Development of a Recombinant Enzyme-Linked Immunosorbent Assay for Detection of Antibodies against Epstein-Barr Virus Nuclear Antigens 2A and 2B

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The baculovirus expression system was used to produce full-length Epstein-Barr virus nuclear antigens (EBNAs) 2A and 2B. Recombinant baculoviruses that contained the EBNA-2A- and EBNA-2B-encoding sequences were constructed. The proteins were expressed in *Spodoptera frugiperda* SF-9 cells infected with the recombinant viruses and were characterized by using monoclonal and human polyclonal antibodies by immunoblotting and immunofluorescence techniques. Partially purified extracts of the EBNA-2A- and EBNA-2B-infected insect cells were used to establish a new enzyme-linked immunosorbent assay for the detection of antibodies against EBNA-2A and EBNA-2B. Preferential reactivity toward the type A or type B EBNA-2 protein was observed in 36% of serum specimens from Swiss patients with acute infectious mononucleosis and in 81% of Swiss patients with latent Epstein-Barr virus infection. Of the patients in the latter group, sera from 76% reacted preferentially with EBNA-2A, sera from 5% reacted preferentially with EBNA-2B, sera from 12% showed similar reactivities against both antigens, and sera from 7% were nonreactive.

Epstein-Barr virus (EBV) is associated with at least four forms of human cancer: African Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkins's disease, and immunoblastic lymphomas of the non-Hodgkins's type (8, 10, 41). Although a number of viruses are known to cause tumors in animals, only four—hepatitis B virus, human papillomavirus types 16 and 18, human T-cell leukemia virus type I, and EBV—show a direct association to human cancer (14). EBV encodes about 100 proteins, but only EBV nuclear antigens (EBNAs) 1 to 6 and the latent membrane proteins LMP-1, -2A, and -2B are consistently expressed in B lymphocytes immortalized in vitro; see Middleton et al. (21) for a review. Except for EBNA-4, expression of all these proteins has been demonstrated to be indispensable for efficient transformation of human cells by EBV (38). Two naturally occurring types of EBV, designated A and B, have been identified and shown to differ in their ability to immortalize B lymphocytes (3). In vitro, EBV type A transforms cells with a greater efficiency and the transformed cells grow initially faster than cells infected with EBV type B strains (26). Further differentiation has been established by nucleotide sequence divergence and serological reactivity. EBV type A has a worldwide distribution and is especially prevalent in the Caucasian population. EBV type B seems to be more common in Central Africa, La Réunion, and New Guinea (2, 15, 42, 43). Recent data from PCR studies by Sculley et al. (34) and

Sixbey et al. (36) have revealed an elevated incidence of B-type EBV in immunocompromised individuals and in healthy populations in certain regions of the United States (34, 36).

Differences between the two EBV types with regard to the EBNA family of proteins have been reported (31). Cells infected with the two types of virus express antigenically distinct allelic forms of EBNA-2, designated EBNA-2A and EBNA-2B (32). The two EBNA-2 proteins have 64% sequence identity and a major internal deletion difference that removes a large part of the polyproline stretch of the type A EBNA-2 of the prototype B95-8 EBV isolate (3).

The presence of antibodies to EBNA-1, EBNA-2A, and EBNA-2B in human sera has been demonstrated mostly by immunoblot analysis of cellular extracts (30, 42). Titers can be determined by indirect immunofluorescence assays (IFA) or anticomplement immunofluorescence (ACIF) on fixed cells (9, 35, 39). An EBNA-2A enzyme-linked immunosorbent assay (ELISA) based on polyproline has been reported, but the results obtained by that method did not correlate with the results of ACIF or immunoblotting for EBNA-2A (17). The most reliable means of differentiating between the two EBV subtypes has been PCR with EBNA-2A- or EBNA-2B-specific primers (15, 36). However, the variability of the nucleotide sequence of the EBNA-2 gene between different isolates often causes problems in evaluating the results. IFA on cells transfected with EBNA-2 expression vectors can be used to determine anti-EBNA-2A and anti-EBNA-2B antibodies, but the titers are often too low to allow a reliable classification.

In the study described here, we developed and evaluated an ELISA method for the determination of immunoglobulin G (IgG) antibodies to EBNA-2A and EBNA-2B. The method is based on a partially purified preparation of recombinant EBNA-2A and EBNA-2B proteins expressed in insect cells.

