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Immunobiochemical Characterization with Monoclonal Antibodies of Epstein-Barr Virus-Associated Early Antigens in Chemically Induced Cells

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Five monoclonal antibodies which are reactive to early antigens of Epstein-Barr virus have been produced by using somatic cell hybridization techniques. The specificity of the monoclonal antibodies to early antigens was demonstrated by indirect immunofluorescence, which showed that the antigens were localized to the nucleus of early antigen-induced Raji cells. Additional indirect immunofluorescence studies showed that like patient antisera to diffuse-staining early antigen, the monoclonal antibodies gave positive staining reactions after methanol fixation. One of the antibodies, 1150-4, was positive by the anti-complement immunofluorescence technique but differed with Epstein-Barr virus-associated nuclear antigen-positive patient sera in that it only stained induced cells. Different fixation methods were found to alter dramatically the appearance of the nuclear staining reactions produced by the monoclonal antibodies. Immunoprecipitation and immunoblot experiments revealed that monoclonal antibodies 1108-1 and 1129-1 recognized two polypeptides of 55,000 and 50,000 daltons (p55;50), 1173-6 and 1180-2 recognized just p50, and 1150-4 identified a 65,000-dalton nuclear protein. Immunobiochemical characterization of these viral antigens showed that p55 is a phosphoprotein, and p55;50 has strong DNA-binding activity preferentially to single-stranded DNA. Elucidation of the role of these nuclear proteins in Epstein-Barr virus infection and the events associated with Epstein-Barr virus-directed lymphocyte transformation may provide significant information on the pathogenicity of this important human virus.

The Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis (IM) (13) and is associated with two human neoplasms, African Burkitt's lymphoma (35) and nasopharyngeal carcinoma (20). Several EBV-related antigens associated with early or late functions of the viral genome have been identified by indirect immunofluorescence (IF) procedures by using sera from patients and healthy viral carriers. These antigens include the early antigen (EA) (11), EBV-associated nuclear antigen (28), membrane antigens (5), and viral capsid antigen (9). Of these, EA is diagnostically the most valuable since positive EA titers are seen only transiently during the acute phases of IM (14). Positive EA titers are also detected in cancer patients with African Burkitt's lymphoma and nasopharyngeal carcinoma and are indicative of abortive EBV replication in the tumor cells (10).

By using IF techniques, EA was first identified in African Burkitt's lymphoma cell lines grown in arginine-deficient medium (12). More recently, investigators have shown that EA may be virally induced with P3HR-1 virus (15) and chemically induced by various reagents, including phorbol 12-myristate 13-acetate (TPA) (34), sodium butyrate (23), halogenated pyrimidines (7), intercalating chemicals (33), culture fluid of *Fusobacterium nucleatum* (16), and anti-human immunoglobulin M (IgM) antibodies (32). IF studies by Henle et al. (10) have shown that EA is responsible for two distinct staining patterns: a diffuse (D) pattern detectable in both the nucleus and cytoplasm and a restricted (R) pattern localized to the cytoplasm and sensitive to methanol fixation. EA expression has been shown to be independent of DNA synthesis, as first demonstrated by Gergely et al. (8). EA can thus be distinguished from late viral products when cells are induced in the presence of a DNA inhibitor such as phosphonoacetic acid (26, 31). Immunoprecipitation experiments with metabolically labeled induced cells and high-titer anti-EA$^+$ sera have shown that EA is composed of several polypeptides which are undetectable in EBV-negative RAMOS cells treated in a similar manner (2, 3, 6, 17, 19, 25). The identity of the antigenic polypeptides responsible for the D and R staining patterns is presently unknown. In view of the significance of EA in the diagnosis of IM and EBV-associated tumors, I attempted to produce monoclonal antibodies to EA by using affinity-purified antigen. My success in producing EA-specific monoclonal antibodies was attributable to a novel method of immunization and my ability to screen the hybridoma supernatants for reactivity to intracellular antigens by IF methods. Immunobiochemical studies with these monoclonal antibodies demonstrated that certain EA polypeptides are phosphoproteins and have DNA-binding activity.

MATERIALS AND METHODS

Cell lines. The EBV nonproducer Raji, producer P3HR-1, and EBV-negative RAMOS cell lines were maintained at 37°C in a humidified 5% CO_2 incubator. All cultures grew in stationary suspension culture in RPMI 1640 medium containing 15% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures were routinely passaged 1:4 twice weekly.

Human sera. High-titer anti-EA$^+$ sera were obtained through the kind generosity of George Klein, Karolinska Institute, Stockholm, Sweden and Werner Henle, The Children's Hospital of Philadelphia, Philadelphia, Pa. The serum from the Karolinska Institute laboratory consisted of pooled sera from six patients with African Burkitt's lymphoma with high anti-EA, -viral capsid antigen, and -EBV-associated nuclear antigen titers. The Children's Hospital laboratory supplied sera pooled from two sets of patients who had either high anti-EA-D$^+$ or high anti-EA-R$^+$ titers.

IM sera drawn at the onset of clinical symptoms were

obtained from the Northwestern University Student Health Center (Chicago, Ill.), courtesy of Alice Brandfonbrener. EBV-negative sera were obtained from screened volunteers.

