Rescue of Epstein-Barr Virus from Somatic Cell Hybrids of Burkitt Lymphoblastoid Cells

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A Burkitt lymphoblastoid cell line, P3J-HR-1, was fused and hybridized to a human sternal marrow cell line. The somatic cell hybrids were negative when examined for Epstein-Barr virus (EBV) markers. When the hybrid cells were exposed to 5-iododeoxyuridine, both EBV-specific antigens and virus particles were induced as determined by the immunofluorescence test and by electron microscopy. The data presented suggest that the EBV genome can be transferred from a lymphoblastoid cell to another cell type during cell hybridization, that the EBV genome can persist in the hybrid cells for long periods of time, and that synthesis of the virus can be induced in the heterokaryons.

Inactivated Sendai virus has been used to induce cell fusion in several laboratories (7, 12, 21). The procedure has been applied to cancer research where somatic cell hybridization (3, 15) has proven to be a fruitful technique in many studies. The rescue of simian virus 40 from transformed cells after they were fused to potentially susceptible cells (6, 16) has greatly stimulated use of the method in virus laboratories. The method, when applied to papovavirus systems, has already allowed observations that suggest that, in somatic cell hybrids of normal and virus-transformed cells, virus-specific tumor and transplantation antigens can be transferred into the hybrid and can persist in the hybrid cells for long periods of time (5, 26).

The association of a herpesvirus designated the Epstein-Barr virus (EBV) with many human lymphoblastoid cell lines has been reported (8). A small percentage of the cells contain the EBV (9) as well as antigens which can be detected by the immunofluorescence test (13). There is also some evidence that the EBV genome is integrated in the host cell genome (19, 27).

Recent findings have indicated that Burkitt lymphoblastoid cells can be fused and hybridized to both mouse and human cells (R. Glaser and F. J. O'Neill, *Science*, *in press*). In this report, further data is presented concerning the properties of the Burkitt lymphoblastoid somatic cell hybrids and the induction of synthesis of EBV-specific antigens and virus particles using the drug 5-iododeoxyuridine (IUDR).

MATERIALS AND METHODS

Cells. The human sternal marrow cell line, D98/AH-2, (D98) (25) was maintained in Eagle medium supplemented with 10% fetal calf serum, 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 1 μ g of fungizone/ml, 10 units of mycostatin/ml, and 0.075% NaHCO₃ in 8-oz (ca. 0.24 liter) glass prescription bottles at 37 C. The Burkitt lymphoblastoid cell line, P3J-HR-1, (HR-1) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 1 μ g of fungizone/ml, 10 units of mycostatin/ml, and 0.075% NaHCO₃ in 8-oz (ca. 0.24 liter) glass prescription bottles at 35 C.

Fusion and hybridization. The method for fusion of D98 cells to HR-1 cells has been previously described (R. Glaser and F. J. O'Neill, *Science*, *in press*). The procedure was as follows: 2×10^6 D98 cells were seeded into tissue culture plates and grown for 24 hr at 37 C. HR-1 cells were prepared at a concentration of 10^7 cells in 0.2 ml in Eagle medium without serum. The D98 cells were washed with phosphate-buffered saline (PBS). Sendai virus (400-1,000 hemagglutinating units in 0.2 ml of PBS) was inactivated with ultraviolet light and added to the center of the cell sheet and to the HR-1 cell suspension. Both the HR-1 and D98 cells were cooled on ice for 5 min. Then the cell suspension was placed on the monolayer cultures and cooled for 5 min on ice, and the cultures were placed at 37 C for 3 to 4 hr in an atmosphere of 5% CO₂. Three days after fusion, the normal Eagle medium was replaced with the selective HAT medium containing aminopterin and thymidine (17), supplemented with 10% fetal calf serum, 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 1 μ g of fungizone/ml, 10 units of mycostatin/ml, and 0.225% NaHCO₃. The cells were maintained in HAT medium in 100-mm

tissue culture plates in an atmosphere of 5% CO₂ at 37 C.

Somatic cell hybrids. After the D98 cells had died and colonies of hybrid cells (D98/HR-1) were observed, the cells were trypsinized and cloned in 35-mm tissue culture plates. The cells were maintained continuously in HAT selective medium at 37 C in 250-ml plastic tissue culture flasks throughout the study.

Treatment with IUDR. The D98/HR-1 cells were trypsinized and grown on 18-mm glass cover slips in 60-mm plastic tissue culture plates. When cell monolayers were obtained, the HAT medium was replaced with 5 ml of Eagle medium containing 40 µg of IUDR/ml. The cells were incubated at 37 C for 3 days, at which time the Eagle medium containing the drug was replaced with normal Eagle medium for an additional 3 days.