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The assay allows for a simple differentiation of antibodies preferentially reactive against EBNA-2A or EBNA-2B from patients in the late phase of acute EBV infection and in the latent stage of EBV infection.

MATERIALS AND METHODS

Plasmids. Plasmid pEΔA24 was constructed as described previously by Ricksten et al. (27). The EBNA-2A-containing *Xho*I-*Bgl*II fragment of pEΔA24 (corresponding to sequence coordinates 48,435 to 50,628 of B95-8 EBV DNA) was excised and ligated into the unique *Bam*HI site of the pVL941 vector (18) by using *Bgl*II linkers, resulting in plasmid pVL941-E2A (see Fig. 1). The EBNA-2B-containing *Hind*III-*Sma*I fragment (corresponding to nucleotides 48,039 to 50,558 of B95-8 EBV DNA) was excised from the pJH65B5 plasmid (kindly provided by G. Bornkamm) and was subcloned into the pGEM-3Zf(+) vector (Promega). To shorten the translational leader sequence, a segment of the insert was amplified from position -27 relative to the EBNA-2B translation initiation site to a position just downstream of the unique *Bst*EII site in the gene. The 5' PCR primer contained a *Hind*III-*Bam*HI linker at its 5' end and an internal sequence that converted the translation initiation signal to an *Nde*I restriction site (CATATG). The PCR fragment was cleaved with *Hind*III and *Bst*EII and was cloned back into the gene, replacing the original sequence. Subsequently, the EBNA-2B-containing *Bam*HI-*Kpn*I fragment of this plasmid was excised and cloned between the corresponding sites of pVL941, to produce the pVL941-E2B plasmid (see Fig. 1).

Cells and virus. The *Autographa californica* nuclear polyhedrosis virus and *Spodoptera frugiperda* SF-9 cells were kindly provided by Max D. Summers (Texas A&M University). SF-9 cells were propagated as monolayer cultures at 27°C in Tc-100 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (Amimed, Muttens, Switzerland) and was subcultured two to three times per week. In short-term cultures, a 0.1% mixture of penicillin, streptomycin, and amphotericin B (antibiotic mixture, catalog no. A7292; Sigma) was added to the medium. EBV-transformed B-lymphoid lines (B95-8, P3HR1, Raji, Jijoye) were grown as suspension cultures in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum and 0.1% antibiotic mixture and were subcultured twice a week.

Isolation of recombinant baculoviruses. A total of 2 µg of pVL941-E2A or pVL941-E2B DNA and 1 µg of wild-type *A. californica* nuclear polyhedrosis virus DNA were cotransfected into 2×10^6 SF-9 cells by the calcium phosphate precipitation method described by Summers and Smith (37). Culture supernatants were collected 5 days after transfection, and serial dilutions were plated with SF-9 cells into 96-well plates. After 5 days, the cells were screened for the production of recombinant virus by dot blot analysis. The supernatants from positive wells were used for further enrichment of recombinant virus by limiting dilutions and hybridization analysis. Recombinant virus clones were finally isolated by picking nonoccluded plaques. The virus was amplified by infecting SF-9 cells, and cell supernatants were collected 3 days after infection (37). Virus stocks were stored at 4°C after determination of the virus titers.

IFA analysis. SF-9 cells in the logarithmic growth phase were infected at a multiplicity of infection of 2 PFU per cell with pVL941-E2A or pVL941-E2B recombinant viruses. Cells were harvested at 48 h postinfection, washed once with

Hank's buffered salt solution without calcium (Amimed), mixed with uninfected cells at a ratio of 1:1, and applied to the slides at 5×10^4 cells per well. The slides were air dried, fixed in acetone-methanol (4:1 mixture) for 10 min at 4°C, dried in air, and stored lyophilized at 4°C. Human serum specimens were preabsorbed by mixing them with equal amounts of a fresh, uninfected SF-9 cell suspension concentrated 1:10 in Veronal-buffered saline and by incubating the mixture for 30 min at 37°C. After centrifugation for 5 min at $1,000 \times g$, the supernatants were further diluted in Veronal-buffered saline. Purified mouse anti-EBNA-2 monoclonal antibodies PE-2 and 115E were directly diluted 1:5 in Veronal-buffered saline. PE-2 is known to react with an epitope that is present on both EBNA-2A and EBNA-2B, whereas 115E is reactive only with EBNA-2A (24, 41). The slides were incubated with the diluted sera for 1 h at 37°C. Fluorescein isothiocyanate-conjugated rabbit anti-human IgG or rabbit anti-mouse IgG (Dako, Glostrup, Denmark), respectively, were diluted 1:40 in Veronal-buffered saline and applied to the washed slides. After incubation for 1 h at 37°C, the slides were mounted in Tris-glycerol buffer (pH 9.0) and were examined with a fluorescence microscope.

