Identification of Epstein–Barr Virus-induced Polypeptides in P3HR-1 Cells by Protein Immunoblot

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SUMMARY

The protein immunoblot technique was used to identify Epstein–Barr virus-specific antigens present in sodium butyrate-induced P3HR-1 cells. Using sera from patients with either nasopharyngeal carcinoma or arthritis, 16 polypeptides were detected ranging in molecular weight from 22K to 140K. Each of the anti-EA-, anti-VCA-positive sera were found to contain antibodies to different subsets of the antigens. A 72K protein was identified which was consistent with the nuclear antigen (EBNA), and culturing cells in the presence of disodium phosphonoacetate allowed identification of 140K and 22K antigens as late viral products. Treatment of cells with sodium butyrate revealed that expression of some antigens increased in parallel with the time of incubation of the cells in butyrate while other antigens either appeared early and then decreased in intensity or were only present after a number of days of butyrate treatment. One of the antigens which decreased with the time cells were treated with butyrate was EBNA.

INTRODUCTION

Permissive infection of cells by Epstein–Barr virus (EBV) results in the expression of a number of virus-induced antigen complexes. These antigens were originally defined by indirect immunofluorescence procedures and have been termed viral capsid antigens (VCA), membrane antigens (MA) and early antigens (EA) (Pearson, 1980). EA can be subdivided into two distinct staining patterns: a diffuse (D) pattern detectable in both the nucleus and cytoplasm and a restricted (R) pattern localized in the cytoplasm (Henle et al., 1971). Expression of EA has been shown to be independent of DNA synthesis (Gergely et al., 1971) and can be distinguished from late viral products if cells are induced in the presence of a DNA inhibitor.

Biochemical characterization of EBV-specific antigens has been hampered by the lack of a cell line fully permissive for viral replication. This, however, has been partially overcome by the use of a variety of chemical inducers which can increase the proportion of cells entering the productive cycle. These inducers include halogenated pyrimidines (Hampar et al., 1973), n-butyrate (Luka et al., 1979) and phorbol esters (zur Hausen et al., 1978). Employing chemically induced cells, many groups have attempted to identify the individual polypeptides which constitute the EA and VCA complexes by radioimmunoprecipitation. This technique, however, has a number of disadvantages; not all antigens may incorporate radioisotope, coprecipitation of cellular proteins may occur due to their association with EBV-specific antigens, and many antigens may not be able to be precipitated either because they are unavailable to react with antibodies or because they are bound to cellular components. We have attempted to overcome these drawbacks by utilizing the protein immunoblot technique to identify EA and VCA polypeptides in P3HR-1 cells induced with sodium butyrate.

METHODS

Cells. The P3HR-1 and BJAB cells used in this study were grown on RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum at 37°C with 5% CO₂. Cells were allowed to grow to 1.5 x 10⁶ cells/ml, then cut to a cell density of 5 x 10⁴ cells/ml and 5 mM-sodium butyrate (J. T. Baker Chemical, Phillipsburg, N.J., U.S.A.) added. The cells were allowed to grow under normal conditions, then collected by centrifugation 3 days
after the addition of butyrate and stored at −80 °C. Cells used for the preparation of cytoplasmic and nuclear extracts were stored at −80 °C in nuclei isolation buffer (0:25 M-sucrose, 60 mM-KCl, 15 mM-NaCl, 5 mM-MgCl2, 10 mM-Tris-HCl pH 7.4, 0.1 mM-PMSF) with glycerol added to 25% (v/v).

**Treatment with sodium butyrate and disodium phosphonooacetate (PAA).** P3HR-1 cells at a starting concentration of 5 × 10^6 cells/ml were incubated with 5 mM-sodium butyrate for up to 7 days, with aliquots (2 × 10^7 cells) being taken at day 0, day 1, day 2, day 4 and day 7. Cells from each sample were collected by centrifugation and stored at −80 °C. Cells incubated with PAA (Sigma) were treated similarly except that 200 μg/ml PAA was added along with the sodium butyrate. Two percent of the cells were VCA-positive and this increased with butyrate treatment to 10% by day 2, 40% by day 4 and 50 to 60% by day 7.

