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Identification of an Epstein-Barr Virus Nuclear Antigen by Fluoroimmunoelectrophoresis and Radioimmunoelectrophoresis

BRUCE C. STRNAD,* TIMOTHY C. SCHUSTER, RALPH F. HOPKINS III, RUSSELL H. NEUBAUER, AND HARVEY RABIN

Biological Carcinogenesis Program, Frederick Cancer Research Center, Frederick, Maryland 21701

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A 65,000-dalton (65K) antigen found in Raji cells by fluoroimmunoelectrophoresis and radioimmunoelectrophoresis has been identified as an Epstein-Barr virus nuclear antigen (EBNA). This identification is based on the following evidence. The 65K antigen is detected in Raji cells but not in three Epstein-Barr virus (−) human B cell lines. It is not detected with EBNA (−) sera. The 65K antigen is found predominantly in the nucleus and co-elutes with EBNA during partial purification by DNA-Sepharose and Blue Dextran-Sepharose chromatography. Finally, the partially purified 65K antigen is an effective absorbant of EBNA antibody as measured in an anticomplement immunofluorescence assay. Antigens with molecular weights of 72, 70, and 73K have been detected in B95-8, P3HR-1, and Namalwa cells, respectively. These antigens are the likely homologues of the 65K Raji EBNA. In addition, an Epstein-Barr virus-associated, 81K DNA-binding antigen has been detected in both B95-8 and Raji cells.

Four Epstein-Barr virus (EBV) antigens have been defined on the basis of the staining patterns observed in various immunoflourescence assays (8, 9, 11, 25). These antigens are membrane antigen, virus capsid antigen, early antigen, and nuclear antigen (EBNA). Immunoprecipitation experiments performed in several laboratories have demonstrated that membrane, virus caspid, and early antigen each consist of more than one protein (3, 5, 10, 17, 18, 20, 23, 28, 30). Recently, a 48,000-dalton (48K) protein has been identified as an EBNA on the basis of immunoprecipitation experiments and biochemical purification of the antigen (14, 15). Identification of EBNA by classical radioimmunoprecipitation techniques has been difficult for three reasons. First, EBNA constitutes less than one-thousandth of the total cellular protein. Second, the in vivo radiolabeling of constitutive intracellular proteins yields proteins of relatively low specific activity. Third, human sera which effectively precipitate EBNA are rare in our experience. Therefore, to identify EBNA we turned to the techniques of radioimmunoelectrophoresis (RIE) and fluoroimmunoelectrophoresis (FIE). Using these techniques, we have identified a second EBNA with a molecular weight of 65K in Raji cells. Antigens with similar molecular weights were also detected in B95-8, P3HR-1, and Namalwa cells.

MATERIALS AND METHODS

Cell lines and growth conditions. All B lymphocyte cell lines were grown in RPMI 1640 medium containing 10% decomplemented fetal bovine serum supplemented with 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). The cells were grown at 37°C in an atmosphere of 95% air, 5% CO_2. B95-8 cells, an EBV producer line, were grown in the above medium supplemented with 100 μg of phosphonoacetic acid (Richmond Organics) per ml to block late viral protein synthesis (29). However, the B95-8 cells were 2 to 3% early antigen (+) at the time of harvest. P3HR-1 (N) is an EBV-positive nonproducer subline of P3HR-1 obtained from Berge Hampar and was negative for all EBV antigens except EBNA. P3HR-1 (P) is an EBV producer line obtained from Meihan Nonoyama. BJAB and Ramos are EBV-negative B cell lines. B1-19 is an EBV-positive converted BJAB line obtained from Harald zur Hausen (7), and B1-19-2 is an EBNA-negative subline of B1-19 cloned in our laboratory. Both P3HR-1 (P) and B1-19 were passaged at daily intervals before harvest to minimize the number of early antigen (+) virus capsid antigen (+) cells. The Raji and Namalwa cell lines are EBV-positive, nonproducer cell lines.

Description of human sera. Sera 1 and 12 were from individuals who were negative for antibodies to all EBV antigens. Sera 6 and 11 were from clinically normal individuals and had EBNA antibody titers of 1/80 and 1/160, respectively, in the anticomplement immunofluorescence assay (ACIF) performed on Raji cells (25). Sera 9 and 10 were pooled human AB+ sera

purchased from GIBCO and had EBNA antibody titers of 1/40 and 1/80, respectively. All of the sera were negative for nonspecific antinuclear antibody when tested on Ramos and BJAB cells by ACIF.