Induction of EA. Cultures of Raji and RAMOS cells at a concentration of 8×10^5 cells per ml were chemically induced at 37°C for 3 days in fresh medium containing 50 ng of TPA (P-L Biochemicals, Inc., Milwaukee, Wis.) per ml and 25 μg of iododeoxyuridine (IUdr) (Sigma Chemical Co., St. Louis, Mo.) per ml. For these studies, a 100× stock solution of IUdr dissolved in alkalinized distilled water was used. After membrane filtration, this stock solution was found to be stable at 4°C for 6 months. The TPA was prepared by dissolving 5 mg into 100 ml of dimethyl sulfoxide. This 1,000× stock solution was then portioned under sterile conditions into 2-ml provials (Dynatech Laboratories, Alexandria, Va.) and stored at −85°C for up to 6 months. Since the TPA was found to be labile to freeze-thawing, each vial was used only once. For comparison, Raji cells were also induced by viral superinfection with P3HR-1 supernatants as described previously (15).

Purification of viral antigens. Of the pooled high-titer anti-EA^+ human sera, 8 ml was placed over a 7.5-ml protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) affinity column to purify the IgG. After a 50-ml wash with buffer (pH 7.0) containing 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, and 0.15 M NaCl, the IgG was eluted as a sharp peak by using buffer (pH 2.3) containing 0.05 M sodium acetate and 0.15 M NaCl. The IgG was immediately brought to neutral pH and dialyzed overnight against coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl [pH 8.3]). Approximately 10 mg of IgG was then coupled to 1 g of cyanogen bromide-activated Sepharose 4B beads (Pharmacia). 2-Aminoethanol (1 M) was used to block the remaining active sites on the beads. The IgG-coupled Sepharose beads were then incubated overnight at 4°C with 10 mg of bovine serum albumin (BSA) in phosphate-buffered saline (PBS) by using an end-over-end rotator.

Chemically induced Raji cells (3 liters) were washed twice in PBS at 4°C and resuspended in 50 ml of lysis buffer (150 mM NaCl, 20 mM Tris [pH 8.0], 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 1% aprotinin). The cells were sonicated three times at 15-s intervals, and the lysate was clarified by ultracentrifugation at 30,000 rpm for 30 min at 4°C. The lysate was first incubated overnight at 4°C in an end-over-end rotator with IgG-coupled Sepharose beads prepared from an EBV-negative serum donor to remove the proteins which bound to the Sepharose beads. After these beads were pelleted, the lysate was incubated overnight at 4°C as above with the high-titer anti-EA^+ IgG-coupled Sepharose beads. The antigen-antibody-Sepharose (Ag-Ab-S) beads were then washed three times with buffer containing 0.15 M NaCl and 0.1 M Tris (pH 8.0) and three times with buffer containing 0.25 M LiCl and 0.1 M Tris (pH 8.0) to remove nonspecifically bound proteins. The beads were then divided into six portions and were frozen at −85°C in 0.5 ml of PBS.

Production of hybridomas. A portion of the Ag-Ab-S beads was emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) and inoculated by subcutaneous injection at multiple sites into three BALB/c mice. On day 14, the mice received subcutaneously a second aliquot which had been emulsified in incomplete Freund adjuvant and on day 28, they were given a final intraperitoneal injection of Ag-Ab-S beads suspended in PBS. The mice were killed 4 days later, and the immune splenocytes were fused with NS-1 mouse myeloma cells as described elsewhere (27). Antibody-secreting hybridoma cells were cloned on 0.5% Noble agar as described by A. L. Epstein and C. V. Clevenger (*in* I. Bekhor, ed., *Recent Advances in Non-Histone Chromosomal Protein Research*, in press).

IF. Hybridoma supernatants were examined by IF techniques by using fixed-cell preparations of chemically induced Raji and control RAMOS cells. To examine large numbers of supernatants in the required time, it was necessary to develop a rapid screening procedure. Induced cells were washed in PBS containing 1 mg of BSA per ml and 0.02% sodium azide and pipetted dropwise at a concentration of 5×10^6 cells per ml onto Teflon-coated printed microscope slides containing 10 5-mm wells per slide (Cel-Line Associates, Inc., Newfield, N.J.). After the cells settled to the surface of the glass, the overlying fluid was quickly removed by aspiration, and the cells were dried on the slide by a gentle stream of warm air. The slides were then immediately fixed in 2% paraformaldehyde (no. 4018; Polysciences, Inc., Warrington, Pa.) in PBS for 15 min at room temperature. After fixation, the slides were rinsed in PBS and placed in acetone at −20°C for 3 min to make the cells permeable. After a final PBS rinse to remove the acetone, the slides were stored at 4°C in PBS containing 0.02% sodium azide. Alternatively, the slides were fixed in acetone or methanol-acetone (1:1) at −20°C for 5 min, air dried, and stored at −20°C.

For the screening procedures, 35 μl of each supernatant was pipetted on a well of the printed microscope slide preparations by using a different tip for each supernatant. After 60 min of incubation at 37°C in a humidified chamber, the slides were rinsed three times in PBS and again incubated for 30 min at 37°C with 20 μl of a 1:20 dilution of fluorescein-conjugated goat anti-mouse IgG antibody which was $F(ab')_2$ fragment specific (Cappel, Malvern, Pa.). The slides were then rinsed three times in PBS, counterstained with Evans blue for 5 min at room temperature by using a freshly prepared solution containing 50 μl of a 1% stock solution of Evans blue in 80 ml of PBS, rinsed a final time in PBS, and mounted with cover slips by using a 1:1 solution of glycerol-PBS. The slides were then examined by epifluorescence microscopy with a Leitz Orthoplan microscope with Ploemopak 2.1 fluorescence illuminator, HBO 100 mercury lamp, and a ×50 water immersion objection. The anti-complement immunofluorescence (ACIF) procedure, by which cells were fixed with methanol-acetone, was performed as previously described (28).

