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Activation of Epstein–Barr Virus by 5-Bromodeoxyuridine in "Virus-Free" Human Cells

(complement-fixing antigen/immunofluorescence/leukocytes)

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ABSTRACT Treatment of three human lymphoid cell lines, free of detectable Epstein–Barr virus, with 5-bromodeoxyuridine resulted in activation of virus synthesis in up to 8% of the cells. The induction of infectious virus could be demonstrated by cocultivation experiments with peripheral leukocytes. These studies demonstrated that the entire viral genome may persist in at least a portion of the lymphoid cells.

In recent years, many human lymphoid cell lines have been established from patients with various malignant and nonmalignant diseases, and also from normal donors. 0.1–5% of these cells generally contain Epstein–Barr virus (EB virus), detectable by immunofluorescence or electronmicroscopy. A few of these lymphoid cell lines contain no detectable EB virus, e.g., Raji cells derived from a Burkitt lymphoma (1), $NHDL_3$ cells from normal human leukocytes (2), and NC_{37} cells from leukocytes of a patient with pneumonia (Jensen, unpublished data). Although these cells appear to be free of detectable EB virus, the presence of the viral genome has been demonstrated by cloning experiments (3) and by nucleic acid hybridization studies (4, 5). Furthermore, these cells also contain soluble complement-fixing (CF) antigens that are EB virus-related (6–8).

The present study was undertaken to develop 5-bromodeoxyuridine (BrdU)-resistant lymphoid cell lines that are EB virus-negative for cell fusion experiments. During the course of this work, it became apparent that the BrdU-treated cells showed the presence of EB virus antigens. Further studies indicated that induction of virus synthesis by BrdU was reproducible. This report gives an account of our preliminary observations. Hampar *et al.* (9) in a separate and independent study obtained essentially similar findings.

METHODS

Cell Cultures. NC_{37}, $NHDL_3$, and Raji cells were grown in static suspension cultures in McCoy's 5a medium with 20% unheated fetal-calf serum and neomycin sulfate. The medium was replaced every 2–3 days with or without BrdU. All three cell lines were derived from male subjects and the cells had a male karyotype.

Immunofluorescence Tests. Cell smears were prepared on slides, air-dried, fixed in acetone for 5 min, and treated with human serum B 76 (diluted 1:10) for 1 hr at 37°C. The cell smears were washed in saline and stained with antiserum to human globulin that was prepared in the goat and labeled with fluorescein-isothiocyanate (Hyland), mounted with Elvanol, and examined by ultraviolet microscopy.

Complement-Fixation Tests (CF). Untreated and BrdU-treated lymphoid cells were pelleted and washed in veronal-buffered saline (pH 7.8); 10% cell suspensions in veronal-buffered saline were disrupted by ultrasonic vibration. After centrifugation at 3500 rpm for 25 min, the supernatant was titrated for CF antigen content by the microtiter test; a known positive human serum B 76, reactive with both virion and soluble antigens of EB virus, was used (7). All appropriate controls were included in each test.

