0022-538X/78/0025-0138$02.00/0
JOURNAL OF VIROLOGY, Jan. 1978, p. 138-145

Vol. 25, No. 1
Printed in U.S.A.

# Cytofluorometry of Lymphocytes Infected with Epstein-Barr Virus: Effect of Phosphonoacetic Acid on Nucleic Acid

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Received for publication 13 July 1977

DNA synthesis in Epstein-Barr virus (EBV)-infected lymphocytes was inhibited by phosphonoacetic acid (PAA) as measured by [$^3$H]thymidine incorporation. PAA, at a concentration of 200 μg/ml, inhibited [$^3$H]thymidine incorporation by human umbilical cord lymphocytes infected with EBV strain P94 but had little effect on DNA synthesis in mitogen-stimulated cells. Transformed cell lines did not develop from infected cord cell cultures treated with 100 μg of PAA per ml. Cytofluorometric analysis showed marked increases in cellular nucleic acid content (RNA plus DNA) as early as 9 days after infection of cord cells in the absence of PAA and before significant enhancement of [$^3$H]thymidine incorporation became apparent. Moreover, EBV led to increases in cellular nucleic acid even when 200 μg of PAA per ml was added to cell cultures before infection. The apparent discrepancy between results obtained by [$^3$H]thymidine incorporation and cytofluorometry is explained either by significant inhibition of cellular DNA polymerases by PAA or by a block at the $G_2$ + M phase of the cell cycle. The data suggest that EBV initiates alterations in cellular nucleic acid synthesis or cell division without prior replication of viral DNA by virus-induced DNA polymerases.

Transformation of human lymphocytes by Epstein-Barr virus (EBV) confers upon these cells the ability to proliferate indefinitely in vitro. Transformed, or immortalized (20), lymphocytes possess surface properties of B-lymphocytes (18) and contain EBV nuclear antigen (19) and multiple copies of the EBV genome (16). Most of the EBV DNA exists intracellularly as covalently closed circles (1, 10) and replicates during the S phase of the cell cycle (4). The average number of genome copies per cell remains remarkably constant over long periods, with replication probably mediated by host cell DNA polymerases (4, 21).

Little is known, however, of the early cycles of viral DNA replication during transformation. Recent data suggest that a single virus particle is capable of transforming a lymphocyte (5). Studies by Thorley-Lawson and Strominger (22) with phosphonoacetic acid (PAA), an inhibitor of virus-induced DNA polymerase, were interpreted as showing that viral DNA synthesis mediated by virus-specific polymerase is necessary for transformation. Such a PAA-sensitive, EBV-induced enzyme has since been identified and partially purified (15). PAA inhibited the normal stimulation of cellular DNA synthesis that follows EBV infection of human adult lymphocytes (22). We have confirmed this finding and show here that PAA also inhibits [$^3$H]thymidine ([$^3$H]TdR) incorporation in human umbilical cord lymphocytes infected with EBV.

We have also utilized flow cytofluorometry to determine the nucleic acid content of transforming cord lymphocytes (3, 24). We show that infection with EBV resulted in increased cellular nucleic acid (RNA-plus-DNA) content even in the presence of high concentrations of PAA. These results indicate that EBV induces fundamental alterations in the control of lymphocyte nucleic acid synthesis or cell division without replication of viral DNA by the virus-induced DNA polymerase.

(This study was presented in part at the National Meeting of the American Society for Clinical Investigation, Washington, D.C., 1 to 2 May 1977.)

## MATERIALS AND METHODS

**Materials.** A stock solution of PAA (Bodman Chemicals, Narveth, Pa.) was prepared to a concentration of 20 mg/ml in distilled water and adjusted to pH 7.4 with 1 N NaOH. Cells were cultured in RPMI 1640 medium (Grand Island Biological Co., Grand

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Island, N.Y.) supplemented with 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 20% heat-inactivated fetal calf serum (Grand Island Biological Co.).

**Mononuclear cord cells.** Human umbilical cord blood in 10- to 50-ml portions was collected into sterile tubes containing 2,500 U of preservative-free heparin (Fisher Scientific Co., Pittsburgh, Pa.), and mononuclear cells were separated on Ficoll-Hypaque gradients (LSM solution; Bionetics Laboratory Products, Kensington, Md.) by a modification of the Boyum method (2). Heparinized blood was diluted with 2 volumes of RPMI 1640 medium, layered over 0.5 volume of LSM solution, and centrifuged for 40 min at 400 × *g*. Cells collected from the interface were washed three times in RPMI and resuspended in growth medium; over 99% of the cells were mononuclear. For Cytofluorograf experiments, cells were incubated overnight in a 75-$cm^2$ flask (Falcon 3024; Falcon Plastics, Oxnard, Calif.) at 37°C, effectively removing the adherent cell population.