Preparation of whole cell SDS lysates. A total of 1×10^8 cells were washed three times in phosphate-buffered saline (PBS) and suspended in 0.8 ml of water. The cells were lysed by the addition of 0.1 ml of 10% sodium dodecyl sulfate (SDS), and the DNA was sheared by passage through a 27G needle. The protein content of the lysates was determined by the method of Lowry et al. (13), and the lysates were stored at −70°C before use.

Iodination of proteins. Protein A (Pharmacia Fine Chemicals, Inc.) and human immunoglobulin IgM and IgG (Cappel Laboratories) were iodinated by the following procedure. Twenty-five microliters of protein solution at 1 mg/ml of 0.4 M Tris-hydrochloride–4 mM EDTA (pH 7.4) (RB) were mixed with an additional 25 μl of RB. Ten microliters containing 1 mCi of $Na^{125}I$ (New England Nuclear Corp.; carrier-free in 0.1 M NaOH) was added, followed by 10 μl of chloramine T at 2.5 mg/ml of RB. After a 60-s shaking at room temperature, the reaction was terminated by the addition of 25 μl of sodium metabisulfite at 2.5 mg/ml of RB. The reaction mixture was chromatographed on a 10-ml G-25 Sephadex column, poured in a 10-ml disposable pipette, and equilibrated with PBS–1% bovine serum albumin. Fractions (0.5 ml) were collected, and the fractions in the leading portion of the excluded peak were pooled. Specific activities of 1×10^7 to 2×10^7 cpm/μg were routinely obtained.

RIE. RIE was performed by a modified procedure of Adair et al. (1). Whole cell SDS lysates and ^{125}I-labeled human IgM and IgG standards were disassociated in 1.4% SDS–0.5 M β-mercaptoethanol at 80°C for 10 min. The samples were electrophoresed in the discontinuous gel system described by Laemmli (12). The gel consisted of a 3% acrylamide–0.08% bisacrylamide stacking gel (1.5 cm long) and an 8% acrylamide–0.2% bisacrylamide running gel (8.5 cm long). Both gels and the electrode buffer contained 0.1% SDS. After electrophoresis the SDS was eluted from the gel by washing in 3 1-liter volumes of PBS, followed by 2 1-liter volumes of PBS, containing 1% bovine serum albumin and 0.02% NaN_3. Each of these washes lasted 0.5 h. Then gel was covered with human sera diluted 1:2 in PBS–1% bovine serum albumin–0.02% NaN_3 and incubated at 37°C in a humid atmosphere for 1 h. After incubation, the gel was washed with 6 250-ml volumes of PBS–0.02% NaN_3 for a total of 1.5 days at room temperature. Then the gel was covered with a solution of ^{125}I-protein A (2×10^6 cpm/ml of PBS–1% bovine serum albumin) and incubated in a humid atmosphere at 37°C for 0.5 h. The gel was washed with 2 200-ml volumes of PBS–1% bovine serum albumin and with 5 200-ml volumes of PBS over a 4-h period. The gel was dried down under vacuum and placed on Kodak XR-2 X-ray film and a Kodak X Omatic Regular intensifying screen. The film was exposed overnight and developed. The molecular weights of the antigens detected in the cell lysates were determined by linear extrapolation from the mobilities of the iodinated μ chain (76K) and γ chain (50K) of the IgM and IgG standards co-electrophoresed with the lysates.

Preparation of fluorescein-labeled human IgM and IgG standards. A 0.5-ml amount of either human IgG or human IgM at 3 mg of protein per ml was dialyzed against 2 100-ml volumes of 0.025 M carbonate buffer (pH 9.4) at 5°C and then against 10 ml of 0.025 M carbonate buffer containing 100 μg of fluorescein isothiocyanate (FITC; Sigma Chemical Co.) per ml for 2 days at room temperature. Finally, the immunoglobulin was dialyzed against 3 250-ml volumes of PBS at 5°C and stored at −70°C.