Titers of IgG and IgM antibodies to the virus capsid antigen were determined by IFAs on slides coated with B95-8 or P3HR-1 cells, respectively, as described by Henle and Henle (8). Antibody titers against "total EBNA" (except EBNA-6) were determined by ACIF on slides containing Raji cells (25). A commercial ACIF assay (EBV Combi Test; Institute Virion Ltd.) was used to measure simultaneously complement-fixing antibodies to virus capsid antigen, early antigen, and total EBNA (except EBNA-2) on slides coated with P3HR-1 cells. This assay allows differentiation of acute and latent infection in a single step, as described elsewhere (4).

Human sera. Fifteen defined control serum specimens were kindly provided by J. W. Gratama and were used for validation of the ELISA system. The EBV carrier status of the patients who provided these serum specimens had previously been analyzed by spontaneous outgrowth assays of peripheral blood mononuclear cells and cord blood lymphocyte transformation assays of throat wash samples. The chance to detect simultaneous carriage of multiple transforming EBV strains was maximized by harvesting and expanding lymphoblastoid cell lines from individual wells separately. The resulting cell lines were classified as carrying type A versus type B EBV by immunoblotting ("Ebnotyping" [7, 23]). This group of 15 EBV genotyped serum specimens was analyzed by routine laboratory tests which included IFA analysis of IgG and IgM antibodies to virus capsid antigen, ACIF analysis of antibodies to EBNA and to EBNA or virus capsid antigen plus early antigen (EBV Combi Test), and ELISA analysis of IgM antibodies to virus capsid antigen. All sera were negative by IFA for IgM antibodies to virus capsid antigen, positive by IFA for IgG antibodies to virus capsid antigen (titer range, 320 to 5,120), and positive by ACIF for total EBNA in both Raji and P3HR-1 ACIF.

A total of 186 serum specimens were taken from a routine serological laboratory in Switzerland, heat inactivated for 30 min at 56°C, and stored at -20°C. All serum specimens were analyzed with the same panel of routine laboratory tests as described above. The results for the patients were then assigned to one of three diagnostic groups. The EBV-negative group consisted of 26 individuals that showed no clinical or laboratory signs of EBV infection. The group with acute EBV infection included 56 acutely infected patients at

different stages of infectious mononucleosis (IM) by the criteria of Evans (5). All serum specimens from patients in this group contained IgM antibodies to virus capsid antigen and 9 contained anti-EBNA antibodies in the standard ACIF test. Six of the nine ACIF EBNA-positive serum specimens were 100% positive in the P3HR-1 ACIF assay (EBV Combi Test), which is indicative of anti-EBNA-1 antibodies. The group with latent EBV infection consisted of 104 patients without clinical or laboratory signs of acute IM but with elevated levels of anti-virus capsid antigen IgG and anti-EBNA antibodies in IFA and ACIF (persistent or latent EBV infection). All samples were EBNA positive in the EBV Combi Test and negative in the IFA for IgM antibodies to virus capsid antigen.

Immunoblotting analysis. Protein extracts of whole cells and partially purified protein fractions were analyzed by discontinuous sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) (16) and were electrophoretically transferred to nitrocellulose membranes. After preincubation in 5% phosphate-buffered saline (PBS)-milk, the membranes were incubated with human sera diluted 1:100 or monoclonal antibodies PE-2 and 115E diluted 1:500 in PBS-milk. The membranes were washed and incubated with horseradish peroxidase-conjugated rabbit anti-mouse or rabbit anti-human IgG (Dako) diluted 1:500 in PBS-milk. After washing with 0.3% PBS-Tween 20, the filters were stained with a solution containing diaminobenzidine (Fluka, Buchs, Switzerland) and H_2O_2 .

