Relationship between Epstein–Barr Virus Nuclear Antigen and DNA Genome Number in Superinfected and Induced Lymphoblastoid Cell Lines

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SUMMARY

The relative contents of Epstein–Barr virus (EBV)-associated nuclear antigen (EBNA) and average numbers of EBV genome equivalents per cell were determined (i) in Raji cells superinfected with P3HR-1 virus, (ii) in Raji cells induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and sodium n-butyrate and (iii) in P3HR-1 cells induced by TPA and sodium n-butyrate. This treatment was followed by increases in the percentage of early antigen (in both Raji and P3HR-1 cells) and virus capsid antigen-positive cells (in P3HR-1 cells) and increases of approximately 20-fold in the average number of EBV DNA equivalents in superinfected Raji cells and in TPA- and sodium n-butyrate-induced P3HR-1 cells. However, the content of EBNA in these cells dropped. This was revealed by a decrease in both the complement-fixing antigen content and in the proportion of EBNA-positive cells as determined by anti-complement immunofluorescence. Thus, the positive correlation found previously between the content of EBNA and the number of EBV genome equivalents per cell in proliferating lymphoblastoid cultures does not seem to apply to the situation in either superinfected Raji cells or in P3HR-1 cells induced by TPA and sodium n-butyrate.

INTRODUCTION

Epstein–Barr virus nuclear antigen (EBNA) is uniformly present in Epstein–Barr virus (EBV) genome-positive cells (Reedman & Klein, 1973). It has a strong affinity for double-stranded DNA and chromosomes (Luka et al., 1977; Ohno et al., 1977) and preliminary evidence suggests that it has higher affinity for EBV DNA than for other DNAs of viral and cellular origin (Hirsch et al., 1979). It stimulates the template activity of chromatin for transcription in vitro (Kamata et al., 1979) and is apparently associated with protein kinase activity (Kamata et al., 1981). Upon microinjection into contact-inhibited 3T3 fibroblasts, EBNA stimulates a new cycle of cell DNA synthesis (Klein et al., 1979). Taken together, these findings indicate that EBNA may play an essential regulatory role in the establishment and maintenance of cellular transformation by EBV. Since, except for the poorly defined lymphocyte-detected membrane antigen (LYDMA) (Svedmyr & Jondal, 1975), EBNA is the only virus-induced product known to be expressed in proliferating EBV-carrying cells, it seems that it is not only involved in the induction and maintenance of cellular transformation but may also play a regulatory role in the expression of the viral genome.

A positive linear correlation between the number of EBV genome equivalents per cell and the amount of EBNA in a collection of exponentially growing EBV-non-producer cell lines has recently been demonstrated by Ernberg et al. (1977) and by Shapiro et al. (1979). A similar positive relationship between the contents of viral DNA and virus-associated nuclear antigen has also been observed for a set of herpesvirus ates-carrying marmoset lymphoid lines (Johnson et al., 1981).

The present experiments were undertaken to examine the relationship between EBNA content and the number of EBV genome equivalents per cell upon induction of EBV early
antigen (EA), which was used as a criterion of virus cycle activation. Three experimental systems in which EA had been efficiently induced were studied: (i) superinfection of Raji cells with P3HR-1 virus; (ii) induction of Raji cells with a mixture of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and sodium n-butyrate; (iii) induction of P3HR-1 cells with a mixture of TPA and n-butyrate. No increase in EBNA content was detected either in the superinfected Raji cells or induced Raji or P3HR-1 cells.

METHODS

Cell lines. Human lymphoblastoid cell lines Raji, Ramos and P3HR-1 were the same as described in earlier studies (Břicháček et al., 1981). They were passed and cultivated as described previously (Vonka et al., 1972). The eosin exclusion method was used for differentiating between viable and dead cells.

For induction experiments, exponentially growing Raji and P3HR-1 cells were sedimented by centrifugation and resuspended at a density of $5 \times 10^5$ cells/ml in fresh medium containing 3 mm-sodium n-butyrate (Merck) and $3.2 \times 10^{-5}$ mm-TPA (Sigma).

