Distinction between Epstein-Barr Virus Type A (EBNA 2A) and Type B (EBNA 2B) Isolates Extends to the EBNA 3 Family of Nuclear Proteins

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The Epstein-Barr virus (EBV) nuclear antigens EBNA 3a, 3b, and 3c have recently been mapped to adjacent reading frames in the *Bam*HI L and E fragments of the B95.8 EBV genome. We studied by immunoblotting the expression of the family of EBNA 3 proteins in a panel of 20 EBV-transformed lymphoblastoid cell lines (LCLs) carrying either type A (EBNA 2A-encoding) or type B (EBNA 2B-encoding) virus isolates. Certain human sera from donors naturally infected with type A isolates detected the EBNA 3a, 3b, and 3c proteins in all type A virus-transformed LCLs (with a single exception in which EBNA 3b was not detected) but detected only EBNA 3a in LCLs carrying type B isolates. These results were confirmed with human and murine antibodies with specific reactivity against sequences of the type A EBNA 3a, 3b, or 3c expressed in bacterial fusion proteins. Conversely, selected human sera from donors naturally infected with type B strains of EBV identified the EBNA 3a encoded by both types of isolates plus two novel EBNA proteins present only in type B, and not in type A, virus-transformed LCLs; these novel proteins appear to be the type B homologs of EBNA 3b and 3c. The distinction between type A and type B EBV isolates therefore extends beyond the EBNA 2 gene to the EBNA 3 family of proteins. This has important implications with respect to the evolutionary origin of these two EBV types and also places in a new light recent studies which identified differences between type A and type B transformants in terms of growth phenotype (A. B. Rickinson, L. S. Young, and M. Rowe, *J. Virol.* 61:1310-1317, 1987) and of detection by EBV-specific cytotoxic T cells (D. J. Moss, I. S. Misko, S. R. Burrows, K. Burman, R. McCarthy, and T. B. Sculley, *Nature [London]* 331:719-721, 1988).

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus which infects resting B cells in vitro, transforming them into immortal lymphoblastoid cell lines (LCLs). Every cell within an LCL carries multiple copies of the viral genome, and viral gene expression is generally restricted to a limited number of latent genes. The EBV latent gene products include six nuclear proteins (EBNAs) encoded by highly spliced messages from different regions of the genome (5, 6); a latent membrane protein mapping to the BNLF-1a, -b, and -c, reading frames (14); and a putative terminal protein encoded by a spliced transcript spanning the terminal repeats in circularized episomal viral DNA, which may also be a membrane protein (22). Of the six nuclear antigens, EBNA 1 is encoded by an exon in the *Bam*HI K fragment of the viral DNA (9, 12, 16, 37) and EBNA 2 is encoded by an exon in the *Bam*HI Y and H fragments (4, 8, 17, 26, 34). A leader protein, EBNA-LP, so-called because it may be expressed from the leader sequence of a bicistronic message for EBNA 2 (39), is encoded by multiple exons derived from the *Bam*HI W repeats and the *Bam*HI Y fragment; this EBNA has also been designated EBNA 5 (7, 11). The remaining three EBNA proteins are derived from a family of related genes in the *Bam*HI E fragment. EBNA 3a has been mapped to the reading frames BLRF3 and BERF1 (18, 19, 31), EBNA 3b maps to BERF2a and BERF2b (27, 35), and EBNA 3c maps to BERF3 and BERF4 (2, 28, 31, 35).

EBNA 2 exists as two antigenically different alleles, EBNA 2A and EBNA 2B, which share just 50% amino acid

homology (1, 4, 20). The majority of wild-type EBV isolates in Western communities carry the EBNA 2A gene. The EBNA 2B-carrying isolates appear to be largely restricted to areas of equatorial Africa and Papua New Guinea (41, 42). The existence of two distinct groups of EBV isolates, hereafter referred to as type A and type B viruses, has been exploited in certain studies aimed at determining the function of EBNA 2. Thus, consistent with the presumed function of EBNA 2 in B-cell transformation (24, 36), we have previously shown that the growth kinetics of LCLs differ according to whether they carry type A or type B strains of EBV (30). In separate studies, Moss et al. (25) isolated HLA-restricted cytotoxic T-cell clones which kill LCLs carrying type A EBV isolates but not LCLs carrying type B isolates. On this basis, it was suggested that EBNA 2 serves as a target antigen for the EBV-specific cytotoxic response. The interpretation of these earlier observations, based on comparisons between type A and type B strains of EBV, is now questioned by new data presented here. We studied the antigenicity of the latent proteins expressed in a panel of LCLs transformed by different type A and type B viruses and showed that the distinction between these two types of EBVs is not restricted to EBNA 2 but extends to the EBNA 3 protein family.

