5 0.001 | 5 | -- |
| 52 nil nil nil 5 -- |
| B84-15¶ (squirrel monkey) | Parent 0.017 | 2,506 0.147 | 5 | + 6 2 |
| 9 0.040 | 998 0.025 | 5 | + 15 2 |
| 13 0.062 | 9,980 0.161 | 5 | + 5 9 |
| 15 nil nil nil 5 -- |
| 21 0.018 | 5,000 0.278 | 5 | -- |
| 30 0.052 | 5,000 0.096 | 5 | -- |
| 32 0.010 | 100 0.010 | 5 | -- |
| B95-8¶ (marmoset) | Parent 0.103 | 50,000 0.485 | 3 | + 44 11 |
| 18 0.170 | 158,150 0.930 | 1 | + 82 6 |
| 20 0.127 | 99,800 0.786 | 3 | + 43 3 |
| 21 0.222 | 99,800 0.450 | 1 | + ND** |
| 22 0.144 | 50,000 0.347 | 7 | + 25 10 |
| 29 0.080 | 158,150 1.977 | 1 | + 42 15 |

* An aliquot of the same supernatant fluid was tested for infectious virus and, after 60X concentration by centrifugation, was examined for viral particles.  
† Fraction of cells with immunofluorescence based on counts of 1,000 cells.  
§ Infectivity = no. 50% transforming units/ml.  
¶ Yield of EBV = No. 50% transforming units/ml 
Fraction cells with VCA × 10⁶ cells/ml.  
¶ The data are based on examination of 883L clones at 20 mo, B84-15 clones at 4 mo, and B95-8 clones at 3 mo after derivation.  
** ND = not done; although this preparation had many enveloped and naked virions a differential count was not made.

2 In this paragraph the statements are based on the following median values for human, squirrel monkey, and marmoset clones, respectively: viral capsid antigens: 0.018, 0.040, and 0.144; infectious virus per milliliter: 84, 5,000, and 99,800; yield of infectious virus per cell with capsid antigen: 0.004, 0.096, and 0.786.
the cell number at the time of virus harvest is considered to be 10⁶/ml for all clones and the number of activated cells per milliliter is obtained by applying the percent cells with viral capsid antigen to 10⁶/ml. Squirrel monkey clones contained approximately twice the number of cells with viral capsid antigen as human clones; however, the squirrel monkey clones yielded on the average 27 times more infectious virus. Marmoset clones contained 8 times more cells with VCA than human clones and, furthermore, these cells yielded approximately 200 times more infectious virus than human clones. Thus, enhanced virus production by simian clones resulted from both an increased proportion of activated cells and an increased yield of virus from each activated cell. For each species the median yield of infectious cell-free virus by productive daughter clones closely paralleled the yield of virus by the parent although there was some variation among individual clones of each species. Human transformants all yielded less than 10 infectious units per 1,000 VCA-positive cells; the median for productive squirrel monkey clones was 96 infectious U/1,000 virogenic cells (range 10–278 TD₅₀/1,000 VCA-positive cells) while for marmoset clones it was 786 infectious U/1,000 VCA-positive cells (range 347–1,977 TD₅₀/1,000).

Attempts to Rescue Biologically Active Virus from Nonproductive Clones.—Thus

![Image of EB virus particles](image)
far we have not been able to rescue biologically active virus from two nonproducer clones (human clone 52 and squirrel monkey clone 15) by treatment with BUdR or by X irradiation followed by co-cultivation with primary marmoset leukocytes (Table VII). BUdR treatment caused the appearance of antigens detectable by immunofluorescence in 1-2% of cells in the nonproducer squirrel monkey clone but not in the human clone. Neither infectious virus nor viral particles detectable by electron microscopy were found in fluids of nonproducer clones after BUdR treatment. The content of infectious virus from the BUdR-treated productive marmoset clone was reduced to 4% of the value without drug treatment.

**TABLE VII**

*Attempt to Induce Nonproducer Clones to Release Infectious Virus by Treatment with 5'-Bromodeoxyuridine*

| Line          | Clone | Control BUdR-treated* |
|---------------|-------|-----------------------|
|               |       | **Antigens detected by immunofluorescence** | **Early** + viral capsid | **Infectious virus** | **Antigens detected by immunofluorescence** | **Early** + viral capsid | **Infectious virus** |
|               |       | Viral capsid |       |       | Viral capsid |       |       |
| 883 L human   | Clone 52 | nil         | nil  | nil  | nil       | nil  | nil  |
| B84-15 squirrel monkey | Clone 15 | nil         | nil  | nil  | 1.0       | 2.2  | nil  |
| B9S-8 marmoset | Clone 18 | 15.0        | 28.6 | 3.7  | 18.4      | 25.0 | 2.3  |

(1) A serum with only antibodies against viral capsid antigen (SW), (2) and a serum pool which contained antibodies against early antigens (3), and viral capsid antigens were used. Number is the percent cells with antigens. 1,000 cells enumerated per slide.