**Virus.** EBV strain P94 was concentrated from spent medium of P94 cell cultures, a marmoset lymphocyte line transformed by B95-8 virus (13). Cells were sedimented at 600 × *g* for 10 min, and the supernatant fluid was centrifuged at 13,000 × *g* for 90 min. The pellet from the high-speed centrifugation was resuspended in RPMI (1% original culture volume), clarified at 400 × *g* for 10 min, and passed through a 0.8-μm membrane filter (Millipore Corp., Bedford, Mass.). Fetal calf serum (20%) was added, and portions were stored at −70°C. The preparation contained $10^3$ transforming units per ml by end-point dilution.

EBV strain IM22 was prepared from IM22 cells, a human lymphocyte line derived from the peripheral blood of a patient with infectious mononucleosis. Cells ($10^9$) were sedimented at 400 × *g* for 10 min and washed three times in RPMI. The cells were then passed through four rapid freeze-thaw cycles at −78°C; the resultant suspension was clarified at 500 × *g* for 5 min and passed through a 0.8-μm Millipore filter. The lymphocyte-transforming ability of this preparation, assessed by stimulation of [$^3$H]TdR incorporation after infection of cord lymphocyte cultures (20), was approximately equal to that of the P94 virus stock.

**Infection of cord mononuclear cells.** Cells ($10^7$) were suspended in 0.5 ml of medium, and 0.5 ml of virus (medium for mock infection) was added. The cells were blended in a Vortex mixer, placed at 37°C for 90 min, diluted to 2 × $10^6$ cells per ml, and placed in 25-$cm^2$ culture flasks (Falcon 3013) at 37°C in an atmosphere of 5% $CO_2$. When required, PAA was added before infection and maintained in the medium for the duration of the experiments. Medium was totally replaced every 5 days. Morphological evidence of transformation included visible cell clumping, proliferation, and acid production.

**[$^3$H]TdR incorporation.** The method for [$^3$H]TdR incorporation was a modification of that of Robinson and Miller (20). Five days after feeding, 0.4 ml was withdrawn from each tissue culture flask, and of this, 0.1 ml was placed into each of four wells of a MicroTest II tissue culture plate (Falcon 3040). [$^3$H]TdR (1 μCi; Schwarz/Mann, Orangeburg, N.Y.; specific activity, 1.9 Ci/mmol) was added to each well, and the plate was incubated for 4 h. The contents of the plate were transferred to glass-fiber filters with a MASH II automatic sample harvester (Microbiological Associates, Bethesda, Md.), and filters were washed three times with normal saline solution. Dried filters were suspended in PPO (2,5-diphenyloxazole; 5 g/liter)-dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)-benzene; 60 g/liter]-toluene scintillation fluid, and $^3$H was counted in a Packard Tri-Carb liquid scintillation counter. Results are expressed as stimulation index, which was calculated as follows: stimulation index equals cpm incorporated by infected cells/cpm incorporated by uninfected control cells, where cpm indicates counts per minute.

**Inhibition of transforming virus production by PAA.** P94 cells were washed three times in medium and then suspended in medium supplemented with 0 to 200 μg of PAA per ml. After 1 week, the cells were washed twice and resuspended in medium containing PAA. After an additional week, virus was harvested from the supernatant fluid of each culture as described above for P94 virus. The transforming ability of each sample was assessed by [$^3$H]TdR incorporation of cord cells 6 weeks after infection (20).

**Mitogen stimulation of cord cells.** Cord cells (3 × $10^6$) were cultured in tissue culture tubes (Falcon 2054, 12 by 75 mm) with 2.5 μg of phytohemagglutinin (PHA; Burroughs Wellcome Co.) per ml or 2 μg of pokeweed mitogen (Grand Island Biological Co.) per ml. When appropriate, PAA was added to the cell cultures before addition of the mitogen. [$^3$H]TdR incorporation or nucleic acid content was determined after 3 to 5 days of culture.