MATERIALS AND METHODS

Cell lines. This work involved a panel of 20 LCLs, each transformed with a different strain of EBV (10 type A and 10 type B viruses). Most LCLs were established by infection of adult seronegative blood samples (CD, CR, JD, PR, and RE)

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or of umbilical cord blood samples (C1 and C2) with type A or type B viruses derived from Burkitt's lymphoma cells (30). Otherwise, the Zost.1 LCL was obtained by spontaneous outgrowth *in vitro* from the circulating B cells of a patient with recurrent herpes zoster; this line carries a type A EBV. The Hmy2 (10) and IM12 (Q. Y. Yao, P. Ogan, M. Rowe, M. Wood, and A. B. Rickinson, *Int. J. Cancer*, in press) LCLs were similarly obtained by spontaneous outgrowth from a patient with myeloma and from a patient with infectious mononucleosis, respectively; these LCLs carry different type B EBVs. The B95.8 marmoset LCL was used as a prototype type A-infected line.

Mouse MAbs and polyclonal antibodies. The β -galactosidase fusion proteins containing sequences from EBNA 3a (codons 538 to 687 of BERF1), EBNA 3b (codons 84 to 730 of BERF2b), or EBNA 3c (codons 319 to 661 of BERF4) are described elsewhere (18, 27, 28). BALB/c mice were primed intraperitoneally with 100 μ g of fusion protein in complete Freund's adjuvant and boosted with two further intraperitoneal injections of 50 μ g of fusion protein with alum adjuvant at 3-week intervals. On day 3 after the final antigen injection, sera were collected for polyclonal antibodies and spleen cells were collected for the generation of monoclonal antibodies (MAbs). Immune spleen cells were fused with the nonsecreting mouse myeloma line X63-Ag8.653, and antibody-producing hybridomas were selected as described elsewhere (32). The specificity of the MAbs and polyclonal antibodies was confirmed by immunoprecipitation with lysates of well-characterized EBV-positive cell lines. The T2.78 MAb reacts specifically with EBNA 3a in immunoprecipitation assays but is only weakly reactive in immunoblotting tests and is nonreactive in immunofluorescence tests. MAbs specific for EBNA 3b and 3c were not available at the time of this study, but specific polyclonal mouse sera were obtained and these sera were used to identify EBNA 3b and 3c by immunoprecipitation.

Human sera. Sera from healthy individuals and rheumatoid arthritis patients in England and from healthy individuals and Burkitt's lymphoma patients in Kenya were screened by immunoblotting with nuclear extracts of well-characterized EBV-positive (type A and type B) and EBV-negative cell lines for specific reactivity with the EBNA 3a, 3b, and 3c family of high-molecular-weight EBNA. As a result of this screening, a reference serum (RS22) from a patient with rheumatoid arthritis was identified which showed strong reactivity with all the EBNA, together with particularly low background staining with EBV-negative cell extracts in the 120- to 200-kilodalton (kDa) region of immunoblots. A similar reference serum, PG, was obtained from a patient with chronic lymphocytic leukemia in whom 2% of the leukemic cells were EBNA positive (23). Both the RS22 and the PG sera were from patients infected with type A strains of EBV. Other sera were found which showed preferential or exclusive reactivity with only one of the family of EBNA 3 proteins. These sera included MS, from a healthy EBV-seropositive caucasian carrying a type A virus, and JUMA, from a Kenyan Burkitt's lymphoma patient also carrying a type A virus; both these sera showed strong reactivity with EBNA 3c in the absence of detectable antibodies to EBNA 3a and 3b. The sera of interest from donors carrying type B strains of EBV included the NZ serum from a healthy Kenyan donor (41) and the AM (33) and WC (15) sera from patients with chronic infectious mononucleosis.