**Sera.** The EBV titres of the sera used are shown in Table 1. Anti-EBNA titres were measured according to the method of Reedman & Klein (1973). Anti-VCA titres were measured as described by Henle & Henle (1966). Anti-EA titres were determined by the method of Klein & Dombos (1973). In brief, EA was induced in Raji cells by their infection with 25 μg/ml 5-iododeoxyuridine (IUdR) for 2 days, after which the cells were fixed in either acetone or methanol. Cells fixed in methanol, and thereby lacking restricted early antigen, were used to titrate antibodies to diffuse early antigen (EA-D), while cells fixed in acetone, which contained both restricted and diffuse early antigen (EA-D + R), were used to titrate sera to both restricted and diffuse early antigens.

Monoclonal antibodies directed against membrane antigens and early antigens were purchased through Bio-Tech Research Laboratories Inc. (Rockville, Md., U.S.A.). Antibody 2F5.6 was directed against the 320K/350K glycoprotein of B95 virus-infected cultures, but did not react with the P3HR-1 cell line and was used as a control. Monoclonal antibody B10.3 reacted by immunofluorescence with the membrane of all virus-producing cell lines (Qualtiere et al., 1982). Antibody K9 was specific for an 85K EA-R component and antibody R3 a 50K to 52K EA-D component (Pearson et al., 1983).

**Preparation of cell fractions.** Frozen cells were allowed to thaw and were collected by centrifugation at 2000 g for 5 min. The cells were suspended in nuclei isolation buffer containing 0.5% (v/v) Triton X-100, left on ice for 5 min and collected by centrifugation at 2000 g for 5 min. This process was repeated twice more, and the final pellet represented purified nuclei. The pooled supernatants from each of these centrifugations represented the cytosol fraction.

A nuclear extract was prepared by suspending purified nuclei in 0.4 M-NaCl, 20 mM-Tris- HCl (pH 7.4), 0.1 mM-PMSF and incubating them at 4 °C for 5 min. The nuclei were collected by centrifugation. This extraction of nuclei was repeated and the two extracts were pooled, the combined preparation being clarified by centrifugation (10000 g for 20 min). The resulting pellet was then suspended in 1 M-NaCl, 20 mM-Tris-HCl (pH 7.4), 0.1 mM-PMSF, and the procedure described above was repeated. The whole process was again repeated with 2 M-NaCl, 20 mM-Tris-HCl (pH 7.4), 0.1 mM-PMSF.

**Preparation of whole cell lysate.** Frozen cell pellets were dispersed by sonication in 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.1 mM-PMSF, 10 mM-sodium phosphate (pH 6.8). The samples were then placed in a boiling water-bath for 2 min, allowed to cool, and centrifuged at 15000 g for 5 min. Samples were stored at −20 °C. Twenty to 40 μl samples (150 μg of protein) were used for electrophoresis.

**Polyacrylamide gel electrophoresis.** Composition and electrophoresis of 10% (w/v) polyacrylamide-SDS slab gels were essentially as reported by Laemmli (1970). Electrophoresis was performed at 100 V for 16 h at 0 to 4 °C.

**Protein immunoblot.** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose paper (Bio-Rad) and detection of antigens with antibody and radiiodinated Protein A (70 to 100 μCi/μg) (New England Nuclear) were performed essentially as described by Burnette (1981), except that the transfer buffer contained 0.05% SDS. When monoclonal antibodies were used, the nitrocellulose sheets were incubated with rabbit antimouse Ig for 1 h, then washed, prior to incubation with Protein A. Samples of protein standards (Pharmacia) were iodinated by using 125I and Iodo-Beads (Pierce Chemical Co., Rockford, Ill., U.S.A.). These were subjected to electrophoresis alongside the cell extracts and were similarly transferred to nitrocellulose paper. The symbol K has been used to denote 10^3 mol. wt.