Serological characterization of antibody isotypes. Hybridoma supernatants from 4-day cultures were concentrated 10 to 20 times in B15 minicon concentrators (Amicon Corp., Lexington, Mass.) and tested in double-diffusion Ouchterlony plates against rabbit anti-mouse immunoglobulin heavy chain subclass antisera (Miles Laboratories, Inc., Elkhart, Ind.). The precipitin bands were read after 2 to 3 days of incubation in a well-humidified 37°C incubator.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Raji and control RAMOS cells on day 3 of chemical induction were metabolically labeled overnight in methionine- and leucine-free RPMI 1640 medium containing 5% fetal calf serum, antibiotics, 30 μCi of [^{35}S]methionine per ml, and 10 μCi of [^{14}C]leucine per ml. Alternatively, the cells were labeled as above in phosphate-free medium containing 5% dialyzed fetal calf serum, antibiotics, and 200 μCi of $^{32}P_i$ per ml. After being labeled, the cells were washed twice with PBS and resuspended in lysis buffer at 4°C. The cells were sonicated three times for 15-s intervals, ultracentrifuged at 30,000 rpm for 30 min, and divided into 1-ml portions in

plastic tubes (no. 2052 [12 by 75 mm]; Falcon Plastics, Oxnard, Calif.). A total of 1 ml of hybridoma supernatant, 10 μl of human serum, or 15 μl of mouse serum was added, and the tubes were incubated overnight at 4°C with constant shaking. The antigen-antibody complexes were removed from solution by the addition of protein-A Sepharose beads (Pharmacia) which were pretreated consecutively with 10 mg of BSA per ml in PBS and cold Raji lysate to prevent the nonspecific binding of radiolabeled proteins. The immunoprecipitates were washed two times in buffer containing 0.15 M NaCl and 0.1 M Tris (pH 8.0) and three times in buffer containing 0.25 M LiCl and 0.1 M Tris (pH 8.0). The immunoprecipitates were then switched to a new set of tubes before being denatured in 65 μl of sodium dodecyl sulfate (SDS) sample buffer (0.07 M Tris [pH 6.8], 11% glycerol, 0.003% bromophenol blue, 3% SDS, and 5% 2-mercaptoethanol).

The samples were then analyzed by SDS-polyacrylamide gel electrophoresis with a 5 to 15% gradient separating gel and a 3.0% stacking gel as described by Laemmli (21). After electrophoresis, the gels were processed for fluorography by the method of Chamberlain (4), dried under vacuum, and then exposed to Cronex 4 X-ray film (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) at −70°C before development.

Immunoblot analysis. Raji lymphoma cells (2.5×10^7) were washed twice with PBS and dissolved in 2 ml of SDS sample buffer. Repeated vortexing and sonication were required to completely dissolve the cells and reduce the viscosity of the lysate. After being boiled for 90 s, 30-μl samples were loaded onto a 10% polyacrylamide gel and subjected to electrophoresis for 3.5 h with 20 mA of current as described by Laemmli (21). Molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were routinely run with each gel. The proteins were then transferred electrophoretically to nitrocellulose paper (Hahy paper; Millipore Corp., Bedford, Mass) using an Electroblot apparatus (E-C Apparatus Corp., St. Petersburg, Fla.) set at 0.2 A for 10 h. The chamber buffer contained 24 mM Tris hydrochloride (pH 8.3), 192 mM glycine, 20% methanol, and 0.1% SDS. After the transfer was completed, the section of the nitrocellulose paper which contained the molecular weight markers was cut and stained for 1 h with a 0.1% amido black solution containing methanol-dH_2O-glacial acetic acid (5:5:1). The remaining piece of nitrocellulose paper containing four lanes of cellular proteins was rinsed three times in PBS containing 0.5% Nonidet P-40 to remove excess SDS and then blocked for 3 h in freshly prepared 10× Denhardt solution (0.2% Ficoll 400, 0.2% BSA, and 0.2% polyvinyl pyrrolidone 360) to prevent the nonspecific binding of antibody to the nitrocellulose paper. The four lanes were then carefully separated with a scalpel blade, and each strip was incubated in plastic, heat-sealable Scotchpak pouches (4 by 6 in. [ca. 10 by 12 cm]) containing 10 ml of hybridoma supernatant and 0.02% sodium azide for 3 days at room temperature with constant mixing. No more than two strips per dish were then rinsed in glass dishes (8 by 8 in. [ca. 20 by 20 cm]) for 4 h by using several changes of PBS and then incubated overnight as above with 10^6 cpm of ^{125}I-labeled goat anti-mouse antibody (New England Nuclear Corp., Boston, Mass.) diluted in 10 ml of PBS containing 1 mg of BSA per ml and 0.02% sodium azide. The following day, the strips were washed in PBS for 6 h (three times per hour) as described above. The nitrocellulose strips were finally placed on Saran Wrap, blotted dry with filter paper, and sandwiched with a second piece of Saran Wrap. The enclosed strips were placed between an intensifying screen (Cronex Hi-Plus; Dupont) and Cronex 4 X-ray film in an X-ray holder (8 by 10 in. [ca. 20 by 25 cm]; Eastman Kodak) and exposed for 1 to 3 days at −70°C. The molecular weights of the radioactive bands were determined by placing the amido black-stained marker nitrocellulose strips in juxtaposition to the developed X-ray films.

