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Transforming activity of Epstein–Barr virus obtained by superinfection of Raji cells

(immortalization/karyotype)

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ABSTRACT **Epstein–Barr virus obtained by superinfection of Raji cells with Epstein–Barr virus recovered from P3HR1 cells (HR1 virus) transformed human lymphocytes, but it did not superinfect Raji cells. A human lymphoblastoid cell line, HLB, established by such transformation contained 22 Epstein–Barr virus genomes per cell and Epstein–Barr virus-associated nuclear antigen, and a few cells contained early or viral capsid antigen complexes. Chromosomal analysis revealed that HLB cells were diploid with normal female karyotypes. Replication of Epstein–Barr virus DNA and inhibition of host cell DNA synthesis were observed in HLB cells after superinfection with HR1 virus.**

Epstein–Barr virus (EBV) is associated with two human tumors, Burkitt's lymphoma and nasopharyngeal carcinoma, and is the principal cause of heterophile-positive infectious mononucleosis (1–3). Lymphoblastoid cell lines have been established from tumors and peripheral blood of patients with these diseases. Furthermore, infection with EBV converts peripheral lymphocytes of uninfected individuals into established lymphoblastoid cell lines (4), demonstrating a potential capacity of EBV oncogenicity. EBV DNA has been detected in these established cell lines, irrespective of their origin or method of establishment. In many human lymphoblastoid cells, EBV genomes exist in a repressed state; one of these cell lines, Raji, established from an African patient with Burkitt's lymphoma, is free of detectable EBV particles or antigens associated with virus production (early antigen, viral capsid antigen), although it retains 50–60 EBV genomes per cell in a stable manner. Some other lymphoblastoid cell lines, in contrast, spontaneously produce infectious EBV. Many human lines have receptor sites for EBV and several of these respond to superinfection with an increase in numbers of viral antigen-positive cells. Superinfection of Raji cells with EBV recovered from culture fluids of virus-producing P3HR1 cells (HR1 virus) causes lytic or semipermissive infection (5). A large amount of EBV DNA and EBV particles is produced under conditions of complete inhibition of host cell DNA synthesis. EBV particles (SI Raji virus) detected in cells and supernatant fluid of superinfected Raji cells, however, fail to cause subsequent superinfection of Raji cells although they contain unit-length EBV DNA (5).

This paper reports the transformation of human cord blood lymphocytes by SI Raji virus and some properties of a lymphoblastoid cell line, HLB, established after infection of human cord blood lymphocytes with SI Raji virus.

MATERIALS AND METHODS

Cell Lines. P3HR1 and Raji cells were grown in RPMI 1640 medium containing 10% calf serum. HLB cells, which were established by infection of human cord blood lymphocytes with SI Raji virus, were maintained in RPMI 1640 medium supplemented with 8% fetal calf serum. The medium contained 100 units of penicillin and 50 μg of streptomycin per ml.

Superinfection of Raji and HLB Cells. Raji cells and HLB cells were superinfected with HR1 virus in minimal essential medium lacking phosphate with 2% fetal calf serum as reported previously (5).

Test for Transforming Activity of SI Raji Virus. Human cord blood was collected into syringes rinsed with preservative-free heparin and dextran was added to a final concentration of 1%. After the blood stood at 37° for 1 hr, cell-rich plasma was collected and the mononuclear leukocytes were separated from plasma on Ficoll–Hypaque gradients (6). Isolated leukocyte populations were incubated overnight in a humidified CO_2 incubator at the concentration of 1×10^6 cells per ml in RPMI 1640 medium supplemented with 20% fetal calf serum. Leukocytes were collected by centrifugation at 1000 rpm for 10 min and resuspended in supernatant fluid of superinfected Raji cells (48 hr after superinfection) that had been made cell free by filtration through a nitrocellulose membrane filter (0.45 μm pore size). After 1 hr of adsorption with occasional shaking, infected cells were washed once and then maintained at 10^6 cells per ml at 37° in RPMI 1640 medium containing 20% fetal calf serum. Half the medium was replaced with fresh medium weekly.

