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INDUCTION OF EPSTEIN-BARR VIRUS ANTIGENS AND DNA-POLYMERASE ACTIVITIES IN P3HR-1 CELL LINE AND ITS SUBLINE PASSAGED IN THE PRESENCE OF PHOSPHONOFORMATE

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Summary. — Lymphoblastoid cell line P3PFA was derived from P3HR-1 cells by long-term cultivation in the presence of phosphonoformate (PFA). Spontaneous production of Epstein-Barr virus antigens in PFA-selected subline was markedly reduced in comparison with the original cell line. Induction of early antigen (EA) and viral capsid antigen (VCA) syntheses with 5-iodo-2-deoxyuridine (IUDR), n-butyrate and 12-o-tetradecanoyl phorbol-13-acetate (TPA) was significantly less efficient in P3PFA than P3HR-1 cells. This was most likely associated with reduction in the number of viral genome copies per P3PFA cell that decreased more than one hundred times in comparison with P3HR-1 line. The synthesis of EA in both cell lines was not inhibited in the presence of PFA. On the other hand, PFA inhibited the synthesis of VCA by 95 and 30% in P3HR-1 and P3PFA, respectively. The relative resistance of VCA synthesis to PFA in P3PFA cells was not due to the presence of a drug-resistant virus-specific DNA-polymerase. It is noteworthy that the activity of virus-specific enzyme was markedly reduced, while cellular enzyme activity was significantly enhanced in induced P3PFA cells when compared to P3HR-1 cells treated in the same way.

Key words: *Epstein-Barr virus*; *virus induction*; *phosphonoformate*; *DNA-polymerase*

Introduction

Epstein-Barr virus (EBV) persists in lymphoblastoid cell lines in a partially repressed form. In producer cell lines like P3HR-1 (Hinuma and Grace, 1967) or B95-8 (Miller and Lipman, 1973) spontaneous activation of virus growth cycle occurs in a low percentage of cells. In non-producer lymphoblastoid cell lines (Durr *et al.*, 1970; Pulvertaft, 1965) no spontaneous production of viral antigens except of EBNA (Reedman and Klein, 1973) can be detected. In both, viral antigen synthesis can be induced by various chemicals (Gergely *et al.*, 1971*a*; ZurHausen *et al.*, 1978; Luka *et al.*, 1979; Tovey *et al.*, 1978; Tovey *et al.* 1979) and in the non-producer cell lines also

by superinfection with P3HR-1 virus (Gergely *et al.*, 1971*b*). In producer and superinfected cell lines induction of complete viral cycle can be achieved (Hampar *et al.*, 1974; Fresen *et al.*, 1978). Upon chemical induction of non-producer cells only abortive cycle is observed restricted to early antigen (EA) synthesis (Gergely *et al.*, 1971*a*; Luka *et al.*, 1979). This is indicative of involvement of viral and/or cellular regulatory functions in the induction process.

In present work we studied the induction of virus antigens in P3HR-1 cell line and its subline P3PFA which had been isolated by long term passaging P3HR-1 cells in the presence of phosphonoformate (PFA). Because PFA has been described to be a potent inhibitor of herpesvirus-specific DNA-polymerases (Helgstrand *et al.*, 1978) an attention was paid in our experiments to the properties of DNA polymerases in non-induced and induced cultures. Two criteria were used for characterizing the enzymes: in vitro sensitivity to PFA and the effect of amonium sulphate on the enzyme activity. This substance has been described to reduce the activity of cellular polymerase but to stimulate the virus-specific enzyme (Feighny *et al.*, 1980).

Materials and Methods

Cells. Virus producer lymphoblastoid cell line P3HR-1 (Hinuma and Grace, 1967) and its subline (P3PFA), which had been derived from P3HR-1 cells by six month-passaging (one passage a week, split 1 : 10) in the presence of 50 μg PFA per ml, were employed. PFA was kindly provided by Dr. D. Zelená, Research Institute of Antibiotics and Biotransformations, Prague, Czechoslovakia. The concentration used had no effect on the viability of cells. The cell lines were cultivated as described previously (Vonka *et al.*, 1972).