DNA-cellulose radioimmunoassay. Single- and double-stranded DNA-cellulose were prepared by the method of Moss et al. (24). Calf thymus DNA (Sigma) was used as the DNA source. Single-stranded DNA was prepared as described by Alberts and Herrick (1). Briefly, 10 mg of single- or double-stranded DNA was covalently linked to 2.5 g of Whatman F11 cellulose. A stock solution of DNA-cellulose was then prepared by resuspending the 2.5 g of single- or double-stranded DNA-cellulose in 25 ml of PBS containing 1 mg of BSA per ml and 0.02% sodium azide.

For the radioimmunoassay procedure, 1-ml portions of cold Raji, induced Raji, and induced RAMOS cellular lysates were incubated overnight at 4°C with 250 μl of DNA-cellulose by using constant vortexing (The Big Vortexer; Kraft Apparatus, Inc., Mineola, N.Y.). For these experiments, the cells were lysed in buffer containing 2 M NaCl to solubilize completely the EA polypeptides. The lysates were then diluted 10 times with buffer minus NaCl to reduce the salt concentration to 0.2 M. The DNA-cellulose was then washed twice with wash buffer (0.15 M NaCl, 0.1 M Tris [pH 8.0]) and then incubated at 4°C with 1 ml of monoclonal antibody supernatant for 2 h by using constant vortexing. After being washed three times, the DNA-cellulose was incubated at 4°C for 1 h with 100,000 cpm of ^{125}I-labeled goat anti-mouse IgG (New England Nuclear). The DNA-cellulose was then washed four times, transferred to vials, and counted in a gamma counter. Each reaction was tested in triplicate to determine the statistical significance of the data.

RESULTS

Chemical induction of EAs. Several induction methods were studied to maximize EA expression in Raji cells. Superinfection with P3HR-1 virus and chemical treatment with either TPA or IUdr induced no greater than 10% of the cells. By comparison, the combination of TPA and IUdr synergistically induced 60 to 80% of the Raji cells. This high level of induction was obtained consistently once it was discovered that the TPA was labile to freeze-thawing procedures.

Immunobiochemical identification of EA polypeptides by using high-titer patient sera. Immunoprecipitation procedures with high-titer anti-EA$^+$ sera and labeled lysates of induced Raji cells were performed to identify EA-specific polypeptides. Labeled lysates of induced RAMOS cells were used as negative controls in these experiments. Seven major polypeptide bands were immunoprecipitated by pooled African Burkitt's lymphoma sera obtained from the Karolinska Institute laboratory (Fig. 1, lane 2). Pooled high-titer anti-EA-D$^+$ sera obtained from the Childrens Hospital laboratory immunoprecipitated the same seven bands (Fig. 1, lane 4). Both sets of sera immunoprecipitated predominantly p85, p35, and p55 in descending order. Pooled anti-EA-R$^+$ sera consistently gave negative results in these experiments (Fig. 1, lane 6).

To determine whether IM sera drawn at the onset of clinical symptoms immunoprecipitated the same polypeptides, additional immunoprecipitation experiments were performed. The immunoprecipitation patterns of sera obtained from eight acutely ill IM patients were compared with that of the pooled African Burkitt's lymphoma sera (Fig. 2). In all

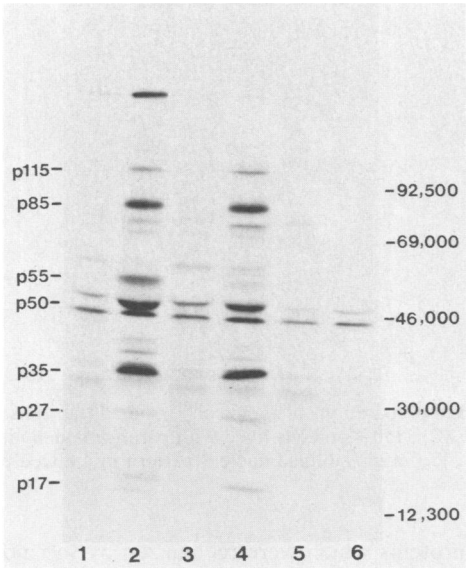

FIG. 1. Immunoprecipitation of [^{35}S]methionine- and [^{14}C]leucine-radiolabeled EA polypeptides by high-titer anti-EA$^+$ sera. Lanes 1 and 2, chemically induced RAMOS and Raji cells, respectively, with pooled African Burkitt's lymphoma sera; lanes 3 and 4, chemically induced RAMOS and Raji cells, respectively, with pooled high-titer anti-EA-D$^+$ sera; lanes 5 and 6, chemically induced RAMOS and Raji cells, respectively, with pooled high-titer anti-EA-R$^+$ sera.

cases, p85 was the most prominant polypeptide immunoprecipitated by the IM sera. The p115, p55, and p35 polypeptides were immunoprecipitated to various degrees and appeared to be independent of the immunoprecipitation of p85. The amount of antibody to p35, however, did appear to be inversely related to the quantity of anti-p55 in these sera. The significance of these results can only be assessed after testing a larger number of IM sera.

Generation of EA-specific monoclonal antibodies. EA polypeptides were affinity purified from cellular lysates of induced Raji cells by using the pooled high-titer African Burkitt's lymphoma sera supplied by the Karolinska Institute laboratory. For the affinity purification procedure, the human sera were placed over a protein A-Sepharose column to isolate the IgG fraction. After being eluted from protein A, the IgG was coupled to cyanogen bromide-activated Sepharose beads. The IgG-linked Sepharose beads were used to affinity purify the EA polypeptides from cellular lysates of induced Raji cells. EA linked to the human IgG-Sepharose beads were then used to immunize mice for the hybridoma procedure.