Calculation of EBV Genome Content. EBV-specific cRNA was prepared *in vitro* from EBV DNA by using *Escherichia coli* RNA polymerase. Alkaline-denatured DNA in 0.90 M NaCl/0.090 M sodium citrate was passed through nitrocellulose membrane filters and single-stranded DNA was fixed to it by baking at 80° under reduced pressure for 2 hr. cRNA (1.5×10^5 cpm) in 1 ml of 0.90 M NaCl/0.090 M sodium citrate containing 0.11% sodium dodecyl sulfate was incubated with DNA filter at 66° overnight. The filters were treated with RNase and washed with 0.30 M NaCl/0.030 M sodium citrate, and the amount of hybridized cRNA was determined by measuring the radioactivity of hybridized DNA filters in a liquid scintillation counting system. The amount of DNA on each filter was determined by diphenylamine reaction. Human DNA from Simpson cells was used to obtain background counts.

Detection of EBV-Associated Antigens. Smears of cells were dried on glass slides and fixed with cold acetone. An indirect immunofluorescence technique was used to detect early antigen and viral capsid antigen (7, 8). EBV-determined nuclear antigen was detected by anticomplement immunofluorescence tests (9).

Chromosome Analysis. Metaphase spreads from both Raji and HLB cell cultures were prepared from cells treated with

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Abbreviation: EBV, Epstein–Barr virus.

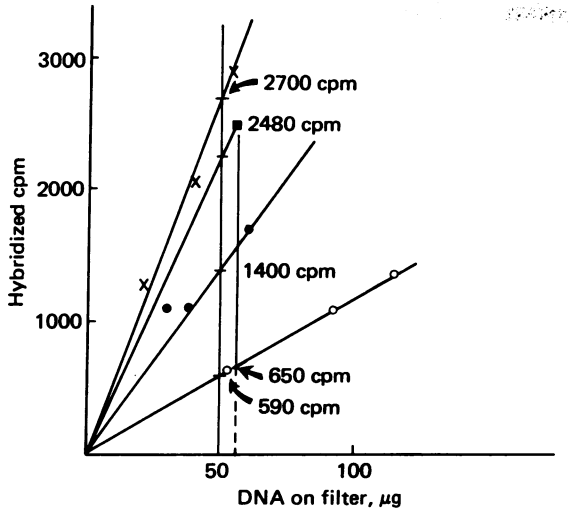

FIG. 1. Detection of EBV DNA in HLB cells by cRNA hybridization. DNAs from Raji cells, HLB cells, and Simpson cells were fixed to nitrocellulose membrane filters and hybridized with EBV-specific cRNA prepared with *E. coli* RNA polymerase. EBV genome content was calculated from the hybridized counts at 50 μg of each DNA. ×, Hybridized with Raji DNA; ●, hybridized with HLB DNA; ○, hybridized with Simpson DNA; ■, hybridized with Simpson DNA (55 μg) plus EBV DNA (62 ng) to make 45 genomes per cell.

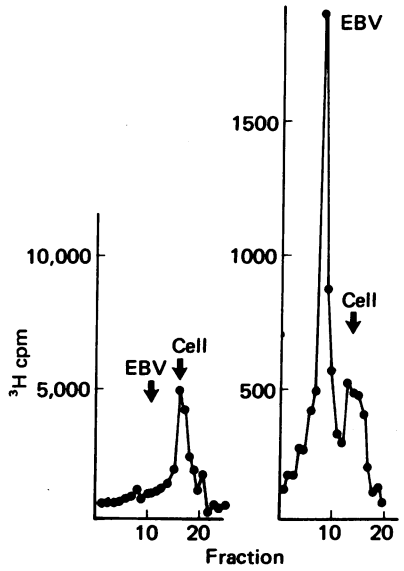

FIG. 2. EBV DNA replication and inhibition of host cell DNA synthesis after superinfection of HLB cells with HR1 virus. Infected (*Right*) and noninfected (*Left*) HLB cells were incubated with 20 μCi of [^{3}H]thymidine per ml between 10 and 48 hr after infection. They were lysed by incubation at 37° with 100 μg of proteinase K per ml and 1% Sarkosyl NL 97 for 15 hr. The density of labeled DNA in the lysate was determined by CsCl density gradient centrifugation. Density of fractions collected was obtained from refractive indexes.