Induction of EBV antigens. For induction of viral antigen synthesis two procedures were used: a) 24 hr pulse with 5-iodo-2-deoxyuridine (IUDR, Sigma) at the concentration of 20 μg/ml in the presence of hypoxanthine (Sigma) and aminopterine (Sigma) at concentrations of 14 and 0.4 μg/ml, respectively (Long *et al.*, 1974). b) Addition of the mixture of n-butyrate (Merck) and 12-o-tetradecanoylphorbol-13-acetate (TPA, Sigma) to a final concentration of 3 mmol/l and 20 ng/ml, respectively (Ooka and Calender, 1980). On 3rd and 5th days after induction, the samples were collected for immunofluorescence (IF) tests. The determination of the DNA-polymerase activities was made on day 3 only.

Detection of virus antigens by immunofluorescence (IF) test. IF test was performed on air dried smears fixed in cold acetone for 10 min. Viral capsid antigen (VCA) was stained in indirect IF test (Henle and Henle, 1966). Human serum diluted 1 : 10 possessing antibody against VCA (titre 1 : 160), but not against EA, and goat antihuman IgG conjugated with fluoresceine isothiocyanate (FITC) (Hyland, Deerfeeld) were used. Early antigen (EA) was stained by direct IF technique (Roubal *et al.*, 1980) using FITC-conjugated human serum containing antibody against EA (titre 1 : 40). The dilution used was 1 : 40.

Determination of DNA-polymerase activity. DNA-polymerase activity was determined in nuclear extracts of untreated cells and butyrate-TPA-treated cells, 3 days after addition of the drugs. Preparation of nuclear extracts, activation of calf thymus DNA and the polymerase assay were performed as described by Feighny *et al.* (1980). DNA-polymerase activity was monitored by incorporation of ^{3}H-thymidine triphosphate (TTP) into acidoprecipitable fraction and expressed as cpm per mg protein. The reaction was linear for at least 20 min. All assays for determination of DNA-polymerase activity were run for 10 min. In order to distinguish between cellular and viral DNA-polymerases, the assay was performed in parallel in the absence or in the presence of 100 mmol/l amonium sulphate (AS) (Lachema, Brno) and various concentrations of PFA.

Reassociation kinetics experiments. EBV DNA isolated from TPA induced P3HR-1 cells (ZurHausen *et al.*, 1978) was labelled by the nick translation reaction (Rigby *et al.*, 1977) with ^{32}P-GTP to the specific activity of 10^{8} cpm/μg DNA. The reassociation kinetic of ^{32}P-labelled

probe EBV DNA was followed up in the presence of test and control DNAs. The DNA isolated from EBV genome-free Ramos cells and the same DNA supplemented with EBV DNA were used as a negative or positive control, respectively. The reassociation kinetics data were processed as described by Břicháček *et al.* (1981). For the calculation of the number of viral genomes per cell it had been supposed, that molecular weight of EBV DNA was 10^8 and of cellular DNA 4×10^{12}.

Results

Induction of EBV antigens

The effects of PFA on the spontaneous and induced synthesis of EA and VCA in P3HR-1 line and its subline P3PFA grown for prolonged period of time in the presence of PFA are shown in Figures 1 and 2, respectively. As can be seen, spontaneous production of viral antigens was markedly decreased in P3PFA subline when compared with the parental P3HR-1 line.

