Immunological studies on the Epstein–Barr virus encoded alkaline deoxyribonuclease found in virus-producing lymphoblastoid cells

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Antisera were raised against a purified recombinant form of the Epstein–Barr virus (EBV) alkaline deoxyribonuclease (DNase) expressed in Escherichia coli. These sera were shown to be reactive with lymphoblastoid cells expressing EBV antigens (B95-8, P3HR-I and Raji). Immunostaining studies of cells expressing EBV antigens revealed that the DNase was a component of the restricted early antigen complex of EBV. Western blot analysis of these chemically induced cells revealed that the polypeptide associated with the EBV DNase has an Mr of approximately 55000, slightly greater than that of the recombinant form, suggesting that the protein undergoes some form of post-translational modification during virus replication. The DNase enzymic activities observed in B95-8, P3HR-1 and Raji cells following chemical induction were neutralized using the specific antiserum. A detailed examination of protein extracts from the nude mouse-passaged nasopharyngeal carcinoma cell line C-15 failed to detect any antigenic or biochemical evidence for the presence of the DNase. Immunostaining of biopsies of oral 'hairy' leukoplakia with the antisera against EBV DNase revealed high level expression in the more differentiated spinous layers of the epithelium, a pattern of reactivity identical to that observed for other lytic cycle antigens.

Epstein–Barr virus (EBV) (Epstein et al., 1964) is the aetiological agent of infectious mononucleosis (IM) (Henle et al., 1974) and is associated with the development of a number of tumours: endemic Burkitt's lymphoma (BL) (Epstein et al., 1964), undifferentiated nasopharyngeal carcinoma (NPC) (Henle et al., 1970) and B cell lymphomas in immunodeficient individuals (Cleary et al., 1986).

EBV genome-positive B cell lines, such as the virus producer line P3HR-1, may be treated with chemical agents such as 12-O-tetradecanoylphorbol 13-acetate (TPA), which leads to the synthesis of early antigens (EA), followed by virus capsid antigens (VCA) and then the production of infectious virus (zur Hausen et al., 1978). Under these conditions, non-producer cell lines, such as Raji, express only EA; however, if these cells are superinfected with virus from producer cell lines, viral antigens are synthesized and infectious virus is produced (Yajima et al., 1978).

Like other herpesviruses, EBV induces a novel alkaline deoxyribonuclease (DNase) activity in infected cells (Clough, 1979). This activity has been demonstrated in the non-producer cell line Raji superinfected with HR-1 viruses and in chemically treated producer and non-producer B cell lines (Cheng et al., 1980a). Studies have shown that sera from patients with nasopharyngeal carcinoma neutralize EBV DNase activity, and this neutralizing activity has been used for the early diagnosis (Cheng et al., 1980b; Chen et al., 1982, 1987) and prognosis of the disease (Tan et al., 1982; Chen et al., 1985; Hsu et al., 1988). Neutralizing antibodies are not observed in the sera of healthy EBV-seropositive and -seronegative donors or patients with BL or IM.

Little is known about the involvement of the DNase in EBV replication; certain evidence suggests that the enzyme may be involved in the utilization of host cell DNA, providing the virus with deoxyribonucleotides by the degradation of cellular DNA (Feighny et al., 1981). In the case of herpes simplex virus (HSV), the DNase is a major viral product synthesized in infected cells which can be observed after PAGE of infected cell extracts (Banks et al., 1983). The HSV DNase has both 5' and 3' exonuclease and endonuclease activities (Hoffmann & Cheng, 1979) but, like the EBV enzyme, its precise role in viral replication remains unclear.

We have recently reported the expression of the EBV DNase in Escherichia coli and its subsequent purification and characterization (Baylis et al., 1989). We describe
here the production of antisera to the recombinant EBV DNase and show them to be useful reagents in examining the expression of the authentic DNase in lymphoblastoid cells expressing EBV antigens.