Colcemid (1 μg/ml) for 4 hr before harvest. Cell spreads on glass slides were stained with Giemsa. Twenty spreads of each preparation were analyzed and karyotyped (10).

RESULTS

Establishment of human lymphoblastoid cells

It has been reported that superinfection of Raji cells with EBV obtained from P3HR1 cell cultures induced the production of EBV particles (SI Raji virus) but the virus thus produced did not superinfect Raji cells (5). In this study, however, we found that infection of human cord blood lymphocytes *in vitro* with SI Raji virus resulted in the establishment of lymphoblastoid cell lines that grew continuously *in vitro*. In five transformation experiments with different preparations of both SI Raji virus and human lymphocytes, clumped cells consistently appeared within 5–8 weeks and lymphoblastoid cell lines were established. A 1:10 dilution of supernatant fluid from superinfected Raji cells still had the transformation activity of SI Raji virus although no transformation occurred with a 1:100 dilution. Under the same conditions a 1:1000 dilution of supernatant fluid from B95-8 cell cultures retained transforming activity. No evidence of transformation was observed in uninfected fresh cord lymphocyte cultures or in the cultures directly exposed to HR1 virus (10° to 10^{-5} dilutions of concentrated virus used for superinfection of Raji cells).

HLB cells established by SI Raji virus infection

One cell line (HLB) established by infection of human cord blood lymphocytes with SI Raji virus was further characterized. As shown in Fig. 1, 22 EBV genomes per cell were detected in HLB cells by hybridization of EBV-specific cRNA with HLB cell DNA on nitrocellulose membrane filters. In accordance with results of cRNA hybridization, more than 95% of HLB cells contained EBV-determined nuclear antigen in anticomplement immunofluorescence tests. Early antigen and viral capsid antigen were demonstrated in less than 0.1% of the cell population. HLB cells were susceptible to superinfection by HR1 virus and EBV DNA replication under conditions of inhibition of host cell DNA synthesis, similar to Raji cells superinfected with HR1 virus was observed (Fig. 2).

Karyotypes of HLB and Raji cells were analyzed to confirm that the establishment of the HLB cell line was the result of

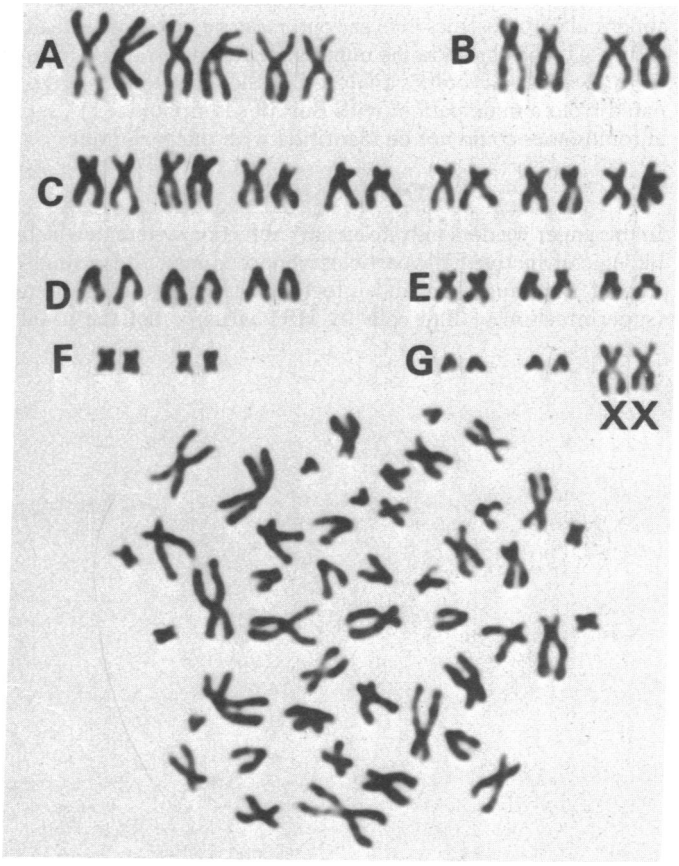

FIG. 3. Karyotype of HLB cells.