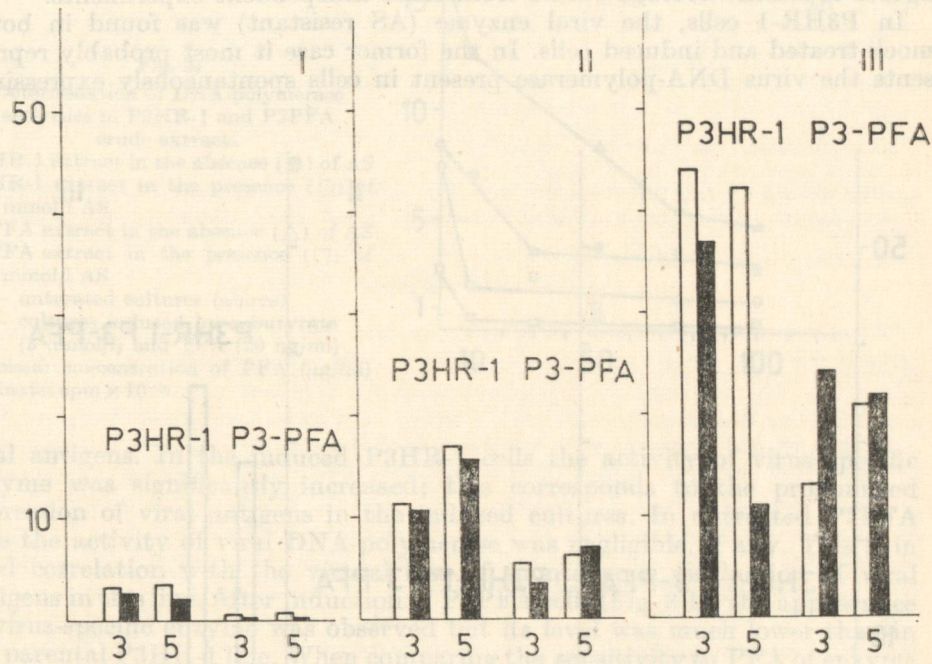

Fig. 1.

Effect of PFA on induction of EA in P3HR-1 line and its P3PFA subline
Empty column — in the absence of PFA
Black column — in the presence of PFA
I — uninduced controls
II — cultures induced with the mixture of IUDR, hypoxantine and aminopterine
III — cultures induced with the mixture of n-butyrate and TPA
Abscissa: days after induction
Ordinate: percentage of IF-positive cells

This property of P3PFA cells remained stable even after several months of passaging in the absence of PFA (results not shown). In addition, the syntheses of both EA and VCA were less inducible in P3PFA cells than in the parental line. As shown in Fig. 1 the EA production in both the original cells and the PFA-subline was not blocked in the presence of PFA. This indicates that the first phase of productive cycle runs independently of EBV DNA--polymerase. While in the butyrate-TPA treated P3HR-1 cells PFA reduced the number of VCA positive cells by 95%, the corresponding drop in the case of P3PFA subline amounted only to 30%.

Determination of DNA-polymerase activity

DNA-polymerase activity was determined in mock-treated and butyrate--TPA-induced P3PHR-1 and P3PFA cells after treatment for 3 days. It was assayed in the absence and the presence of 100 mmol/l AS and various concentrations of PFA. The results are summarized in Figs. 3-I, -II. The figures represent average values from three independent experiments.

In P3HR-1 cells, the viral enzyme (AS resistant) was found in both mock-treated and induced cells. In the former case it most probably represents the virus DNA-polymerase present in cells spontaneously expressing