Rheumatoid arthritis sera, including RS22, were obtained from H. Gaston, Department of Rheumatology, University of Birmingham Medical School. The PG serum was a kind

gift from N. Lewin, Karolinska Institute, Sweden. Kenyan sera, including NZ, were obtained from H. Rupani during an earlier collaborative study (41), and the AM and WC sera were obtained from G. Lenoir (IARC, Lyon, France) and M. Allday (Department of Virology, Royal Postgraduate Medical School, London), respectively.

Affinity purification of antibodies from human sera. The β -galactosidase fusion proteins containing sequences of EBNA 3a, 3b, or 3c were partially purified from bacterial lysates by salt precipitation with 40% ammonium sulfate (32). Affinity chromatography columns were prepared by coupling 10 mg of partially purified fusion protein to 1.0-ml prepacked activated columns (HEMA-Cart; Anachem, Luton, United Kingdom) according to the instructions of the manufacturer. RS22 human serum (1 ml) was diluted with 3 ml of 10 mM Tris-buffered saline (pH 7.5) (TBS) and recirculated through a series of fusion protein affinity columns for 4 h each. Each column was washed extensively with TBS-0.3% Tween 20-0.1% bovine serum albumin before specifically bound antibodies were eluted with 50 mM diethylamine (pH 11)-150 mM NaCl, which was recirculated through each column for 30 min. The eluted antibodies were neutralized with dilute HCl and were diluted in TBS containing 5% reconstituted dried milk (TBS-milk). Antibodies eluted from the EBNA 3b column were further purified by a second round of affinity column chromatography. The affinity-purified antibodies were used to probe immunoblots at a final dilution of 1:50 (anti-EBNA 3a) or 1:10 (anti-EBNA 3b and anti-EBNA 3c).

Electrophoresis and immunoblotting. Nuclear fractions were prepared by incubating cells in nuclear extraction buffer (10 mM Tris buffer [pH 7.5], 10 mM NaCl, 3 mM magnesium acetate, 2% sucrose, 0.7% Nonidet P-40) for 5 min at 0°C and washing them once in nuclear extraction buffer without Nonidet P-40 detergent. The nuclear pellets were solubilized by sonication in electrophoresis sample buffer (0.05 M Tris buffer [pH 6.8], 2% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and boiled for 3 min. Aliquots of these samples (10⁶ cells per lane) were separated by discontinuous gel electrophoresis (21) with a stacking gel of 5% acrylamide and a resolving gel of 7.5% acrylamide and were transferred onto nitrocellulose at 85 V for 5 h in a cooled Bio-Rad Transblot apparatus. Molecular weight markers (SDS-6H; Sigma Chemical Co., St. Louis, Mo.) were visualized by staining with 10% ponceau (Sigma) in 3% trichloroacetic acid, before the excess protein-binding sites on the nitrocellulose were blocked by incubation for 2 h in phosphate-buffered saline containing 5% skim milk. The filters were subsequently probed with antibody (human serum diluted 1:100 in phosphate-buffered saline-milk or affinity-purified antibodies diluted 1:10 or 1:50) for 16 h at 4°C, and specifically bound antibody was detected with ¹²⁵I-labeled staphylococcal protein A as described elsewhere (33).

Immunoprecipitation. Monospecific mouse antibodies were used to specifically immunoprecipitate EBNA 3a, 3b, or 3c from whole-cell lysates in RIPA buffer (11). Immunoprecipitates recovered in electrophoresis sample buffer were separated by electrophoresis, blotted onto nitrocellulose filters, and detected with an appropriate human serum probe (RS22) as described above.