**RESULTS**

**Identification of EBV-specific antigens**

P3HR-1 cells were treated with sodium butyrate for 3 days as described. An SDS extract was prepared from these cells, and layered across the top of the 10% polyacrylamide-SDS slab gel. Proteins were separated by electrophoresis, then electrophoretically transferred to nitrocellulose paper. After blocking with bovine serum albumin, the paper was cut into strips and each strip incubated with an individual serum (Table 1). Two seronegative control sera, three anti-EBNA+, anti-VCA-positive, anti-EA-negative sera, and nine anti-EBNA+, anti-VCA+, anti-EA-positive sera were used to identify EBV-specific antigens and the results are shown in Fig. 1. The anti-EBNA+, anti-VCA+, anti-EA-positive sera reacted with 16 different polypeptides (assuming
Identification of EBV-induced antigens

Fig. 1. Detection of EBV-specific antigens in P3HR-1 cells by protein immunoblot. An SDS extract was prepared from P3HR-1 cells which had been incubated with sodium butyrate for 3 days. This extract (1.5 mg protein) was applied across the top of a 10% polyacrylamide–SDS slab gel. Proteins were separated by electrophoresis then transferred to nitrocellulose paper. The paper was cut into strips and each strip incubated with an individual serum. The strips were realigned prior to exposure to X-ray film. Serum samples: 1, S.B.; 2, G.L.; 3, D.S.; 4, D.M.; 5, RO17; 6, RO53; 7, RO41; 8, A14; 9, RO36; 10, A39; 11, A22; 12, A31; 13, RO71; 14, A20. Iodinated protein standards were included in the gel and the molecular weights of each of the antigens were determined by comparison to these standards.

Table 1. Antibody titres of test sera

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* From clinically normal individuals.
† From individuals diagnosed as having nasopharyngeal carcinoma (Moss et al., 1983a).
‡ From individuals diagnosed as having either osteo- or rheumatoid arthritis (Moss et al., 1983b).
Table 2. Reaction of sera with EBV-specific antigens

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* The order of the sera matches Fig. 1.
† The molecular weights of the antigens are given \( \times 10^{-3} \).

that the 44 to 48K antigen was composed of two polypeptides) ranging in molecular weight from 140K to 22K. Only one serum (RO41) reacted with all of these antigens, while the others reacted with various combinations of each of the antigens (Table 2). The anti-EBNA-, anti-VCA-positive, anti-EA-negative sera all detected the 72K and 22K antigens. The 72K antigen has been previously shown to represent EBNA (Strnad et al., 1981; Sculley et al., 1984a, b) while the 22K antigen may be a VCA component. None of the 16 polypeptides was identified by either of the control sera, nor were they detected in BJAB cells which had been treated with sodium butyrate for 3 days (results not shown) indicating that they were all EBV-specific.

Sera RO53 and RO36 which had high titres of EA-D, reacted strongly with EBNA and the 44 to 48K antigens, suggesting that the 44 to 48K antigens may be components of the EA-D complex. This contention is supported by the observation that serum RO71, which contains only antibodies to EA-R did not react with the 44 to 48K antigens.