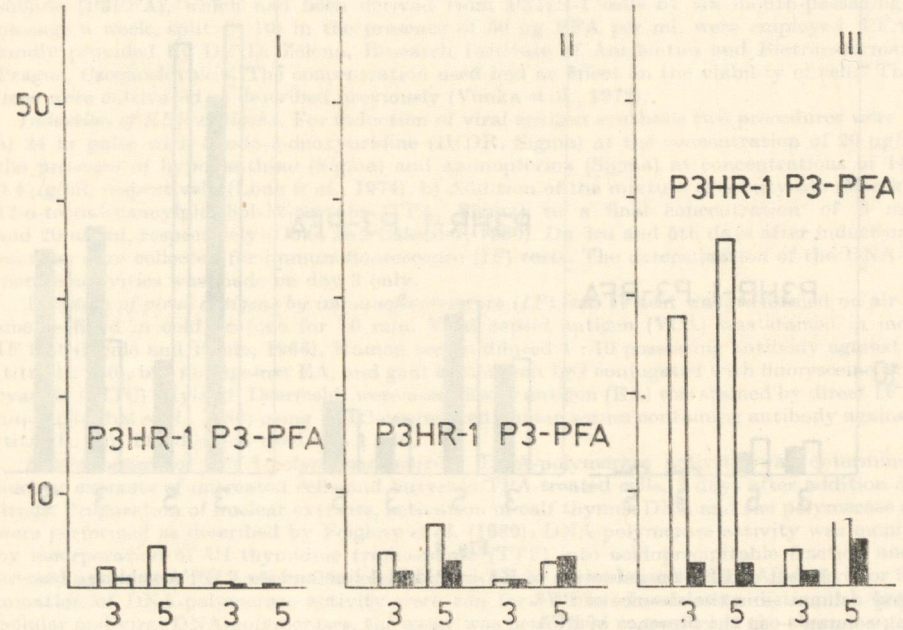

Fig. 2.
Effect of PFA on induction of VCA in P3HR-1 line and P3PFA subline
For explanations see Fig. 1.

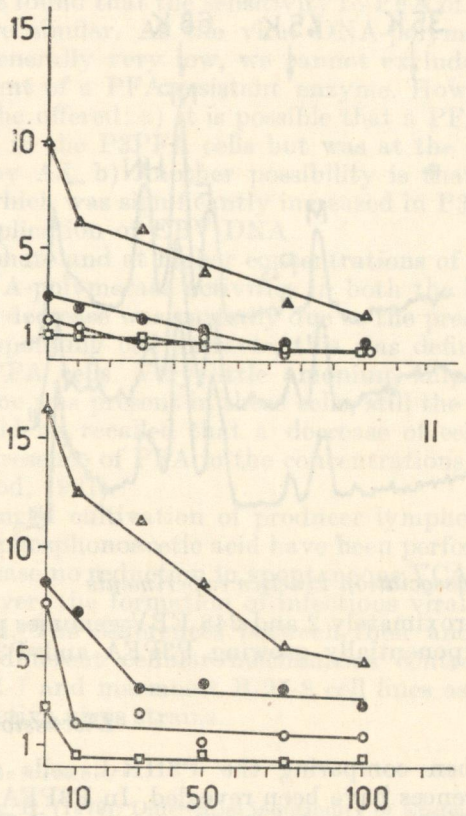

Fig. 3.
Determination of DNA-polymerase activities in P3HR-1 and P3PFA crude extracts
P3HR-1 extract in the absence (●) of AS
P3HR-1 extract in the presence (○) of 100 mmol/l AS
P3PFA extract in the absence (△) of AS
P3PFA extract in the presence (□) of 100 mmol/l AS
I — untreated cultures (above)
II — cultures induced by n-butyrate (3 mmol/l) and TPA (20 ng/ml)
Abscissa: concentration of PFA (μg/ml)
Ordinate: cpm × 10^{-3}

viral antigens. In the induced P3HR-1 cells the activity of virus specific enzyme was significantly increased; this corresponds to the pronounced expression of viral antigens in the induced cultures. In untreated P3PFA cells the activity of viral DNA-polymerase was negligible, if any. This is in good correlation with the virtual loss of spontaneous production of viral antigens in this line. After induction of P3PFA cells (Fig. 3-II) the appearance of virus-specific enzyme was observed but its level was much lower than in the parental P3HR-1 line. When comparing the sensitivity to PFA of enzyme activities present in induced P3HR-1 and P3PFA cells, the following observations were made: in the absence of AS the enzyme activities from P3HR-1 and P3PFA cells were cut down to 50 per cent by 30 and 50 μgPFA/ml, respectively. On the other hand, in the presence of AS the 50 per cent reduction was achieved at 6—7 μgPFA/ml with both enzyme preparations. Interestingly enough, the levels of cellular DNA-polymerase activities in both control and induced P3PFA cells were significantly higher than in P3HR-1 line. This was observed in all three repeated experiments.