Preparation of EBNA-2-containing cell extracts. A total of 3×10^7 SF-9 cells were seeded in 150-cm² culture flasks (Nunc) and were infected with pVL941-E2A or pVL941-E2B recombinant viruses at 2 PFU per cell. The cells were harvested at 48 h after infection, washed in PBS, and homogenized on ice in hypotonic buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5], 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], dithiothreitol [DTT], 0.5% aprotinin) by pipetting up and down. The homogenates were cleared by centrifugation at $2,000 \times g$ for 20 min at 2°C to separate the cytoplasmic fractions from the pelleting nuclei. Ammonium sulfate (Fluka) was slowly added to the supernatants to a saturation level of 30%, and the mixture was incubated for 1 h at 4°C on a rotator and centrifuged at $4,000 \times g$ for 30 min at 0°C. Ammonium sulfate was added to the supernatants to obtain a final saturation of 60%. The extracts were precipitated overnight at 4°C and centrifuged at $4,000 \times g$ for 30 min at 0°C. The pellet was dissolved in buffer A (20 mM HEPES [pH 7.15], 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, 10% glycerol, 0.5% aprotinin) and desalted on a Sephadex G-20M desalting column (Pharmacia, Uppsala, Sweden) that was equilibrated with buffer A. The EBNA-2A- and EBNA-2B-containing extracts were stored at -30°C. The protein concentration was measured by the method of Bradford (1) by using bovine serum albumin as a standard.

ELISA. Microtiter plates (Immunoplate II; Nunc) were incubated at 4°C for 18 h with 200 μ l (per well) of a solution containing 1.5 μ g of pVL941-E2A virus, pVL941-E2B virus, or mock-infected SF-9 cell extract per ml in 100 mM sodium carbonate (pH 9.6). The coated plates were rinsed six times with washing solution (150 mM NaCl [pH 7.5], 0.1% NaN_3 , 0.05% Tween 20), air dried, sealed, and stored desiccated at 4°C. The ELISAs were performed by incubating aliquots of human serum specimens, diluted 1:100 in PBS containing 0.1% NaN_3 and 0.1% Tween 20, in duplicate wells (200 μ l) for 1 h at 37°C in a humid chamber. After six washes with

washing solution, 200 μ l of alkaline phosphatase-conjugated goat anti-human IgG antibody (Tago), diluted 1:2,000 in PBS-Tween 20 containing 0.2% casein (Fluka), was added to the wells and incubation of the plates was continued at 37°C for 1 h. The plates were washed and developed with 1% p-nitrophenylphosphate (Merck, Darmstadt, Germany) in 200 μ l of substrate buffer (3.5% diethanolamine, 0.56% HCl, 0.01% $MgCl_2 \cdot 6H_2O$ [pH 9.8]) for 20 min at room temperature on a shaker. The reaction was interrupted by the addition of 50 μ l of 1.5 N NaOH, and the absorption at 405 nm (optical density at 405 nm [OD₄₀₅]) of the reaction solution was measured. The cutoff value was set at a point calculated from the average OD₄₀₅ + (2.1 $\times$ standard deviation) for the 186 routine human serum specimens against uninfected SF-9 control antigen.

The anti-viral capsid antigen IgM antibody titers in human sera were determined by using a commercial ELISA kit (Ortho Diagnostic Systems) (19).

RESULTS

Expression of EBNA-2A and EBNA-2B in insect cells. DNA fragments encoding the full-length EBNA-2A or EBNA-2B protein were cloned into the pVL941 baculovirus vector and were transferred to the *A. californica* nuclear polyhedrosis virus as described above and in Fig. 1. SF-9 cells infected with the recombinant viruses exhibited a bright, nuclear fluorescence with a maximum intensity at 48 h postinfection in the IFA with the PE-2 monoclonal antibody (Fig. 2). The patterns and distributions of the fluorescence were the same in EBNA-2A- and EBNA-2B-expressing cells. The fluorescence was drastically reduced in intensity at 72 h and was found mainly in the cytoplasm, indicating that a rapid degradation or leakage of the recombinant proteins occurred in the dying cells. Cells infected with the wild-type virus were negative in the IFA with the PE-2 monoclonal antibody at all time points. With the 115E monoclonal antibody, reactivity was found only in cells infected with EBNA-2A recombinant baculovirus (data not shown).