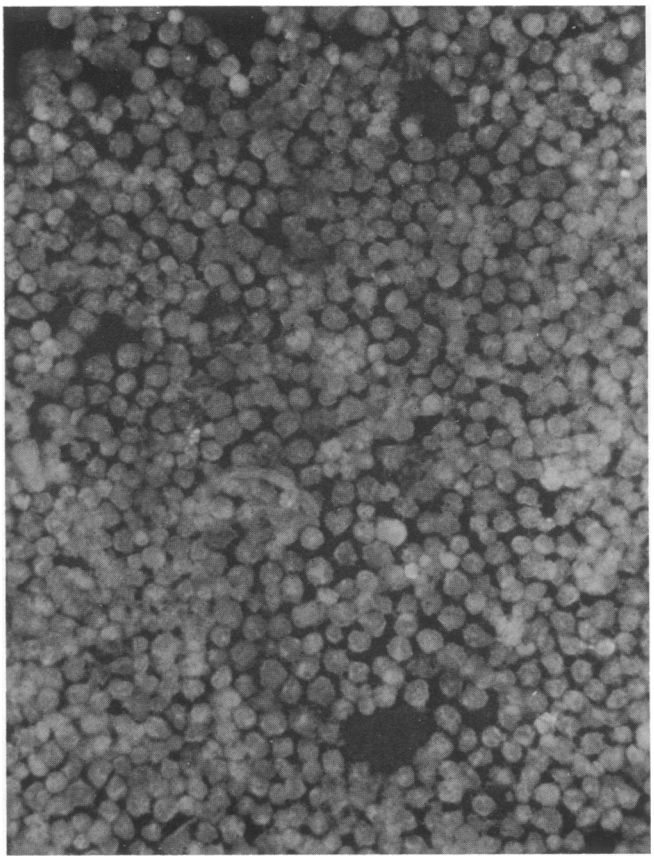
RESULTS

Treatment of these three cell lines with BrdU resulted in the appearance of immunofluorescence-positive cells. As seen in Fig. 1, untreated controls contained no cells with detectable EB virus antigens. Immunofluorescence observed after treatment with BrdU (Fig. 2) involved both cytoplasm and nucleus. When treated cells were maintained on drug-free medium for 7–10 days, about $1/3$ of the immunofluorescence-positive cells showed distinct nuclear staining. The specificity of the immunofluorescence reaction was determined as follows. BrdU caused no autofluorescence of the treated cells. Human sera free of detectable antibodies to EB virus, but positive for antibodies to herpes simplex or cytomegalo virus failed to react with treated cells. Only EB virus-positive sera from patients recovering from infectious mononucleosis were reactive, while their pre-illness sera were negative. Induction of EB virus fluorescence by BrdU could be completely inhibited by the addition of 50 μg/ml of thymidine at the time of drug treatment.

Activation of viral antigen production was depended on the time and dose of treatment with BrdU, within the range of 5–25 μg/ml, as summarized in Table 1. The first antigen-positive cells appeared 3–4 days after treatment and had a maximum amount of antigen after 7–10 days. Concentrations of BrdU in excess of 25 μg/ml caused the death of 50% or more of the cells after 5–7 days of continuous treatment.

The CF antigen activity was determined 7–10 days after continuous treatment. It can be seen that treatment with BrdU resulted in 4- to 8-fold enhancement of CF antigen activity. The low CF activity of untreated cells has been reported to represent soluble, EB virus-related antigens (7, 8). The enhanced CF activity induced by BrdU may be due to increased soluble and/or viral antigens.

Abbreviations: EB virus, Epstein-Barr Virus; CF, complement fixing.



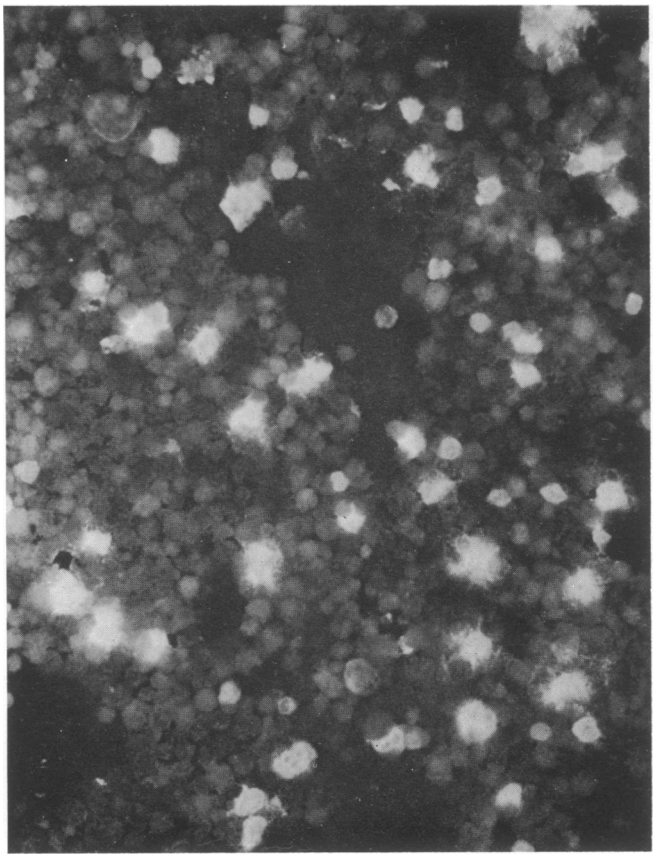


FIG. 1 (*left*). Untreated NC_{37} cells, acetone fixed, ×100. Note absence of immunofluorescence.
FIG. 2 (*right*). BrdU-treated NC_{37} cells, acetone fixed, ×100, showing many immunofluorescent cells.