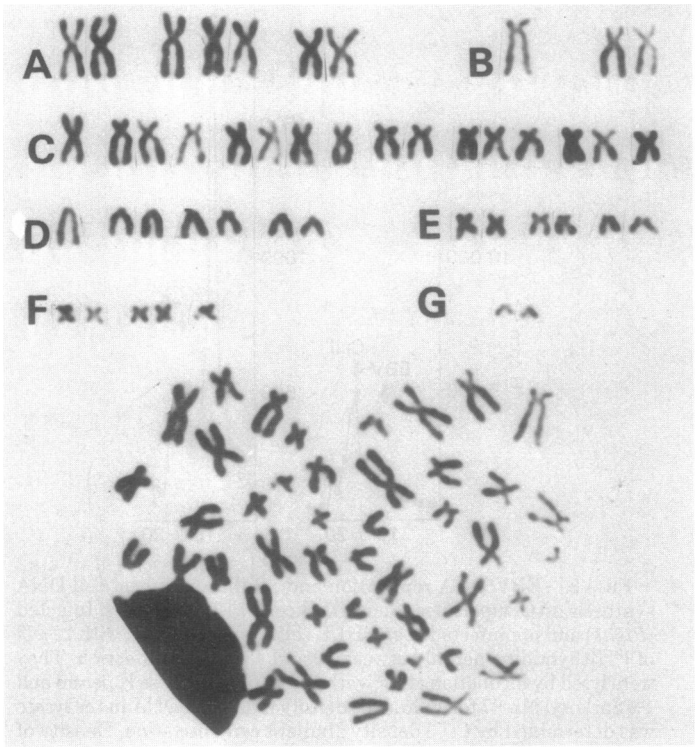

FIG. 4. Karyotype of Raji cells.

immortalization of human cord blood lymphocytes rather than contamination with Raji cells. The HLB cell chromosome spreads analyzed were diploid, with normal female karyotypes (Fig. 3). Although the majority of Raji cells were diploid, none of 20 karyotypes was normal; they contained extra, missing, or abnormal chromosomes in every chromosome group other than E (Fig. 4). The chromosome numbers of Raji cells ranged from 42 to 55 in 20 karyotypes analyzed. Although Raji cells originated from a male patient with Burkitt's lymphoma (11), sex chromosomes could not be identified with this technique.

DISCUSSION

In this paper we demonstrate an EBV infection system in which biologically active EBV particles were produced after a single step of lytic and cytocidal infection. However, this system (superinfection of Raji cells by HR1 virus) is not the usual permissive system because progeny virions exhibited an altered capability of infection from parental HR1 virus. HR1 virus is a unique strain of EBV which is characterized as a superinfecting virus rather than a transforming virus. Superinfecting but nontransforming EBV (HR1 virus) was converted into nonsuperinfecting but transforming EBV (SI Raji virus) through superinfection of Raji cells with HR1 virus.

The question concerning the origin of SI Raji virus remains to be answered. Two possibilities are considered here: (*i*) SI Raji virus DNA is the replicational result of parental HR1 virus DNA. In this case, HR1 virus DNA must be modified or partially deleted during replication of virus DNA, which results in production of defective virus with transforming activity. (*ii*) SI Raji virus DNA is derived from resident EBV DNA in Raji cells. Raji cells carry constant numbers of EBV genomes per cell, the expression of which is regulated so that the cells maintain the transformed state but the virus does not enter the lytic cycle. This regulatory mechanism may be destroyed by HR1 virus infection leading to the replication of latent EBV DNA.

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