Immunofluorescence techniques. Both the direct and indirect immunofluorescence tests were used to detect EBV-specific antigens. Cover slips with monolayers of IUDR-treated cells were washed with tris(hydroxymethyl)aminomethane (Tris)-buffered saline, pH 7.4, air dried, and fixed in acetone for 3 min. For the direct test, the cells were rehydrated in Tris and adsorbed with human serum positive for EBV antigens (by immunofluorescence) previously conjugated to fluorescein isothiocyanate (FITC) (obtained from Charles Pfizer & Co.) for 30 min, washed three times with Tris, two times with distilled water, air dried, and mounted in elvanol on glass slides.

For the indirect test, acetone-fixed cells were rehydrated in Tris, adsorbed with human EBV-positive serum (tested on HR-1 cells) for 30 min, washed three times with Tris, and reabsorbed with rabbit anti-human immunoglobulin G (IgG)-FITC (Hyland) for 30 min. The cells were washed as before and mounted. Preparations were examined for EBV-specific fluorescence with a Zeiss microscope with an ultraviolet light source.

Serum-blocking experiments. Cover slips with monolayer D98/HR-1 cell cultures that had been treated with IUDR were prepared, washed, and fixed as previously described. The cells were adsorbed with EBV-specific antiserum for 30 min, washed as before, and reabsorbed with EBV-specific antiserum conjugated to FITC, washed, dried, and mounted. The cells were then examined for EBV-specific antigens by using the immunofluorescence method.

Electron microscopy. Cultures of D98/HR-1 cells were grown in 250-ml plastic tissue culture flasks and treated with IUDR as previously described. At 3, 5, 7 and 10 days after removal of IUDR, the cell monolayers were washed with PBS, pH 7.3, and scraped off the glass with a rubber policeman. Any floating cells in the culture were combined with the cells from the monolayer and fixed in Karnovsky's fixative (14) overnight at 4 C. The specimens were postfixed for 2 hr with Dalton's chrome-osmium (4) (containing 2% osmium tetroxide), dehydrated, and embedded in Spurr's low-viscosity plastic (24). Thin sections were prepared by using an LKB ultratome; the sections were stained with 0.5% uranyl acetate in 50% meth-

anol and Reynold's lead (22), placed on naked copper grids, and examined with an Hitachi HU-12 electron microscope at 75 kv.

RESULTS

General properties of somatic cell hybrids. When D98 cells were grown in selective HAT medium, the cells died within 2 to 3 weeks. Briefly, D98 cells do not contain the enzyme IMP pyrophosphorylase and are not able to utilize hypoxanthine in the "scavenger" pathway of deoxyribonucleic acid (DNA) synthesis. The HAT medium contains aminopterin and thymidine which selectively inhibits de novo purine synthesis but allows cells capable of synthesizing DNA, utilizing preformed purines, to grow (25). However, after fusing the D98 cells to the Burkitt lymphoblastoid cell line HR-1 (a cell line which only grows in suspension *in vitro*), the resulting heterokaryons were able to grow in the selective medium, due to the synthesis of the enzyme IMP pyrophosphorylase mediated by the genome of the HR-1 parent. The fused cells developed into somatic cell hybrids and were cloned. Detailed chromosome studies were performed. The modal numbers of the D98/HR-1 clones examined were approximately equal to the sum of the modal numbers of the D98 and HR-1 parental cell lines (R. Glaser and F. J. O'Neill, Science, *in press*).

The morphology of the D98/HR-1 cells, which grow as monolayers, was unlike the morphology of the D98 or the HR-1 cells, presumably due to the complement of chromosomes supplied by both parental lines. The hybrids were larger in size, and the growth patterns on a glass or plastic surface were different (Fig. 1 and 2). The D98 cells were contact inhibited (Fig. 3). In contrast, the D98/HR-1 cells were observed to grow frequently in multilayered foci (Fig. 4).

EBV specific antigens. When cover slips of D98/HR-1 cells were fixed in acetone and tested for EBV-specific antigens by the direct and indirect immunofluorescence test, no evidence of virus-specific antigens was found. These tests were performed at 2, 3, 4 and 8 months after fusion.

Induction of EBV-specific antigens after treatment with IUDR. Cover slip cultures of D98, human embryonic lung (HEL), and D98/HR-1 cells clone (cl) 1, 2, 3, and 8 were prepared. Attempts were made to induce EBV-specific markers by using 40 µg of IUDR/ml in Eagle medium. The cells were treated for 3 days after which the medium containing IUDR was replaced with normal medium for an additional 3 days. Acetone-fixed D98, HEL, and D98/HR-1 cells were tested for EBV-specific antigens by using the direct and indirect immunofluorescence tests. The results are

FIG. 1. Photomicrograph of D98 cells stained with hematoxylin and eosin. $\times 527$.