**Flow cytofluorometric determination of cell nucleic acid content.** The principles and instrumentation of flow cytofluorometry have been described (24). Briefly, infected lymphocytes were stained in suspension with propidium iodide, a fluorescent nucleic acid stain (3, 9), and then were passed into a model 4802A Cytofluorograf (Bio-Physics Systems, Mahopec, N.Y.). This instrument passes cells through a 448-nm argon ion laser beam. Each cell generates a light-scatter signal proportionate to cell size and a fluorescent pulse proportionate to cell nucleic acid content. These light signals are converted to pulses by a photomultiplier tube, amplified, and analyzed for intensity. Only cells displaying normal light-scatter properties and with a diameter of 8 to 35 μm are counted. The intensity distribution of fluorescence is stored in a multichannel pulse-height analyzer. The instrument analyzes 10,000 cells in several minutes and produces a measure of relative cell nucleic acid content (DNA plus RNA).

**Nucleic acid staining for cytofluorometry.** Cells were pelleted at 400 × *g* for 5 min and suspended in 50% methanol at 0°C for 30 min. The cells were then washed once with 30% methanol and once again with phosphate-buffered solution, then suspended in 0.05 mg of propidium iodide (Calbiochem) per ml in 1.12% sodium citrate. After 1 h at 0°C, cells were washed once and suspended in phosphate-buffered solution for analysis by the Cytofluorograf. The cells were blended in a Vortex mixer frequently during the staining procedure, and significant cell clumping was excluded by direct microscopic examination.

**Virus inactivation.** Virus stock was UV inactivated by a 30-min exposure at 20 cm from a G15 TB germicidal lamp (General Electric). Neutralization by antibody (14) was accomplished by addition of 10% heat-inactivated infectious mononucleosis serum containing an anti-viral capsid antigen antibody titer of 1:640, followed by incubation at 37°C for 60 min. Viral capsid antigen antibody titer was determined as previously described (6).

## RESULTS

**P94 virus production in PAA.** PAA inhibits productive replication of EBV DNA (7, 21, 26) and production of infectious virus (17). Inhibition of transforming virus production established the sensitivity of the P94-specified DNA polymerase to PAA. Cord mononuclear cells were infected with virus prepared from P94 cell cultures that had been maintained in various concentrations of PAA for 2 weeks. [$^3$H]TdR incorporation, measured 6 weeks after infection, is shown in Fig. 1. PAA at 100 μg/ml resulted in a significant reduction in transforming activity, and 200 μg/ml completely inhibited production of transforming virus.

**Transformation of cord lymphocytes maintained in PAA.** Cord cell cultures infected with P94 virus were maintained in concentrations of PAA ranging from 0 to 200 μg/ml. [$^3$H]TdR incorporation by these cultures was assessed at intervals after infection, as shown in Fig. 2. PAA caused a dose-related inhibition of [$^3$H]TdR incorporation. At day 26 after infection, a sixfold increase in [$^3$H]TdR incorporation

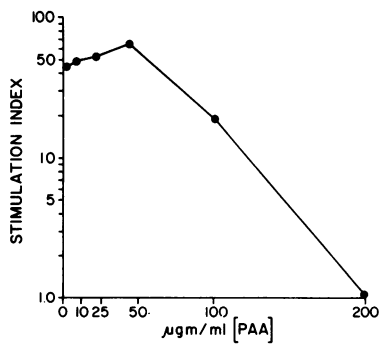


FIG. 1. *Inhibition of P94 virus production by PAA. P94 cells were grown in various concentrations of PAA for 2 weeks, and virus was harvested from the spent media. Cord mononuclear cells were infected with the virus preparations and cultured for 6 weeks, and [$^3$H]TdR incorporation was determined. Stimulation index equals cpm incorporated by infected cells/cpm incorporated by uninfected cells, where cpm indicates counts per minute. Each point represents the mean of two replicate cultures. PAA concentrations of 100 μg/ml or greater markedly diminished production of transforming virus.*

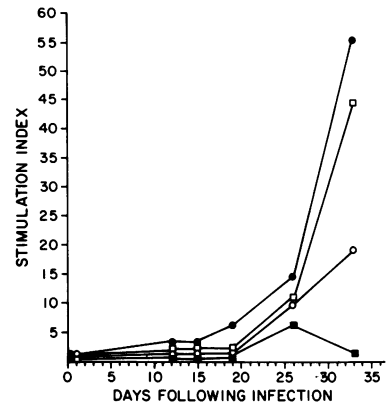


FIG. 2. *P94 transformation of human cord lymphocytes in PAA. Cord mononuclear cells were infected with P94 virus and maintained continuously in various concentrations of PAA. [$^3$H]TdR incorporation was assessed periodically. Symbols: ●, no PAA; □, PAA at 50 μg/ml; ○, PAA at 100 μg/ml; ■, PAA at 200 μg/ml. PAA caused a dose-related inhibition of [$^3$H]TdR incorporation at each time tested.*

by cells infected in 200 μg of PAA per ml was at the limits of significance. By day 33, there was no difference between this culture and uninfected control cells.

