Expression of Epstein–Barr Viral Capsid, Complement Fixing, and Nuclear Antigens in Stationary and Exponential Phase Cultures

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Three continuous lymphoblastoid cell lines, 2 productive of nucleocapsids and 1 nonproductive line, were studied for their content of Epstein–Barr viral (EBV) antigens during transition from stationary to logarithmic phase growth. As a preliminary step, viable cells were separated from degenerating ones in discontinuous gradients of serum albumin. Viral capsid antigens were found in both living and dead cells of the 2 producer lines; however, complement fixing (CF) antigens and nuclear antigen were detected only in viable cell subpopulations. The content of antigen detectable in extracts of viable cells by complement fixation remained constant in replicating and resting cultures; further, all viable cells of the 3 lines demonstrated intranuclear antigen by anticomplement immunofluorescence in all stages of growth. In contrast, the proportion of cells with viral capsid antigen in the producer lines increased 7- to 24-fold following entry of resting populations into the phase of exponential growth.

The results suggest that expression of viral capsid antigens is discontinuous and is initiated in response to events in log phase, possibly DNA synthesis or mitosis. Expression of the complement fixing and nuclear antigens is continuous in viable cells. These findings emphasize the intimate relationship of the CF and nuclear antigen to the transformed state and suggest that study of this antigen complex will shed light on the mechanisms of lymphocyte transformation by EBV.

INTRODUCTION

A central problem in tumor virology is the relationship between the expression of viral specific information and the stage of growth of the transformed cell. This
problem was perhaps first appreciated by Noyes and Mellors who found that in Shope papilloma of the cottontail rabbit viral antigens were present in the nondividing differentiated keratinized layers of the tumor, although such antigens were not detectable in the rapidly proliferating deeper epithelial cells (1). They postulated that the proliferating cells contained viral nucleic acids but did not produce viral specific proteins.

The present experiments were undertaken to determine whether the phase of cellular proliferation studied in vitro affected the expression of Epstein–Barr virus (EBV) antigens. This system was of particular interest for two reasons: first, spontaneous activation of the EBV genome leading to formation of nucleocapsids occurs in only a small fraction of the population of certain productive cell lines, although cloning experiments indicate that all cells in these lines contain at least one complete copy of the viral genome (2–4). It is not known whether physiologic events leading to the spontaneous initiation of synthesis of EBV particles in a few cells occur in log phase, stationary phase, or during cell death. Second, cells transformed by EBV are of lymphoid origin and there is now considerable evidence relating viral replication to active growth of lymphoid cells. For example, replication of several viruses is enhanced in primary cultures of lymphocytes activated by phytohemagglutinin (5–7). Specific antigen stimulation of lymphocytes may also result in enhanced virus growth. This is apparently the case for dengue virus which replicates to a greater extent in cultured lymphocytes from immune primates than from nonimmune monkeys, presumably because of the blastogenic properties of the infecting virus (8). Furthermore, experimental graft versus host disease in mice with its attendant lymphoproliferation leads to replication of RNA tumor viruses (9, 10). Thus events affecting lymphocyte growth in vivo may affect expression of an endogenous viral genome.

METHODS

Cells and Media

Three lymphoblastoid cell lines, 2 productive and 1 nonproductive of nucleocapsids were examined. From the P3J-HR1 clone of Burkitt lymphoma cells single cell subclones were derived in antiserum to EBV (11). One clone (No. 8) in which the level of viral capsid antigen was 1–4% of randomly growing cells was used. The B95-8 line was derived following exposure of cotton-top marmoset peripheral blood leukocytes to an EBV strain from an infectious mononucleosis patient (12). Raji, originally from a Burkitt lymphoma patient, produced neither viral capsid antigens nor nucleocapsids, but the EBV genome, detectable by methods of nucleic acid hybridization, is associated with Raji chromosomes (13a, 13b). Media for all experiments was RPMI 1640 (Grand Island Biological Co.) plus 10% heated (56°C for 1/2 hr) fetal bovine serum, penicillin (50 μg/ml), streptomycin (50 μg/ml), and amphotericin B (1.0 mg/ml). Stock cultures of each line were maintained by weekly two to one splits.