Procedure for FIE. Whole cell SDS lysates and FITC-conjugated IgM and IgG standards were disassociated and electrophoresed as previously described (27). The immunofluorescence assay for antigen bands was performed by a modified procedure of Towbin et al. (31). After electrophoresis, the gel slab was washed three times (0.5 h/wash) in 500 ml of 0.025 M Tris–0.192 M glycine–20% methanol (pH 8.5) (TGM) to remove SDS. The following multilayered transfer assembly was put together in a tray of TGM buffer so that bubbles were not introduced between the filter paper and the gel. A rectangular metal anode (13 by 20 cm) was placed in the bottom of the tray, followed by a Scotch Brite pad, a piece of Whatman 3MM paper, a sheet of HAWP filter paper (Millipore Corp.), the acrylamide gel slab, another piece of 3MM paper, another Scotch Brite pad, and finally a rectangular metal cathode. The layers were held together with nylon clamps. The assembly was placed in a tank of TGM buffer with the electrodes in a vertical position so that bubbles formed during transfer would escape upward. Electrophoretic transfer was carried out toward the anode at 6 V for 4.5 h with the electrodes 1.5 cm apart. Control experiments performed with ^3H-amino acid–labeled SDS cell lysates indicated that 80% of the extract protein was removed from the gel by this procedure and about 65% of the electro-eluted proteins were bound to the HAWP paper. The overall efficiency of transfer was therefore about 50%. After transfer, the HAWP filter paper was stored overnight in PBS at 5°C. Residual binding sites on the paper were blocked by incubation for 2 h at 37°C in decomplemented calf serum diluted 1:2 in PBS. Then the calf serum was removed, and the paper was covered with human serum diluted 1:2 in decomplemented calf serum and incubated at 37°C for 1 h. After washing six times with 100 ml of PBS (10 min/wash), the paper was covered with FITC-conjugated goat antihuman IgG, heavy and light chain specific (Hyland Diagnostics; 5 to 7 μg of fluorescein per mg of protein) diluted 1:20 in PBS. The paper was incubated with the FITC conjugate for 1 h at 37°C, washed six times with 100 ml of PBS (10 min/wash), and photographed under UV light.

The paper was photographed with a Polaroid MP-4 Land camera equipped with a Wratten no. 12 gelatin filter and Polaroid Type 55 Positive/Negative 4 × 5 Land film. Illumination was provided by two Mineralight R-52 UV units (Ultraviolet Products, Inc.) located on each side of the paper at 45° angles to the paper surface and at a distance of 20 cm. Exposure times ranged from 2 to 7 min depending on the inten-

sity of the antigen bands. The molecular weights of the antigens were determined by linear extrapolation from the mobilities of the FITC-conjugated μ chain (76K) and γ chain (50K) of the IgM and IgG standards co-electrophoresed with the lysates.

Preparation of whole cell, nuclear, and cytoplasmic lysates from Raji cells. Freshly harvested Raji cells were washed three times in 10 volumes of PBS. For the preparation of whole cell lysates, the cells were washed one time in 10 volumes of 0.15 M NaCl-20 mM Tris-hydrochloride (pH 7.4)-1 mM EDTA-1 mM 2-mercaptoethanol-0.5 mM phenylmethylsulfonyl fluoride (buffer A), suspended in 7 volumes of buffer A, sonicated twice for 15 s, and dialyzed against Veronal buffer at 5°C. For the preparation of nuclear and cytoplasmic lysates, the cells were suspended in 6 volumes of 0.01 M Tris-hydrochloride-0.01 M NaCl-0.002 M $MgCl_2$ (pH 7.6) (HB buffer) for 10 min on ice. The cells were lysed by the addition of 1 volume of HB buffer containing 1% Nonidet P-40, incubated for 5 min on ice, and centrifuged at 200 × *g* for 5 min at 5°C. The cytoplasmic lysate was dialyzed against Veronal buffer. The nuclear pellet was suspended in 7 volumes of Veronal buffer, sonicated for 15 s three times, and dialyzed against Veronal buffer. The protein concentration in the lysates was determined by the method of Lowry et al. (13), and the titer of complement-fixing (CF) antigen was determined by a micro ^{51}Cr release CF assay (4, 16).

Biochemical fractionation of EBNA by DNA-Sepharose and Blue Dextran-Sepharose chromatography. Chromatographic separation of EBNA on a DNA-Sepharose column was performed as previously described (19). The fractions which eluted from the DNA affinity column with 0.5 M NaCl contained all of the measurable CF activity and all of the activity in the acid-fixed nuclear binding assay (21). The 0.5 M NaCl eluate was diluted to 0.15 M NaCl by the addition of buffer A-10% glycerol without NaCl. The diluted eluate was passed through a Blue Dextran-Sepharose column (2.4 by 0.9 cm; 8.6 mg of Blue Dextran bound per ml of packed Sepharose) prepared as described by Ryan and Vestling (26) and equilibrated in buffer A-10% glycerol. The column was eluted in succession with 6-ml volumes of buffer A-10% glycerol containing 0.5, 1.0, or 2.0 M NaCl. Samples of all of the column fractions were dialyzed first against Veronal buffer for the determination of protein and CF activity and then against 0.02% SDS for FIE.

