Acta virol. **32** : 349—352, 1988

UNCHANGED EXPRESSION OF EPSTEIN-BARR VIRUS-DETERMINED NUCLEAR ANTIGEN-1 IN PRODUCTIVE VIRUS CYCLE

Z. NOVÁKOVÁ, V. KRCHŇÁK[1], J. ROUBAL

Department of Experimental Virology, Institute of Sera and Vaccines, 101 03 Prague, and [1]Pharmaceuticals Prague, Czechoslovakia

Received August 20, 1987

Summary. — The level of the Epstein-Barr virus (EBV)-determined nuclear antigen (EBNA-1) encoded by the *Bam HI-K* fragment of EBV DNA remained unchanged in P3HR-1 cells after induction of the productive cycle of virus replication by sodium-n-butyrate and 12-o-tetradecanoylphorbol-13-acetate (TPA). This was shown by the same capacity of cell-free extracts from untreated and treated P3HR-1 cells to absorb anti-EBNA-1 antibody from a known human serum.

Key words: Epstein-Barr virus, virus nuclear antigen (EBNA-1), synthetic peptide

Epstein-Barr virus (EBV) is capable of immortalizing B lymphocytes both in vivo (Gerber *et al.*, 1969) and in vitro (Miller *et al.*, 1971). The immortalized cells always contain EBV DNA (Adams, 1979) and express latent membrane antigen (Moss *et al.*, 1981) and the proteins of the EBV-determined nuclear antigen complex (EBNA complex) (Kallin *et al.*, 1986). In some cell lines, so-called virus producers, a certain proportion of cells spontaneously express also the early antigen (EA) and the viral capsid antigen (VCA) (Miller and Lipman, 1973; Hinuma and Grace, 1967).

Two of the EBNA complex components, EBNA-1 and EBNA-2, have been studied extensively. EBNA-1, encoded by the *Bam HI-K* fragment of EBV DNA (Summers *et al.*, 1981) is required in trans for maintaining EBV genomes in the form of intracellularly replicating plasmids (Yates *et al.*, 1985). EBNA-2 is coded for by the *Bam HI-YH* region of B95-8 EBV DNA (Dillner *et al.*, 1985) and seems to be important for the establishment of cell immortality but not for its maintenance. This conclusion is mainly based on its absence from P3HR-1 cells, which produce transformation-defective virus that has the particular DNA region deleted (Rowe *et al.*, 1985).

Until recently, the EB virus-determined nuclear antigens have been studied as a complex. Newly developed tools provide a means of exploring their behavior separately. In the present work we studied EBNA-1 expression

after induction of the productive virus cycle. We expected to obtain some information as to whether this virus-genome product is a factor of negative or positive regulation.

The virus-producer Burkitt lymphoma (BL) P3HR-1 cell line (Hinuma and Grace, 1967) and the EBV-negative BL cell line Ramos (Klein *et al.*, 1975), cultivated as described elsewhere (Vonka *et al.*, 1972), were used troughout. Entry of P3HR-1 cells into the productive virus cycle was enhanced by cultivating them in a growth medium containing 3 mmol/l n-butyrate and 20 ng/ml of 12-o-tetradecanoylphorbol-13 acetate (Anisimová *et al.*, 1984). Untreated P3HR-1 cells and both untreated and drug-treated Ramos cells were used as controls. On day 3 after the addition of inducers samples were withdrawn to determine the number of cells containing EA and VCA on acetone-fixed smears by the indirect immunofluorescence technique (Henle and Henle, 1966). The remaining cells were pelleted, washed twice with 150 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.4, and 1 mmol/l EDTA, and frozen at −70 °C for at least 48 hours. The pellets were then resuspended in 1 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.4, and 1 mmol/l EDTA and centrifuged at 30,000 rev/min in rotor 50 Ti, Beckman ultracentrifuge L5-65, for 1 hr. Supernatants from all samples were adjusted to the same protein concentration. The content of EBNA-1 in the samples was determined by their capacity to absorb anti-EBNA-1 antibody from a human serum of known anti-EBNA-1 titre. In the test, 0.1 ml volumes of two-fold dilutions of cell-free extracts were mixed with 0.1 ml of serum diluted 1 : 100 and incubated 1 hr at 37 °C. After this the amount of free antibody was determined by the ELISA using the synthetic peptide as an antigen. The composition of buffers and the ELISA procedure were described elsewhere (Vestergaard *et al.*, 1977). The peptide corresponding to the gly-ala-rich region of EBNA-1 (Rhodes *et al.*-1985) was synthesized by the solid phase technique (Merrifield, 1963) and before use was conjugated to bovine serum albumin (BSA). Specific reactivity of the conjugate with EBNA-1 antibody was verified by pretesting it against the panel of both positive and negative sera (results not shown). Micro ELISA plates (Dynatech) were coated with 2 μg of the conjugate/well. Free binding sites in the plates were blocked by 1 % BSA. The peptide was then reacted with sorbed samples of the human serum and reaction intensity was monitored using swine anti-human IgG conjugated with horse-radish peroxidase (Sevac, Prague).