Cell Growth Curves and Separation of Cellular Populations

Cells of the 3 lines used in the present experiments were manipulated in a manner previously described for derivation of cultures in stationary phase (14). This consisted of inoculation of cells at low density and subsequent omission of fresh medium. Cell growth was initiated following addition of 2 parts of fresh complete
medium to 1 part of 1 wk stock culture. The starting cell density was approximately $3 \times 10^5$ viable cells/ml. Daily counts of viable and dead cells were made by the method of tripan blue exclusion. Stationary phase cells were defined as 12-day-old cultures. To obtain logarithmic phase populations, viable stationary phase cells were first separated from dead cells by means of discontinuous gradients of bovine serum albumin (15). Approximately $2 \times 10^8$ cells were washed once in Puck’s saline (0.1 M NaCl, 5 $\times$ 10$^{-3}$ M KCl, 5 $\times$ 10$^{-3}$ M glucose) and resuspended in 1 ml of sterile 33% (v/v) albumin (Pentex) in Hank’s balanced salt solution. One milliliter each of 23, 20, 17, and 10% albumin solutions were added and the gradient was centrifuged for 45 min at 17,000 rpm in the Spinco SW65 rotor at 5°C. Each cell band was collected individually by aspiration with a capillary pipette. The two bands of viable cells at the interfaces between 10 and 17% and 17 and 20% albumin were pooled, washed once in warm complete medium, and reconstituted at $3 \times 10^7$ cells/ml, and returned to 35°C. Thereafter, daily cell counts and estimates of the rate of DNA synthesis were made.

Rate of DNA Synthesis

Duplicate aliquots of 0.9 ml of cell suspension were placed in prewarmed (37°C) tubes containing 1.1 ml of medium and 2 $\mu$Ci/ml of $^{14}$C-thymidine (specific activity 56 mCi/mM). After 30 min at 37°C incorporation of radioactivity was stopped by addition of 10 ml of cold (5°C) Puck’s saline. Cells were washed two times in Puck’s saline, collected by centrifugation and resuspended in 2 ml of cold 5% trichloroacetic acid. The insoluble material was collected onto cellulose acetate filters and radioactivity determined. The rate of DNA synthesis was expressed as cpm/10$^5$ cells.

Epstein–Barr Viral Antigens

Viral capsid antigen was detected on cells fixed in acetone for 10 min at room temperature. The indirect immunofluorescence technique was employed with fluorescein conjugated rabbit antihuman gamma G globulin (Antibodies, Inc.) (16). Cells (1000–2000) were enumerated per slide. Complement fixing antigens were prepared from cell extracts (17). Cells were washed twice and resuspended in veronal buffered saline pH 7.2 with 0.1% bovine serum albumin at a concentration of $5 \times 10^7$/ml. Cells were lysed by 3 cycles of alternate freezing and thawing and debris removed by centrifugation at 800 g for 10 min. The supernatant fluids were used as antigen in microtiter complement fixation tests employing 1.3 50% hemolytic units of complement. The anticomplement immunofluorescence technique of Reedman and Klein was used to study EB nuclear antigens (EBNA) in acetone fixed preparations (18). An indirect test with fluorescein conjugated rabbit antihuman B Ig globulin (Hyland Laboratories) was performed. Cell spreads were washed with Hank’s balanced salt solution pH 6.9. Antisera to EBV used in this test contained as a source of complement 10% (vol/vol) fresh human serum, lacking EBV antibodies.

Antisera

All 3 antigens (viral capsid, complement fixing, and EBNA) were studied with the same pair of human reference sera. The antibody positive serum (RM) was from a healthy adult with a history of mononucleosis 20 years ago. The antibody negative control serum (LH) used in each test also was from a healthy adult. The
sera were used at a 1:10 dilution to study viral capsid antigens and the intranuclear antigen and at a 1:4 dilution to measure complement fixing antigens. In selected experiments viral capsid antigens were also determined with 1:10 dilution of pooled positive (titer 1:80) postinoculation cotton-top marmoset sera; pooled antibody negative preinfection marmoset sera served as a control (19). The simian sera were anticomplementary and could not be used in the CF or anticomplement immunofluorescence test.