EA-specific monoclonal antibodies were produced by the fusion of primed BALB/c splenocytes and mouse myeloma NS-1 cells. From three fusions, five monoclonal antibodies designated 1108-1, 1129-1, 1150-4, 1173-6, and 1180-2 of the IgG1 heavy chain isotype were generated which showed EA specificity by IF. IF studies with supernatants from these hybridoma clones showed positive reactivity with chemically induced Raji cells. The EA specificity of these monoclonal antibodies was demonstrated by their negative reactivity with induced and noninduced RAMOS cells and noninduced Raji cells which do not express EA. ACIF studies with EBV-negative human serum as the complement source showed that only monoclonal antibody 1150-4 was positive by this procedure in induced Raji cells. The finding that 1150-4 was negative by ACIF in noninduced Raji cells indicates that it recognized an antigen other than EBNA.

To determine the effects of fixation on the immunofluorescence staining reactions of the monoclonal antibodies, chemically induced Raji cells were fixed by three different methods: paraformaldehyde-acetone; acetone; and methanol-acetone. The reactivity of the monoclonal antibodies was unaffected by the different fixatives, with the exception of 1129-1, which gave a decreased intensity of staining after acetone and methanol-acetone fixation.

The IF staining pattern of monoclonal antibody 1108-1 on chemically induced Raji cells fixed with paraformaldehyde-acetone is shown in Fig. 3A. Monoclonal antibodies 1129-1, 1173-6, and 1180-2 stained identically to 1108-1, all of which gave a bright, homogeneous nuclear and, in certain cells, nucleolar immunofluorescence pattern. Occasional cells showed a coarse granular nuclear staining, which was the predominant pattern obtained with acetone-fixed preparations (Fig. 3B). Monoclonal antibody 1150-4 gave a similar nuclear immunofluorescence pattern in chemically induced Raji cells but was less intense in appearance (Fig. 3C). Monoclonal antibody 1150-4 also reacted with another nuclear antigen expressed in Raji and control RAMOS cells which characteristically had a sparsely speckled nuclear pattern (Fig. 3C).

Immunobiochemical characterization of EA polypeptides recognized by monoclonal antibodies. To determine the identity of the antigens recognized by the monoclonal antibodies, immunoprecipitation and immunoblot analyses were performed with lysates of chemically induced cells. The immunoprecipitation results with monoclonal antibody 1108-1 revealed that 1108-1 recognized the EA-specific p55 and p50 polypeptides (Fig. 4, lane 4). Overexposed autoradiograms of the immunoprecipitate produced by 1108-1 revealed the presence of two minor bands located between p55 and p50 and the presence of light bands at 88,000 and 32,000 daltons. Immunoprecipitation analysis with sera from the hyperimmunized mice demonstrated that affinity-purified EA acted as an effective immunogen. As shown in Fig. 4, the mice responded specifically to p85, p35, and p55;50 (lane 2) but also reacted to other induced antigens unrelated to EBV (lane 1).

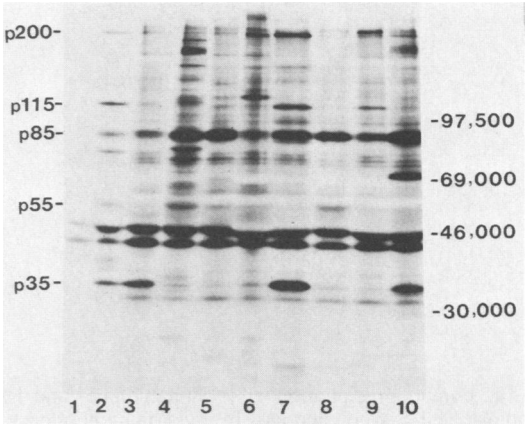

FIG. 2. Immunoprecipitation of [^{35}S]methionine- and [^{14}C]leucine-radiolabeled EA by IM sera. Lanes 1 and 2, chemically induced RAMOS and Raji cells, respectively, with pooled high-titer EA-D$^+$ sera; lanes 3 to 10, chemically induced Raji cells with sera drawn from eight IM patients at the time of clinical presentation.

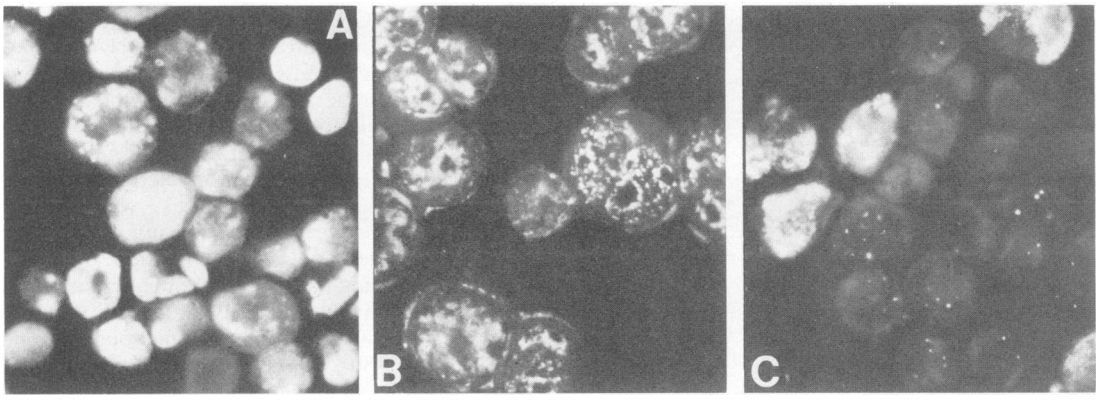

FIG. 3. Indirect IF staining patterns of EA-specific monoclonal antibodies on fixed preparations of chemically induced Raji cells. (A) 1108-1 on cells fixed with paraformaldehyde-acetone; (B) 1108-1 on acetone-fixed cells; (C) 1150-4 on cells fixed with paraformaldehyde-acetone. Note the appearance of two staining patterns with monoclonal antibody 1150-4 (C); a bright, diffuse nuclear pattern in induced cells and a sparsely speckled nuclear pattern in noninduced cells.

