DNA Synthesis in EB Virus-Containing Burkitt Lymphoma Cultures during a Temperature Cycling Procedure (34619)

BRUCE A. MAURER, J. LESLIE GLICK, AND JUN MINOWADA
(Introduced by G. E. Moore)

Cell Laboratories, Roswell Park Memorial Institute (New York State Department of Health), Buffalo, New York 14203

A herpes-type virus has been detected in nearly all of the lymphoblastoid cell lines derived from Burkitt's lymphoma (BL) and is now referred to as EB virus (EBV) after Epstein and Barr (1). A temperature cycling procedure (2) or incubation at 32° (3) has been demonstrated to increase the number of EBV-containing cells and the EBV content in Burkitt lymphoma cell lines relative to cultures maintained at 37°. Since this appeared to be a reproducible method of enhancing the replication of this herpes-type virus, the pattern of DNA synthesis of EBV-containing and "EBV-free" BL cell lines was studied during temperature cycling and temperature reduction (32°) experiments.

Materials and Methods. Cell lines. Two permanent lymphoblastoid cell lines derived from Burkitt's lymphoma were selected for this study; P3JHR1K (HR1K) which is an EBV-containing clone established by Hinuma (4) and derived from the P3J cell line of Pulvertaft (unpublished data), and P-1 Raji (P1R), also established by Pulvertaft.

The P1R cell line was free of EBV as determined by electron microscopy, although a few fluorescent positive cells by an indirect immunofluorescent technique have been observed. The positive relationship between virus-containing cells and an indirect immunofluorescent technique has been previously established (5). Since the incidence of virus-containing cells in P1R culture probably falls well below the sensitivity of the electron microscopic and indirect fluorescent antibody techniques, the term "virus-free" will be used in this report. These cells were grown in spinner or stationary culture vessels in RPMI medium 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (50 μgm/ml).

Temperature cycling. An equal number of stationary HR1K and P1R cultures, each dispensed from a common pool of cells, were incubated at 37°. After 3 days incubation at 37°, one-half of each set of cultures was transferred to 32°. After approximately 2 weeks, the cultures at 32° were reincubated at 37°. Several times during this procedure, duplicate HR1K and P1R cultures from each incubator were selected at random and exposed to methyl-labeled ^{3}H-thymidine (sp. act. 6.7 Ci/m*M*) at a concentration of 0.5 μCi/ml. After a 24-hr exposure, each culture was treated as follows: total and viable cell counts were determined on duplicate samples using the trypan blue dye-exclusion test; duplicate smears were prepared for a direct or indirect immunofluorescent test for EBV-related antigen; and DNA was extracted. Duplicate cultures from each condition were routinely examined for cell density and viability, and all cultures were fed accordingly. Thus, the cultures were fed so as to provide for an optimum environment for cell growth.

Immunofluorescent tests for EBV-related antigen. The cells were concentrated by low-speed centrifugation and thin smears prepared on 10-× 35-mm coverslips from the resuspended cell pellet. The smears were air dried and fixed in acetone for 10 min. In the direct test, the smears were stained for 30 min at 37° with fluorescein isothiocyanate-conjugated human 7S globulin. The globulin was extracted from pooled human serum by two precipitations with ammonium sulfate at 50% saturation followed by Sephadex G-200 column chromatography. The 7S was conjugated to flourescein (on 10% celite,

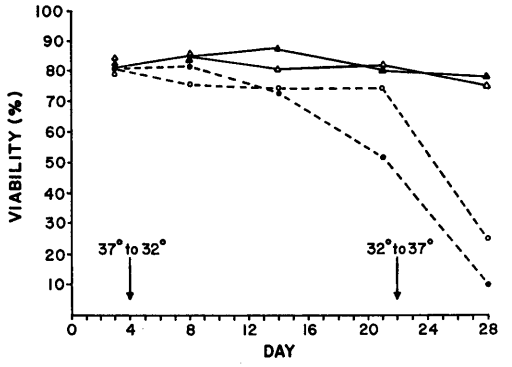

FIG. 1. Viability of temperature cycled and noncycled HR1K and P1R cultures. Each value represents an average of two determinations of duplicate cultures. The arrows indicate the time at which the indicated temperature shift was performed. ▲——▲, noncycled P1R cultures; ●- - -●, cycled P1R cultures; △—△, noncycled HR1K cultures; ○- - -○, cycled HR1K cultures.