Using the chromatographically purified recombinant EBV DNase, antisera were raised in mice inoculated once intravenously with 1.0 μg of protein in Freund's incomplete adjuvant every 2 weeks and then once intravenously with 1.0 μg of protein. The mouse hyperimmune antiserum raised against the recombinant EBV DNase was shown to react with the recombinant protein in both Western blot and enzyme neutralization tests (data not shown).

In order to confirm the authenticity of the recombinant DNase purified from *E. coli*, the antisera raised against the antigen were used to examine lymphoblastoid cells expressing EBV antigens. The cell lines used included B95-8 (a marmoset B cell EBV producer line), Raji (a Burkitt's tumour non-producer B cell line), P3HR-1 (a Burkitt's tumour producer B cell line), the virus produced is non-transforming), Namalwa (a latently infected B cell line from a Burkitt's tumour) and DG-75 and Ball-1 cells (both EBV-negative human B cell lines) (Adams, 1979). Cells were maintained as described previously (Arrand et al., 1983). EBV antigen production was induced by treating cultures at a starting density of 3 × 10⁵ cells/ml with TPA and 5-bromodeoxyuridine (BUDR) at a final concentration of 50 ng/ml and 50 μg/ml respectively (Bauer, 1983). Cells were maintained for 2 to 3 days and EBV induction was monitored by following the expression of EA and VCA using the indirect immunofluorescence procedure (Henle & Henle, 1966). Upon harvesting, cells were washed twice with phosphate-buffered saline and resuspended in extraction buffer (50 mM-Tris–HCl pH 7.5, 3 mM-2-mercaptoethanol) at a final cell density of 1 × 10⁷ cells/ml. The extracts were briefly sonicated at 0°C and their specific DNase activity was determined (Baylis et al., 1989).

TPA and BUDR treatment of Raji, B95-8 and P3HR-1 cells resulted in the induction of DNase activity. In Raji cells there was a 6-9-fold increase in specific enzyme activity, B95-8 cells showed a 6-7-fold increase and in P3HR-1 cells the increase was 3-1-fold, as compared to non-induced controls. The increase in DNase activity correlated with the levels of cells shown to be EA/VCA-positive when examined by indirect immunofluorescence with EA+/VCA+ human sera (data not shown).

The ability of the antisera to neutralize the DNase activity observed in the chemically induced cell lines was determined as described previously (Baylis et al., 1989). The results of the enzyme neutralization are shown in Fig. 1(a). The antisera raised against the recombinant protein were found to neutralize the DNase activity observed in B95-8, Raji and P3HR-1 cells induced with TPA and BUDR but not the activity observed in the EBV-negative cell line Ball-1 after chemical treatment. The low level of neutralization observed (particularly in the case of the P3HR-1 cells) is a reflection of the relatively low level of induction of the EBV-encoded enzyme activity with a high background of endogenous cellular DNases. It is, however, clear that the antisera are capable of recognizing authentic EBV DNase.

In order to identify the specific polypeptide associated with the DNase in lymphoblastoid cells expressing EBV antigens, extracts were prepared from B95-8, P3HR-1 and Raji cells treated with TPA and BUDR and examined by Western blot analysis. Fig. 1(b) clearly shows the presence of a polypeptide detected in induced B95-8 and Raji cells and at a lower level in P3HR-1 cells. No reaction was observed in non-induced cells, the cell lines Namalwa or Ball-1 or in an identical blot probed with a preimmune mouse serum (results not shown). The hyperimmune antiserum detected a polypeptide with a size of 55K in all the induced EBV-positive lymphoblastoid cell lines, which is larger than the protein size predicted (52-5K) for the BGLF5 open reading frame (Baer et al., 1984) and the protein expressed in *E. coli* and purified to homogeneity (Baylis et al., 1989). The purification of a DNase activity from Raji cells has recently been reported and a 58K polypeptide was identified by silver staining. The apparent observed difference in Mₙ may be due to the different gel systems used (Hwang et al., 1990). The larger size of the native protein in lymphoblastoid cells suggests that the protein undergoes some form of post-translational modification which may not occur in the prokaryotic system. The DNases from HSV-1 and HSV-2 have predicted sizes of 67-4K and 66-1K respectively, however, the purified polypeptides have Mₛ, estimated from their electrophoretic mobility, of 85K to 90K, and have since been shown to be post-translationally phosphorylated (Banks et al., 1985). The size discrepancy in the case of the HSV DNase may also be explained in part by the high proline content determined from the sequence analysis. The EBV DNase does not have a high proline content nor have we been able to demonstrate phosphorylation of the DNase in chemically induced lymphoblastoid cells. It is possible that the EBV DNase may be post-translationally modified in some other way.