Appearance of antigens after induction with sodium butyrate

P3HR-1 cells were incubated with sodium butyrate for 7 days, with aliquots of these cells being taken at day 0, day 1, day 2, day 4 and day 7. SDS extracts were prepared and electrophoresed on 10% polyacrylamide–SDS slab gels. The proteins were then transferred to nitrocellulose paper and incubated with three anti-EBNA-, anti-VCA-, anti-EA-positive sera (Fig. 2). The results show that some of the antigens present prior to the treatment with sodium butyrate decreased with increased duration of the cells in sodium butyrate. The most notable was EBNA, where each of the sera showed a marked decrease in the reaction with EBNA after the cells had been incubated with sodium butyrate for 2 days. By day 7 only RO53, which initially gave a strong reaction with EBNA, was able to detect the antigen. Antigens with molecular weights of 40K and 50K increased in intensity to day 2 but then began to diminish. Some antigens (120K, 22K) showed an increase parallel with the time of the cells in sodium butyrate, while others only appeared after 2 days in sodium butyrate (42K, 38K, 36K). The differential appearance of each of these antigens, after treatment of cells with sodium butyrate, stresses the need to examine cells at various times after induction especially if inducers other than sodium butyrate cause a similar phenomenon.

It is possible that some of the late appearing antigens may arise by proteolysis or biochemical modification of pre-existing antigens since their appearance is concomitant with the disappearance of other antigens. This is particularly noticeable with the decrease in the 50K
Identification of EBV-induced antigens

antigen and appearance of the 42K antigen. Likewise the concomitant disappearance of the 40K and 38K antigens with the appearance of the 36K antigen suggests that they may be related.

Induction of EBV-specific antigens in the presence of PAA

P3HR-1 cells which had been cultured in the presence of both sodium butyrate and PAA were sampled at various times. SDS extracts were prepared from these samples and the proteins separated by gel electrophoresis, transferred to nitrocellulose paper, then incubated with anti-EBNA-, anti-VCA-, anti-EA positive sera. Fig. 3 shows essentially the same results as depicted in Fig. 2 except that induction of the 140K and 22K antigens were inhibited by PAA. The sensitivity of expression of the 22K antigen to PAA as well as its detection by all of the sera containing anti-VCA antibodies indicates that it is a VCA-related component. The sensitivity of the 140K antigen to PAA demonstrates that it is also a late viral product. Expression of a 41K protein, which was only detected by serum A14, also appeared to be sensitive to PAA, but because this antigen was only detected with a single serum its significance is obscure. All of the other antigens, including those induced late after incubation of the cells with sodium butyrate were unaffected by the presence of PAA, indicating that they were early antigens.

Cellular localization of the antigens

Cytoplasmic and nuclear extracts were prepared from P3HR-1 cells which had previously been incubated with sodium butyrate for 3 days. Samples from each of the extracts, as well as the remaining pellet, were subjected to electrophoresis then transfer to nitrocellulose paper. Antigens were identified by employing two anti-EBNA-, anti-VCA-, anti-EA-positive sera (Fig. 4). Five of the antigens (90K, 67K, 38K, 36K and 22K) were almost undetectable in the cytoplasmic extract, whereas the remaining antigens, including EBNA, were present in both
Fig. 3. Time course of P3HR-1 cells in sodium butyrate in the presence of PAA. P3HR-1 cells were incubated with both sodium butyrate and PAA and samples were taken at time 0 (1), day 1 (2), day 2 (3), day 4 (4) and day 7 (5). SDS extracts were prepared from these samples and electrophoresed on 10% polyacrylamide-SDS gels. Proteins were then transferred to nitrocellulose papers and the papers incubated with sera: (a) RO17, (b) A14, (c) RO53. The lane marked 'P' contains a sample of P3HR-1 cells incubated with sodium butyrate for 4 days in the absence of PAA. The lane marked 'S' contains iodinated protein standards.

Fig. 4. Cellular localization of EBV-specific antigens. P3HR-1 cells incubated with sodium butyrate for 3 days were used as the source of antigens. Lane 1, cytosol; lane 2, 0.4 M-NaCl nuclear extract; lane 3, 1.0 M-NaCl nuclear extract; lane 4, 2.0 M-NaCl nuclear extract; lane 5, chromatin remaining after 2 M-NaCl extraction. All samples were electrophoresed on 10% polyacrylamide-SDS gels. The proteins were either stained with Coomassie Brilliant Blue (a), or transferred to nitrocellulose and antigens were detected with serum RO17 (b) or serum RO53 (c). Lanes marked 'S' contain iodinated protein standards.
Identification of EBV-induced antigens