Calbiochem) and the unconjugated fluorescein separated by Sephadex G-25 column chromatography. In the indirect test, the smears were first stained with human 7S globulin for 30 min at 37°, washed twice with saline for 5 min, and then stained with goat antihuman gamma-globulin conjugated with flourescein isothiocyanate (Hyland). Both reagents for the indirect test were twice adsorbed with rabbit liver powder and the reagent for the direct test was also adsorbed with 1.0% FCS.

The smears were washed twice for 5 min with saline and mounted in 10% glycerin on thin (<1.0 mm) glass slides. A minimum of 500 cells per smear were counted employing an American Optical Fluorolume fluorescent microscope system. The cells used in this report did not synthesize enough IgG to interfere with the indirect immunofluorescent test.

DNA extraction and isotope counting procedures. The DNA was extracted from approximately 10^7 cells by either a modified perchloric acid technique of Ogur and Rosen (6) or a modified SDS phenol extraction procedure of Dulbecco, Hartwell, and Vogt (7). The extracted DNA was quantitated by employing the factor of Sueoka and Cheng for denatured DNA (8) or the diphenylamine reaction (9). Duplicate 0.2-ml samples of labeled DNA were quantitatively examined in a Packard Tricarb liquid scintillation counter.

Results. Viability and EBV antigen composition of temperature cycled HR1K and P1R cultures. The data in Fig. 1 demonstrates the viability of cycled (32°) and noncycled (37°) P1R and HR1K cultures. They indicate that temperature cycling had a deleterious effect both on the P1R and HR1K cultures, whereas the noncycled cultures maintained a viability between 70 and 90%. The proportion of P1R cultures which fluoresced using the direct test (Fig. 2) was low and relatively constant (1%) in the noncycled cultures and increased to a maximum of about 10% in the cycled cultures. The incidence of fluorescent cells remained relatively stable in the noncycled HR1K cultures, whereas that in the cycled cultures increased from 5 to 40%. Since P1R cells do not contain detectable EBV by electron microscopy, an indirect immunofluorescent test was also performed on cells from both cycled and noncycled P1R and HR1K cells. The P1R cells from the cycled cultures as well as the noncycled cultures did not fluoresce. The incidence of fluorescent cells in the cycled but not the noncycled HR1K cultures

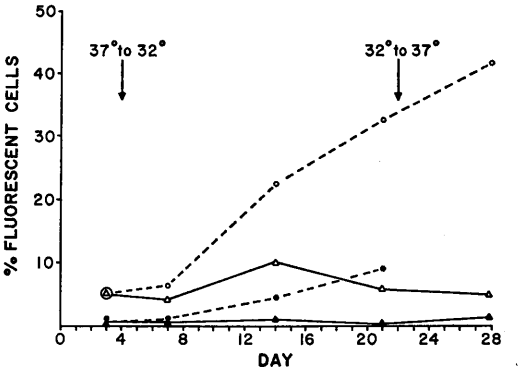

FIG. 2. Immunofluorescent study of temperature cycled and noncycled HR1K and P1R cultures. Each value represents an average of two determinations of duplicate cultures examined by a direct immunofluorescent test. The arrows indicate the time at which the indicated temperature shift was performed. ▲—▲ noncycled P1R cultures; ●- - -●, cycled P1R cultures; △—△, noncycled HR1K cultures; ○- - -○, cycled HR1K cultures.