The observation that an EBV-specific polypeptide could be detected in Raji cells following TPA and BUDR treatment suggests that the EBV DNase is an EA synthesized in the absence of virus DNA replication and virion synthesis. The family of EAs has been divided into diffuse (D) and restricted (R) components based on two criteria: the EA-R is sensitive to methanol fixation and is cytoplasmic (Henle et al., 1971). However, in practice, the difficulty in identifying the location of EBV antigens
Fig. 1. (a) Neutralization of the EBV DNase from lymphoblastoid cell lines using hyperimmune mouse serum. Volumes (2 µl) of cell extracts (prepared as described in the text) were incubated with various volumes of antiserum for 30 min at room temperature and enzyme activity was determined. Neutralization of induced B95-8 (●), Raji (▲) and P3HR-1 (▲) cells. No neutralization was observed in extracts from Ball-1 cells (○). (b) Western blot analysis of the EBV DNase in lymphoblastoid cells. Extracts from a series of uninduced and induced cells were run on a 10% polyacrylamide gel at a concentration of 2 x 10⁵ to 3 x 10⁶ cells per lane and electrophoretically transferred to Immobilon P. The blot was incubated with the primary mouse serum at a 1:100 dilution overnight at 4 °C. Protein reactivity was detected using a naphthol AS-MX Fast Blue BB substrate (Sigma). Uninduced cells: Ball-1 (lane 1); Namalwa (lane 3); B95-8 (lane 5); P3HR-1 (lane 7); Raji (lane 9). TPA- and BUdR-induced: Ball-1 (lane 2); Namalwa (lane 4); B95-8 (lane 6); P3HR-1 (lane 8); Raji (lane 10). Mr markers are indicated.

Fig. 2. Immunostaining analysis of EBV-infected and non-infected cell lines. Induced cell line B95-8 (a), cell line DG-75 (b) and induced cell line Raji (c) were fixed in acetone, and induced Raji cells (d) were fixed in methanol. Following fixation, cells were reacted with hyperimmune mouse serum to the EBV DNase at a dilution of 1:200 and stained using the alkaline phosphatase monoclonal antibody anti-alkaline phosphatase technique (Cordell et al., 1984). Bar markers represent 100 µm.