Untreated virus-producer P3HR-1 cells spontaneously expressed EA and VCA in 2.7 per cent of the cell population. On day 3 after the addition of n-butyrate and TPA the proportion of antigen-positive P3HR-1 cells increased to 28.4 per cent. No EA and VCA was detected in untreated or drug--treated BV-negative Ramos cells. As shown in Fig. 1, extracts from P3HR-1 were able to bind effectively anti-EBNA-1 antibody, reducing the amount of antibody available for reaction with EBNA-1 peptide. The efficiency of absorbtion decreased with the increasing dilution of cell extracts, but was the same for both untreated and drug-induced cells. Similarly treated EBV-negative Ramos cells did not exhibit any reactivity with anti-EBNA-1 antibody. These results indicate that the concentration of EBNA-1 in P3HR-1 cells was not influenced by the entry of a significant proportion of the cells into the productive virus cycle.

Boguszaková *et al.* (1983) studied EBNA expression, after the induction of productive virus cycle, using complement fixation (CF) and anticomplement immunofluorescence (ACIF); i.e., they measured the expression of the whole EBNA complex. On the third day after the addition of butyrate and TPA the CF activity per 10^8 cells decreased by about 30 per cent in both Raji and P3HR-1 cells and the number of ACIF-positive Raji cells also dropped. This decrease was, however, less marked or undetectable when the activity was normalized to a unit protein weight. The authors conclude that EBNA, unlike the SV-40 virus large T antigen (Tijan, 1981), is not essential

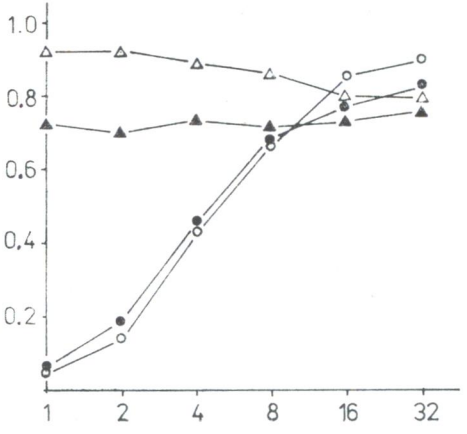

Fig. 1.
Adsorption capacity of cell free extracts from untreated and drug-treated P3HR-1 cells and Ramos cells
Unadsorbed antibodies were measured by ELISA using a synthetic peptide as an antigen.
Untreated P3HR-1 cells ●
Drug-treated P3HR-1 cells ○
Untreated Ramos cells ▲
Drug-treated Ramos cells △
Abscissa: Log_2 dilution of cell-free extracts; ordinate: The amount of unadsorbed EBNA-1 antibody (extinction at 490 nm)

in the course of EBV lytic growth cycle. Our finding that the content of EBNA-1 remained unaltered during the productive virus cycle is in line with this conclusion so far as this particular EBNA subcomponent is concerned.

Weigel *et al.* (1985), using immunoblotting for separate EBNA-1 detection, also report that the polypeptide was expressed with unchanged abundancy after induction of the superinducible HR-1 cell clone, X50-7 cells, and Raji cells. The same was true for EBNA-1 m-RNA. That the role of EBNA-1 is inessential in lytic virus growth is further more evidenced by the results of Volsky *et al.* (1981) and Shapiro *et al.* (1982): experimental EBV infection of some naturally EBV-insusceptible cells resulted in productive virus replication without any detectable EBNA synthesis.

It is believed that EBNA-1 is needed in trans for the maintenance of the EBV genome in the form of replicating plasmid in latently infected cells (Yates *et al.*, 1985). This function is mediated by its interaction with a specific sequence of the EBV genome (Reisman *et al.*, 1985). Recent results suggest that the function is not required for the replication of virus progeny DNA. As EBNA-1 is however, constitutively expressed during the lytic cycle of the virus, the question remains open whether it plays any other regulatory role in this cycle or is expressed "in vain".

References

Adams, A. (1979): The state of virus genome in transformed cells and its relationship to host cell DNA, pp. 155–163. In: Epstein, M. A. and Achong, B. G. (Eds): The *Epstein-Barr virus*, Springer-Verlag, Heidelberg.

Anisimová, E., Prachová, K., Roubal, J. and Vonka, V. (1984): Effect of n-butyrate and phorbol-ester (TPA) on induction of Epstein-Barr virus antigens and cell differentiation. *Arch. Virol.* **81**, 223–237.

Boguszaková, L., Hirsch, I., Břicháček, B., and Vonka, V. (1983): Relationship between Epstein-Barr virus nuclear antigen and DNA genome number in superinfected and induced lymphoblastoid cell lines. *J. gen. Virol.* **64**, 883–894.

Dillner, J., Kallin, B., Ehlin-Henriksson, B., Timar, L. and Klein G. (1985): Characterization of a second Epstein-Barr virus-determined nuclear antigen associated with the Bam HI WYH region of EBV DNA. *Int. J. Cancer* **35**, 359—366.