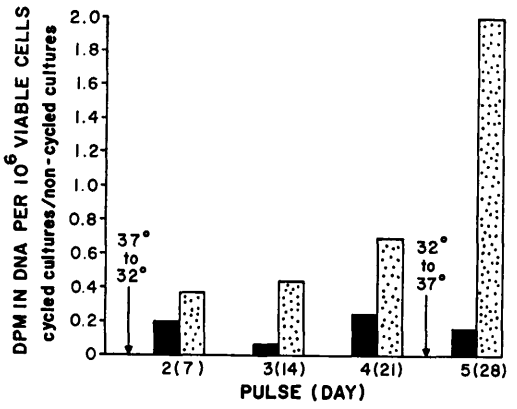

FIG. 3. Ratios of rates of DNA synthesis in temperature cycled and noncycled HR1K and P1R cultures. The DNA was extracted from the cultures after a 24-hr labeling period with ^{3}H-thymidine at a concentration of 0.5 μCi/ml of culture. The arrows indicate the time at which the indicated temperature shift was performed. Shaded bars, P1R cultures; stipled bars, HR1K cultures.

was lower than that observed when the direct test was employed. Both the direct and the indirect test indicated that the temperature cycling procedure enhanced the incidence of fluorescent cells, but the degree of enhancement was higher when the direct test was used.

The effect of temperature cycling on DNA synthesis in HR1K and P1R cells. The rates of DNA synthesis, determined at various times during the temperature cycling procedure, were expressed as the amount of radioactivity in the DNA per 10^6 viable cells. In order to determine the effect of the temperature cycling procedure on DNA synthesis, the rates of DNA synthesis in cycled HR1K cultures were compared, by a ratio, to that occurring at the same time in noncycled HR1K cultures. Ratios were also computed for the P1R cultures. These data, presented in Fig. 3, indicated that cycled P1R cells were synthesizing DNA at about one-fifth the rate as noncycled P1R cells and remained at this low rate throughout the experiment, even though the cultures were returned to 37° prior to pulse 5. Initially, cycled HR1K cultures also synthesized DNA at slower rates than their 37° counterparts, but the inhibition was not as great as that in the cycled P1R cells. As the temperature cycling procedure continued, DNA synthesis in the cycled HR1K cultures proceeded at progressively faster rates. After being reincubated at 37° (pulse 5), the HR1K cultures exhibited a rate of DNA synthesis which was about twice that of the control HR1K cultures. In order to compare the rates of DNA synthesis in the EBV-containing cultures to that in the "virus-free" cultures, rates of DNA synthesis in HR1K cultures were compared, by ratios, to that of P1R cultures for cycled and noncycled cultures. These data are presented in Fig. 4 and demonstrate that the noncycled HR1K cultures were synthesizing DNA at approximately the same rate as noncycled P1R cultures. Cycled HR1K cultures, on the other hand, synthesized DNA at faster rates than cycled P1R cells. Prior to pulse 5 all cycled cultures were returned to 37°, and a comparison of rates of DNA synthesis at this time revealed that HR1K cultures were synthesizing DNA at 12 times that in P1R cultures. This 12-fold increase does not accurately reflect the DNA synthesizing activity of the virus-containing cells because the cycled P1R cultures, although reincubated at 37°, did not synthesize DNA at their normal

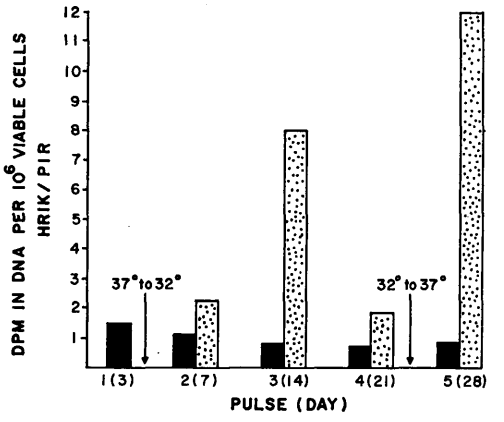

FIG. 4. Ratios of rates of DNA synthesis in temperature cycled and noncycled HR1K and P1R cultures. The DNA was extracted from cycled and noncycled cultures after a 24-hr labeling period with ^{3}H-thymidine at a concentration of 0.5 μCi/ml. The arrows indicate the time at which the indicated transfers were performed. Shaded bars, noncycled cultures; stipled bars, cycled cultures.