The addition of 200 μg of PAA per ml to previously transformed cord lymphocytes did not cause an immediate reduction in [$^3$H]TdR incorporation.

In four replicate experiments, outgrowth of transformed cell lines did not occur in any culture infected and maintained for 4 weeks or more in 100-μg/ml or greater concentrations of PAA. Despite this, clumping of cells and a tendency of the medium to become acid were noted in all cultures within 1 week of infection.

**Mitogen stimulation of cord lymphocytes maintained in PAA.** Since the inhibition of [$^3$H]TdR incorporation by PAA (Fig. 2) could have been due to nonspecific suppression of cell growth, the effect of PAA on mitogen stimulation of cord lymphocytes was also determined. Cord mononuclear cells were tested for [$^3$H]TdR incorporation after 4 to 5 days of culture in 2.5 μg of PHA per ml or 2.0 μg of pokeweed mitogen per ml (Fig. 3). PAA at 200 μg/ml had a small but definite inhibitory effect on mitogen stimulation, although similar concentrations greatly inhibited the stimulation of [$^3$H]TdR incorporation produced by infection with P94 virus. This inhibition of mitogen stimulation was most likely due to inhibition of cellular DNA polymerases by PAA, as described previously (8, 15). The toxicity of PAA was more pronounced at 400 μg/ml.

**Cytofluorometric analysis of mitogen-**

**stimulated lymphocytes.** Significant stimulation of [$^3$H]TdR incorporation by infected cultures was not detected until over 20 days after infection (Fig. 1). Because of the uncertain effects of prolonged cell exposure to PAA during this time, cytofluorometric determination of cell nucleic acid content was chosen as a potentially more sensitive indicator of cell proliferation (23). The nucleic acid content of cord cells stimulated by PHA is shown in Fig. 4. Stimulated cells were recorded in the higher channels of the instrument at the right of the histogram, as compared with unstimulated cord cells. This was due to increased numbers of cells with greater nucleic acid content relative to unstimulated cells. PHA-stimulated cells maintained in PAA were recorded in the intermediate channels, which indicated reduced nucleic acid content relative to cells stimulated in the absence of PAA. This result is explained by inhibition of cellular DNA polymerases by PAA (8, 15).

Data from a replicate experiment appear in Fig. 5. The cumulative percentages of cells recorded in each channel are presented to facilitate comparison between cultures. PAA causes dose-related inhibition of the increase in cellular nucleic acid produced by stimulation with PHA. Cytofluorometry thus confirmed the toxicity of PAA at 100 to 200 μg/ml.

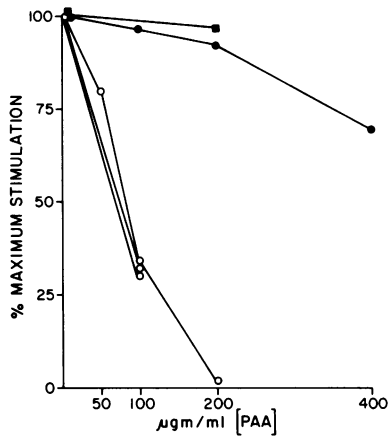


FIG. 3. *PAA inhibition of [$^3$H]TdR incorporation by cord lymphocytes infected with P94 (33 days) or stimulated by mitogens (4 to 5 days). [$^3$H]TdR incorporation was measured at the times indicated. Symbols: ■, pokeweed mitogen-stimulated; ●, PHA-stimulated; ○, virus-infected (three separate experiments). Percent maximal stimulation equals (cpm incorporated by cells stimulated in PAA/cpm incorporated by control stimulated cells) × 100, where cpm indicates counts per minute. The stimulation index for cells stimulated in the absence of PAA in each experiment was: pokeweed mitogen, 9.3; PHA, 24; EBV, 20 to 148. Although a marked difference existed between the degrees of inhibition of mitogen- and virus-stimulated cultures, there was still appreciable inhibition of mitogen stimulation at high PAA concentrations.*