Fig. 5. Detection of antigens with monoclonal antibodies. (a) Extracts prepared from P3HR-1 cells, which had been incubated with sodium butyrate for 3 days, were electrophoresed on 10% polyacrylamide–SDS gels and transferred to nitrocellulose papers. The paper was incubated with either serum RO41 (lane 1) or monoclonal R3 (lane 2). (b) P3HR-1 cells were incubated with sodium butyrate and samples taken at time 0 (1), day 2 (2), day 4 (3) and day 7 (4). SDS extracts were prepared, electrophoresed on 10% polyacrylamide–SDS gels, transferred to nitrocellulose paper, and incubated with the R3 monoclonal antibody.

cytoplasmic and nuclear extracts. Notably, the 44 to 48K antigens which reacted with the high-titred EA-D sera (RO53 and RO36) were prominent in both cytoplasmic and nuclear extracts. Many of the antigens (including the 22K VCA component) were found to be associated with the nuclear pellet after extraction with a 2 M-sodium chloride solution, suggesting that they may have a high avidity for cellular DNA.

Identification of components with monoclonal antibodies

An SDS extract was prepared from P3HR-1 cells which had been cultured in the presence of sodium butyrate for 3 days. Samples of the extract were subjected to electrophoresis, transferred to nitrocellulose papers and the papers then incubated with monoclonal antibodies directed against either membrane antigens or early antigens (see Methods).

Of these antibodies, only R3, which was specific for a 50 to 52K EA-D antigen, reacted in the immunoblots (Fig. 5a). R3 reacted with the 50K and 44 to 48K antigens which is consistent with the results presented by Pearson et al. (1983), and demonstrates that these antigens are EA-D components.

Immunoblots, containing extracts of P3HR-1 cells that had been cultured with sodium butyrate for different periods of time were incubated with R3 (Fig. 5b). The results show that the 50K and 44 to 48K antigens were maximally induced between 2 and 4 days of sodium butyrate treatment of the cells. After day 4 both antigens decreased, with the monoclonal antibody not detecting any low molecular weight breakdown products.

DISCUSSION

Using anti-VCA-, anti-EA-positive sera in the protein immunoblot technique, 16 EBV-specific polypeptides were identified in P3HR-1 cells induced with sodium butyrate. Although this technique has several advantages over radioimmunoprecipitation, there are some
limitations. Solubilization of proteins and electrophoresis in SDS causes complete denaturation of antigens so that some antigens may not be detected if all of their epitopes are contained within their tertiary structure. This may be one reason for the relatively few VCA components that were identified. Only two proteins, with molecular weights of 140K and 22K, were found to be late antigens judged by their sensitivity to PAA. The 140K antigen was only identified by eight of the 12 anti-VCA-positive sera whereas all 12 of the sera reacted with the 22K antigen. Because the 22K antigen reacted with all of the anti-VCA-positive sera, appeared to be present in the cells in reasonable amounts, and could be easily separated from the other antigens by gel filtration under denaturing conditions, it would be a good candidate for evaluation in an enzyme-linked immunosorbent assay (ELISA) for measuring antibodies to VCA.

Of the nine anti-VCA- and anti-EA-positive sera used, only one contained antibodies which reacted with the full spectrum of EBV-specific polypeptides. That sera contain antibodies against different subsets of the EA complex was first reported by Henle et al. (1971), who classified EA antigens into EA-R and EA-D subcomponents. The present results, however, indicate that sera may contain antibodies reactive against select individual components of the EA-R and EA-D complexes. This phenomenon may explain many of the discrepancies in work published by various groups (Kallin et al., 1979; Kawanashi et al., 1981; Mueller-Lantzsch et al., 1979). This observation was also made by Bayliss et al. (1983), who suggested the use of pooled sera to overcome the problem.