RESULTS

In the first set of experiments, a panel of LCLs infected with different strains of EBV (10 type A and 10 type B

FIG. 1. Immunoblots of nuclear extracts from LCLs transformed with different strains of EBV probed with human sera RS22 (A) and PG (B). Lane 1 contains an EBV-negative control line, BL40. Lanes 2 to 7 contain LCLs transformed with type A strains of EBV: lane 2, B95.8; lane 3, C2-OBA; lane 4, C2-KYU; lane 5, C2-BL37; lane 6, PR-WW1; and lane 7, Zost.1. Lanes 8 to 14 contain LCLs transformed with type B strains of EBV: lane 8, C-JIJOYE.BL; lane 9, C1-MUK; lane 10, CD-ELI; lane 11, PR-CHEP; lane 12, CR-WW2; lane 13, Hmy2; and lane 14, RE-AG876. The positions of the molecular size markers (in kilodaltons) are indicated to the right of each blot.

transformants) was studied by Western immunoblotting with the human serum (RS22) which contained antibodies to each of the six EBNA. Figure 1A shows a representative blot with nuclear extracts of a control EBV-negative lymphoma line (lane 1), six type A transformants (lanes 2 to 7), and seven type B transformants (lanes 8 to 14). Each LCL showed a pattern of EBNA bands that was characteristic for the particular transforming strain of EBV present. The type A transformants displayed a doublet of EBNA 1 and EBNA

2 in the 75- to 90-kDa size range and, with the exception of the BL37 strain of EBV, EBNA 1 was always the lower of these two EBNA bands. In the type B transformants, only the EBNA 1 band was detected since the RS22 serum reacts with EBNA 2A and has no detectable cross-reactivity with EBNA 2B. An MAb specific for a common determinant on EBNA 2A/B (PE2 [40]) was used to demonstrate the expression of EBNA 2B in all LCLs transformed with type B viruses (data not shown; but see Fig. 4B and C, showing

FIG. 2. Immunoblots of EBNA 3 proteins immunoprecipitated from RIPA lysates of LCLs and probed with human serum RS22. (A) Total cell extract (lane T) and immunoprecipitates (lanes a, b, c, and o) from the LCL C2-OBA.BL. The immunoprecipitations were performed with T2.78, an anti-EBNA 3a monoclonal antibody (lane a), a mouse polyclonal anti-EBNA 3b serum (lane b), a mouse polyclonal anti-EBNA 3c serum (lane c), and an irrelevant MAb (lane o). The positions of molecular size markers (in kilodaltons) are indicated between panels A and B. (B) Immunoprecipitates of EBNA 3a, EBNA 3b, and EBNA 3c isolated from RIPA lysates of three type A transformants and three type B transformants. The LCLs were C2-BL37 (lanes 1), C2-KYU (lanes 2), B95.8 (lanes 3), JC5 (lanes 4), C1-MUK (lanes 5), and CR-WW2 (lanes 6).

results with EBNA 2B-reactive human sera). EBNA-LP was present in all the LCLs, ranging in size from 32 kDa (C1-MUK.BL) to 95 kDa (Hmy2), but these bands were sometimes obscured by the prominent set of EA lytic cycle antigens in the 45- to 55-kDa region of the blot in those LCLs that were not tightly latent.

The reactivity of the RS22 serum with the EBNA 3 family of proteins provided the observation which initiated this study. Thus, as seen in Fig. 1A, the RS22 serum detected two or three EBNA 3 bands in the 140- to 165-kDa region of the blot in all type A transformants but detected only a single band in type B transformants. Furthermore, the single band in the type B transformants was generally weaker than the corresponding band in type A transformants. This difference in EBNA 3 expression did not correlate with the expression of the 45- to 55-kDa set of early-antigen bands present in some LCLs and was therefore unrelated to virus producer status. Another human serum (PG), which also contained antibodies to all six EBNAs, gave a similar pattern of results with respect to the number of EBNA 3 bands in type A and type B transformants (Fig. 1B), although the relative intensities of the bands differed from those stained with RS22 serum. Upon prolonged exposure of these blots, very weak bands in the size range 165 to 185 kDa (Fig. 1B, lanes 12 to 14) were noted in some type B transformants but not in any of the type A transformants or in the EBV-negative control; further experiments designed to determine the identity of these much weaker bands are described later.