P3HR-1 virus for superinfection of Raji cells was obtained from the fluid phase of P3HR-1 cultures kept at 33 °C for a period of 10 to 14 days. Superinfection was carried out as described by Yajima & Nonoyama (1976), except that the superinfected cells were cultivated in minimal essential medium containing phosphate.

Sera and immunofluorescence. Serum M.T. (from a tonsillar carcinoma patient) and serum F.S. (from an infectious mononucleosis patient) and serum F.S. (from a nasopharyngeal carcinoma patient) were kindly provided by Dr O. Šibl (Bulovka Hospital, Prague). Their reactivities with the various EBV antigens (virus capsid, early, and nuclear) as determined by indirect and anticomplement immunofluorescence (ACIF) (Reedman & Klein, 1973; Vonka et al., 1977) were as follows: M.T., VCA 1:40, EA < 1:10, EBNA 1:160; F.C., VCA 1:160, EA < 1:10, EBNA < 1:10; F.S., VCA 1:320, EA 1:320, EBNA 1:320. M.T. serum was also examined in the radioimmuno-precipitation test (Kallin et al., 1979) with extracts of TPA- and n-butyrate-induced Raji and P3HR-1 cells that had been labelled with a $^{14}$C-amino acid mixture. No components of EA were detected.

Quantitative determination of EBNA. A sensitive micromodification of the complement-fixing (CF) assay based on lysis of $^{14}$Cr-labelled sheep erythrocytes was used to quantify EBNA in cell extracts (Shapiro et al., 1979). The antigen titre was expressed as the reciprocal of the dilution of antigen leading to 50% release of $^{14}$Cr in the presence of an antibody excess and 1-5 units of complement. For comparison of different antigen preparations, the antigen titre was expressed as the reciprocal of the dilution of antigen leading to 50% release of $^{14}$Cr in the presence of an antibody excess and 1-5 units of complement.

Several procedures for extracting EBNA from untreated lymphoblastoid cells were tested for their efficiency. These were (i) extraction by 0.1 M-glycine buffer at pH 9-5 (Ablashi et al., 1979), (ii) extraction by 1.7 M-NaCl (Luka et al., 1977) and (iii) extraction in physiological conditions by freezing and thawing (Luka et al., 1980). The first, second and third procedures yielded 21, 29, and 26 antigen units per 10^8 of exponentially growing Raji cells per ml or 3.9, 3.3 and 3.2 antigen units/mg of Raji cell protein, respectively. The total yield of CF antigen was not substantially increased (approx. 10%) after extraction of the high salt sediment with 4 M-guanidine hydrochloride (Pikler et al., 1978).

Determination of number of EBV genomes per cell. The average number of EBV genomes per cell was determined by reassociation kinetics using the nick-translated $^3$H-labelled EBV DNA probe as described in detail previously (Břicháček et al., 1981).

RESULTS

Specificity of CF test for monitoring EBNA

EB virus EA and viral capsid antigens (VCA) were efficiently induced in Raji cells superinfected with P3HR-1 EB virus (Fig. 1 a) and in P3HR-1 cells treated with a TPA-n-butyrate mixture (Fig. 1 c). Only EA was induced in Raji cells treated with TPA-n-butyrate (Fig. 1 b).

Both VCA and EA are known to fix complement in the presence of specific antibody (Vonka...
EBNA upon EBV activation

Fig. 1. Indirect immunofluorescence of VCA, and VCA and EA in (a) Raji cells superinfected with P3HR-1 virus, (b) Raji cells induced by TPA and n-butyrate, and (c) P3HR-1 cells induced by TPA and n-butyrate. ●, Serum F.C. (VCA+, EA-, EBNA-); ○, serum F.S. (VCA+, EA+, EBNA+).