Gerber, P., Whang-Peng, J. and Monroe, J. H. (1969): Transformation and chromosome changes induced by Epstein-Barr virus in normal human leucocyte cultures. *Proc. natn. Acad. Sci. U.S.A.* **63**, 740—747.

Henle, W. and Henle, G. (1966): Immunofluorescence in cells derived from Burkitt's lymphoma. *J. Bacteriol.* **91**, 1248—1256.

Hinuma, Y., and Grace, J. T. (1967): Cloning of immunoglobulin producing human leukemia and lymphoma cell lines in long-term cultures. *Proc. Soc. exp. Biol. Med.* **124**, 107—111.

Kallin, B., Dillner, J., Ernberg, I., Ehlin-Henriksson, B., Rosen, A., Henle, W., Henle G., and Klein, G. (1986): Four virally determined nuclear antigens are expressed in Epstein-Barr virus-transformed cells. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1499—1503.

Klein, G., Giovanella, B., Westman, A., Stehlin, J. G. and Mumford, D. (1975): An EBV-genome negative cell line established from an American Burkitt lymphoma; receptor characteristics, EBV-infectability and permanent conversion into EBV-positive sublines by in vitro infection. *Intervirology* **5**, 319—334.

Merrifield, R. B. (1963): Solid phase peptide synthesis. I. The synthesis of tetrapeptide. *J. Am. chem. Soc.* **85**, 2149—2154.

Miller, G., and Lipman, M. (1973): Release of infectious EBV by marmoset transformed leucocytes. *Proc. natn. Acad. Sci. U.S.A.* **70**, 190—194.

Miller, G., Lisco, H., Kohn, H. I., Stitt, D. and Enders, J. F. (1971): Establishment of cell lines from normal adult human blood lymphocytes by exposure to Epstein-Barr virus and neutralization by human sera with Epstein-Barr virus antibody. *Proc. Soc. exp. Biol. Med.* **137**, 1456—1459.

Moss, D. J., Rickinson, A. B., Wallace, L. E., and Epstein, M. A. (1981): Sequential appearance of Epstein-Barr virus nuclear and lymphocyte-determined membrane antigen in B-cell transformation. *Nature* (*London*) **291**, 664—666.

Reisman, D., Yates, J. and Sugden, B. (1985): A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. *Mol. Cell Biol.* **5**, 1822—1832.

Rhodes, G., Carson, A. D., Valbracht, J., Houghten, R. and Vanghan, H. J. (1985): Human immune responses to synthetic peptides from the Epstein-Barr virus nuclear antigen. — *J. Immunol.* **134**, 211—216.

Rowe, D., Heston, L., Metlay, J. and Miller G. (1985): Identification and expression of nuclear antigen from the genomic region of the Jijoye strain of Epstein-Barr virus that is missing in its nonimmortalizing deletion mutant P3HR-1. *Proc. nant. Acad. Sci. U.S.A.* **82**, 7429—7433.

Shapiro, I. M., Volsky, D. J., Saemundsen, A. K., Anisimová, E. and Klein, G. (1982): Infection of the human T cell-derived leukemia line Molt-4 by Epstein-Barr virus: induction of EBV--determines antigens and virus reproduction. *Virology* **120**, 171—181.

Summers, W. C., Grogan, E., Shedd, D., Robert, M., Lui, G. and Miller, G. (1982): Stable expression in mouse cells of nuclear neoantigen after transfer of 3.4 megadalton cloned fragment of Epstein-Barr virus DNA. *Proc. nant. Acad. Sci. U.S.A.* **79**, 5688—5692.

Tijan, R. (1981): T antigen binding and the control of SV 40 gene expression. *Cell* **26**, 1—2.

Vestergaard B. F., Graubale, P. C. I. and Spangaard, H. (1977): Titration of herpes simplex virus antibodies in human sera by the enzyme-linked immunosorbent assay (ELISA). *Acta pathol. microbiol. Scand.* (*B*) **85**, 466—468.

Volsky, D. J., Klein, G., Volsky, B. and Shapiro, I. (1981): Production of infectious Epstein-Barr virus in mouse lymphocytes. *Nature* (*London*) **293**, 399—401.

Vonka, V., Vlčková, I., Závadová, H., Kouba, K., Lazovská, J. and Duben, J. (1972): Antibodies to Epstein-Barr virus capsid antigen and to soluble antigen of lymphoblastoid cell lines in infectious mononucleosis patients. *Int. J. Cancer* **9**, 529—535.

Weigel, R., Fischer, D. K., Heston, L. and Miller, G. (1985): Constitutive expression of Epstein--Barr virus-encoded RNAs and nuclear antigen during latency and after induction of Epstein--Barr virus replication. *J. Virol.* **53**, 254—259.

Yates, J. L., Warren, W., and Sugden, B. (1985): Stable replication of plasmid derived from Epstein-Barr virus in various mammalian cells. *Nature* (*London*) **313**, 812—815.