RESULTS

Derivation of Stationary Phase Cultures of Viable Cells

An initial objective of these studies was to manipulate in vitro cultural conditions to derive viable cell populations at rest. This was done by adding fresh medium to one week-old stock cultures following which similar growth curves were obtained for the 3 lines. A lag period of approximately 24 hr was followed by logarithmic growth which lasted from 3 to 4 days, thereafter there was a slow decline in the number of viable cells and an increase in the number of degenerating ones. By Day 12, a resting phase had been established, with approximately 40–50% of the cells remaining viable (Fig. 1).

The next major objective was a physical separation of degenerating cells from viable ones. This step was necessary because EBV nucleocapsids and viral capsid antigens are found in cells which are dead, degenerating, or markedly altered mor-

![Fig. 1. Cell growth curve of three lymphoblastoid cell lines. Stock cultures were diluted to contain 3 × 10⁶ cells/ml with fresh medium. Daily counts of viable and dead cells made by method of trypan blue exclusion. (a) Clone 8; (b) Raji; (c) B95-8. ○○ viable cells; ●● dead cells.](image-url)
physically (20) and we wished to study the recruitment of new virus producer cells into the population. This separation was accomplished using discontinuous gradients of bovine serum albumin: Viable cells were found in the upper bands of the gradient while nearly all cells in the pellet were dead as judged by trypan blue staining. Cell separations of Clone 8 were carried out during Days 4, 8, and 12 of the growth curve. On Day 4 only 7% of the cells were in the pellet; this fraction increased to 22% on Day 8 and 44% on Day 12 (Table 1). Thus, as the proportion of dead cells in the culture increased the fraction of cells found in the pellet of the gradient increased. A similar separation of viable from dead cells could be achieved for each of the three lines.

*The morphology of cells* studied in Giemsa-stained preparations or by phase contrast microscopy reflected the stage of growth of the population. Cells in bands 1–3 appeared to be in active growth on Day 4 and were resting on Day 12 as determined by morphologic criteria. During log phase, viable cells had a median diameter of 22–26 μm, abundant cytoplasm, and approximately 3% were in mitosis. By Day 12, viable cells were 14–16 μm, contained scant cytoplasm, dense nuclear chromatin, the nucleolus was not readily apparent, and no mitotic cells were found.

*Distribution of Virus-Specific Antigens and Nucleocapsids in Viable and Degenerating Cells*

We next examined expression of EBV in viable and dead subpopulations. Table 2 illustrates data obtained with Clone 8 at the end of logarithmic growth, eight

### Table 1

|       | Albumin concentration at interface | Clone 8 | Raji | B95-8 |
|-------|----------------------------------|---------|------|-------|
|       | Viable (%) | Total (%) | Viable (%) | Total (%) | Viable (%) | Total (%) |
| 1     | 10–17%    | 100      | 100  | 99    |
| 2     | 17–20%    | 99       | 100  | 99    |
| 3     | 20–23%    | 97       | 96   | 96    |
| 4     | 23–33%    | 90       | 84   | 96    |
| 5     | pellet    | 13       | 68   | 9     |

### Table 2

| Band | Percent cells viable | Percent of cells with VCAa | Percent of cells with EBNAa | Titer of CF antigenb | EBY Nucleocapsidsd visualized by electron microscopy |
|------|----------------------|-----------------------------|-----------------------------|---------------------|--------------------------------------------------|
| 1–3  | 99                   | 2.4                         | 100                         | 8                   | 0/324                                            |
| 5    | 1                    | 4.3                         | <10                         | <1                  | 8/326                                            |

a VCA = viral capsid antigen; 1000 cells counted.
b EBNA = EB nuclear antigen.
c Per 0.025 ml of extract prepared from 5 × 10² cells/ml.
d Number cells with EBV nucleocapsids/No. cell profiles studied.
days after subculture. Intracellular immunofluorescent antigens were found in both viable and dead cells. However, qualitative differences were noted in the intracellular immunofluorescent staining of living and dead cells for capsid antigen (Fig. 2). Intracellular antigen was restricted to the cytoplasm of approximately 60% of viable cells in log phase; by contrast the staining was generalized in 95% of dead cells found in the pellet of the gradient. An electron microscopic survey was made by M. Lipman for viral particles in more than 300 cell profiles of viable and dead cells. EBV nucleocapsids were detected only in the markedly degenerated cells found in the pellet of the albumin gradient.