Fig. 2. Complement-fixing activity of extracts from (a) non-induced Raji cells, (b) non-induced P3HR-1 cells, (c) Raji cells induced by TPA and n-butyrate and (d) P3HR-1 cells induced by TPA and n-butyrate on the third day after treatment. For the percentages of VCA-positive cells, and VCA-positive, EA-positive cells, see Fig. 1. The $^{51}$Cr-release CF reactions were done with serum F.C. (VCA+, EA-, EBNA-) (△), serum M.T. (VCA+, EA-, EBNA+) (○) and serum F.S. (VCA+, EA+, EBNA+) (□).

et al., 1970b; Lenoir et al., 1975). Since quantitative changes in EBNA content were measured by the CF method in this study, it was necessary to determine whether and how the CF activities of EA and VCA interfere with that of EBNA. With the use of VCA+, EA- and EBNA- serum, negligible CF activity was detected in Raji cells (Fig. 2a), P3HR-1 cells (Fig. 2b) and in Raji
cells induced by TPA and n-butyrate (Fig. 2c). Some CF activity was observed only in cell extracts obtained from P3HR-1 cells induced by TPA and n-butyrate (Fig. 2d) and containing a high proportion of VCA-positive cells (see Fig. 1c).

Upon appearance of EA, we observed an increased CF antigen content in cell extracts using VCA+, EA+, EBNA+ serum in comparison with VCA+, EA-, EBNA+ serum (Fig. 2b, c, d). Hence, significant amounts of EA CF activity were present in the respective cells. To rule out the interference of EA CF activity and to diminish to negligible levels the possible interference of VCA, subsequent experiments were carried out with the VCA+, EA-, EBNA+ serum that possessed a high EBNA antibody titre and a low VCA antibody titre and was free of EA antibody as determined by both IF and radioimmunoprecipitation. The CF antigen titres obtained under these conditions were considered to express the content of EBNA.

**Number of EBV genome equivalents per cell and amount of EBNA**

The average number of EBV genome equivalents per cell increased from 45 to 800 after superinfection of Raji cells with P3HR-1 virus (Fig. 3b) and from 75 to 1300 after induction of P3HR-1 cells with TPA and n-butyrate (Fig. 3d). A very small, if any, increase was observed in TPA and n-butyrate-induced Raji cells (Fig. 3b).

However, a marked loss of CF activity extractable from superinfected or induced cells followed the EB virus cycle activation (Fig. 3a, c). The outcome of the CF test might be influenced by the efficiency of EBNA extraction from cells in different physiological conditions. Therefore, ACIF was used to monitor the proportion of EBNA-positive cells in the respective cell populations. Results of a representative experiment with mock-treated, superinfected and induced Raji cells are shown in Table 1 and Fig. 4. In this experiment, cell viability, EBNA positivity and CF activity of corresponding cell extracts were determined in parallel. When comparing the relative amounts of CF units (normalized to 10^8 cells/ml) and the counts of cells
positive for EBNA in ACIF, it seems clear that in all systems they were decreasing with almost the same kinetics. The decrease in EBNA content following the activation of EB virus cycle was less marked or undetectable, when CF activity was normalized to a unit protein weight (1 mg). This was apparently associated with the release of proteins from dead or dying cells as revealed by the gradually decreasing amounts of protein content per $10^8$ cells/ml in the course of the observation period. In the mock-treated cells the percentage of viable and EBNA-positive cells were close to each other throughout the observation period. The superinfected and induced cells were losing their viability more rapidly than the mock-treated cells; moreover, in the superinfected and induced cells the cell viability was decreasing faster than the EBNA positivity. Thus, in these cultures the number of EBNA-positive cells exceeded the number of viable cells by a factor of 2 to 3.

No marked differences in the intensity of EBNA staining were observed among the fractions of EBNA-positive untreated, superinfected or induced cells (Fig. 4).

