The major immunogenic epitopes of Epstein–Barr virus (EBV) nuclear antigen 1 are encoded by sequence domains which vary among nasopharyngeal carcinoma biopsies and EBV-associated cell lines

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Introduction

Epstein–Barr virus (EBV), a ubiquitous human herpes virus, infects about 90% of the world’s population. EBV shows a dual tropism for B cells and epithelial cells, establishing persistent infections, normally for the lifetime of the host and without symptoms. However, EBV infection is also associated with African Burkitt’s lymphoma (BL) and nasopharyngeal carcinoma (NPC) in Southern Chinese males (for a review see Rickinson & Kieff, 1996). Recently, additional benign or malignant proliferative syndromes of other cell origins, including Hodgkin’s disease (Herbst et al., 1991; Pallesen et al., 1991), T cell lymphoma (Jones et al., 1988; Harabuchi et al., 1990; Su et al., 1991), carcinoma (Leyvraz et al., 1985) and lymphoepithelioma (Dimery et al., 1988) of the thymus and gastric carcinoma (Imai et al., 1994; Tokunaga et al., 1993) were also reported to be associated with EBV infection.

In vitro, EBV infects and transforms B lymphocytes. Lymphoblastoid cell lines (LCLs) derived from EBV-infected B cells express eight, well characterized viral protein-encoding genes, six encoding nuclear antigens (EBNA-1, -2, -3A, -3B, -3C and LP) and two encoding latent membrane proteins (LMP-1 and LMP-2), as well as two untranslated RNAs (EBER1 and EBER2). EBNA-1 is the only viral protein required for the maintenance of latent EBV infection (Reisman et al., 1985), and is expressed in all EBV-associated malignant tissues (Fahracek et al., 1988). EBNA-1 may, therefore, play a critical role in the onset, progression or maintenance of these tumours (Horner et al., 1995). This hypothesis was supported further by the fact that two independent lines of EBNA-1 transgenic mice succumbed to monoclonal B cell lymphoma (Wilson et al., 1996).

EBNA-1 initiates DNA replication at the latent replication origin, oriP, and maintains EBV DNA molecules in cells by binding specifically, as assayed by prolonged detection of those molecules within proliferating cells (Middleton & Sugden, 1994). It can also activate transcription from some
heterologous and native EBV promoters, where multiple EBNA-1 binding sites are positioned within 10 kb of those promoters (Gahn & Sugden, 1995). The predicted amino acid sequence of EBNA-1, which consists of 641 amino acids (aa), can be separated into unique N- and C-terminal domains joined by internal, glycine/alanine-rich short repeat sequences (Baer et al., 1984). Several studies have been carried out to map functional domains within EBNA-1, including dimerization, transactivation, nuclear localization, DNA looping and RNA binding (Ambinder et al., 1991; Chen et al., 1993; Frappier & O’Donnell, 1994; Snudden et al., 1994; Yates & Camilo, 1988). Most of the domains associated with these activities were localized to the C terminus of EBNA-1, except for RNA binding, which involved the RGG motifs at aa 34–55, 330–349 and 355–376 (Snudden et al., 1994; Fig. 1).

Recently, sequence variations within the functionally important unique domains of EBNA-1 were reported by various groups (Wrightham et al., 1995; Snudden et al., 1995). It was proposed that this polymorphism could be critical for the apparent evasion of host CTL responses by EBNA-1, and for disease progression, or it might be important for EBNA-1 transactivation ability within different cell types. However, no obvious functional differences between these variants has been documented.

In this study, we used the highly purified C-terminal region of EBNA-1 to derive a panel of monoclonal antibodies (MAbs) from BALB/c mice and analyse the dominant epitopes. The major epitopes were mapped to small regions and were similar to those IgA-responsive epitopes previously identified in the serum of NPC patients (Chen et al., 1996). The results of this study indicate that the highly immunogenic region of EBNA-1 contains multiple sequence variations. In order to examine whether the sequence variations identified in NPC tissue are tumour-specific or due to geographical variation of the virus, we also analysed the nucleotide sequence within the C terminus of EBNA-1 from NPC biopsies in Taiwan and from different cell lines.