Absorption of EBNA antibody. Partial purification of EBNA for absorption experiments was achieved by DNA-Sepharose chromatography of Raji cell extracts performed as described above, followed by dialysis against 0.02% SDS and electrophoretic separation of the proteins in the 0.5 M NaCl eluate on an 8% SDS-acrylamide gel slab (12, 27). Two tracks on each slab contained FITC-conjugated IgM and IgG standards. After separation on acrylamide gel slabs, the proteins were electrophoretically transferred to HAWP filter paper as described for FIE. The filter papers were blocked with 50% calf serum for 2 h at 37°C, and the μ and γ chain standards were visualized under UV light. The positions of these standards were marked on the paper and served as guides for cutting out horizontal strips of paper (2.5 mm by 12 cm) containing five different molecular weight ranges of protein. These strips of filter paper were cut vertically into 0.5-cm-long segments. One segment of the strips containing the 65K antigen was incubated with EBNA-positive human serum and FITC antihuman IgG as described for FIE to monitor the accuracy of the slicing procedure. The remaining segments were used to absorb 0.2-ml volumes of serum 11 diluted 1:10 in PBS-0.02% NaN_3. Each 0.2-ml sample of diluted serum was absorbed sequentially with four strips of paper containing a particular molecular weight range of protein. Each absorption was performed with segments from one strip at 5°C for 1 day. Then the serum sample was transferred with a capillary pipette to the segments of the next strip. After four absorptions the samples were coded and diluted serially in PBS. The titers of EBNA antibody were determined by the ACIF assay on Raji cells using unabsorbed serum 11 as the control. The slides were read independently by three individuals, and then the results were decoded.

RESULTS

Detection of EBNA-associated antigens by RIE. The use of RIE for the detection of antigens has two advantages. First, a high-specific-activity ^{125}I-protein A probe can be used, and second, with the antigen immobilized in a gel, the unbound immunoglobulins can be washed away, thereby permitting the use of sera with normal titers of EBNA antibody without decreasing sensitivity. Figure 1 illustrates the

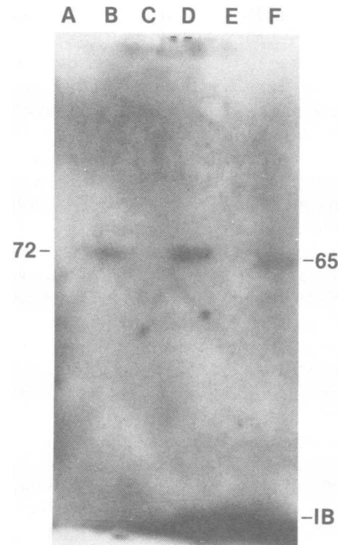

FIG. 1. *RIE on SDS whole cell lysates with pooled human EBNA (+) serum 10. The tracks contain 78 µg of protein from: A, Ramos; B, B95-8/PAA; C, BJAB; D, P3HR-1 (N); E, B1-19-2; F, Raji. The numbers in the margins indicate the molecular weights in thousands. IB indicates the ion boundary.*

results obtained when three EBNA (+) and three EBNA (−) whole cell SDS lysates were assayed by RIE with pooled human serum 10. Single antigen bands were detected in B95-8/PAA, P3HR-1 (N), and Raji at 72, 70, and 65K, respectively. Antigen bands were not detected in Ramos, BJAB, and B1-19-2. The same antigen pattern was obtained when pooled human serum 9 was tested. EBNA (+) serum 6 also detected the 72, 70, and 65K antigens and, in addition, detected antigens in B95-8/PAA at 81, 51, and 44K and in Raji at 81K (data not shown). Antigens were not detected in these lysates by RIE when EBNA (−) serum 1 was employed.

The RIE assay suffered from two technical limitations. The first problem was the high ^{125}I-protein A background which limited exposures to 1 to 2 days and which sometimes gave irregular background blotches that obscured the band pattern. The second limitation was that only the antigen on the surface of the gel could be detected since the incubation times with serum and ^{125}I-protein A were purposely kept short to minimize background. To eliminate these problems, we turned to the technique of FIE.

Detection of EBNA-associated antigens by FIE. The electrophoretic transfer of separated cell proteins to filter paper facilitated the subsequent washing steps of the immunoassay. Furthermore, FIE was more sensitive than RIE as evidenced by the appearance of several antigen bands which were not detected by RIE. This increase in sensitivity was probably due both to an increase in antigen accessibility and to lower background with the FITC-conjugated antihuman IgG probe. Pooled human serum 9 (Fig. 2A) gave an antigen pattern which was identical to the RIE pattern obtained with this serum. Antigens were detected at 72, 70, and 65K in B95-8/PAA, P3HR-1, and Raji cells, respectively. Pooled human serum 10 gave a pattern similar to serum 9, except that a faint additional antigen band was detected in B95-8/PAA and Raji at 81K (data not shown). Figure 2B and C show the FIE results with two individual EBNA (+) sera, 6 and 11. Serum 6 (Fig. 2B) detected antigens at 81, 72, 51, 48, 44, and 39K in B95-8/PAA, at 70K in P3HR-1, and at 81 and 65K in Raji. Serum 11 (Fig. 2C) detected antigens at 81 and 72K in B95-8/PAA and at 70 and 65K in P3HR-1 (N) and Raji, respectively. An EBNA (−) serum did not detect antigens in these lysates by FIE.