*Logarithmic phase cultures treated with 5'-bromodeoxyuridine (25 μg/ml) or RPMI medium as a control for 40 h. Cells were washed three times and replaced in fresh medium. They were tested for IF antigens and infectious virus 1 wk after removal of the drug.

†Log_{10} 50% transforming doses per 0.2 ml.

Exposure of the two nonproducer clones to 8,000 R did not cause the appearance of antigens detectable by immunofluorescence 3 or 7 days after irradiation. These lethally X-irradiated cells were unable to cause transformation of primary marmoset leukocytes. As a positive control lethally X-irradiated producer cells of marmoset clone 22 caused transformation of primary marmoset leukocytes.

With some human lymphoblastoid lines similar methods have proved capable of demonstrating small amounts of biologically active virus (12, 17-19). If rescue experiments had been successful with the nonproducer clones, they would have indicated that even those clones contain information for elaboration of biologically active virus.

*Attempt to Superinfect Nonproducer Clones.*—Superinfection of the two nonproducer clones was attempted in order to investigate the possibility of a helper-
virus phenomenon. EB virus was concentrated and partially purified from line B95-8 by the procedure used to prepare virions for electron microscopy. Each nonproducer clone at a cell concentration of \(10^7/\text{ml}\) was resuspended in 1 ml of virus containing \(10^{8.0}\ T\Delta_00/\text{ml}\). After 4 h the inoculum was removed, and the cells were washed and resuspended in fresh medium at \(5 \times 10^6\) cells/ml. Cells harvested 3 and 7 days after superinfection demonstrated neither viral capsid antigen nor early antigens, and supernatant fluids from cultures 7 days after superinfection did not contain infectious EBV.

**DISCUSSION**

*Differences in the Yield of Infectious EBV by Clones of Human, Squirrel Monkey, and Marmoset Lymphoblastoid Cell Lines.*—Our results indicate that daughter clones, with the exception of the two nonproducer clones encountered, resemble their parent in the amount of infectious virus released. Thus differences between human and simian lines are not primarily due to effects of particular subpopulations of cells, but to differences which reside in most, though perhaps not all, individual transformed cells. Since differences between the lines can be generalized to differences between single clones, and since all three lines were transformed by the same EBV strain, it seems reasonable to conclude that there are cellular mechanisms which regulate the expression of the viral genome in EBV-transformed cells. The results presented indicate that there are quantitative, if not qualitative, differences in these mechanisms among primate species. The data do not allow a choice between the two alternatives which are that the human cells lack facilitating factor(s) or that they possess inhibitory factor(s). Both types of processes, namely "cytoplasmic essential replication factors" and "repressors," have been described in comparative studies of cell lysis and cell transformation after infection with SV40 (20, 21). In fact, the clonal lines of SV40-transformed human embryo lung cells, described by Kit and Dubbs, behaved similarly to clonal lines of EBV-transformed human lymphoid cells. Nearly all clones contained the SV40 genetic information needed for production of infectious virus, but less than one in one hundred thousand SV40-transformed human cells actually formed infectious centers.

Data obtained in earlier work in our laboratory also provide evidence that differences in the production of infectious EBV by transformants of different species is due to cell variation and not to genetic variation in the virus (1). One could argue that human line 883L was transformed by a low-yielding virus variant, that squirrel monkey line B84-15 was transformed by an intermediate virus, and that line B95-8 was transformed by a highly productive variant. This argument is excluded by previous findings that transformants from several individuals of each species all behave similarly: low yields from human cells, high from marmoset cells, and intermediate from squirrel monkey cells. Another possibility is that passage in marmoset cells permanently modifies the virus to a high yielding state. This possibility is unlikely, for marmoset-grown virus is non-
productive in human cells, and EB virus obtained from throat washings and which has never been in cell culture leads to productive transformation in marmoset cells and nonproductive transformation of human cells (22). A third hypothesis, which seems implausible but which is difficult to exclude experimentally, is that there is a very high mutation and back-mutation rate in the property of the virus responsible for high and low yield and that human cells are selectively transformed by low-yielding virus variants and simian cells are transformed by high yielding virus.