Methods

**Cell culture and clinical samples.** B95-8 cells (Miller et al., 1972), which contain the prototype EBV sequence, and three BL cell lines, Raji (Pulvertaft et al., 1965), P3HR1 (Hinuma & Grace, 1967) and Akata (Takada et al., 1991), were maintained in RPMI-1640 medium supplemented with 10% foetal calf serum. BJAB (Menezes et al., 1975) is an EBV-negative BL cell line. 293 cells were grown in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 10% foetal calf serum. 293-EBNA is a cell line which expresses B95-8 EBNA-1 constitutively and which was generated by co-transfection of pCMV-EBNA together with a vector containing the neomycin-resistance gene (from Invitrogen). Two spontaneous LCLs, LCL2(JB) and LCL28(KF), were derived from two Australian healthy individuals and provided by D. Moss. NPC-KT was derived by fusion of primary cells from a Japanese NPC biopsy with GHPRT-AD-AH cells and selected with HAT medium. The epithelial-like, hybrid NPC-KT cells were EBNA-positive (Takimoto et al., 1984). DNA samples of NPC biopsies were collected from the Hospital of National Taiwan University.

**Plasmid constructs.** Some GST fusion constructs, including GST/EBNA-1(390–498) and GST/EBNA-1(390–499) have been described elsewhere (Chen et al., 1996). GST/EBNA-1(390–498) (pCW7-2) was generated using 5’ primer LMRC25 (GAAGATCTTCATCCGGGTCTCCACCGG) and 3’ primer CCGGATCCTCGGTATCCTTTCTACGTG (aa 498) to amplify the EBNA-1 sequence from pMRC72 (Chen et al., 1993). The PCR product was digested with BamHI and BglII and cloned into pGHI418 (a derivative of pGEX). GST/EBNA-1(390–499) (pCW7-2) was generated using 5’ primer LMRC25 and 3’ primer AAACTGCAGGATCCTCACTCCTGCCCTTC to amplify the EBNA-1 sequence from an NPC tissue DNA (#EM86–577). After digestion with BamHI and BglII, the DNA fragment was cloned into pGHI418. Another His-tagged construct, EBNA-1(459–617) (pMRC112), was amplified by 5’ primer GGAGGCAAATCTAC and pRA362 (Ambinder et al., 1991) as the DNA template, the DNA product was digested with NdeI and XhoI and cloned into pET21a (Novagen).

**Purification of recombinant EBNA-1 proteins.** Recombinant EBNA-1 proteins were purified with glutathione beads or nickel columns according to manufacturers’ protocols (Pharmacia and Novagen). For preparation of the highly purified EBNA-1 as antigen for immunization, GST-EBNA-1(390–499) was purified through glutathione beads, cleaved by factor Xa to remove the GST, then passed through a heparin column and monQ-Sepharose.

**Immunization of animals.** Six-week-old BALB/c mice were immunized with 10 µg doses of purified EBNA-1(390–499), first intraperitoneally with antigen mixed with an equal volume of complete Freund’s adjuvant and then boosted fortnightly with several doses of EBNA-1 only. The antibody responses were examined by Western immunoblot using EBNA-1(390–499) as antigen. To generate hybridoma cells, spleens were removed 3 days after the final booster and dissociated. Splenic lymphocytes were obtained and fused with 5 x 10^6 NS-1 cells using polyethylene glycol as described previously (Tsai et al., 1991).

**PCR and sequence analysis.** Oligonucleotide primers including BamHI or BglII restriction sites were designed to amplify the unique C-terminal region of EBNA-1. The sequences and positions of these primers are as follows: LMRC18 (aa 358, 5’ primer), 5’ CTAGCTCGAGGAGGAGGAGGAG; LMRC14(aa 641, 3’ primer), 5’ AACTGCAGGATCCTCACTCCTGCCCTTC; LMRC20 (aa 477, 5’ primer).
primer), 5' CCGGATCCAAATTTGAGAACAATGCA; LMRC21 (aa 498, 3' primer) 5' CCGGATCCCGGTAGTCTCTTTCTACGTG; LMRC48 (aa 543, 5' primer), 5' CGTCTCCCTTTTGGAAATG; LMRC49 (aa 581, 3' primer), 5' GTCTTTAATCCGATCTCC.

The PCR products were purified and sequenced directly or after cloning into pGEM3Z. Sequence reactions were performed using a Therm Sequenase kit (Amersham) and analysed on a 373 DNA sequencing system (Perkin Elmer).