The results of SDS-PAGE and immunoblotting analysis of SF-9 cells infected with the recombinant baculoviruses by using the PE-2 monoclonal antibody are shown in Fig. 3. Cells expressing EBNA-2A contained a strong band that migrated with the same mobility as the EBNA-2 band in the B95-8 cells at 85 kDa. Extracts of EBNA-2B-expressing insect cells contained a PE-2-reactive band with a mobility corresponding to an apparent molecular mass of 72 kDa, which was similar to that of the EBNA-2B protein in the Jijoye B-cell line (29). Bands with higher mobilities found in both EBNA-2A and EBNA-2B baculovirus-infected SF-9 cells presumably were breakdown products of the full-length proteins, since they were not seen in uninfected or wild-type virus-infected cells. Dilution experiments indicated that recombinant baculovirus-infected cells expressed EBNA-2A and EBNA-2B at 100-fold or higher levels than the EBV-transformed B-cell lines. The levels of EBNA-2 were, however, not high enough for the protein to be detected by Coomassie blue staining of the gels. Again, the 115E monoclonal antibody reacted only with extracts from EBNA-2A-infected cells (data not shown).

ELISA for detection of anti-EBNA-2A and anti-EBNA-2B antibodies. With $(NH_4)_2SO_4$, most of the monoclonal antibody PE-2-reactive material from the EBNA-2A and EBNA-2B cytoplasmic extracts precipitated in the interval between 30 and 60% saturation. Desalted protein fractions could be stored at -30°C without any loss of immune

FIG. 1. Expression vectors encoding EBNA-2A and EBNA-2B for the baculovirus system. The major differences between the two alleles in the polyproline repeat are shown at the bottom (amino acid sequences were derived from reference 3).

reactivity. The yield was approximately 500 μg of protein from 10^6 SF-9 cells infected with the recombinant baculoviruses (48 h postinfection). Uninfected SF-9 cells yielded 250 μg of protein per 10^6 cells when subjected to the same purification procedure. The sensitivity and specificity of the EBNA-2 ELISA were found to be optimal when the microplates were coated with 0.3 μg of antigen per well (data not shown).

None of the human serum specimens tested so far has shown significant reactivity in assays with antigen preparations from uninfected SF-9 cells (Fig. 4). For the 186 human serum specimens tested against the negative control antigen, the average OD₄₀₅ was 0.104, with a standard deviation of 0.073; therefore, the cutoff limit was set to 0.3, with a grey zone from 0.2 to 0.3. The preferential reactivities of anti-EBNA-2 antibodies in human sera toward the A and B subtypes were determined from the average OD₄₀₅ value of at least two independent assays of EBNA-2A and EBNA-2B performed under standard conditions. To facilitate a classification of the sera, an index was calculated by dividing the OD₄₀₅ value obtained in the anti-EBNA-2A assay by that obtained in the anti-EBNA-2B assay. From the results obtained with the subtype-defined sera, the limiting values for the preferential reactivities of the anti-EBNA-2 antibodies were determined as follows. An index value greater than 1.5 (for sera with an OD₄₀₅ value greater than 0.3 in the EBNA-2A ELISA) was considered preferentially reactive against EBNA-2A-type virus. An index value less than 0.67 (for sera with an OD₄₀₅ value greater than 0.3 in the EBNA-2B ELISA) was considered preferentially reactive against EBNA-2B-type virus. Sera with index values of between 0.67 and 1.5 were considered similarly reactive toward both antigens and were not further classified.

Analysis of human sera. A total of 201 serum specimens were examined for the presence of IgG antibodies against EBNA-2A, EBNA-2B, and mock-infected SF-9 cell extracts by the ELISA at a standard dilution of 1:100 as described above and summarized in Table 1. The results are averages of at least two separate determinations. In the group of subtype-defined sera, all patients were previously analyzed for their infecting EBV subtype by a nonserological method (23). Five serum specimens from patients with known EBV-type A infection, as determined by Ebnotyping, were also preferentially reactive against EBNA-2A in the ELISA.

resulting in index values ranging from 1.55 to 2.84. Ten known type B serum specimens were also preferentially reactive against EBNA-2B in the assay; their index values ranged from 0.24 to 0.64.