Monoclonal antibody 1129-1 gave the same immunoprecipitation pattern as 1108-1 (Fig. 5, lane 2), and 1173-6 was found to immunoprecipitate p50 only. Monoclonal antibodies 1150-4 and 1180-2 failed to give positive results in these experiments (Fig. 5, lanes 3 and 5, respectively). p55 of the p55;50 complex immunoprecipitated by monoclonal antibodies 1108-1 and 1129-1 was found to be a phosphoprotein (Fig. 5B). When serum from a patient with acute IM was used to immunoprecipitate EA polypeptides labeled with $^{32}P_i$, p55 was found to be the major EA polypeptide to be phosphorylated in induced Raji cells (Fig. 5, lane 6).

Immunoblot procedures with monoclonal antibodies 1173-6 and 1180-2 demonstrated that these reagents were specifically directed against p50 (Fig. 6). Monoclonal antibody 1150-4, however, was found to recognize a 65,000-dalton nuclear protein (Fig. 6C) expressed in chemically induced Raji cells.

EA p55;50 is a DNA-binding protein with specificity for single-stranded DNA. To determine whether any of the nuclear proteins which were recognized by the monoclonal antibodies had DNA-binding activity, a DNA-cellulose radioimmunoassay developed in my laboratory was performed. Cold lysates from induced cell cultures were incubated with either single- or double-stranded calf thymus DNA-cellulose. Binding of p55;50 or p65 was then determined by quantifying the amount of monoclonal antibody that bound to the DNA-cellulose by using an ^{125}I-labeled goat anti-mouse IgG probe. Monoclonal antibodies 1108-1 and 1129-1 showed significant binding activity in the single-stranded DNA-cellulose tubes (12 to 20 times greater than background) (Table 1). Both antibodies also had significant binding in the double-stranded DNA-cellulose tubes, but for 1108-1, this activity was only four times over background.

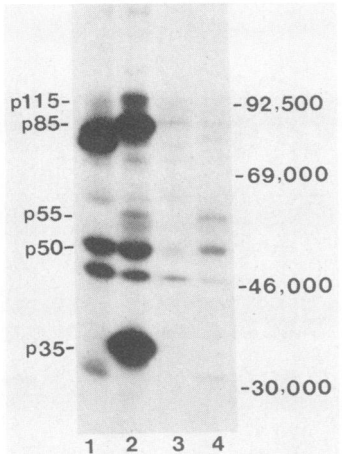

FIG. 4. Immunoprecipitation of [^{35}S]methionine- and [^{14}C]leucine-radiolabeled EA polypeptides by hyperimmune mouse serum and monoclonal antibody 1108-1. Lanes 1 and 2, chemically induced RAMOS and Raji cells, respectively, with hyperimmune mouse serum; lanes 3 and 4, chemically induced RAMOS and Raji cells, respectively, with monoclonal antibody 1108-1. Note that monoclonal antibody 1108-1 specifically immunoprecipitated p55;50.

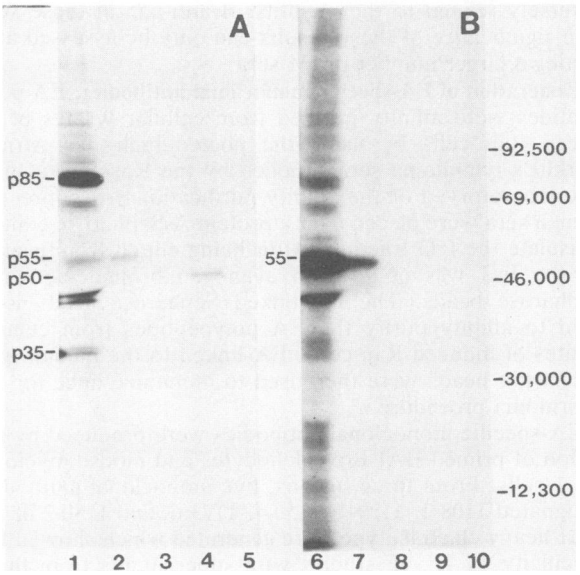

FIG. 5. Immunoprecipitation of [^{35}S]methionine- and [^{14}C]leucine-radiolabeled (A) and ^{32}P-radiolabeled (B) EA polypeptides from chemically induced Raji cells. Lanes 1 and 6, IM patient serum; lanes 2 and 7, 1129-1; lane 3 and 8, 1150-4; lanes 4 and 9, 1173-6; lanes 5 and 10, 1180-2. From these results, the EA p55 recognized by monoclonal antibody 1108-1 was shown to be a phosphoprotein. EA p85, p50, and p35 were not phosphorylated by using these methods.