The results obtained by RIE and FIE on whole cell lysates can be summarized as follows. All four EBNA (+) sera tested detected antigens between 65 and 72K in Raji, P3HR-1, and B95-8/PAA cells. Three of these sera detected a second fainter antigen band at 81K in B95-8/PAA cells, and two of these sera detected a similar antigen in Raji cells. A single serum detected four additional antigen bands between 39 and 51K in B95-8/PAA cells. Antigens were not detected in three EBNA (−) cell lines, and they were not detected with EBNA (−) sera. In all experiments, numerous faint bands were seen which were of equal intensity in both the tracks containing the EBNA (−) and those containing the EBNA (+) cell extracts. These represent background nonspecific binding of the FITC an-

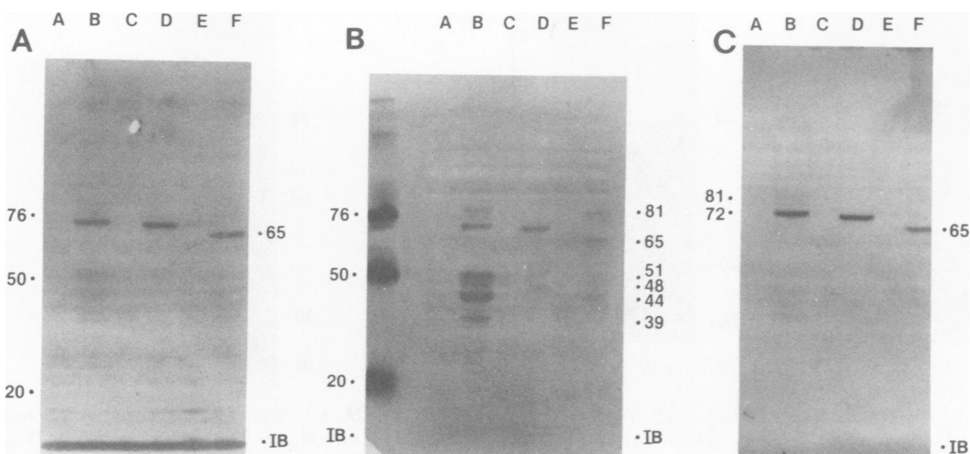

FIG. 2. *FIE on SDS whole cell lysates with pooled human EBNA (+) serum 9 (A), EBNA (+) serum 6 (B), and EBNA (+) serum 11 (C). The tracks contain 78 µg of protein from: A, Ramos; B, B95-8/PAA, C, BJAB; D, P3HR-2 (N); E, B1-19-2; F, Raji. The numbers in the margins indicate the molecular weights in thousands. In (A) and (B) the weights on the left are those of the standards. The unlabeled track on the left in (B) shows the µ chain, γ chain, and light-chain standards. IB indicates the ion boundary.*

tihuman IgG conjugate to extract proteins.

The antigens most consistently associated with EBNA by RIE and FIE were the antigens with molecular weight between 65 and 72K, depending on the virus-transformed cell line used. A second antigen with a molecular weight of 81K was detected by three of the four EBNA (+) sera in Raji or B95-8/PAA cells or both. It was unlikely that the four antigens detected by FIE between 39 and 51K were associated with EBNA because they were only detected with a single serum and only in B95-8 cells.

The association between EBNA and the 70K antigen detected in P3HR-1 cells was further strengthened by FIE using the B1-19 cell line. The B1-19 line was initially cloned in the laboratory of Harald zur Hausen from a culture of BJAB cells infected with P3HR-1 virus (7). Most of the cells in the B1-19 culture were EBNA (+) but some were EBNA (−), and B1-19-2 was an EBNA (−) cell line cloned from the B1-19 population in our laboratory. Figure 3A shows that the 70K antigen was present in P3HR-1 (P), an EBV producer line, and absent from BJAB. The 70K antigen was present in the EBNA (+) B1-19 line, cloned from P3HR-1-infected BJAB, and absent from the EBNA (−) B1-19-2 line, cloned from B1-19. The 72K antigen detected in B95-8/PAA cells could conceivably be an early antigen since the cells were 2 to 3% positive for early antigen. However, FIE on the EBNA (+), nonproducer cell line, Namalwa (Fig. 3B, track C) indicates that an antigen of similar molecular weight (i.e., 73K) was detected in these early antigen (−) cells.