None of the 26 human serum specimens in the group of EBV-negative samples showed any reactivity to EBNA-2A or EBNA-2B, as judged from the OD₄₀₅ obtained in the ELISA (Fig. 4). The average OD₄₀₅ with negative control wells was 0.075 (standard deviation [s] = 0.033), that with the EBNA-2A antigen was 0.091 (*s* = 0.035), and that with the EBNA-2B antigen was 0.102 (*s* = 0.044). The results for 56 serum specimens from the group of patients with acute EBV infections (acute IM) and for 104 serum specimens from the group of patients with latent EBV infections are summarized in Table 1 and Fig. 4. In the group of patients with acute IM, 19 of 56 serum specimens could be classified on the basis of the measured OD₄₀₅ values as preferentially reactive against EBNA-2A (one serum specimen had a borderline value), one serum specimen reacted preferentially against EBNA-2B (borderline value), and 11 serum specimens were EBNA-2 nonreactive. The remaining 25 serum specimens had significantly elevated levels (OD₄₀₅ of greater than 0.3 in the ELISA) of anti-EBNA-2 antibodies which showed similar reactivities against both EBNA-2A and EBNA-2B antigens. A total of 79 of 104 serum specimens from the group of latently infected individuals preferentially reacted against EBNA-2A (3 serum specimens had borderline values), 5 serum specimens reacted preferentially against EBNA-2B (2 serum specimens had borderline values), 13 serum specimens had elevated levels of anti-EBNA-2 antibodies with similar reactivities against both antigen types, and 7 serum specimens were EBNA-2 nonreactive at a dilution of 1:100. With selected human sera, preferential reactivity toward EBNA-2A- or EBNA-2B-expressing insect cells and its extracts could be observed by IFA and immunoblotting (data not shown). Because of the increased background levels, however, these methods were not routinely applied.

DISCUSSION

Only very small amounts of the EBNA proteins are expressed in EBV-infected B lymphocytes, making it necessary to use highly sensitive methods for their detection. The

FIG. 2. Immunofluorescence photographs of *S. frugiperda* SF-9 insect cells stained with mouse anti-EBNA-2 monoclonal antibody PE-2 and counterstained with Evans blue. (A) Wild-type baculovirus infected cells. Recombinant EBNA-2B infected cells were examined at 24 h (B), 48 h (C), or 72 h (D) after infection. Magnification, $\times 400$.

commonly used method for the determination of antibodies to "total" EBNA has been by ACIF with EBV-transformed Burkitt's lymphoma cell lines (e.g., Raji cells) as targets (25). The development of transfected cell lines that specifically express only one of the known EBNA proteins has allowed more precise evaluation of the anti-EBNA antibody pattern in EBV-infected patients (27, 32, 35, 42). However, the diagnosis still must rely on the IFA technique, which requires an experienced examiner and is unsuitable for screening purposes. The EBNA-2A protein is phosphorylated at serine and threonine residues (24), a fact that does not allow production of an immunogenic, recombinant protein in bacterial systems. Expression of EBNA-2A in the baculovirus system was reported recently (33). We expressed EBNA-2A and EBNA-2B as full-length, nonfusion proteins in insect cells and used partially purified antigens to develop an ELISA method that allows the specific determination of antibodies against these proteins. Recombinant proteins produced in insect cells with baculovirus vectors seem to undergo posttranslational modifications very similar to those of proteins produced in mammalian cells. In many cases, such proteins have been found to be immunogenically and functionally similar to their native counterparts.

Recombinant EBNA-2A and EBNA-2B were both recog-

FIG. 3. Confirmation of protein expression by immunoblots stained with anti-EBNA-2-specific monoclonal antibody PE-2 from the following cellular extracts: EBNA-2A positive lymphoblastoid cell line B95-8 (lane 1); cytoplasmic extract from EBNA-2A expressing SF-9 cells at 24 h (lane 2), 48 h (lane 3), and 72 h (lane 4) postinfection; nuclear extract of the same cells as in lane 2 (lane 5); wild-type baculovirus-infected SF-9 cells (lane 6); EBV-positive, but EBNA-2-deleted Burkitt's lymphoma-derived cell line P3HR-1 (lane 7); EBNA-2B positive Burkitt's lymphoma-derived cell line Jijoye (lane 8); cytoplasmic extract from EBNA-2B-expressing SF-9 cells at 48 h (lane 9) and 72 h (lane 10) postinfection; nuclear extract of the same cells as in lane 9 (lane 11); molecular mass markers (lanes M). The apparent sizes are given on the left (in kilodaltons).