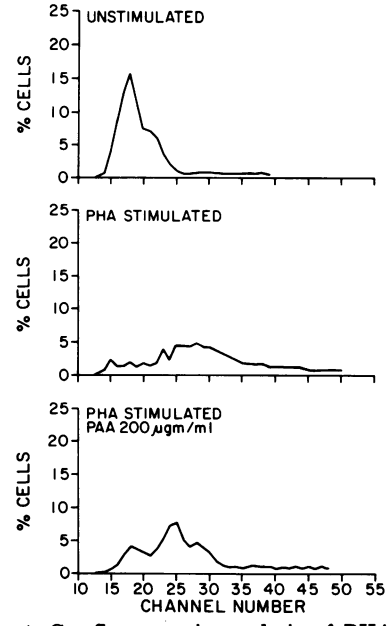


FIG. 4. *Cytofluorometric analysis of PHA-stimulated cord lymphocytes. The histograms depict the distribution of nucleic acid content in populations of cord mononuclear cells. Each cell is assigned by the Cytofluorograf to a given channel, depending on the intensity of the fluorescence generated by that cell as it passes through the argon-ion laser beam. The intensity of fluorescence is proportionate to the cellular nucleic acid content. The percentage of the total cell population recorded in each channel of the Cytofluorograf appears on the ordinate. Stimulated cells were recorded in the higher channels, reflecting increased nucleic acid content. Cells stimulated in 200 μg of PAA per ml displayed an intermediate distribution of nucleic acid content. Analyzed after 3 days of culture.*

**Cytofluorometric analysis of EBV-infected lymphocytes.** Analysis of cord cell cultures 12 days after infection with P94 virus generated the histograms shown in Fig. 6. As with mitogen-stimulated cells, P94-infected cells were recorded in the higher channels, a result that indicated increased cell nucleic acid content relative to uninfected control cells. No significant enhancement of [$^3$H]TdR incorporation was present this soon after infection (Fig. 2). The increased nucleic acid was not due to nucleic acid contained in the virus inoculum, since cytofluorometry did not reveal any increase in the nucleic acid content of lymphocytes 24 h after infection.

Cells infected with P94 virus and maintained in a 200-μg/ml concentration of PAA also dem-

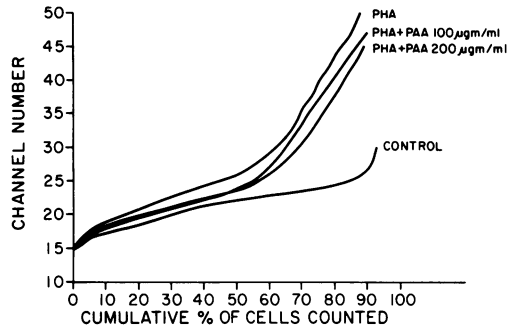


FIG. 5. *Cytofluorometric analysis of PHA-stimulated cord lymphocytes. Channel number is presented as a function of the cumulative fraction of the total cell population recorded by each channel. Cells were stimulated with PHA in PAA at 0, 100, or 200 μg/ml (PAA 0, PAA 100, and PAA 200, respectively); control cord cells were unstimulated. An increased fraction of stimulated cells was recorded in the higher channels, indicating relatively increased nucleic acid content. By chi-square analysis, the nucleic acid distributions differ as follows: PHA versus PHA + PAA 100: $\chi^2 > 169$ ($P < 0.001$); PHA versus PHA + PAA 200: $\chi^2 = 169$ ($P < 0.001$); PHA + PAA 100 versus PHA + PAA 200: $\chi^2 = 144$ ($P < 0.001$). PAA therefore caused dose-related inhibition of cell nucleic-acid content. Analyzed after 3 days of culture.*