in lymphoid cells results in the use of methanol fixation being the major criterion applied to differentiate EA-D from EA-R. Fig. 2 shows the differential reactivity of hyperimmune serum to the EBV DNase in immunostaining of EBV-infected cell lines using previously described techniques (Henle et al., 1971; Cordell et al., 1984). No reaction was observed in the EBV-negative cell line DG-75, nor with mouse preimmune sera with EBV-transformed cells, but staining was seen in chemically induced Raji and B95-8 cells fixed with acetone.
Staining was abolished in identical preparations of Raji cells which had been methanol-fixed, which suggests that the EBV DNase belongs to the EA-R complex. We have also shown by immunofluorescence and confocal microscopy that the protein is largely nuclear (S. A. Baylis & E. Littler, unpublished results). Hence it appears that the classical definition of EA-D and EA-R, which relied upon clinical antisera and hence detected the presence of a complex mixture of EBV proteins, may be only generally true of the mixture; our data, however, clearly show the alkaline exonuclease to be a nuclear, methanol-sensitive protein. Interestingly, it has been reported that serum antibodies to the EA-D component of EBV-infected cells have been found to be of prognostic value for NPC and IM patients, whereas serum antibodies to the EA-R component appear to be prognostically important in BL (Henle et al., 1971; Okano et al., 1988). These findings directly contradict our observations that the EBV DNase is a component of the EBV EA-R complex and our observations, and those of others, that antibodies (IgG and IgA) to the EBV DNase are good diagnostic markers for NPC. Other such differences are likely to come to light when other polypeptides of the EA complex have been characterized more fully. The use of specific sera to individual proteins such as the alkaline exonuclease should help clarify the prognostic value of patients' sera.

Owing to the inability to culture cell lines derived from NPC tumours (in attempts to understand more about the involvement of EBV in tumour development) many groups have analysed biopsy material transplanted and passaged in nude mice, the best characterized of which is the C15 'tumour'. Transcripts have been detected from a small subset of the EBV latent proteins, EBNA-1, and latent membrane protein (LMP) (Busson et al., 1988) and, in addition, low levels of transcripts thought to correspond to the EBV DNase have also been reported (Hitt et al., 1989). In order to confirm the presence of EBV DNase protein, we examined the C15 tumour with the specific antiserum using a variety of approaches. We failed to detect the DNase in Western blots of proteins extracted from the C15 tumour under conditions in which we could detect the EBV LMP (data not shown). In addition, although DNase assays on extracts obtained from the C15 tumour detected enzyme activity, it could not be neutralized by our hyperimmune serum and hence it is likely to have been of host cell origin. We have previously reported a failure to detect certain other lytic cycle antigens in the C15 tumour and in fresh biopsies of NPC (Young et al., 1988). Although the presence of transcripts in NPC biopsies mapping to the region of the EBV DNase have been reported (Tugwood et al., 1987), our own preliminary investigations using in situ immunology failed to detect any protein (S. A. Baylis, J. F. Lees & E. Littler, data not shown). Our findings confirm these results and would suggest that little or no expression of the EBV DNase protein occurs in the C15 tumour; however, the significance of the presence of transcripts for the DNase remains unknown.

Oral 'hairy' leukoplakia (HL), a lesion which appears on the lateral border of the tongue in AIDS patients, is now recognized as an exaggerated focus of EBV replication in epithelial cells (Greenspan et al., 1985). In situ hybridization to EBV DNA and immunohistochemical staining of lytic cycle antigens (VCA, EA, membrane antigens) have revealed replicating EBV distributed focally in the more differentiated spinous layers of HL (Greenspan et al., 1985; Young et al., 1989a). Thus this lesion is the only instance in vivo where the full replicative cycle of EBV has been convincingly demonstrated to date. Immunostaining of HL with the hyperimmune mouse serum to EBV DNase demonstrated reactivity in the spinous layers of the lesion (Fig. 3a) identical in histological location to that observed with a monoclonal antibody against the restricted component of EA (Fig. 3b). However, within individual cells of the spinous layer the DNase antiserum gave nuclear staining as opposed to
the cytoplasmic staining shown by the monoclonal antibody R63. That the EBV DNase is expressed in HL emphasizes the role of the enzyme in virus replication in vivo. This is consistent with previous work in which sera from AIDS patients were found to have EBV DNase neutralizing activity (Bardy et al., 1989).

In conclusion, the immune response of NPC patients with the EBV DNase suggests that it may be interesting to study the location of the cells expressing the protein and to investigate its pathological relevance. We believe that the hyperimmune antiserum described here which reacts with the EBV DNase is a good reagent to allow immunological and biochemical characterization of the authentic enzyme produced in lymphoid and epithelial cells.

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