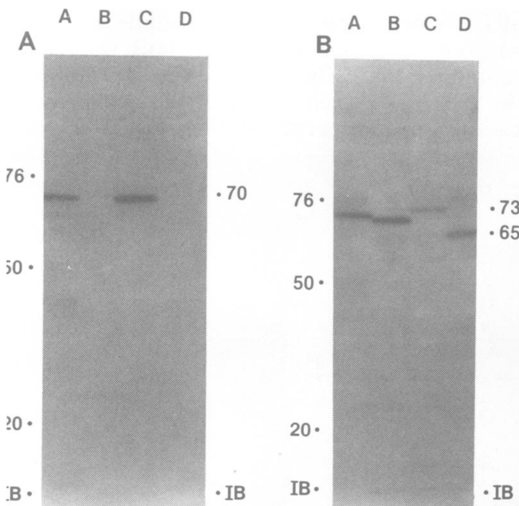

FIG. 3. *(A) FIE on SDS whole cell lysates with pooled human EBNA (+) serum 9. The tracks contain 78 μg of protein from: A, P3HR-1 (P); B, BJAB; C, B1-19; D, B1-19-2. (B) FIE on SDS whole cell lysates with pooled human EBNA (+) serum 9. The tracks contain 78 μg of protein from: A, B95-8/PAA; B, P3HR-1 (P); C, Namalwa; D, Raji. The numbers in the margins indicate the molecular weights in thousands. The weights of the standards are on the left. IB indicates the ion boundary.*

Biochemical characterization of the 65K antigen detected in Raji cells by FIE. The antigens detected between 65 and 73K by FIE were strongly associated with EBNA since an antigen in this weight range was found in all four EBNA (+) cell lines tested. Accordingly, we decided to examine the biochemical properties of the antigen. In these studies, we focused our attention on the 65K antigen found in Raji cells, since EBNA can readily be detected in Raji cell extracts by a microcomplement fixation assay without interference from other viral antigens (16).

Whole cell, nuclear, and cytoplasmic lysates were prepared from Raji cells. The lysates were first assayed for the EBNA-associated CF antigen and then tested by FIE. The specific activities (C_{50} U/mg of protein) of the whole cell, nuclear, and cytoplasmic lysates were 118, 185, and 40, respectively. The FIE results in Fig. 4 indicated that the 65K antigen was preferentially associated with the nuclear lysate (track B) and almost undetectable in the cytoplasmic lysate (track C).

EBNA has been reported to bind and elute from both DNA and Blue Dextran affinity columns, resulting in a substantial purification of

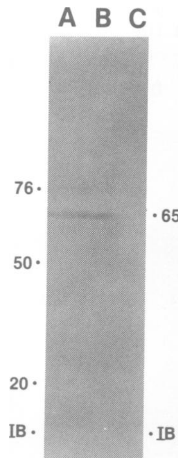

FIG. 4. *FIE on Raji lysates with pooled human EBNA (+) serum 10. The tracks contain 77 μg of protein from: (A) sonically treated, whole cell; (B) sonically treated nuclear material; (C) cytoplasmic lysate. The numbers in the margins indicate the molecular weights in thousands. The weights of the standards are on the left. IB indicates the ion boundary.*

the antigen (2, 16). Accordingly, we prepared a crude Raji cell sonic extract and chromatographed it on DNA-Sepharose and Blue Dextran-Sepharose affinity columns. As Table 1 indicates, all of the measurable CF activity eluted from DNA-Sepharose, with 0.5 M NaCl giving a sevenfold purification of the antigen. The 0.5 M NaCl eluate was chromatographed on a Blue Dextran-Sepharose column, and all of the recoverable CF activity eluted with 2 M NaCl for a total purification of 46-fold. Samples of each column eluate were concentrated, and the same amount of protein from each eluate was assayed for the 65 and 81K antigens by FIE. Since the same amount of protein was electrophoresed in each track, the intensity of the antigen band should correspond to the relative concentration of antigen in a particular fraction. Figure 5 indicates that both the 65 and 81K antigens were found in the 0.5 M NaCl eluate from DNA-Sepharose (track D). However, the 65K antigen was the predominant antigen found in the 2 M NaCl eluate from Blue Dextran-Sepharose (track I). Furthermore, the intensity of the 65K antigen band increased at each step of EBNA purification as measured by CF, as would be expected if both the CF and FIE assays measured the same antigen. Since less than 0.5% of the initial sonically treated cell protein was found in the 2 M NaCl eluate from Blue Dextran-Sepharose, the probability of this association being fortuitious is extremely remote. In contrast, the 81K antigen elutes from the Blue Dextran column over the entire range of salt concentrations employed. Several other faint antigen bands were observed in the 2 M NaCl Blue Dextran eluate. These antigens had molecular weights less than 65K and the pattern of decreasing intensity with decreasing molecular weight which they exhibited suggests that they represent processed or degraded products of the 65K antigen.