FIG. 4. ELISA with baculovirus-produced EBNA-2A and EBNA-2B. Anti-EBNA-2 IgG antibodies in the sera from four groups of individuals were measured in terms of OD₄₀₅ values. (A) Serum specimens with OD₄₀₅ values for EBNA-2A (E2A) greater than OD₄₀₅ values for EBNA-2B (E2B); the points at the upper ends of the lines represent the values obtained in the EBNA-2A ELISA; the points at the lower ends of the lines represent values obtained in the EBNA-2B ELISA. (B) Serum specimens with OD₄₀₅ values for EBNA-2B (E2B) greater than OD₄₀₅ values for EBNA-2A (E2A), where the upper point of each line represents the result for EBNA-2B and the lower point represents the result for EBNA-2A. The dots at the bottom of both diagrams represent the results obtained with the SF-9 control antigen. Group 1, 15 subtype-defined serum specimens; group 2, 26 EBV-negative serum specimens; group 3, 56 serum specimens from patients with acute EBV infection; group 4, 104 serum specimens from individuals with latent EBV infection. The cutoff value is indicated with the solid lines at an OD₄₀₅ of 0.3.

TABLE 1. Comparison of ELISA results at a standard dilution of 1:100 for sera from four groups of individuals^a

Human sera	No. (%) of serum specimens				
	EBV-A infected	EBV-B infected	EBV negative	Acute infection	Latent infection
EBNA-2A > EBNA-2B	5	0	0 (0)	19 (34)	79 (76)
EBNA-2A < EBNA-2B	0	10	0 (0)	1 (2)	5 (5)
EBNA-2A ≈ EBNA-2B ^b	0	0	0 (0)	25 (45)	13 (12)
EBV-2 nonreactive ^c	0	0	26 (100)	11 (19)	7 (7)
Total	5	10	26 (100)	56 (100)	104 (100)

^a A total of 203 serum specimens were examined. Fifteen subtype-defined serum specimens (as defined by Ebnotyping as described by Grata and colleagues [7, 23]) divided into EBV type A and EBV type B subsets were used to validate the ELISA, 26 serum specimens were EBV negative, 56 serum specimens were from patients with acute EBV infection, and 104 serum specimens were from individuals with latent EBV infections.

^b 0.67 < index (EBNA-2A/EBNA-2B) < 1.50.

^c OD₄₀₅ < 0.3 for both antigens.

nized by the PE-2 mouse monoclonal antibody by IFA (Fig. 2) and immunoblotting (Fig. 3) analyses. Cross-reactivity indicates the presence of at least one common epitope on the two proteins. This is expected, since the overall identity of EBNA-2A and EBNA-2B at the amino acid level is 64% (3). It is also reflected by the fact that EBV-positive human sera always reacted with both proteins in the ELISA, although in different ratios. Type-specific epitopes, however, are also present in EBNA-2A and EBNA-2B, as demonstrated by preferential reactivity toward one of the two antigens in sera from 83 of the 104 patients with latent EBV infection. Type-specific epitopes have been shown to be located near the carboxyl terminus, which seems to be the main immunogenic part of the EBNA-2 protein (28). Additionally, monoclonal antibody 115E was found to react only with EBNA-2A.

The EBNA-2-expressing insect cells exhibited a bright fluorescence in IFA with a large number of serum specimens from patients with latent EBV infection. However, about 50% of all human serum specimens tested also gave a significant fluorescence with uninfected SF-9 cells. Pread-sorption of the samples with a suspension of fresh SF-9 cells removed the reactivity (data not shown). The difference in anti-EBNA-2A and anti-EBNA-2B antibody levels of the adsorbed sera as determined by IFA was, however, often too small to permit a definite estimation of the subtype of the infecting virus. The IFA technique is, therefore, considered to be unsuitable for screening large numbers of samples.