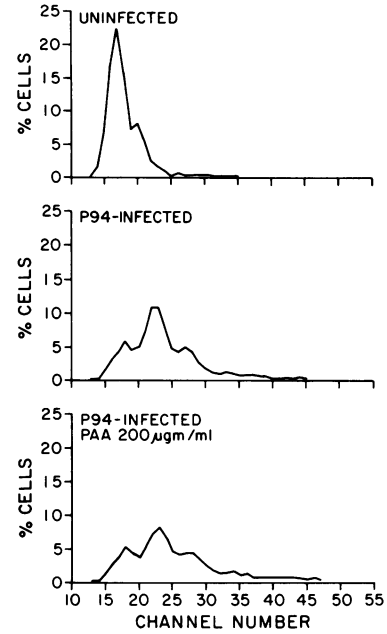


FIG. 6. *Cytofluorometric analysis of P94-infected cord lymphocytes. See legend to Fig. 4. Infection with P94 virus induced a significant increase in cord cell nucleic acid content, even in PAA at 200 μg/ml. Analyzed 12 days after infection.*

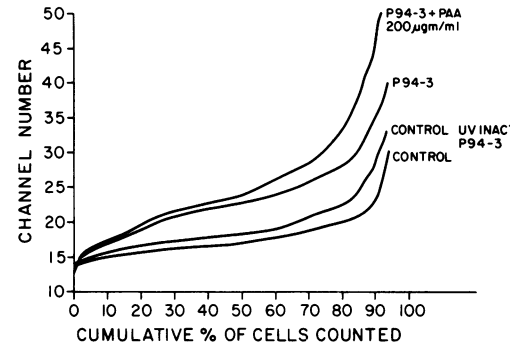


FIG. 7. *Cytofluorometric analysis of P94-infected cord lymphocytes. See legend to Fig. 5. P94-3, cells infected with P94 virus; P94-3 + PAA, cells infected and maintained in PAA at 200 μg/ml; control UV-inactivated P94-3, cells infected with P94 virus previously exposed to UV light; control, uninfected cord cells. The nucleic acid content of cells infected with P94 virus increased relative to control cells; the nucleic acid content of cells infected in PAA was enhanced relative to that of cells infected in the absence of PAA (see Table 1). UV treatment of virus before infection greatly reduced the increase in cell nucleic acid content (Table 2). Analyzed 12 days after infection.*

onstrated enhanced nucleic acid content (Fig. 6). When the cumulative percentage of cells counted in each channel was analyzed, cells infected in the presence of PAA contained relatively more nucleic acid than cells infected in the absence of PAA (Fig. 7). The nucleic acid content of cells infected in PAA was increased in three replicate experiments (Table 1). In each, cells infected with P94 in PAA had greater nucleic acid content than cells infected in the absence of PAA.

Cord cells infected with IM22 virus were also studied with the Cytofluorograf (Fig. 8). Results differed from those described above for P94 virus. Cells infected with IM22 virus in the absence of PAA contained relatively more nucleic acid than uninfected control cells, before any significant increases in [$^3$H]TdR incorporation occurred. PAA caused a reduction in the nucleic acid content of infected cells relative to cells infected in the absence of PAA, although the nucleic acid content of PAA-treated infected cells was significantly increased relative to uninfected control cells. Such partial reduction of nucleic acid content occurred in three replicate experiments (Table 1).

In summary, both P94 virus and IM22 virus induced significant increases in the nucleic acid content of cord mononuclear cells, even in the presence of high concentrations of PAA (200 μg/ml). PAA caused a reduction in the nucleic acid content of IM22-infected cells to a level about the same as in mitogen-stimulated cultures. On the other hand, PAA actually en-

TABLE 1. *Cytofluorometric analysis of PAA effect on lymphocyte transformation by two strains of EBV*[a]

| EBV strain | Expt | Day[b] | Control %[c] | Virus %[d] | Virus + PAA %[e] | Channel no. | Cells counted[f] |
|---|---|---|---|---|---|---|---|
| P94 | 1 | 12 | 29.3 | 80.0 | 82.2 | 19 | 6,672 |
| | 2 | 9 | 27.3 | 29.5 | 55.9 | 22 | 5,461 |
| | 3 | 12 | 29.6 | 35.5 | 42.7 | 19 | 4,300 |
| | Mean | | 28.7 | 51.7 | 60.3 | | |
| IM22 | 4 | 16 | 29.1 | 70.9 | 42.0 | 25 | 28,430 |
| | 3 | 12 | 29.6 | 48.7 | 46.8 | 19 | 11,369 |
| | 5 | 16 | 32.2 | 84.3 | 41.7 | 26 | 19,858 |
| | Mean | | 30.3 | 68.0 | 43.5 | | |

[a] The point of analysis was arbitrarily defined as the Cytofluorograf channel by which 70% of uninfected control cells had been recorded. The specific channel number varied between experiments. The percentage of cells not recorded by this channel was then calculated for each cell culture in each experiment. In the unstimulated control sample, for each experiment, approximately 30% of the cells remained unrecorded. The other percentages expressed, therefore, represent that fraction of cells that were unrecorded and thus possessed a greater nucleic acid content than those that were recorded by the chosen channel.