Identity of individual EBNA 3 bands. To identify the individual EBNA 3 bands in each of the transformants studied, we raised murine antibodies to β -galactosidase fusion proteins containing amino acid sequences of EBNA 3a (18), EBNA 3b (27), or EBNA 3c (28). An MAb, T2.78, was produced that was reactive with EBNA 3a in immuno-

precipitation assays, and polyclonal antisera were obtained that were similarly reactive with either EBNA 3b or EBNA 3c. These reagents were used to immunoprecipitate the respective EBNA 3 proteins from RIPA lysates of each of the LCLs studied. The immunoprecipitates were isolated on protein A-Sepharose, electrophoresed, and blotted and then detected by probing with the polyclonal human serum RS22. The specificity of the reagents is illustrated by the results obtained with the LCL C2-OBA.BL (Fig. 2A). This LCL carries a type A virus and displayed three EBNA 3 bands of 144, 152, and 157 kDa apparent molecular size which were identified from the immunoprecipitation studies as being EBNA 3a, 3c, and 3b, respectively. Figure 2B shows representative immunoprecipitation results obtained with six other LCLs (three type A transformants and three type B transformants). These data identify the single band in the type B transformants as EBNA 3a. Furthermore, lane 1 of Fig. 2B (C2-BL37) shows that in those type A transformants in which only two EBNA 3 bands were detected in whole nuclear extracts with the polyspecific sera (Fig. 1, lane 5), both EBNA 3b and EBNA 3c proteins are usually expressed but they comigrate with the same apparent molecular weight under the gel conditions used here.

The difference in EBNA 3 expression between type A and type B transformants was studied further by using human sera that were monospecific for one of the three EBNA 3 proteins. Affinity-purified anti-EBNA 3a antibodies obtained from serum RS22 by affinity chromatography with a column of immobilized EBNA 3a- β -galactosidase fusion protein confirmed the presence of EBNA 3a protein in 10 of 10 type A and 10 of 10 type B transformants. Figure 3A shows a representative blot of the same panel of lines as in Fig. 1 probed with the affinity-purified anti-EBNA 3a reagent. Interestingly, the affinity-purified antibodies from serum

FIG. 3. Immunoblots of nuclear extracts from type A and type B transformants probed with affinity-purified anti-EBNA 3a from serum RS22 (A), affinity-purified anti-EBNA 3b from serum RS22 (B), and serum JUMA, which contains antibodies to EBNA 3c but lacks antibodies to EBNA 3a and EBNA 3b (C). Lanes 1 to 14 are identical to those in Fig. 1. The positions of molecular size markers (in kilodaltons) are indicated to the right of each blot.

RS22 did not show the preferential reactivity for EBNA 3a of type A transformants that was displayed by the whole serum (compare with Fig. 1A and 2B). A second affinity column containing immobilized EBNA 3b- β -galactosidase fusion protein was similarly used to isolate anti-EBNA 3b antibodies from serum RS22; Fig. 3B illustrates the specificity of this reagent. The EBNA 3b antigen was detected in 9 of 10 type A transformants but not in any of 10 type B transformants. The EBNA 3b protein showed little variation in molecular size from one type A virus to another. In contrast, EBNA 3c showed a relatively large variation in apparent molecular size among different type A viruses, as illustrated by the blot in Fig. 3C which is probed with an unpurified human serum, JUMA. This serum reacted strongly with EBNA 1, EBNA 2A, and EBNA 3c but was nonreactive with EBNA 3a and EBNA 3b. The EBNA 3c antigen was detected in 10 of 10 type A transformants but not in any of the 10 type B transformants. Identical results were obtained with an EBNA 3c-reactive serum from a healthy donor (MS) and with affinity-purified anti-EBNA 3c antibodies from RS22 (data not shown).