TABLE I. Relationship Between the Incidence of EBV-Containing Cells and the Rate of DNA Synthesis in HR1K and P1R Cultures.

% EBV containing cells[a]	CPM in DNA per 10^6 viable cells[b] (HR1K/P1R)
6.2 ± 0.94 (10)[c]	1.10 ± 0.16 (10)
13.6 ± 0.69 (6)	1.45 ± 0.11 (6)
17.2 ± 1.22 (5)	1.91 ± 0.078 (6)

[a] Average % ± SE of fluorescent cells in HR1K cells at 37° using an indirect immunofluorescent technique. P1R cultures were negative.

[b] Average ratio ± SE of the rates of DNA synthesis for HR1K and P1R cultures at 37°.

[c] The numbers in parentheses are the number of samples used in the calculation of the standard error.

(37°) rates (Fig. 3, pulse 5).

It was possible that the observed variations in the rate of DNA synthesis in HR1K cultures might have been due to a more temperature resistant DNA synthetic mechanism. Consequently, the rates of DNA synthesis of HR1K and P1R cultures were determined in three temperature cycling experiments in which the incidence of virus-containing cells in noncycled HR1K cultures was a variable. The data presented in Table I indicated that the incidence of fluorescent cells in noncycled HR1K cultures were significantly different and their corresponding rates of DNA synthesis were faster than that in noncycled P1R cultures. The rates of DNA synthesis were directly proportional to the incidence of virus-containing cells.

Discussion and Summary. The temperature cycling or temperature reduction procedures applied to EBV-containing Burkitt lymphoma cells usually produce cultures which have a higher incidence of EBV-containing cells than nontreated cultures. The magnitude and kinetics of the response was not predictable. It appears that other unknown factors are involved in this stimulation. Henle (10) has suggested that the level of arginine in serum may have an effect upon the level of virus-containing cells in Burkitt lymphoma cell cultures. Minowada *et al.* (2) has indicated that an active period of cell proliferation accompanied by the synthesis of immunoglobulin may be prerequisite to the synthesis of EBV antigen.

"EBV-free" Burkitt lymphoma cells exhibited a loss in viability and a reduction in the rate of DNA synthesis when subjected to these procedures. The direct immunofluorescent test using fluorescein-labeled human 7S globulin reacted with both cells and debris in P1R cultures, and the incidence of this fluorescence increased when the cells were subjected to a temperature cycling procedure. When P1R cells from the same culture were examined by the indirect test, the cells did not fluoresce, and in subsequent experiments in which only the indirect test was used, fluorescent P1R cells were rarely seen. In comparison, when HR1K cells were examined by the direct and indirect tests, the incidence of positive cells in noncycled cultures was the same in both tests and, although there was an increase in the incidence of fluorescent cells in the cycled cultures in both tests, the incidence was significantly less with the indirect test. We have compared the incidence of virus-containing cells by the indirect test to that determined by electron microscopy in several Burkitt lymphoma cell lines and have found a good correlation (unpublished data). Minowada *et al.* (2) has reported that some nonspecific fluorescence was observed with the direct test, especially in old or damaged cultures. Thus, in addition to EBV-containing cells, the direct test used in this report involved the binding of labeled protein to dead cells and debris, and this did not occur or was not detected, when an indirect test was used.