[b] After infection.

[c] Uninfected control cord cells not recorded by chosen channel.

[d] Cells, infected with either strain of EBV in the absence of PAA, not recorded by chosen channel.

[e] Cells, infected in 200 μg of PAA per ml, not recorded by chosen channel.

[f] Mean number of cells counted from each culture bottle; range, 883 to 29,789.

hanced the nucleic acid content of cells infected with P94 virus.

Additional experiments were performed to confirm the virus-specific nature of the increased cell nucleic acid content produced by infection with either virus preparation. UV treatment of virus significantly inhibited the increase in cellular nucleic acid (Table 2, Fig. 7), as did virus neutralization with acute infectious mononucleosis serum known to contain a high titer of antibody to viral capsid antigen.

## DISCUSSION

PAA is a potent inhibitor of DNA polymerase activity induced by most herpesviruses, including EBV (11, 12, 15). The EBV-induced DNA polymerase is required for synthesis of late viral antigens (17, 21), productive replication of viral DNA (7, 21, 26), and production of infectious virus (17). This last finding is confirmed by the demonstration that PAA inhibits production of transforming virus by the P94 cell line. The role of the viral DNA polymerase in transformation, however, remains speculative.

Thorley-Lawson and Strominger reported that PAA inhibited [$^3$H]TdR incorporation by adult B-lymphocytes infected with EBV (22). Our data, based on mixed umbilical cord mononuclear cells, agree closely with this earlier study. The clear difference between the magnitudes of inhibition of EBV-stimulated cultures and mitogen-stimulated cultures suggests that this is a virus-specific phenomenon. Additionally, PAA, at 100 to 200 μg/ml, prevented the establishment of transformed cell lines from infected lymphocyte cultures. It would seem that replication of viral DNA by the virus-induced DNA polymerase is a necessary prelude to transformation (22).

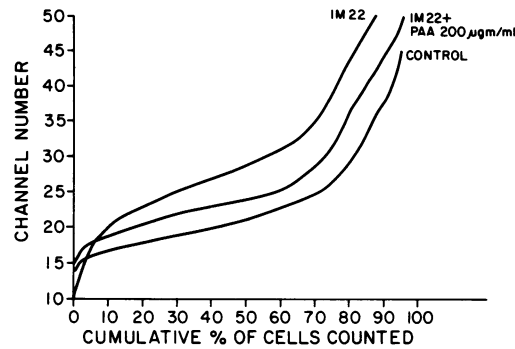


FIG. 8. *Cytofluorometric analysis of IM22-infected cord lymphocytes. See legend to Fig. 5. IM22, cells infected with IM22 virus; IM22 + PAA, cells infected and maintained in PAA at 200 μg/ml; control, uninfected cord cells. Cells infected in PAA had an increased nucleic acid content relative to uninfected control cells but were intermediate in nucleic acid content between uninfected cells and cells infected in the absence of PAA (Table 1). Analyzed 16 days after infection.*

Cytofluorometric analysis, however, yields data apparently at variance with this concept. The nucleic acid content of cord lymphocytes was increased after infection with EBV, even in the presence of high concentrations of PAA. Identical concentrations of PAA totally inhibited production of transforming virus by the original P94 virus-producing cell line. Propidium

TABLE 2. *UV inactivation and neutralization of EBV*[a]

| Expt | EBV strain | Control % | Virus % | UV-inactivated[b] virus | VCA+[c] | VCA−[d] | Channel no. |
|---|---|---|---|---|---|---|---|
| UV inactivation | P94 | 29.3 | 80.0 | 41.1 | | | 19 |
| | IM22 | 32.2 | 84.3 | 32.2 | | | 26 |
| Antiserum neutralization | P94 | 31.1 | 69.3 | | 37.1 | 79.5 | 19 |
| | IM22 | 32.2 | 84.3 | | 31.5 | | 26 |

[a] Results were analyzed as in Table 1.
[b] Cells infected with virus inactivated as described in the text.
[c] Cells infected with virus neutralized by viral capsid antigen (VCA)-positive serum.
[d] Cells infected with virus treated in identical fashion with VCA-negative serum.

iodide binds to both DNA and RNA. Although other workers have found the major contribution to fluorescence measured to be due to binding of the dye to DNA (9), the absence of distinct $G_2$ + M peaks in our histograms suggests a significant contribution from RNA binding as well. It is unlikely, however, that the nucleic acid increases observed were due solely to RNA.