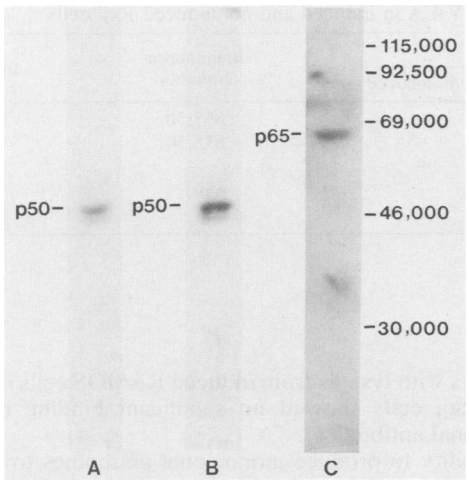

FIG. 6. Immunoblot analysis of monoclonal antibodies (A) 1173-6, (B) 1180-2, and (C) 1150-4 by using cellular lysates from chemically induced Raji cells.

Monoclonal antibody 1150-4 had no measurable activity in this assay, whereas monoclonal antibodies 1173-6 and 1180-2 had low activity (two to three times over background). S1 nuclease treatment (5,000 U/10 mg of bound DNA) for 1 h at 37°C released only a small amount of DNA (0.2 mg) from the double-stranded DNA-cellulose and did not significantly change the results (data not shown). Hence, the binding of 1129-1 and 1108-1 in the double-stranded DNA-cellulose tubes was not exclusively due to the presence of isolated single-stranded areas in these preparations. Of interest, the low activities of monoclonal antibodies 1173-6 and 1180-2 compared with those of 1108-1 and 1129-1 indicates that either p50 is dissociated from the p55;50 complex or that the antibody-binding sites of p50 are unavailable after DNA binding. Studies are presently being performed to characterize further the specificity of binding of p55;50.

DISCUSSION

Five monoclonal antibodies to the EA of EBV have been produced. IF studies have shown that these antibodies recognize nuclear antigens expressed in chemically induced Raji cells but fail to stain noninduced Raji cells and EBV-negative RAMOS cells after chemical induction. The antigens recognized by the monoclonal antibodies were resistant to methanol fixation and hence were found to be similar to those stained by anti-EA-D$^+$ IM and African Burkitt's lymphoma sera. Different fixation methods were found to dramatically alter the staining of EA by the monoclonal antibodies (Fig. 3). Paraformaldehyde-acetone-fixed preparations, which had superior preservation of cellular morphology, characteristically had bright, homogeneous or diffuse nuclear and nucleolar staining patterns, whereas acetone- and methanol-acetone-fixed cells had a less bright, coarsely granular nuclear staining reaction. Classically, the EA-D or diffuse IF staining pattern first described by Henle et al. (10) is composed of both nuclear and cytoplasmic staining. As noted by Henle et al. (10), the first evidence of D after superinfection by Raji cells appears in the nucleus and only later extends to the cytoplasm. It is therefore likely that the nuclear IF staining pattern seen with the monoclonal antibodies described in this study represents an early D reactivity.

One of the monoclonal antibodies, 1150-4, was positive by ACIF. Since its reactivity was confined to chemically induced Raji cells, 1150-4 is distinguishable from anti-EBV-associated nuclear antigen sera which stain 100% of noninduced EBV-positive cells by the same technique (28). Of interest, 1150-4 also was found to cross-react with a cellular antigen expressed in noninduced EBV-positive and -negative lymphoma cells, in which it produced a sparsely speckled nuclear pattern. The discovery of a host nuclear antigen with antigenic properties in common with EA raises intriguing questions about its possible role in transformed cells. Additional studies aimed at uncovering the specificity of binding of 1150-4 in various normal and transformed cells are presently being performed.

Immunoprecipitation experiments with metabolically labeled, induced Raji cells showed that two monoclonal antibodies, 1108-1 and 1129-1, recognize the same EA-specific polypeptides, namely, p55 and p50. These results were rather surprising since p55 and p50 are not the major polypeptides immunoprecipitated by the pooled high-titer anti-EA-D$^+$ sera (Fig. 1 and 2). Like the hyperimmune sera drawn from the mice used in the hybridoma experiments (Fig. 4), the anti-EA-D$^+$ sera were found to react predominantly with p85 and p35. My inability to generate monoclonal antibodies to these EA polypeptides may be due to the selection of IF as the initial screening procedure in the hybridoma experiments. It is possible that, unlike p55;50, p85 and p35, as well as other EA polypeptides, may be irreversibly altered by fixation, making them undetectable by present IF techniques. To test this possibility, hybridoma experiments with immunoprecipitation as the primary method of antibody screening would have to be performed.

Immunoprecipitation of ^{32}P-labeled proteins from induced Raji cells revealed that EA polypeptide p55 is a phosphopro-

TABLE 1. DNA-binding activity of EA polypeptides as measured by DNA-cellullose radioimmunoassay with monoclonal antibodies

Monoclonal antibody	Cellular lysate	DNA-binding activity (mean cpm ± SD) for:	
		Double-stranded DNA	Single-stranded DNA
1108-1	Raji, induced	1,551 ± 630	7,620 ± 663
	Raji	434 ± 86	417 ± 134
	RAMOS, induced	228 ± 61	163 ± 58
1129-1	Raji, induced	5,051 ± 1561	4,996 ± 419
	Raji	661 ± 110	602 ± 91
	RAMOS, induced	716 ± 134	476 ± 252
1150-4	Raji, induced	524 ± 56	443 ± 8
	Raji	391 ± 84	183 ± 20
	RAMOS, induced	136 ± 18	101 ± 10
1173-6	Raji, induced	840 ± 214	1,676 ± 258
	Raji	224 ± 88	597 ± 192
	RAMOS, induced	158 ± 35	196 ± 45
1180-2	Raji, induced	1,198 ± 28	1,404 ± 96
	Raji	214 ± 44	341 ± 86
	RAMOS, induced	242 ± 33	199 ± 8
NS-1	Raji, induced	386 ± 115	88 ± 30
	Raji	199 ± 24	45 ± 18
	RAMOS, induced	162 ± 63	19 ± 8