Because of a strong non-specific ACIF staining with the negative serum in induced P3HR-1 cells, we were not able to follow the changes in the content of EBNA-positive cells in this popula-

![Fig. 4. Anticomplement immunofluorescence (ACIF) of Raji cells with serum M.T. (VCA+, EA-, EBNA+) seen at: (a) 2, (b) 4, (c) 6, (d) 8 and (e) 10 days after passage; (g) 0, (h) 2 and (i) 4 days after induction with TPA and n-butyrate; (j) 0, (k) 2 and (l) 4 days after superinfection with P3HR-1 virus; (f) as (a) but with EBV-negative human serum. × 150.](image-url)
Table 1. *EBNA content in Raji cells*

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<th>Time after treatment (days)</th>
<th>Viable cells (%)</th>
<th>EBNA-positive cells (%)</th>
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* Anticomplementary immunofluorescence (ACIF).
† 100% corresponds to 18 CF units/10^6 cells/ml.
‡ 100% corresponds to 2.6 CF units/mg protein.

The positive correlation found between the content of EBNA and the number of EBV genome equivalents per cell in proliferating lymphoblastoid cultures (Ernberg *et al.*, 1977; Shapiro *et al.*, 1979) does not seem to apply to the situation upon activation of the virus cycle. We detected a significant decrease in EBNA content accompanying an increase in the number of EBV genome equivalents and appearance of EA following either superinfection of Raji cells or induction of P3HR-1 cells. Our data thus suggest that EBNA formation is not essential in the course of EBV lytic growth cycle. This seems to be corroborated by recent findings in naturally EBV non-susceptible cells onto which EBV receptors had been transplanted prior to EBV infection (Volsky *et al.*, 1981; Shapiro *et al.*, 1982). In at least some of these cells a complete virus cycle was observed without prior EBNA formation. It should be noted, however, that neither in their study nor in our study was the presence of EBNA monitored at very early stages upon infection or induction, during which period important regulatory steps could take place.

As discussed in the recent review (Vonka & Hirsch, 1982) EBNA seems to have a stimulatory effect on some cellular functions such as DNA synthesis and transcription. The suppression of other viral functions in non-producer lines may suggest that EBNA also plays an essential role in the regulation of the EBV replication cycle, e.g. a negative regulatory role by restricting EBV from entering into the lytic cycle or a positive role in the maintenance of EBV episomes within cell nuclei and in the transcription of some viral genes upon induction. Both positive and negative functions in the regulation of SV40 replication have been described for the SV40 T-antigen (Tjian, 1981). If EBNA had a positive regulatory function in EBV growth, an increase in its content could have been expected upon activation of the virus cycle. Because this was not the case in the present series of experiments, EBNA rather seems to play a repressor-like role in the regulation of the EBV replication cycle. It is noteworthy that in the case of the best-studied virus repressor, the lambda repressor, its *de novo* synthesis ceases and the repressor is specifically inactivated upon lambda phage induction (Roberts *et al.*, 1978).
It seems clear that the decrease of EBNA content after induction of the EB virus cycle is associated with the rapid loss of cell viability in these cultures. The disappearance of EBNA activity with the death of cells in ageing cultures has been reported by Suzuki & Hinuma (1975); our data on the mock-treated culture are in agreement with their results. EBNA disappearance in the ageing cultures is probably the cumulative effect of three events: the stopping of synthesis of EBNA in non-growing cultures (Suzuki & Hinuma, 1975; Vonka et al., 1970a), the release of the antigen from dead or dying cells and the inactivation of the antigenicity of cell-associated antigen resulting from the histochemical decomposition of the cell. Nevertheless, in the superinfected or induced cells the number of EBNA-positive cells markedly exceeded the number of viable cells, indicating that a significant proportion of 'specifically' killed cells remains EBNA-positive for some time. This difference may be associated with a possible modification and stabilization of EBNA in the course of the activation of the virus growth cycle. However, the reason for the discrepancy might well be more prosaic: the presence of EBNA in many dead cells in the induced or superinfected cultures may merely reflect the fact that the interval between ceasing synthesis de novo of EBNA and the cell death was generally much shorter than in the ageing cultures.

REFERENCES


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