The apparent discrepancy between data arrived at by cytofluorometry and by [$^3$H]TdR incorporation measurements might be explained if PAA directly inhibited [$^3$H]TdR incorporation in infected cells by interfering with either the thymidine transport system or the functional thymidine kinase. This appears not to be the case, however, since the addition of PAA to previously transformed cord cells did not result in a sudden inhibition of [$^3$H]TdR incorporation.

PAA measurably inhibits cellular DNA polymerases (8, 15). This inhibitory effect is variable among lymphoid cell lines. Inhibition is most pronounced with the $\alpha$-polymerase, which is the predominant DNA polymerase activity found in rapidly proliferating mammalian cells (25). This effect of PAA is reflected in the slight but definite inhibition of [$^3$H]TdR incorporation and decreased nucleic acid content observed in mitogen-stimulated cord cells cultured in PAA. Although mitogen-stimulated cord lymphocytes were studied after 3 to 5 days in culture, EBV-infected cells were cultured for over 3 weeks before significant enhancement of [$^3$H]TdR incorporation could be detected.

It is conceivable that partial but critical inhibition of cellular polymerase activities by PAA over this longer time period could be responsible for the inhibition of [$^3$H]TdR incorporation observed in infected cultures. PAA possesses a long-term cumulative toxicity for cultured lymphoid cell lines (8). Cytofluorometry detected marked changes in cell nucleic acid content relatively soon after infection (9 days) and thus may have disclosed enhanced cell nucleic acid synthesis before the long-term effect of PAA became manifest.

A block in mitosis of infected lymphocytes is a third possible explanation for the discrepancy between [$^3$H]TdR incorporation and cytofluorometric results. Cytofluorometry yields a static image of cellular nucleic acid content, and a block at either the $G_2$ or the M phase of the cell cycle would create increases in nucleic acid. Preliminary DNA-specific cytofluorometric determinations, with RNase treatment of lymphocytes before propidium iodide staining (3), suggest that the $G_2$ + M population is increased by approximately 10% of the total number of cells 12 days after infection with P94 virus (S. Lemon, unpublished data). With flow cytofluorometry, Lehman et al. (J. M. Lehman, I. B. Klein, and L. S. Cram, personal communication) have demonstrated that simian virus 40 induces a block in the $G_2$ + M phase of the cell cycle in infected WI-38 fibroblasts, leading to increases in cellular nucleic acid. Unlike EBV-transformed lymphocytes, however, simian virus 40-transformed cells are often polyploid.

Flow cytofluorometry clearly indicates that EBV induces a major increase in cell nucleic acid content (RNA plus DNA) even when PAA inhibits replication of viral DNA by the virus-induced DNA polymerase. It remains to be shown whether this is due to enhanced nucleic acid synthesis or to a block at the $G_2$ + M phase of the cell cycle.

Of special interest was the finding that PAA actually enhanced the nucleic acid content of lymphocytes infected with P94 but not with IM22 virus. PAA reduced the nucleic acid content of cells infected with IM22 virus relative to cells infected in the absence of PAA, as it reduced the nucleic acid content of lymphocytes stimulated with PHA. This result may be explained by inhibition of cellular DNA polymerases by PAA (8, 15). However, we can only speculate as to why PAA enhanced the nucleic acid content of P94-infected cells. If PAA inhibited a lytic event occurring in a fraction of cells shortly after infection, it might promote latency, thereby improving the chances for cell survival and subsequent transformation. Alternatively, enhanced nucleic acid content would

also be expected were PAA to promote a block in the $G_2$ + M phase of the cell cycle.

## ACKNOWLEDGMENTS

We thank Marshall Logue for expert technical assistance in the operation of the Cytofluorograf, Peter Utsinger for helpful discussion concerning cytofluorometric techniques, and David Crews and Colly Gilbert for technical assistance.

This study was supported by Public Health Service grants PO1-CA16086 and 1PO1-CA19014 awarded by the National Cancer Institute. S.M.L. was the recipient of Public Health Service National Research Service Award 1-F32-AI05125 from the National Institute of Allergy and Infectious Diseases.

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