Table 1 summarizes the EBNA 3a, 3b, and 3c data obtained in the experiments described above. The WW-QWIL LCL, which contains the type A virus strain QMIR-WIL (29), did not express a detectable EBNA 3b protein;

TABLE 1. Relative molecular sizes of EBNA 3a, EBNA 3b, and EBNA 3c proteins detected by immunoblotting and immunoprecipitation experiments

Cell line	Relative molecular size (10^3 kDa)		
	EBNA 3a	EBNA 3b	EBNA 3c
Type A EBV			
B95.8	145	157	149
C2-OBA.BL	144	157	152
C2-KYU.BL	144	157	149
C2-BL37 ^a	143	157	157
PR-WW1.BL	145	157	161
Zost.1	142	155	163
C2-BL60 ^a	151	155	155
C2-BL74 ^a	145	155	155
JD-MUTU	146	157	149
WW-QWIL	147	— ^b	149
Type B EBV			
C-JIJOYE.BL	142	—	—
C1-MUK.BL	143	—	—
CD-ELI.BL	142	—	—
PR-CHEP.BL	143	—	—
CR-WW2.BL	141	—	—
Hmy2	141	—	—
RE-AG876.BL	143	—	—
C2-BL16	143	—	—
C2-BL29	143	—	—
IM12	143	—	—

^a In these transformants, the EBNA 3b and EBNA 3c proteins comigrated with the same apparent molecular size.

^b —, EBNA band not detected.

this result was obtained with two independent LCLs transformed with this strain of EBV. The EBNA 3b protein was also difficult to detect in certain long-established LCLs carrying the B95.8 virus. Additional independent LCLs transformed with the other eight type A strains of EBV showed readily detectable levels of this protein (data not shown).

Identification of putative antigenic variants of EBNA 3b and 3c in type B transformants. All the above experiments were performed with either human sera from donors carrying type A strains of EBV or mouse antibodies raised to polypeptides derived from the amino acid sequences of the EBNA 3a, 3b, and 3c proteins of the prototype type A virus, B95.8. We therefore sought to examine the reactivity of sera from donors carrying type B strains of EBV. Three sera (NZ, AM, and WC) were identified which had reactivity with EBV antigens in the EBNA 3 region of immunoblots and were used to probe blots of nuclear extracts from each of the 20 LCLs that were characterized in the first set of experiments.

Representative blots with the same panel of nuclear extracts as in Fig. 1 and 3 are shown in Fig. 4. The AM and WC sera (Fig. 4B and C), in addition to displaying strong reactivity with EBNA 2 and with lytic cycle antigens, also reacted with EBNA 3a; both the anti-EBNA 2 and the anti-EBNA 3a reactivities were preferentially directed toward the proteins encoded by type B EBV strains.

The NZ serum (Fig. 4A) detected EBNA 1 in all LCLs and also reacted with one or two unidentified bands of variable size between 163 and 183 kDa in each type B transformant but not in any type A transformant or in the EBV-negative control line. These new type B EBV-associated bands corresponded to those bands detected very weakly in some lines by the RS22 and PG sera (Fig. 1B). Note that the prominent band of about 155 kDa detected by the NZ serum in the

FIG. 4. Immunoblots of nuclear extracts probed with sera from donors carrying type B strains of EBV. (A) Serum NZ; (B) serum AM; and (C) serum WC. Novel high-molecular-weight EBNA s were detected in the region indicated by the asterisk to the right of the blots. Lanes 1 to 14 are identical to those in Fig. 1 and 3. The positions of molecular weight markers ($\times 10^3$) are shown to the left of each blot.

Zost.1 type A transformant in Fig. 4A (lane 7) corresponded to a normal cellular protein seen more weakly in all the tracks, and such an intense signal was not observed in repeat experiments. The AM serum (Fig. 4B) also detected some of the type B virus-associated bands but was less sensitive than the NZ serum. The WC serum (Fig. 4C) detected two new bands in most type B transformants, and one of these bands always corresponded to the band detected by the NZ or AM serum or both. The WC serum also detected a 170-kDa band in B95.8 cells which was not detected by either the NZ or the AM serum, but no corresponding reactivity was observed with any of the other type A transformants.