**ELISA.** Hybridoma cell culture supernatants were screened for reactivity with recombinant EBNA-1 fragment 408–641 using an indirect binding ELISA. Purified protein was coated onto 96-well plates (Nunc) at 0.05 µg per well by overnight incubation at 4 °C in 50 mM NaCl, 50 mM Tris–HCl, pH 7.4. Plates were then washed with washing buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 0.05% Tween 20) and blocked with 2.5% skim milk, 1% washing buffer for 1 h at room temperature. After incubation with culture supernatant and washing, secondary detection was by goat anti-mouse Ig–alkaline phosphatase, with p-nitrophenyl phosphate as the substrate. The plates were then examined using an ELISA reader (Sigma 960) at 405 nm. Mouse monoclonal isolates were also determined by ELISA, using subclass-specific reagents (Sigma).

**Immunofluorescence assay (IFA).** Hybridoma culture supernatants were also screened for antibody production by IFA on 293 and 293-EBNA cells. Cells were fixed with 50% aceton and 50% methanol for 20 min at −20 °C. The test supernatants were added to the smears and incubated in a moist chamber at 37 °C for 1 h. The smears were then washed in PBS for 5, 10 and 15 min. FITC-conjugated, goat anti-mouse serum was diluted 1:100, placed onto the smears and incubated at 37 °C for 1 h. After incubation, the smears were washed as above and mounted in 90% phosphate-buffered glycerol solution and examined under a UV microscope.

**Western immunoblotting and immunoprecipitation.** For Western immunoblot analysis, 293-EBNA or other cells were lysed with 2× sample buffer (50 mM Tris, 4% SDS, 20% glycerol, 0.04% bromophenol blue, 200 mM DTT) at 2 × 10⁶ cells/ml and sonicated briefly. Lysates were displayed in 10% polyacrylamide gels and transferred onto Hybond-C membranes. After blocking in Tris-buffered saline (TBS)–5% nonfat dry milk–0.1% Tween 20 for 1 h and several washes with TBS–0.1% Tween 20, the filter was then incubated with culture supernatants at room temperature for 1 h. The filter was washed three times with TBS–0.1% Tween 20 for 15 min each time and then incubated in a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody. The filter was washed three times and developed using an ECL kit (Amersham) and then exposed to Kodak X-ray film. For epitope mapping, purified recombinant EBNA-1 proteins were used instead of cell lysates.

In vitro transcription/translation was carried out using a TNT T7 quick coupled transcription/translation system (Promega) and pRA362 as template [containing EBNA-1(408–641) derived from B95-8]. For immunoprecipitation, 2 µl [35S]methionine-labelled in vitro translation product of EBNA-1(408–641) (Ambinder et al., 1991) was used as antigen. After incubating with culture supernatant, the immunocomplexes were precipitated using protein A–Sepharose and displayed in a 12% SDS gel. The gel was dried and subjected to autoradiography.

**Results**

**Purification of recombinant EBNA-1 protein and production of hybridomas**

EBNA-1(408–641) from the B95-8 EBV strain was expressed as a GST fusion protein (Chen et al., 1996). After
purification using a glutathione column and cleavage by factor Xa to remove the GST portion, EBNA-1 protein was further purified by heparin column and monoQ-Sepharose. The recombinant EBNA-1(408–641) was purified to more than 95% homogeneity as resolved by silver-stained SDS–PAGE (data not shown) and was then used as antigen for immunizing BALB/c mice. After fusion, more than 60 hybridomas were identified by an ELISA screening system as showing strong reactivity with the immunizing protein. Culture supernatants were then examined for the reactivity to EBNA-1 by Western blotting and indirect IFA using both 293 cells and 293-EBNA cells, which express EBNA-1 under the control of CMV promoter. Fourteen clones which showed specific reactivity were characterized further as summarized in Table 1.