Equal numbers of viable cells from the upper bands of the gradient and from degenerating cells in the pellet were examined for complement-fixing antigen. This antigen was detected only in the viable cells. Tests with several additional human sera with high CF titer (\( < 1:512 \)) failed to demonstrate CF antigen in the dead cells. Similar results were obtained in tests for EBNA which was found in nearly 100% of viable cells but in less than 10% of cells in the pellet of the albumin gradient.

Expression of Viral Functions in Stationary and Log Phase Cultures

The levels of viral antigens were measured as stationary cultures entered exponential growth. Viable late stationary phase cells were harvested from bands 1 and 2 of the albumin gradient, were washed and resuspended in fresh prewarmed complete medium at a density of \( 3 \times 10^5 \) cells/ml. Cell number, the rate of DNA synthesis and EB viral antigens were measured sequentially. The results of the parameters of cell growth are found in the upper panels of Figs. 3–5 and the viral antigens are illustrated in the lower panels.

In all 3 lines there was no detectable DNA synthesis in the culture initially. In Clone 8, there was a further 24 hr absence of DNA synthesis. The rate of DNA synthesis was maximum 48–72 hr after subculture for all lines. The cell number doubled approximately 24 hr after the peak of DNA synthesis; the generation time for the first doublings was approximately 18–24 hr for the 3 lines.

In the 2 productive lines viral capsid antigen was at a low level (less than 0.1% for Clone 8 cells and approximately 1.8% for the EBV transformed marmoset cells) during stationary growth (Figs. 3, 5). The level of capsid antigen increased as the cells entered exponential growth to 2.4% for Clone 8 and 13% for B95-8.

In the experiment illustrated for Clone 8 (Fig. 3) there was a lag of 3 days before cell doubling began; during this time viral capsid antigen was at a barely detectable level, and only rose concomitant with exponential growth. No further increase in the fraction of cells with capsid antigen occurred once maximum cell density was reached.

Data similar to those shown in Figs. 3 and 5 were obtained in two additional experiments with Clone 8 and in one duplicate experiment with line B95-8. In one instance cell spreads of Clone 8 were studied simultaneously for capsid antigen with the reference human antisera and with pre- and postinoculation marmoset sera. Similar results were obtained with antibody positive human and simian sera.

The content of complement fixing antigens detectable in crude extracts of the two Burkitt lymphoma lines did not vary significantly from cell rest to exponential growth. The same results were obtained with crude extracts or with supernatants of cell extracts which had been centrifuged at 80,000 g for 1 hr suggesting that most of the antigens measured by CF were “soluble.”
Fig. 2. EB viral capsid antigen in viable and degenerating subpopulations. Logarithmic phase (Day 4) cultures of Clone 8 stained by the indirect immunofluorescence method. Original magnification ×250. Top panel from upper bands of the albumin gradient; the cells are large and the single fluorescent one contains antigen in the cytoplasm. Bottom panel from pellet of the albumin gradient showing small cells and cell fragments; the two fluorescent cells contain antigen throughout cytoplasm and nucleus.
DISCUSSION

Several techniques have permitted the analysis herein reported of the effect of cell growth on the expression of EB viral antigens. It has been relatively easy to manipulate in vitro conditions to derive stationary and logarithmic phase cultures. Cells in these two phases of growth can be readily differentiated on the basis of
Fig. 4. EBV antigens in Raji cells in transition from stationary to logarithmic phase growth (see legend for Fig. 3).

Fig. 5. EBV antigens in B95-8 cells in transition from stationary to logarithmic phase growth (see legend for Fig. 3).
size, morphology, and total DNA synthesis. It was possible to remove degenerating cells from the cultures and thus to eliminate those cells which had already produced nucleocapsids. Finally, a doubly cloned line was studied in one instance to assure that the results reflected different physiologic states of a genetically homogenous population.