Isolation of virus by cell culture requires much time and might not give representative results because of differences in growth properties between the EBV A and B subtypes (26). Identification of viral DNA sequences by PCR is a highly specific, although expensive, method, with the drawback that whole cells must be used as a source of EBV. Furthermore, the sensitivity of the PCR method seems to be limited, as judged from the fact that only 22 to 24% of all mouth washings of healthy seropositive donors were EBNA-2 positive by this technique (15, 36). ELISA systems for detecting antibodies to EBNA-1 have been reported recently (6, 11, 12), but for antibodies to EBNA-2, no such method is available. For these reasons an ELISA that was based on recombinant EBNA-2A and EBNA-2B was established. The capacity of the assay to detect preferential reactivity against EBNA-2A or EBNA-2B was demonstrated with 15 serum specimens with defined EBV subtypes. To evaluate the method, 186 human serum specimens were analyzed and the results were related to those obtained by conventional diagnostic tests for EBV infection. The major-

ity of the serum specimens showed only a very minor reactivity with negative control wells coated with mock-infected SF-9 cell extracts (91% had OD₄₀₅ values of less than 0.2; 5% were between 0.2 and 0.3). This demonstrates that components that react nonspecifically with antibodies present in many human serum specimens were successfully removed from the cell extracts by ammonium sulfate fractionation. Eight serum specimens (4%) showed an increased reactivity (OD₄₀₅ greater than 0.3) with the negative control wells. Five of them were from patients with high levels of anti-EBNA-2 antibodies, but three of these serum specimens were from patients with moderately increased or borderline levels; for these specimens, the unspecific reaction might affect the outcome of the EBNA-2 antibody typing. It is possible that insect cells contain additional endogenous epitopes that interact with a limited number of human serum specimens and are not removed by the purification procedure. Sera from the group of patients that were EBV negative by the other criteria all showed low levels of reactivity against mock-infected and EBNA-2A- and EBNA-2B-containing cell extracts in the ELISA (Fig. 4). The cutoff limit for the presence of EBNA-2 antibodies in the sera was set to an OD₄₀₅ of 0.3 to accommodate all the samples in the EBV-negative group.

Henle et al. (9) and Niederman and Miller (22) demonstrated that antibodies to EBNA-2 appeared early in patients with IM and then showed a tendency to decline or disappear, whereas anti-EBNA-1 antibodies appeared only after the third month and remained at high levels over a long period of time. This agrees well with our finding that only a minority of patients (16%) with acute IM had detectable levels of anti-EBNA-1 antibodies as detected by P3HR-1 ACIF (EBV Combi Test) and at least 81% had antibodies to EBNA-2. In our group of healthy seropositive individuals, all had antibodies to EBNA-1 and 93% had measurable titers of anti-EBNA-2 antibodies. The time to the appearance of anti-EBNA-2A and anti-EBNA-2B IgG antibodies seemed to follow the same course.

The ratio between the EBNA-2A and EBNA-2B ELISA OD₄₀₅ values was used as an index specifying the preferential reactivity against one of the two types of EBNA-2 proteins. To validate the border value for classification of the subtype, defined sera from patients that had been analyzed previously for the infecting EBV subtype by a nonserological method were used. Among the sera from the group of individuals with latent EBV infection, 76% had index values greater than 1.5, suggesting a preferential type A infection, and 5% had index values less than 0.67, suggesting a

preferential type B infection (Table 1). The dominance of the A subtype agrees with the notion that type A virus is much more prevalent in the healthy Caucasian population than type B (43). About 13% of the serum specimens with significantly elevated antibody levels showed similar reactivities with both antigens (index values between 0.65 and 1.5). This might be caused by an immune response directed mainly against EBNA-2 epitopes common to the A and B subtypes. Another explanation might be dual infection with the two types of EBV, which has been shown to occur frequently in human immunodeficiency virus-positive individuals and immunocompromised patients (7, 34). It has also been suggested that more than one virus strain might be involved in the acute phase of IM, but this question is still unresolved (13). Sixbey et al. (36) have found an incidence of double infections of 9% in healthy, EBV-positive individuals, as detected by PCR of mucosal washings. Nevertheless, in the same study (36), only 22% of the healthy adult donors were found to contain EBV DNA in their saliva; the situation in peripheral lymphocytes was not analyzed and might thus differ with respect to the presence of EBV-specific antibodies. In contrast, it has been demonstrated by different methods, including PCR, that individuals with healthy immune systems usually carry only one virus strain (2, 15, 20, 40).

In summary, the ELISA method described in this report allows a qualitative determination of anti-EBNA-2A and anti-EBNA-2B IgG antibodies in sera from individuals with postacute EBV infection and latently infected individuals. Indirect detection of the subtype of the infecting virus seemed to be possible for most individuals. The method provides a fast and relatively cheap diagnostic tool for investigating the distribution of the two virus strains in different human populations and their role in IM and other EBV-associated diseases.

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