GST/EBNA-1 fusions containing different overlapping portions of EBNA-1, as indicated in Fig. 1, were purified through glutathione beads (Fig. 2 A) and transferred onto membranes for epitope mapping of these hybridoma cells. Among these MAbs, 5C11 recognized an epitope between aa 408 and 446, which gave the strongest signal in Western blotting (Fig. 2B). MAbs from six clones (3C2, 4H11, 7A6, 7E4, 8F8 and 8H3) recognized the sequence around aa 446–498. Fig. 2(C) shows the 8H3 pattern. 5F12 and 8F6 reacted with a C-terminal His-tagged EBNA-1(459–617). MAb 6B10 is a clone identified by IFA but which reacted very weakly in the Western blot, and probably recognized a conformational epitope. Another GST fusion construct, GST/E1(390–641)N, which contains the C-terminal region of EBNA-1 amplified from an NPC biopsy, was also examined for reactivity. Although 5C11, 5F12 and 8F6 appeared to react with the B95-8 sequence, none of them recognized the EBNA-1 sequence in NPC (Table 1).

**Immunoprecipitation**

In vitro-translated EBNA-1(408–641) (Ambinder et al., 1991) labelled with [35S]methionine was used in the immunoprecipitation reaction and reacted with the culture supernatant. The immunocomplexes were pulled down using protein A-Sepharose beads and displayed on SDS–PAGE. Two clones, 3C2 and 7B11, failed to immunoprecipitate EBNA-1 (Fig. 3). Among these clones, 3C2 was found to be IgA, as identified using the subclass-specific ELISA system, whereas the rest of the clones examined are IgG1.

**Reactivity of MAbs to EBNA-1 from different cell origins**

Wrightham et al. (1995) reported that two MAbs generated against the B95-8 EBNA-1 sequence can react with B95-8 cells but not with EBNA-1 from other LCL cells. We therefore examined six clones, 3C2, 4H11, 5C11, 5F12, 6B10 and 8H3,
EBNA-1 is a viral protein with multiple important functions for EBV DNA replication during latency. Distinct functional domains have been mapped for DNA binding, dimerization (Chen et al., 1993, 1994), RNA binding (Snudden et al., 1994) and transactivation (Ambinder et al., 1991). The observation of high heterogeneity of the EBNA-1 gene was unexpected and believed to be important for EBV, since Arrand et al. (1989) reported that the overall homology at the nucleotide level across the EcoRI J regions of multiple EBV strains was 99%, and the slight variation was mainly concentrated in noncoding regions. The study carried out by Wrightham et al. (1995) found that two MAbs generated using B95-8 EBNA-1 failed to recognize EBNA-1 within most of the LCLs. By comparing published sequence motifs it was proposed that the polymorphism may affect the putative MHC class I binding epitopes within EBNA-1 (Wrightham et al., 1995). These variations may therefore help cells expressing EBNA-1 escape cytoxic T lymphocyte attack in the EBV-infected individuals.

In this study, we used highly purified recombinant EBNA-1 protein to immunize BALB/c mice. The mouse with a very high EBNA-1 antibody titre was chosen for hybridoma production. We aimed to obtain MAbs which can recognize EBNA-1 in most cells and also antibodies which can distinguish EBNA-1 of different origins. A panel of MAbs against the C terminus of EBNA-1 was generated and their ability to recognize EBNA-1 in different cell lines and a recombinant protein derived from the EBNA-1 sequence in NPC biopsies was analysed. Using different recombinant EBNA-1 fragments in an immunoblot format, we demonstrated that the amino acids between aa 408 and aa 498 are very immunogenic in mice according to the protein fragment recognized by different MAbs (Table 1). This result was similar to that of the IgA

by Western blotting (Fig. 4) and indirect IFA (Fig. 5) for their ability to react with EBNA-1 from different sources. Fifty µg of each cell lysate was first probed with human serum with a high titre of EBNA-1 antibodies to estimate the molecular mass of EBNA-1 (Fig. 4). The polymorphism of molecular mass of EBNA-1 among different cells was believed to be due to a different number of Gly-Gly-Ala repeats within EBNA-1 (Allday & MacGillivray, 1985). BJAB is an EBV-negative BL cell line and served as a negative control. As summarized in Table 2, we found that although 3C2, 4H11 and 8H3 appeared to recognize similar epitopes of EBNA-1 aa 446–498, they reacted variably with EBNA-1 protein from different cell origins. On the other hand, 5C11 (epitope between aa 408 and 446) and 5F12 (epitope between aa 459 and 641) appeared to react well with EBNA-1 in P3HR1, Raji and B95-8 and two LCLs but reacted with Akata weakly. Another MAb, EBNA.OT1x (Chen et al., 1993), which recognizes an epitope between aa 420 and 432, also failed to recognize EBNA-1 in Akata. One interesting observation (shown in Fig. 4) was that the relative band intensities detected by different MAbs could be different. EBNA-1 within P3HR1 seems to react much strongly with MAb 5C11 than other MAbs. This again implies that the highly immunogenic regions of EBNA-1 are highly variable.