Another aspect which has to be taken into account when studying EA and VCA components was observed when a time course of treatment with sodium butyrate was performed. Some of the antigens increased in intensity in parallel with the time the cells had been incubated with sodium butyrate, while others only appeared after a number of days, and some antigens decreased with increasing time of incubation of the cells in sodium butyrate. This variation in the number of antigens detectable in P3HR-1 cells could also account for some discrepancies in the number and intensity of EBV-specific antigens reported by various workers (Kallin et al., 1979; Kawanashi et al., 1981; Roubl et al., 1981; Sugawara et al., 1982; Edson et al., 1983), particularly if a similar situation exists when other chemical inducers are used. Some of the antigens which decreased in intensity did so concomitantly with the appearance of other polypeptides. This was most noticeable with the 40K-38K-36K group of antigens and the disappearance of the 50K antigen and appearance of the 42K antigen. This observation leads to the speculation that the antigens may be cleaved at specific sites to change their molecular weights and possibly their function. The R3 monoclonal antibody, which reacts with the 50K antigen, failed to detect the 42K antigen. This result, however, does not indicate whether these two antigens are related or not since proteolysis of the 50K antigen to give the 42K protein may remove the epitope to which the antibody reacts. Purification of each of these antigens may be the only means of resolving the question.

Another antigen which decreased in intensity, the longer the cells were incubated in sodium butyrate, was EBNA. The decrease in EBNA as the percentage of VCA-positive cells increased suggests that it may be involved in regulating viral replication. A similar conclusion was drawn by Boguszakova et al. (1983) who also determined that EBNA decreased in both the Raji cell line and P3HR-1 cell line when these lines were induced to produce EA and VCA. Another alternative, however, may be that when cells are induced to replicate virus, the production of viral antigens required to maintain the transformed state is shut down. In this respect it would be interesting to know if other EBV-specific antigens, associated with transformed cells (Sculley et al., 1984b) also decrease in cell lines induced to replicate virus.

Comparisons of EBV-specific polypeptides identified in this study with results obtained by other laboratories are difficult because of variations in polyacrylamide gel systems, cell lines and chemical inducers used, and the sera employed. Some specific comparisons, though, can be made: use of the R3 monoclonal antibody demonstrates that the 44 to 48K antigens are equivalent to the 50 to 52K antigens identified by Pearson et al. (1983), and it is likely that these antigens are the same as the 50 to 55K doublet identified by Epstein (1984) with monoclonal antibodies. These antigens exist as a pair of polypeptides and are components of the EA-D complex. Similar antigens have been identified in studies of EBV early antigens (Thorley-
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Lawson et al., 1982). The 140K late antigen may correspond to the 140 to 160K late antigens reported by many groups (Bayliss & Wolf, 1981; Edson et al., 1983; Sugawara et al., 1982; Kallin et al., 1979; Kawanashi et al., 1981), and the 160K major capsid component identified by Baer et al. (1984). Cohen et al. (1984) identified a 140K late antigen in P3HR-1 cells as well as a 23K DNA-binding late antigen component that could correspond to the 22K VCA component identified in this study.

Study of EBV-specific components had been hampered by the complexity of the polypeptides which constitute these antigen groups. Purification of individual polypeptides has been difficult because the only method of identifying the position at which different antigens elute from columns has been radioimmunoprecipitation and this technique is not feasible when using large quantities of cells. This problem has been overcome in part by the production of monoclonal antibodies against some of the antigens (Pearson et al., 1983; Epstein, 1984; Takeda et al., 1983; Kishishita et al., 1984). The usual method of generating monoclonal antibodies had been to use chemically induced cells as the immunogen, so the operator has little control over the antigens against which the antibodies will be generated. By using the immunoblot technique to assay elution of antigens from columns they can be separated and purified, then used either to generate antibodies against specific antigens of interest or in the development of ELISA or radioimmunoassays to replace immunofluorescence assays.

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REFERENCES


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