FIG. 2. Photomicrograph of D98/HR-1 cells stained with hematoxylin and eosin. $\times 527$.

FIG. 3. Photomicrograph of D98 cells growing in cell culture. $\times 280$.

shown in Table 1. All 4 clones of D98/HR-1 cells contained EBV-specific antigens by the direct and indirect immunofluorescence test with a variety of human sera (pretested on HR-1 cells). The sera were from patients with infectious mononucleosis or with nasopharyngeal carcinoma. Cytoplasmic and nuclear fluorescence was observed (Fig. 5 and 6) in from 1 to 20% of the cells. Normal and drug-treated D98 and HEL cells under the same test conditions were negative.

Serum blocking tests. To obtain additional data on the specificity of the reactions observed, serum blocking tests were performed. D98/HR-1 cells, grown on cover slips and treated with IUDR, were exposed to serum containing antibodies to EBV. This was followed by exposure to serum containing antibodies to EBV conjugated to FITC (used in the direct immunofluorescence test). Reactions were observed in cells where the primary (blocking) serum was used at a dilution of 1:10, but no EBV-specific antigens were observed by

fluorescence when undiluted EBV antiserum was used to block the reaction.

To determine if the EBV antiserum used in the serum blocking experiments would block the immunologic reaction in a nonspecific manner, it was tested on rabbit kidney (RK) cells infected with herpes simplex virus type 2 (HSV-2). The EBV-positive serum was adsorbed on acetone-fixed monolayer cell cultures of RK cells infected with HSV-2 (pretested with rabbit anti HSV-2 antiserum and found positive for HSV-2-specific antigens by immunofluorescence). The cells were then adsorbed with rabbit anti-HSV-2 antiserum followed by horse anti-rabbit antiserum conjugated to FITC. The EBV-specific antiserum did not interfere with the detection of the HSV-2-specific antigens.

Synthesis of virus particles. When cultures of D98/HR-1 cells were examined at 3, 5, 7 and 10 days after seeding in tissue culture flasks, no virus particles were observed. However, when D98/

FIG. 4. Photomicrograph of D98/HR-1 cells growing in cell culture. $\times 280$.

HR-1 cells previously treated with IUDR were examined, virus particles were observed in the nuclei of degenerating cells on days 7 and 10 (Fig. 7). Both enveloped (in the cytoplasm) and unenveloped particles were found in many cells examined.

In a second set of experiments, D98/HR-1 cells were pooled from several flasks and either seeded into tissue culture flasks or grown on cover slips. One set of flasks and cover slip monolayer cell cultures was treated with IUDR; the other set was not exposed to the drug. Seven days after removal of IUDR from the treated cultures, both sets of cell cultures were fixed for electron microscopy examination. The cover slip monolayer cells were fixed and examined by the direct immunofluorescence test, by the method already described.

The D98/HR-1 cells treated with IUDR contained virus particles in the nuclei of about 10% of the cells examined and some cells exhibited particles in the cytoplasm; however, no virus particles were observed in the untreated cells. When

the specimens prepared for immunofluorescence were examined, the cells treated with IUDR contained EBV antigens as described previously. No EBV antigens or particles were detected in the untreated cells (Table 2).

DISCUSSION

The results presented in this report and in a preliminary report (R. Glaser and F. J. O'Neill, Science, *in press*) suggest that Burkitt lymphoblastoid cells have been fused and hybridized with both mouse and human cells. The chromosome data, along with the alteration in cell morphology and ability to grow in a selective medium, were the criteria used to support evidence of somatic cell hybridization.

The data obtained from chromosome analysis from earlier work suggested that most, if not all, the HR-1 chromosomes were in the nuclei of the D98/HR-1 cells. In addition, EBV-specific anti-

FIG. 5. Immunofluorescence photomicrograph of EBV antigens localized in the cytoplasm in D98/HR-1 cells treated with 40 μ g of 5-IUDR/ml for 3 days and then examined 3 days after the cells were grown in normal medium. Many of the cells containing EBV antigens were rounded. $\times 527$.

FIG. 6. Immunofluorescence photomicrograph of EBV antigens localized in the nucleus of a D98/HR-1 cell treated with 40 μ g of 5-IUDR/ml for 3 days and then examined 3 days after the cells were grown in normal medium. Note the even distribution of EBV antigens throughout the nucleus. $\times 840$.

