JOURNAL OF VIROLOGY, July 1983, p. 193–201
0022-538X/83/070193-09$02.00/0

Vol. 47, No. 1

Identification of Polypeptide Components of the Epstein-Barr Virus Early Antigen Complex with Monoclonal Antibodies

GARY R. PEARSON,[1]* BEN VROMAN,[1] BOB CHASE,[1] TOM SCULLEY,[1] MARY HUMMEL,[2] AND ELLIOTT KIEFF[2]

Sections of Microbiology and Biochemistry, Department of Cell Biology, Mayo Clinic/Mayo Foundation, Rochester, Minnesota 55905,[1] and Departments of Medicine and Microbiology, Kovler Viral Oncology Laboratories, University of Chicago, Chicago, Illinois 60637[2]

Received 30 December 1982/Accepted 12 April 1983

Three monoclonal antibodies were produced against the Epstein-Barr virus-induced early antigen complex. These antibodies were shown to be specific for the early antigen complex by the fact that they only reacted with cells supporting a permissive or abortive Epstein-Barr virus infection and their synthesis was not affected by inhibitors of viral DNA synthesis. One monoclonal antibody, designated R3, was directed against a diffuse component of the early antigen complex since it reacted by immunofluorescence with cells fixed in acetone or methanol. The other two monoclonal antibodies, designated K8 and K9, reacted with a methanol-sensitive restricted component of this complex. The appearance of the R3 antigen in P_3HR-1 superinfected Raji cells occurred approximately 4 h earlier than the antigen detected by K8. By both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and radioimmunoelectrophoresis, it was determined that the R3 monoclonal antibody recognized two major polypeptides with molecular weights of approximately 50,000 to 52,000, whereas K8 and K9 precipitated a protein of approximately 85,000. The R3 monoclonal antibody also immunoprecipitated an in vitro primary translation product. It was, therefore, possible to map this product to the Epstein-Barr virus DNA *Bam*HI M fragment. These in vitro products were slightly smaller than the in vivo proteins, suggesting that these proteins probably undergo posttranslational modification during the virus replication cycle.

A number of antigen complexes have been identified in Epstein-Barr virus (EBV)-infected cells, mainly by immunofluorescence procedures. These have been designated EBNA for the EBV-induced nuclear antigen, VCA for viral-capsid antigens, MA for cell membrane-associated viral antigens, and EA for EBV-induced early antigens (25). EBNA is a viral-specified nuclear antigen expressed in all latently infected cell lines such as Raji (30). VCA and MA are late viral proteins expressed in permissively infected cells, whereas EA is synthesized early in abortive or permissive infections (25). EA can also be induced in latently infected cell lines by superinfection with P_3HR-1 virus or by treatment with chemical inducers (1, 10, 13, 15, 18, 34, 36). The synthesis of EA is not affected by treating cultures with inhibitors of DNA synthesis, whereas VCA and MA production is inhibited by such treatment (7, 11, 12, 24, 35). The EA complex has been divided further into diffuse (D) and restricted (R) components based on staining patterns with different human sera (14). Antibody to EA(D) causes diffuse staining of the nucleus and cytoplasm. This antigen is expressed in both acetone and methanol-fixed cells. In contrast, EA(R) staining is restricted to the cytoplasm in acetone-fixed cells and is denatured by methanol (14). Although most, if not all, of these antigens are now recognized to be complexes, little is known about the polypeptides that compose each of these antigens.

The EA complex has been of particular interest since antibodies to this complex are frequently present at high titers in patients with EBV-associated diseases as opposed to latently infected but nondiseased control populations (25). The anti-EA reactivity in the sera of patients with infectious mononucleosis and nasopharyngeal carcinoma is directed primarily against the D component, whereas reactivity in sera of patients with African Burkitt's lymphoma is directed mainly against the R component (14). In addition, antibodies to the EA complex are of prognostic importance in patients with EBV-associated malignancies since titers tend to vary with disease course.