Absorption of EBNA antibody by the 65K Raji antigen. Partial biochemical purification of the CF antigen detected in Raji cell extracts demonstrated a close correlation between the CF antigen and the 65K antigen associated with EBNA by FIE. However, since EBNA is defined by nuclear fluorescence in an ACIF assay, absorption experiments were performed to test the association of EBNA with the 65K antigen in a more critical manner. The 65K antigen was partially purified from Raji sonic extracts by DNA-Sepharose chromatography, followed by electrophoresis on SDS-acrylamide gel slabs and transfer of the separated proteins to HAWP filter paper. Proteins bound to the insoluble filter paper support were in a useful form for absorption experiments because small volumes of serum could be absorbed with a minimal amount

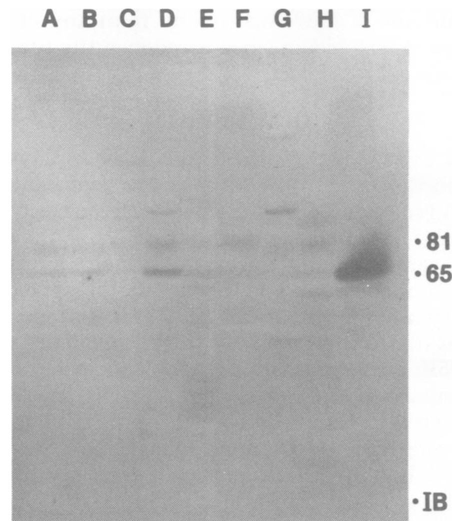

FIG. 5. *FIE on Raji cell fractions with EBNA (+) serum 6. The tracks contain 37 μg of protein from: A, crude Raji cell sonic extract; B, 1.7 M NaCl extraction supernatant; C, DNA-Sepharose 0.15 M NaCl eluate; D, DNA-Sepharose 0.5 M NaCl eluate; E, DNA-Sepharose 2 M NaCl eluate; F, Blue Dextran-Sepharose 0.15 M NaCl eluate; G, Blue Dextran-Sepharose 0.5 M NaCl eluate; H, Blue Dextran-Sepharose 1 M NaCl eluate; I, Blue Dextran-Sepharose 2 M NaCl eluate. The numbers in the margins indicate the molecular weights in thousands. IB indicates the ion boundary.*

TABLE 1. *EBNA purification by DNA-Sepharose and Blue Dextran-Sepharose chromatography*

Fraction	Protein (mg)	C_{50} (U)	Sp act (U/mg)	Fold purification	C_{50} U recovered (%)
Crude Raji cell sonic extract	108.0	10,240	94.8	1.0	100.0
1.7 M NaCl extraction, centrifugation, and dialysis of supernatant	60.6	7,520	124.1	1.3	73.4
0.5 M NaCl eluate of DNA-Sepharose column	6.0	4,000	666.7	7.0	39.1
2 M NaCl eluate of Blue Dextran-Sepharose column	0.346	1,506	4,352.6	45.9	14.7

of dilution. Table 2 illustrates the ACIF results obtained when samples of serum 11 were absorbed with proteins found in five different molecular weight ranges. The unabsorbed serum control had an EBNA antibody titer of 1/160. Although the dilution effect during absorption was minimized by the use of filter paper-bound protein, it was not completely eliminated and it would account for a twofold reduction in apparent titer. All five molecular weight ranges tested exhibited an additional twofold reduction in titer, which probably indicates that there is some nonspecific absorption of antibody to the filter paper. However, it was clear that the 63 to 67K molecular weight range was a more effective absorbant of EBNA antibody than any of the other ranges tested since it reduced the titer an additional fourfold.