**Sequence variations of EBNA-1 from patients with NPC in Taiwan and in different cell lines**

Previously, several studies documented sequence variation within the functionally important C terminus of EBNA-1, although no functional difference has yet been identified (Bhatia et al., 1996; Gutierrez et al., 1997; Wrightham et al., 1995; Snudden et al., 1995). In this study, we found that the dominant immunogenic epitopes of EBNA-1 were located in regions containing variations. Therefore we amplified the C-terminal sequence of EBNA-1 from three BL cell lines, P3HR1, Raji and Akata, two Australian LCLs and two NPC biopsy samples obtained in Taiwan. The sequences are summarized in Fig. 6. We found that the sequences obtained from the two NPC samples and from Akata are very similar, except that aa 585 of Akata is Thr and can be grouped as the variant type V-Val according to the amino acid change at aa 487 of EBNA-1 (Gutierrez et al., 1997). The EBNA-1 sequence of an NPC hybrid cell line established from a Japanese NPC case, NPC-KT, was also examined. The sequence within NPC-KT was grouped into V-Leu, which was not described in any NPC case previously. EBNA-1 sequences in two LCLs contain similar variations to those observed by Wrightham et al. (1995).
Table 2. Reactivity of EBNA-1 MAb with different cell lines

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<tr>
<th>Clone</th>
<th>Western blotting</th>
<th>IFA</th>
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<tr>
<td></td>
<td>3C2</td>
<td>4H11</td>
</tr>
<tr>
<td>B95-8 LCL</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>293-EBNA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCL2(JB)</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>LCL28(KF)</td>
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ND, Not done.

response in NPC patients (Chen et al., 1996). We found that 4H11 (epitope within aa 446–498) seems to react with EBNA-1 sequences from different sources. MAbs 5C11 (epitope within aa 408–446), 5F12 and 8F6 (epitopes within aa 459–617) failed to recognize a recombinant EBNA-1 protein cloned from an NPC patient. This indicates that the highly...
Immuno-epitopes of EBNA-1 are highly variable

Fig. 6. The C-terminal amino acid sequence variations of EBNA-1 observed by sequence analysis of different cell lines and NPC biopsy samples relative to the B95-8 EBNA-1 sequence. A dash indicates no change of amino acids relative to the B95-8 sequence though there was a nucleotide change observed. The four sequence variation types of EBNA-1 identified by Bhatia et al. (1996) grouped according to the amino acid residue at position 487 are also listed.

The amino acid sequences of EBNA-1 were amplified from three BL cell lines, two LCLs, two NPC biopsy samples and compared to that from B95-8. The two NPC biopsy samples obtained in Taiwan share the identical amino acid changes to those observed in Hong Kong. Interestingly, we found that Akata cells, which were derived from a BL biopsy in Japan, showed very similar amino acid sequence changes to those of NPC except that an amino acid substitution at position 585 (Thr → Ile) was not observed in Akata. All these three samples can be grouped as the V-Val subtype of EBNA-1 sequence (Gutiérrez et al., 1997). This also explains why the NPC serum used in Fig. 4 detects EBNA-1 within Akata cells very well, but all the MAbs barely reacted with Akata EBNA-1. The V-Val subtype was not found in BLs in previous studies and was thought to be a subtype that preferred to replicate in epithelial cells (Gutiérrez et al., 1997). It was documented by several studies that the Akata strain of EBV can infect epithelial cells more efficiently than EBV from other sources (Li et al., 1992). This may be attributable to the contribution of EBNA-1. However, it would also need further study of more DNA samples from healthy individuals and patients with different diseases to clarify whether certain subtypes of EBNA-1 sequences are predominant in Asia, or whether the sequence variation is correlated to the oncogenic potential of EBNA-1 within different cell types.

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