A number of candidate polypeptide compo-

nents of EA(D) and EA(R) have been identified by their synthesis early in permissive EBV infections, by the demonstration that their synthesis is not affected by inhibitors of EBV DNA replication, and by their specific precipitation with human sera known to have high antibody titers to EA(D) or EA(R). Early polypeptides with molecular weights ranging from 150,000 (150K) to 165K, 138K to 140K, 120K to 134K, 85K to 103K, 50K to 65K, and 26K to 40K have been identified with anti-EA-positive human sera (2, 3, 6–8, 18–20, 22, 23, 31, 33, 34). However, since these sera also contained antibody reactive with other EBV-specific antigens, no specific polypeptide could be conclusively correlated with EA as defined with immunofluorescence assays. Several of the candidate EA polypeptides have been tentatively mapped to restriction endonuclease fragments of EBV DNA by hybrid selection of early RNA and by demonstrating that the RNA encodes for a polypeptide which is immunoreactive with polyvalent anti-EA serum and is similar in size to a candidate EA polypeptide immunoprecipitated from infected cell extracts (17). Sera specific for individual polypeptides were not available, however, to permit more definitive correlation of infected cell polypeptides with primary in vitro translation products.

We now report the development of monoclonal antibodies reactive with major polypeptides of the EA complex. One of these antibodies is directed against a D component, whereas two other monoclonal antibodies are reactive with an R component. The anti-D monoclonal antibody immunoprecipitated an in vitro primary translation product similar in size to the in vivo protein, thereby making it possible to map this antigen to a specific EBV DNA fragment.

MATERIALS AND METHODS

Cell lines. The lymphoblastoid cell lines used in these experiments include the EBV genome-positive Raji, P_3HR-1, and B-95-8 cell lines and the EBV genome-negative Ramos and BJAB cell lines. All of these cell lines were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum and gentamicin (50 μg/ml). The cells were passaged every 3 to 4 days by reseeding in fresh medium at a concentration of 5×10^5 cells per ml.

Production of monoclonal antibodies to EA. BALB/c mice were hyperimmunized with P_3HR-1 cells activated with 40 ng of tumor-promoting agent (TPA) per ml for 48 h or with P_3HR-1 virus-superinfected Raji cells. Such cultures routinely contained 30 to 40% EA-positive cells. The initial immunization included 20×10^6 cells in complete Freund adjuvant injected intraperitoneally. Three and 5 weeks later, these mice were inoculated intraperitoneally with 20×10^6 cells without adjuvant. Spleens were removed for the production of hybridomas 4 days after the third immunization.

For the production of hybridomas producing antibodies to EBV antigens, 80×10^6 to 100×10^6 spleen cells were fused with an equal number of cells from the mouse plasmacytoma cell line P_3NS-11-AG4-1(NS-1) as previously described (28) with some modifications. Briefly, the cell mixtures were pelleted at $900 \times g$ for 5 min, and the cells were washed one time in medium without fetal calf serum. Medium was drained from the tubes, and the pellets were gently suspended in 0.5 ml of 30% polyethylene glycol 1500 (J. T. Baker Chemical Co., Phillipsburg, N.J.) in medium for 6 min at 37°C. The cells were then diluted with 15 ml of serum-free medium and again centrifuged at $900 \times g$ for 5 min. The cell pellets, suspended to a concentration of 1×10^6 to 2×10^6 cells per ml in 40 ml of HAT medium (0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine in RPMI 1640 medium supplemented with 4.5 g of glucose per liter, 10 ml of 200 mM glutamine, 20% fetal calf serum, and 10% NCTC 135 [GIBCO Laboratories, Grand Island, N.Y.]), were transferred to 75-cm^2 tissue culture flasks and incubated for 48 h at 37°C. The cells were then plated into 96-well microtiter plates at a concentration of 2.0×10^5 to 2.5×10^5 cells per well. Peritoneal mouse macrophages were added as feeder layers. Cultures were refed every 3 days with HAT medium and monitored for the presence of growing clones. Supernatant fluids from wells with visible growth were assayed for antibodies to EBV antigens by immunofluorescence. Positive cultures were cloned and the subclones rescreened for antibody production. Positive clones were grown in Falcon flasks and injected into mice primed with 0.3 ml of pristane for the production of antibody-containing ascitic fluid. The immunoglobulin subclass produced by each clone was determined by radial immunodiffusion.