Table 2 summarizes the new type B virus-associated proteins detected in the complete panel of 10 type B trans-

formants. The nuclear localization of these newly identified proteins was confirmed by fractionation studies. Figure 5 shows a type A transformant (C2-OBA) and a type B transformant (RE-AG876) tested in parallel. Cytoplasmic and nuclear fractions were obtained by differential solubilization in buffer containing Nonidet P-40 detergent and were blotted alongside the total cell extracts for probing with the RS22 and NZ sera. The autoradiographs of the immunoblots were overexposed to confirm that the EBNA 3a, 3b, and 3c proteins were located almost exclusively in the nuclear fraction of C2-OBA cells, even though there was significant leakage of both EBNA 1 and EBNA 2 into the cytoplasmic fraction in this particular experiment. The blot probed with RS22 serum also confirmed the almost exclusive nuclear

TABLE 2. Relative molecular sizes of novel EBNA detected by human sera from donors carrying type B strains of EBV

Cell line	Relative molecular size (10^3 kDa) of putative type B homologs of ":	
	EBNA 3b	EBNA 3c
C-JIJOYE.BL	163	150
C1-MUK.BL	163	178
CD-ELI.BL	163	178
PR-CHEP.BL	164	174
CR-WW2.BL	165	163
Hmy2	163	183
RE-AG876.BL	163	170
C2-BL16	162	178
C2-BL29	162	178
IM12	164	172

^a All bands, with the exception of the 163-kDa band of CR-WW2.BL, were detected by the serum WC. The sera NZ and AM generally reacted preferentially or exclusively with the putative EBNA 3c homolog, except in the C-JIJOYE.BL line, in which they reacted only with the 163-kDa band.

localization of EBNA 3a in the RE-AG876 cells. In addition, these overexposed blots revealed a weak cross-reactivity of the RS22 serum with a band in the RE-AG876 nuclei which corresponded to the novel EBNA strongly detected in these same cells by the NZ serum.

DISCUSSION

This study showed that the distinction between type A and type B EBV isolates, first recognized by differences in EBNA 2 antigenicity, extends to the EBNA 3 family of proteins. There was some antigenic variation between

EBNA 3a proteins of type A and type B transformants. This was demonstrated by several human sera from donors naturally infected with a type A EBV strain, including RS22 (Fig. 1A), showing a strong reactivity with EBNA 3a of type A transformants and a weaker reactivity with EBNA 3a of type B transformants. Conversely two sera, AM and WC, from donors infected with type B EBV strains showed stronger reactivity with EBNA 3a of type B rather than type A transformants (Fig. 4B and C). However, these differences in EBNA 3a antigenicity were minor in comparison with the differences that were observed in the EBNA 3b and 3c proteins encoded by type A and type B EBV isolates. Thus, human antibodies in sera from certain donors naturally infected with type A EBV strains detected both EBNA 3b and 3c in type A transformants but did not detect either of these proteins in any type B transformants (Table 1). This inability to detect EBNA 3b and 3c in type B transformants could be due to (i) major DNA deletions in the EBNA 3b and EBNA 3c genes in type B viruses, (ii) transcriptional regulation of gene expression, or (iii) the existence of antigenically distinct alleles of the EBNA 3b and EBNA 3c genes that segregate with the EBNA 2A and -B alleles.

At present, there are insufficient published data in the literature to discount the possibility of type B virus-associated deletions affecting the EBNA 3b and EBNA 3c genes. However, our most recent data on EBV DNA from several of the transformants used in this work suggest that the EBNA 3b- and EBNA 3c-related sequences are present in type B strains of EBV (L. S. Young, J. Sample, and M. Rowe, unpublished data). It is therefore unlikely that major deletions have removed the EBNA 3b and 3c genes from type B viruses, although the possibility remains that these

FIG. 5. Nuclear localization of EBNA 3a, 3b, and 3c in a type A transformant (C2-OBA.BL) and of the novel high-molecular-weight EBNA in a type B transformant. Two identical blots of total cell extracts (T), cytoplasmic fractions (C), and nuclear pellets (N) were probed either with serum RS22 to detect EBNA 3a, 3b, and 3c or with serum NZ to detect the novel high-molecular-weight band (asterisk). The positions of the molecular weight markers ($\times 10^3$) are shown between the panels.

sequences are present but not expressed in the transformed cell. However, results obtained with sera from three individuals naturally infected with type B virus strains favor the third possibility, i.e., that antigenic variants of EBNA 3b and EBNA 3c exist in type B virus-transformed LCLs. Thus, these particular sera detected two hitherto unidentified high-molecular-weight nuclear antigens (150 to 183 kDa) in each of 10 LCLs carrying different type B virus strains (Table 2). A retrospective evaluation of our earlier published results obtained with the AM serum on immunoblots of spontaneous LCLs established from donors from Kenya and Papua New Guinea (41) showed that the variant proteins were also present in all five type B transformants analyzed (Fig. 3a of reference 41), and examination of the original autoradiograph revealed that these proteins were absent from all five type A transformants run on the same gel (data not shown).