Detection of small amounts of infectious EB virus in culture fluids of BrdU-treated cells was difficult due to the lack of a sensitive assay system. We therefore used a technique that allowed the detection of BrdU-induced, cell-associated virus (10). NC_{37} cells were treated continuously for 7 days with 25 μg/ml of BrdU and were then maintained on drug-free medium for 12 days. Both treated and untreated cells were then lethally x-irradiated (3000 R) and cocultivated with fresh, peripheral leukocytes obtained from a normal, adult female, who did not have detectable CF antibodies to EB virus. Previous attempts to grow the leukocytes of this donor in continuous culture have been unsuccessful. 25×10^6 irradiated cells and an equal number of leukocytes were placed in mixed culture in a 2.5 × 15 cm round-bottom tube, in a total volume of 10 ml.

Separate cultures of BrdU-untreated or treated, irradiated cells, or leukocytes were set up in parallel. All cultures were maintained and observed for a period of 3–10 weeks with regular changes of media. The following results were obtained. None of the individual cultures of leukocytes or irradiated cells remained viable for more than 2–3 weeks. Similarly, none of four mixed cultures of leukocytes and untreated, irradiated NC_{37} cells survived beyond a period of 4 weeks. By contrast, leukocytes in three of four mixed cultures containing BrdU treated, irradiated NC_{37} cells began to multiply after 4–6 weeks and attained a generation time of 36–40 hr. The cells had a blastoid morphology, characteristic of established lymphoid cell lines. All of the examined several hundred cells in metaphase contained the female karyotype. About 0.1–0.3% of the transformed leukocytes contained EB virus anti-

TABLE I. *Induction of EB virus antigens by 5-Bromodeoxyuridine in human cells*

	Immunofluorescence						CF antigens 7–10 days	
	3 days			7–10 days				
	BrdU, μg/ml			BrdU, μg/ml			BrdU, μg/ml	
Cells	0	5	25	0	5	25	0	25
NC_{37}	0*	0.3	0.5	0	2.0	8.0	2†	16
$NHDL_3$	0	0.1	0.3	0	1.0	5.0	1	8
Raji	0	0.1	0.3	0	1.0	5.0	2	8

* Average percent of positive cells.
† CF antigen units/25 μl of cell sonicates.

gens that were detectable by immunofluorescence tests. These results suggest a cell-to-cell transmission of BrdU-induced EB virus from the irradiated cells to adjacent leukocytes, resulting in lymphoproliferation and blastoid transformation.

DISCUSSION

The results reported here demonstrate activation by BrdU of the expression of EB virus genomes in "virus-free" human lymphoid cells. These observations corroborate and extend the evidence obtained by nucleic acid hybridization studies (4, 5), which indicated the presence of EB virus genetic information in such cells. Our demonstration of infectious virus induced by BrdU suggests that the entire viral genome may persist in at least some cells. It is noteworthy, however, that the EB virus genome could not be rescued from untreated, irradiated Raji cells (10) or NC_{37} cells by cocultivation. It is possible that this failure may be due to our inability to achieve fusion between lymphoid cells and leukocytes under the experimental conditions used. However, other possible explanations cannot be ruled out at this time.

The mechanism of action of BrdU-induced EB virus synthesis remains to be determined. Mitomycin C, previously shown to induce SV 40 virus synthesis in virogenic tumor cells (11) failed to activate EB virus (unpublished observations). Inhibition of BrdU activation by thymidine suggests that incorporation of the analog into DNA may be an essential step in the induction process.

It is of particular interest that Lowy *et al.* (12) recently reported induction of murine leukemia virus by BrdU in mouse embryo cell cultures (12).

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