TABLE 1. Induction of EBV-specific antigens in D98/HR-1 cells treated with IUDR as measured by the immunofluorescence test

Cells	Immunofluorescence—Avg percent of positive cells			
	Direct ^a test	Indirect test sera		
		1 ^a	2 ^b	3 ^c
HEL ^d	0	0	0	0
HEL + IUDR ^e	0	0	0	0
D98	0	0	0	0
D98 + IUDR	0	0	0	0
D98/HR-1				
Clone 1	0	0	0	0
Clone 2	0	0	0	0
Clone 3	0	0	0	0
Clone 8	0	0	0	0
D98/HR-1 + IUDR				
Clone 1	10–20	5–15	1–2	0
Clone 2	10–20	5–15	1–2	0
Clone 3	10–20	5–15	1–2	0
Clone 8	10–20	5–15	1–2	0

^a Serums (diluted 1:10) from patients with nasopharyngeal carcinoma.

^b Serum (diluted 1:5) from a patient with infectious mononucleosis.

^c Serum (diluted 1:2) from a normal patient known to be negative for antibodies against EBV.

^d Human embryonic lung cells.

^e 40 µg/ml.

gens, as determined by the immunofluorescence tests, have been induced in the hybrid cells when the cells were treated with IUDR. There are preliminary results (Glaser, *unpublished observations*) that induction can also be accomplished with 5-bromodeoxyuridine (BUDR). The data suggest that at least part of the EBV genome was transferred from the HR-1 cells to the hybrids and has persisted for 10 months without expression of EBV markers, as determined by electron microscopy and by immunofluorescence. These results support current concepts on the integrated state of the EBV genome (11). Cloning and nucleic acid hybridization data support the concept that the EBV genome can persist without expression for long periods of time in what had been presumed to be EBV-negative cells (19, 20). To further support this concept, preliminary data indicate that there are several EBV genomes in each D98/HR-1 cell, as determined by nucleic acid hybridization experiments now being performed (M. Nonoyama, *personal communication*).

An important conclusion can be drawn from the data presented. Namely, the EBV genome can persist and can be activated to synthesize virus

in a cell type other than a lymphoblastoid cell. The possibility exists that the lymphoblastoid cells, in which the EBV is found in vivo, may not be the sole site of infectious virus replication, an observation already made in the case of the Marek's disease herpesvirus (2).

Other investigators have reported the induction of EBV antigens in "normal" lymphoblastoid cell lines with BUDR (10). In the presence of BUDR, both nuclear and cytoplasmic antigens were detected. In addition, ribonucleic acid (RNA) viruses have also been induced in cell lines with BUDR or IUDR (1, 18). Thus, the results presented here concerning induction of EBV antigens in somatic cell hybrids reveal that induction of virus in hybrids may follow a pathway similar to that in virus-transformed cells.

The sera used in the indirect and direct immunofluorescence tests were known to be highly specific. They had been tested against cells infected with other herpesviruses and RNA tumor viruses as well as a variety of cell lines in this laboratory and at Pfizer & Co. (K. Traul, *personal communication*). The data from the serum-blocking experiments and the induction experiments with D98 and HEL cells also support the specificity of the antigens observed in the D98/HR-1 cells.

There was no emperipolesis of the HR-1 cells by the D98 cells as determined by brightfield and phase-contrast microscopy, discounting the possibility of carry-over of lymphoblastoid cells. To further support this, no evidence of the persistence of HR-1 cells was found by visual observations and by chromosome analysis when the cells were cloned. Furthermore, the cells that are positive in the immunofluorescence test and by electron microscopy are epithelial-like in morphology.

Whether the D98/HR-1 cells are transformed is still not clear. The hybrid cells grow in multilayers, indicating a loss of contact inhibition. The D98 cells have never been observed to do this. However, further work is necessary to clarify this point.

The production of EBV particles in the IUDR-treated cells clearly shows that the EBV genome maintained in the hybrid cells are complete enough to code for EBV DNA and capsid proteins, since complete virus particles with dense cores were found. Whether enough information is present to code for the synthesis of "infectious" EBV with a higher oncogenic potential than the EBV presently obtained from lymphoblastoid cells remains to be determined. If infectious, the properties of this virus and its ability to replicate or transform various cell types will represent an important extension of this work.

FIG. 7. Electron micrograph of D98/HR-1 cells treated with 60 μ g of 5-IUDR/ml 7 days after replacement of medium containing IUDR with normal medium. Note particles with dense cores in the nucleus and cytoplasm (arrows). Bar represents 1 μ m.

TABLE 2. Induction of EBV-specific antigens and virus particles in D98/HR-1 cells treated with 5-iododeoxyuridine (IUDR)

Cells	Presence of virus antigens ^a	Presence of virus particles
D98/HR-1	None detected	None detected
D98/HR-1 + IUDR ^b	Present	Present

^a Serum (diluted 1:10) from a patient with nasopharyngeal carcinoma.

^b At 60 μ g/ml for 3 days; cells were fixed for electron microscopy 7 days after the removal of IUDR (performed at 37 C).

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