DISCUSSION

It has been postulated that EBNA plays an important role in the initiation or maintenance of B lymphocyte transformation. This hypothesis was based on the fact that EBNA is the first EBV antigen found during the primary infection of lymphocytes and on the fact that it is the only antigen expressed in all EBV-transformed cell lines (6). In this paper, we present evidence that a 65K antigen is an EBNA. The evidence is summarized as follows. First, the 65K antigen is detected in Raji cells by RIE and FIE, but not in three EBV (−) human B cell lines. Second, the 65K antigen is found preferentially in the nucleus. Third, the antigen coelutes with EBNA during partial purification by DNA-Sepharose and Blue Dextran-Sepharose chromatography. Fourth, the partially purified 65K antigen is an effective absorbant of EBNA antibody as measured by the ACIF assay. In addition, antigens were detected by the same EBV (+) sera at 70K in both producer and nonproducer sublines of P3HR-1 cells, and at 72 and 73K in B95-8 and Namalwa cells, respectively. These antigens are probably the homologs of the 65K antigen detected in Raji. This hypothesis is supported for the P3HR-1 70K antigen by the fact that B1-19, a P3HR-1–converted BJAB, has the 70K antigen, whereas BJAB and B1-19-2, an EBNA (−) clone of B1-19, do not.

TABLE 2. *Absorption of EBNA antibody*

Mol wt range of proteins used for absorption	EBNA antibody titers of absorbed serum by ACIF (reciprocal of serum dilution)				
	10	20	40	80	160
88–95K	+	+	+	−	−
78–84K	+	+	+	−	−
63–67K	+	−	−	−	−
46–50K	+	+	+	−	−
43–46K	+	+	+	−	−
Unabsorbed control	+	+	+	+	+

The 65K antigen detected in Raji cells by RIE and FIE clearly has a mobility on SDS-acrylamide gels different from the 48K EBNA identified by Luka et al. (15). This difference in molecular weight cannot be attributed to the use of different molecular weight standards because the 48K antigen clearly has a mobility faster than the γ chain of IgG (15), whereas the 65K antigen has a mobility substantially less than the γ chain. It is possible that the two antigens have related primary amino acid sequences, the 48K EBNA being a processed or degraded product of the 65K EBNA. Alternatively, it is possible that the two antigens are distinct. There are at least two reasons why we may not have detected the 48K antigen by RIE and FIE. First, since the treatment of Raji extracts with SDS and β-mercaptoethanol inactivates some of the EBNA activity detected by complement fixation, it is possible that the antigenicity of some EBNA components is completely destroyed under the electrophoretic conditions used in the RIE and FIE assays. Second, the 48K EBNA identified by Luka et al. (15) was detected with sera from Burkitt's lymphoma patients with titers of 1/640 in the ACIF assay. None of the sera available to us in sufficient quantities has titers this high.

The hypothesis that EBNA consists of multiple antigenic determinants is supported by studies involving comparative titration of human sera on the nuclear antigens of EBV and the EBV-like viruses of gorillas and baboons. The results indicate that EBNA has at least two determinants, one unique to EBNA and another shared by human, gorilla, and baboon viral nuclear antigens (24). The hypothesis of multiple EBNA determinants is also supported by the results of reciprocal absorption experiments performed in our laboratory. Absorption of certain sera with fixed P3HR-1 or Raji cells completely removed all antibody detected in homologous ACIF reactions but not in heterologous ACIF reactions. These experiments indicate that EBNA consists of multiple antigenic determinants located on either the same of different molecules. The existence of multiple EBNAs is consistent with the hybridization data of Powell et al. (22). In this study, it was shown that Raji and Namalwa contain sufficient polyadenlyated EBV-specific RNA to code for as many as four EBV proteins. It is interesting to consider the 81K antigen in this light. The 81K antigen was detected in Raji or B95-8 or both with three of the four EBV (+) sera tested. This antigen also

binds to DNA-Sepharose columns. However, its chromatographic properties on Blue Dextran columns clearly distinguish it from the 65K antigen. Recent data indicate that the 81K antigen, like the 65K antigen, is found predominantly in the nucleus. Therefore, it is possible that the 81K antigen is a third EBV nuclear antigen. To date, all of the sera tested, which react with the 81K antigen, detect only a faint, slightly diffuse band. The study of this antigen would be facilitated by the identification of a serum which reacted strongly with it by FIE. Four additional antigens with molecular weights between 39 and 51K were detected by FIE. However, it is unlikely that these antigens represent EBNA since they were detected with only one out of four sera tested, and in only one (i.e., B95-8/PAA) of the three EBNA (+) lines tested.

EBNA can be purified 700- to 800-fold by a sequence of DNA-Sepharose, Blue Dextran-Sepharose, and hydroxylapatite chromatography (15). Nevertheless, in our experience, the EBNA-containing fraction from the hydroxylapatite column still contains about 20 protein bands. Additional purification of EBNA for biological studies would be facilitated by immunoaffinity chromatography if an extremely high titered monospecific serum was available in sufficient quantity. In principle, partially purified EBNA can be used to immunize mice for the selection of monoclonal antibodies if the appropriate techniques can be developed for screening large numbers of hybridomas. This work is in progress in our laboratory.

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