When HR1K and P1R cultures were subjected to a temperature cycling or reduction procedure, both exhibited a reduced rate of DNA synthesis compared to their noncycled counterparts. However, the rate of DNA synthesis was less affected in HR1K cultures in which the incidence of virus-containing cells was increased relative to noncycled HR1K cultures. The larger the proportional increase in the incidence of EBV-containing cells, the less the degree of inhibition (Fig. 3). DNA was synthesized in cycled HR1K cultures at a faster rate than cycled P1R cultures,

whereas in noncycled HR1K cultures, DNA synthesis, relative to that in noncycled P1R cultures, was synthesized at similar rates (Fig. 4). These elevated or less inhibited rates of DNA synthesis in cycled HR1K culture (depending upon whether the comparison was to cycled P1R or noncycled HR1K cultures) are not due to a more temperature resistant mechanism of cellular HR1K DNA synthesis because the effects could be related to the incidence of virus-containing cells. The data presented in Table I support this interpretation, because noncycled HR1K cultures synthesized DNA at faster rates than noncycled P1R cultures when there was a higher incidence of EBV-containing cells.

These data have been interpreted to indicate that in HR1K cultures, the cells exist in two forms, a virion-free form which synthesizes DNA at a slower rate than the second form, a virus-producing cell. The virus-producing cell, in addition to virus DNA, may or may not be synthesizing cellular DNA. Cloning experiments (Maurer and Imamura, unpublished data) have indicated that virus-negative cells could give rise to populations of cells which had a similar incidence of virus-containing cells as the parent culture. Also, the recloning of selected negative clones gave rise to populations of cells which had the same incidence of virus-containing cells as the original parent population. These data have been interpreted to indicate that virus-negative cells were "induced" to a virus-productive state. Since such "induced" cells produce virus and cells productively infected with herpes viruses inhibit the synthesis of cellular DNA (11, 12), the DNA synthesized by EBV-containing cells is probably EBV DNA. Attempts, so far, to separate two types of DNA from EBV-containing Burkitt lymphoma cells have not been successful. Such attempts have included equilibrium centrifugation in CsCl density gradients and column chromatography using Sepharose 2B or methylated albumin kieselguhr.

1. Epstein, M. A., and Barr, Y. M., Lancet **1**, 252 (1964).

2. Minowada, J., Moore, G. E., Gerner, R. E., Toshima, S., Takagi, N., and Sandberg, A. A., *in* "Cancer in Africa" (P. Clifford, C. A. Linsell, and G. L. Timms, eds.), p. 177. East African Publishing House, Nairobi, Kenya (1968).

3. Hinuma, Y., Konn, M., Yamaguchi, J., and Grace, J. T., Jr., J. Virol. **1**, 1203 (1967).

4. Hinuma, Y., and Grace, J. T., Jr., Proc. Soc. Exp. Biol. Med. **124**, 107 (1967).

5. Henle, W., Cancer **21**, 580 (1968).

6. Ogur, M., and Rosen, G., Arch. Biochem. **25**, 262 (1950).

7. Dulbecco, R., Hartwell, L. H., and Vogt, M., Proc. Nat. Acad. Sci. U.S.A. **53**, 403 (1965).

8. Sueoka, N., and Cheng, T., J. Mol. Biol. **4**, 161 (1962).

9. Dische, L., *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, p. 285. Academic Press, New York (1955).

10. Henle, W., and Henle, G., J. Virol. **2**, 182 (1968).

11. Kaplan, A. S., and Ben-Porat, T., *in* "Ann. Rev. Microbiol." (C. E. Clifton, S. Raffel and M. P. Starr, eds.), Vol. 22, p. 427. Ann. Rev. Inc., Palo Alto, California (1968).

12. Joklik, W. K. *in* "Molecular Basis of Virology" (H. Fraenkel-Conrat, ed.), p. 576. Reinhold, New York (1968).

Received Sept. 26, 1969. P.S.E.B.M., 1970, Vol. 133.