Several lines of circumstantial evidence suggest that these novel nuclear antigens are the type B homologs of the EBNA 3b and EBNA 3c proteins of type A viruses. First, they are nuclear antigens that are expressed irrespective of the virus producer status of the cell line. Second, they were detected only in type B transformants; nuclear antigens of the same size were not observed in type A LCLs. Third, the size of one of the novel nuclear proteins appears to be relatively constant (162 to 164 kDa), while the other shows considerable size variation (150 to 183 kDa) among different type B isolates; this is exactly analogous to the situation observed with EBNA 3b (155 to 157 kDa) and EBNA 3c (149 to 163 kDa), respectively, in type A transformants. Because the type B sera which recognized these novel nuclear proteins did not show any cross-reactive recognition of EBNA 3b or EBNA 3c in type A LCLs, it was not possible to use these sera to investigate possible antigenic relationships between the two sets of proteins. However, it was interesting that two type A sera which reacted more strongly with EBNA 3c than with EBNA 3b, PG and RS22, showed weak cross-reactivity only with the variable-sized EBNA of type B transformants (Fig. 1B and 5); we provisionally assigned this variable-sized EBNA as the EBNA 3c homolog. Unfortunately, these cross-reactive antibodies could not be isolated by affinity chromatography with the immobilized bacterial fusion protein containing part of the EBNA 3c sequence (codons 319 to 661 of BERF4), presumably because the common antigenic epitope resides outside this particular region of EBNA 3c. The variability in size of the putative EBNA 3c homolog, together with the variability in the size of the EBNA 1 protein in the panel of type B transformants studied here suggest that there are many distinct type B strains of EBV, even though each retains a similar size EBNA 2B protein (33, 41).

One unusual feature of our present results was the failure to detect an EBNA 3b protein in each of two independent LCLs carrying the QMIR-WIL type A virus strain (Table 1). It is not known why EBNA 3b was not detected in these lines. However, it was noted that the EBNA 3b protein was also difficult to detect in a minority of long-established LCLs infected with the B95.8 strain of EBV, notably IB4 (31) and X50-7 (33) (M. Rowe, unpublished data).

The present study strongly suggested that type-specific variation among EBV isolates is not restricted to the EBNA 2 gene but includes the sequences encoding EBNA 3b and EBNA 3c and, to a lesser extent, EBNA 3a. During the preparation of this paper, we learned that antigenic variation among EBNA 3b and 3c proteins had also been noted in a second laboratory (T. B. Sculley, A. Appoloni, D. J. Moss, N. Mueller-Lantczh, I. S. Misko, and D. A. Cooper, sub-

mitted for publication). Recent evidence also indicates differences in the small noncoding RNAs (EBERs) which correlate with the A-B type of EBV (3). The multiple differences now being recognized between the two virus families are compatible with a substantial evolutionary divergence. Such data call for a reappraisal of earlier studies, including our own, that have compared the biological properties of the two types of EBV isolates as a means of investigating the functions of EBNA 2. Thus, we have previously shown that certain facets of the in vitro growth phenotype of established LCLs correlate with the EBNA 2 type of the resident virus (30). While there is much independent evidence indicating a role for EBNA 2 in the initiation of transformation (13, 36, 38), we must concede that differences in the growth phenotype of type A versus type B transformants may, at least in part, be a consequence of their different EBNA 3 proteins. Similarly, Moss et al. (25) recently described EBV-specific cytotoxic T-cell clones which lysed LCLs carrying type A, but not type B, EBV isolates. Although the immediate inference was that such T cells were recognizing target epitopes present on EBNA 2A proteins (25), it is now clear that such results could equally well be explained by recognition of epitopes unique to type A virus-encoded EBNA 3 proteins. Further work is therefore needed to resolve these issues.

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