The spontaneous activation of viral capsid antigen occurred during and following exponential phase growth when there was a seven to more than twentyfold increase in the number of cells with this antigen. Since capsid antigen did not increase until after cell doubling, both DNA synthesis and mitosis may be necessary to trigger its production. In previous studies of the P3HR1 line (the parent of Clone 8) both Gergley and Hampar found that inhibitors of DNA synthesis, such as cytosine arabinoside and 5′ bromodeoxyuridine, depress the level of capsid antigen (21, 22). Both investigators noted that removal of the drugs led to a burst of capsid antigen synthesis. The phenomenon of stimulation of capsid antigen production following reversal of inhibition of DNA synthesis may be due to synchrony of the cells followed by virus activation in the subsequent cell cycle. In future experiments it will be important to define precisely steps leading to spontaneous activation of the viral genome. In particular it will be essential to define, by use of synchronized cell populations, that phase of the cell’s mitotic cycle in which spontaneous expression of EBV specific information is initiated. Our studies do not provide information on the relationship of the cell mitotic cycle to spontaneous induction of EBV; however, Hampar and co-workers have recently indicated that an event in S phase

Fig. 6. EB nuclear antigen and viral capsid antigen in stationary phase, viable cells of a 12 day culture of line B95-8; cells are from the upper three bands of an albumin gradient examined by the indirect anti-complement immunofluorescence technique. Note viral capsid type staining in two cells; remainder of the cells show the characteristic reticular staining of the intranuclear antigen.
is critical in determining the induction of EBV "early antigen" following application of halogenated pyrimidines to the Raji line (23). Does the spontaneous activation of the viral genome in productive cell lines leading to complete viral maturation also occur during the DNA synthetic phase of the cell cycle?

By contrast to viral capsid antigen, expression of CF and nuclear antigens seems to be independent of the phase of cell growth. A constant concentration of CF antigen was found in resting and logarithmic phase cells, although the total amount present increased with the cell number. The concentration of CF antigen in the productive P₃HR1 and in the nonproducer Raji lines was similar. Since Raji does not produce virus particles, this result suggests that the steady level of CF antigen in the productive lines is not due to a combination of antigen synthesis, incorporation into viral particles and release from the cell during logarithmic growth.

Part of the explanation for the difference in expression between viral capsid and complement fixing antigens is that the two likely have different effects on the host cells. While capsid antigen synthesis is accompanied ultimately by cell death, complement fixing antigen production has no effect on cell viability. The anticomplement immunofluorescence test demonstrates this antigen in every cell of a viable population. In fact, cell viability appears necessary for the detection of CF and the nuclear antigen. These antigens are not found in degenerating cells in the pellet of the albumin gradient, but this could be due to loss of soluble proteins due to increased permeability of the cell membranes of degenerating cells.

Continuous expression of the CF antigen may result from initiation of its synthesis during a specific phase of the cycle of exponential phase cells which retain the antigen throughout stationary phase. An alternate hypothesis is that initiation of synthesis of proteins responsible for the complement fixation reaction occurs during both stationary phase and exponential growth. To resolve problems of interpretation posed by the inability to measure the half-life of the antigens, development of methods to study the time of synthesis of EB viral specific proteins is essential. Perhaps the technique of radioimmunoprecipitation will prove useful.

The biologic function of the CF antigen is not known. It is presumed to be coded for by the viral genome, because it is present in all EBV transformed lines regardless of species. However, the origin of the antigen, whether cellular or viral has not been rigorously proved. Small amounts of CF antigen are associated with partially purified preparations of EB virions, but it is not known whether the antigen is a component of the virion per se or whether the reactivity is due to its presence on absorbed cell membranes (25). Complement fixing activity in crude cell extracts is apparently due to at least two soluble components, one of which is inactivated by heating at 56°C (26). As suggested by others, the analogies between the EBV complement fixing antigen complex and the "t" antigen system of the papova viruses are striking (17, 18). In both instances, the antigens are present in an intranuclear location in cells not producing viral particles. The present results demonstrate that in EBV transformed cells the CF antigens are expressed in all phases of cell growth. These results emphasize that delineation of the function of the CF antigen is important to an understanding of transformation of lymphoid cells by EBV, and they support the hypothesis that the CF and nuclear antigens are in some way associated with maintenance of the transformed state. This hypothesis is supported by recent findings that the CF antigens and EBNA are found in Burkitt tumor biopsies; viral capsid antigens are not usually detected directly from the tumors (26, 27).
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