TABLE 2. Characteristics of five monoclonal antibodies to the EBV EA in induced and noninduced Raji cells

Monoclonal antibody	IF[a]		ACIF[b]		Immunoprecipitation	Immunoblot
	Raji	Raji, induced	Raji	Raji, induced		
1108-1	−	+	−	−	p55;50	p55;50
1129-1	−	+	−	−	p55;50	p55;50
1150-4	−[c]	+[c]	−	+		p65
1173-6	−	+	−	−	p50	p50
1180-2	−	+	−	−		p50

[a] Paraformaldehyde-acetone fixation.
[b] Methanol-acetone (1:1) fixation.
[c] Nuclei show sparsely speckled staining pattern.

tein. Both high-titer anti-EA$^+$ IM sera and monoclonal antibody 1129-1 immunoprecipitated ^{32}P-labeled p55 specifically (Fig. 5). Interestingly, p50 was unlabeled by this technique. Phosphotransfer reactions by the methods of Levinson et al. (22) were negative (data not shown), indicating that p55;50 does not have kinase activity. Further studies are required to determine the sites of phosphorylation in p55.

The immunoprecipitation analysis of the binding reactivities of the monoclonal antibodies was greatly complemented by the use of the immunoblot technique. Monoclonal antibodies 1150-4, 1180-2, and initially 1173-6, which gave negative results by immunoprecipitation, were found to give positive reactions by the immunoblot method (Fig. 6). Immunoblot studies showed that monoclonal antibodies 1173-6 and 1180-2 recognized only p50 despite the fact that monoclonal antibodies 1108-1, 1129-1, 1173-6, and 1180-2 all gave identical IF staining reactions in induced Raji cells. This finding was confirmed for monoclonal antibody 1173-6 by immunoprecipitation data. Monoclonal antibody 1150-4 was found to recognize a different EA with a molecular weight of 65,000 daltons. These results show that both immunoprecipitation and immunoblot techniques were necessary to characterize immunobiochemically the EA identified by these monoclonal antibodies. A summary of the immunofluorescence, immunoprecipitation, and immunoblot studies with the five monoclonal antibodies is shown in Table 2.

In previous immunoprecipitation studies on the immunobiochemical characterization of EBV-associated EA, the presence of a large number of polypeptides in metabolically labeled induced cells have been reported. Several of these studies, including those by Kallin et al. (17), Mueller-Lantzsch et al. (25), and Kawanishi et al. (18, 19), reported similar immunoprecipitation patterns with anti-EA-D$^+$ sera as our own. The use of different induction regimens by these investigators did not significantly influence the results. Of specific interest are two groups of investigators, Roubal et al. (29) and Sugawara et al. (30) studying the DNA-binding properties of EA polypeptides, which have observed that among others, 55,000- and 51,000-dalton proteins bound to DNA-cellulose. My studies confirmed the results of these earlier investigations and showed that EA p55;50 has significant DNA-binding activity. Using a newly developed DNA-cellulose radioimmunoassay technique, I demonstrated that the antigen recognized by monoclonal antibody 1108-1 preferentially bound single-stranded DNA (Table 1). Monoclonal antibody 1129-1 showed high reactivity with both double- and single-stranded DNA, and hybridoma clones 1173-6 and 1180-2 showed low reactivity with both forms of DNA. These results indicate that although these monoclonal antibodies are directed against the same polypeptide complex, they bind to different antigenic determinants which affect their binding performance in this assay. Control experiments with lysates from induced RAMOS cells or noninduced Raji cells showed no significant binding by these monoclonal antibodies.

My ability to produce monoclonal antibodies to EA was dependent upon four major factors: the use of TPA and IUdr in the induction regimen; the availability of pooled high-titer anti-EA$^+$ sera; the use of Ag-Ab-S beads as the immunogen; and the large-scale use of IF procedures for initial screening of hybridoma supernatants. The ability to induce in a consistent manner 60% of Raji cells by TPA and IUdr was an important first step in these investigations. Along with the availability of high-titer anti-EA$^+$ sera, the high induction rate greatly enhanced the affinity purification of EA-specific polypeptides. The use of Ag-Ab-S beads for the immunization of the mice was a novel approach which greatly simplified the preparation of the immunogen. Since harsh reagents were not used to dissociate the antigen-antibody complexes, the antigenicity of the affinity-purified proteins was not compromised, and the time required to prepare the immunogen was significantly reduced. Immunoprecipitation analysis of sera obtained from the primed mice revealed that this method was a potent immunization procedure (Fig. 4), although antibodies to cellular antigens unrelated to EBV were also induced by these procedures. These results suggest that this method of immunization may have wide applicability to the generation of monoclonal antibodies. Finally, the large-scale use of IF procedures for the identification of intracellular antigens facilitated the search for EA-specific monoclonal antibodies. This procedure has been used in my laboratory to generate monoclonal antibodies to other nuclear proteins expressed in human leukemia and lymphoma cells (Epstein and Clevenger, in press). The availability of monoclonal antibodies specific for EA polypeptides will enable a more detailed immunobiochemical characterization and the purification of these viral proteins. Elucidation of the chemical nature and role of EA polypeptides in infected cells may provide important information on the pathogenicity and transforming capability of EBV.

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