Since 15 of 17 clones tested produced infectious EBV, it may tentatively be concluded that the majority of cells in the three lines studied contain the complete EB viral genome. However, it has not yet been rigorously demonstrated which viral components are needed for lymphocyte transformation. In experiments in which EBV has been partially purified, transforming activity is associated with the viral particle per se (23–24). It has not yet been conclusively shown whether naked nucleocapsids as well as enveloped particles possess transforming activity. There is no information yet about the proportion of the viral genome which is necessary for transformation. With these reservations it nonetheless seems reasonable to suggest on the basis of our data that enveloped virions are the usual initiators of transformation. We have previously shown that treatment with ether abolishes transformation by EBV. The electron microscopic survey for viral particles performed in conjunction with assays for infectivity demonstrated that enveloped particles were most numerous in the marmoset clones which also yielded the highest titers of infectious virus. By using a semi-quantitative estimate of viral particle counts (25), we calculate that the particle infectivity ratio of EB virions produced in marmoset clones is approximately 40–300 to 1.

In the present experiments it was possible to verify that all extracellular infectious virus was absent from cell suspensions used for cloning. This result supports previous hypotheses that the EBV genome in an intracellular location is associated with all cells. Our studies with clones do not shed light on the molecular basis of the relationships between the viral and cellular genomes, but a recent report by Nonoyama and Pagano suggests that the EBV genome is present on cell chromosomes, though it may not be covalently linked to cellular DNA (26). On the basis of reassociation kinetics between EBV DNA and cell DNA from the nonproductive Raji line, these same investigators have concluded that the Raji cells contain a complete copy of the EBV genome (27).

Significance of the Nonproducer Clones.—Two clones did not release infectious EBV on repeated examinations; nonetheless, these clones contain EBV genetic material. The nonproductive human clone produced capsid antigen for several months, and the nonproductive squirrel monkey clone spontaneously produced EBV antigens detectable by immunofluorescence after treatment with BUdR. Since the yield of infectious virus from human clones is low, the nonyielding human clone might have been shown to release virus if more tests were done or
if concentrated fluids were examined. Failure to detect virus from the squirrel monkey clone is more meaningful since $10^{3.0}-10^{4.0}$ infectious U/0.2 ml were expected by comparison with other clones in that series.

The nonproducer clones may result from either viral or cellular mechanisms. They may have been derived after transformation by defective virions or possibly they may result from excision of part of the viral genome from some cells. As a possible explanation the absence of a helper virus seems less likely since we could not convert the nonproducer clones to yield virus by superinfection. We favor the hypothesis that nonproducer clones result from restrictions imposed by the cell. Cells may differ in the restriction of viral replication not only between species, but also within a species. A nonproducer clone may originate from a different cell type than its producer sisters, for example, a different lymphocyte subtype. Age of the donor may also be a factor since we have demonstrated that transformed human umbilical cord leukocytes rarely produce viral capsid antigens, whereas these antigens are frequently present in transformed leukocytes from adults (1). The observation that in cloned lines the proportion of cells with viral capsid antigen decreases with time and that some producer clones become nonproducers is compatible either with the existence of selection processes which favor nonproducer cells, or with progressive loss of viral information.

The availability of producer and nonproducer sister clones provides starting material for study of the mechanisms of restriction of expression of the EBV genome. Among the questions for future study is whether nonproducer clones are also more restrictive for other viruses, either those of the herpes group or unrelated agents.

SUMMARY

Three lymphoblastoid cell lines, of human, squirrel monkey, and marmoset origin, all transformed by the same strain of Epstein-Barr virus (EBV), differed markedly in their content of infectious virus. Single cell clones were obtained from each line to learn whether these differences were dependent upon factors shared by all cells in each line or upon factors present only in a proportion of the total cell population.

A total of 17 primary clones were examined: 6 human, 6 squirrel monkey, and 5 marmoset. Cloning efficiency on human placental cell feeder layers varied from 16 to 24%. EBV antiserum, present in the cloning suspension, was shown to neutralize all extracellular virus. 15 of the 17 clones released EBV as measured by the transformation assay. Titers of infectious virus released by daughter clones paralleled titers of virus in the parent line. The median virus titers from human, squirrel monkey, and marmoset clones were respectively $10^{3.3}$, $10^{4.6}$, and $10^{4.3}$ 50% transforming doses per 0.2 ml. The median yield of virus from clones of the three species was, respectively, 4, 96, and 786 transforming units per 1,000 cells containing viral capsid antigen.
Two nonproducer clones (one human and one squirrel monkey) did not release infectious virus after treatment with 5'-bromodeoxyuridine, or with X ray followed by co-cultivation with marmoset leukocytes. The nonproducer clones could not be superinfected by biologically active EBV. These results show that differences in production of infectious EBV among the lines tested are reflected in the majority of cells of these lines. The data imply that the mechanism for regulation of the expression of the EBV genome is cellular rather than viral in origin. There are presumably genetic differences among primate species in this regulatory process.

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