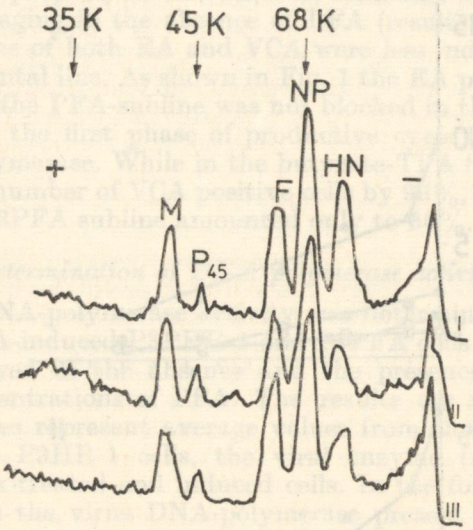

Fig. 4.
Reassociation kinetics of ^{32}P-EBV DNA with DNA from Ramos, P3PFA and P3HR-1 cell extracts
Hybridization kinetics of ^{32}P-EBV DNA with 4 mg of Ramos cell DNA per ml (▲); with mixture of 4 mg of Ramos cell DNA and 0.3 μg EBV DNA per ml (approximately 3 EBV genome equivalents per cell genome) (△); with 3.08 mg of P3HR-1 cell DNA per ml (○); with 3.5 mg of P3PFA cell DNA per ml (●).
Abscissa: reassociation time (in hr).
Ordinate: reciprocal value of single-stranded DNA fraction (1/fss).

Reassociation kinetics experiments

Aproximately 2 and 248 EBV genomes per 1 cellular genome were present in exponentially growing P3PFA and P3HR-1 cells, respectively (Fig. 4).

Discussion

When comparing the P3HR-1 cells and its P3PFA subline, several differences have been revealed. In P3PFA cells, the inducibility of both EA and VCA was reduced. The content of EBV DNA was markedly lower in P3PFA than in the original P3HR-1 cells. It is most likely that this was not only due to the reduction of virus-producing cells but also to the decrease of free virus genome copies in the non-producing cells. Similar observation has also been reported by Yajima *et al.* (1976). As the inducibility of viral antigens in lymphoblastoid cell lines has been found to depend on the number of viral DNA copies per cell (Bister *et al.*, 1979), it is reasonable to assume that the reduction in EBV DNA content is associated with the lower inducibility of P3PFA subline. In the parental cell line the drug-induced synthesis of VCA was reduced by 95% in the presence of PFA, while in P3PFA subline it was only lowered by 30%. As the synthesis of VCA in IUDR-induced lymphoblastoid cell lines has previously been described to be dependent on the function of viral DNA-polymerase (Summers and Klein, 1976) one might expect that in P3PFA subline a subpopulation of cells containing PFA-resistant viral DNA-polymerase was present. This possibility was examined by in vitro measurement of enzyme activities in the extracts from two lines. Activity of the viral enzyme was determined by performing the assay in the presence of 100 mmol/l AS, to the action of which it is not sensitive

(Feighny *et al.*, 1980). However, we found that the sensitivity to PFA of viral polymerases from both lines were similar. As the viral DNA-polymerase activity in P3PFA subline was generally very low, we cannot exclude the presence of a subdetectable amount of a PFA resistant enzyme. However, two alternative explanations can be offered: a) It is possible that a PFA-resistant viral polymerase appeared in the P3PFA cells but was at the same time sensitive to the inhibition by AS. b) Another possibility is that the cellular enzyme—the activity of which was significantly increased in P3PFA cells—might participate in the replication of EBV-DNA.

In the absence of amonium sulphate and at higher concentrations of PFA we observed 50% decrease of DNA-polymerase activities in both the lines. Although one can assume that the decrease was partially due to the presence of the viral enzyme in the corresponding cell extracts, this was definitely not the case on the treated P3PFA cells. Very little amonium sulphate-resistant, i.e. virus-specific, enzyme was present in these cells, still the PFA effect was quite marked. It should be recalled that a decrease of cellular DNA-polymerase activity in the presence of PFA in the concentrations used has been observed (Datta and Hood, 1981).

Similar experiments with prolonged cultivation of producer lymphoblastoid line B-95-8 in the presence of phosphonoacetic acid have been performed by Margalith *et al.* (1980). In this case no reduction in spontaneous VCA production has been observed. However, the formation of infectious viral particles has been strongly supressed. The differences between their and our results may be associated with different cellular mechanisms controlling the EBV growth in human P3HR-1 and marmoset B-95-8 cell lines as well as with the properties of the respective virus strains.

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