Immunofluorescence assays. Culture supernatants were screened for antibodies to EBV antigens by immunofluorescence against acetone-fixed smears of TPA-activated (40 ng/ml) virus-producing cell lines and against EBV genome-positive nonproducer and virus-negative cell lines as previously described (26). If positive, they were screened against VCA-negative, EA-positive cells to identify those monoclonal antibodies directed against EA components. EA-positive, VCA-negative cell smears were fixed in both methanol and acetone to distinguish those antibodies directed against the D component of the EA complex (methanol resistant) from those directed against the R component (methanol sensitive).

Cell extraction and immune precipitation. Viable B-95-8 cells at a concentration of 2×10^6 per ml were incubated in the presence of 40 ng of TPA per ml or 40 ng of TPA plus 150 μg of phosphonoacetic acid (PAA) per ml for 24 h. The cells were then centrifuged at $900 \times g$ for 15 min, washed once with methionine-free medium, and suspended in methionine-free medium containing TPA or TPA plus PAA at a concentration of 2×10^6 cells per ml. [^{35}S]methionine was then added to each flask at a final concentration of 50 μCi/ml, and the cultures were incubated at 37°C for 18 h. The cells were then washed three times in medium and extracted with 0.5% Nonidet P-40 in 0.02 M Tris–0.3 M NaCl, 1.0 mM $CaCl_2$–0.5 mM $MgCl_2$–2 mM EDTA–10% glycerol–2.0 mM phenylmethylsulfonyl fluoride, pH

TABLE 1. Reactivity of different cell lines with anti-EA monoclonal antibodies

Monoclonal antibody[a]	Reactivity						
	Raji	Activated Raji[b]	P_3HR-1-Raji[c]	P_3HR-1	P_3HR-1 + PAA[d]	Ramos	BJAB
R3[e]	−	+	+	+	+	−	−
K8[f]	−	+	+	+	+	−	−
K9[f]	−	+	+	+	+	−	−

[a] Ascitic fluids containing monoclonal antibodies were diluted 1:100 or 1:1,000 before testing in immunofluorescence assays.

[b] Raji cells activated in the presence of 20 ng of TPA per ml and 3 mM sodium butyrate for 48 to 72 h. Approximately 10% of the cells were positive for EA as determined with human reference sera.

[c] P_3HR-1 virus-superinfected Raji cells.

[d] Cultures cultivated in the presence of 150 μg of PAA per ml for 72 h. These cultures were negative for VCA as determined with human reference sera.

[e] When positive, this monoclonal antibody was positive on cells fixed in acetone or methanol.

[f] When positive, these monoclonal antibodies were positive on cells fixed in acetone but negative on cells fixed in methanol.

9.0 (21). For radioimmunoprecipitation, portions of the extracts containing 2.5×10^6 cpm of ^{35}S were incubated overnight at 4°C with 0.2 ml of a 1:50 dilution of the appropriate antibody-positive or antibody-negative ascitic fluid or with human control sera diluted in the extraction buffer. Immune complexes were removed by precipitation with protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) (29). The immune complexes were then analyzed for the presence of EBV-specific polypeptides by polyacrylamide gel electrophoresis, using a 7.5 to 15% exponential gradient and a 3% stacking gel (29). The ^{14}C-labeled proteins used as molecular weight standards were myosin (210,000), phosphorylase B (92,500), bovine serum albumin (67,000), ovalbumin (45,000), and carbonic anhydrase (30,000) (New England Nuclear Corp., Boston, Mass.).

Radioimmunoelectrophoresis. Samples of Raji, Ramos, and TPA-activated B-95-8 cells were solubilized in 2% wt/vol sodium dodecyl sulfate (SDS)–1% (vol/vol) 2-mercaptoethanol–0.1 mM phenylmethylsulfonyl fluoride–10 mM sodium phosphate, pH 6.8. Approximately 20 μl (150 μg of protein) of each preparation was then subjected to electrophoresis in 10% (wt/wt) polyacrylamide–SDS slab gels. Electrophoresis was at 100 V for 16 h at 0 to 4°C. Electrophoretic transfer of protein from SDS-polyacrylamide gels to nitrocellulose paper was performed according to Burnette (5). Radioiodinated or alkaline phosphatase-labeled rabbit antibody to mouse immunoglobulin G (IgG) was used to identify immune reactions between the monoclonal antibodies and viral proteins on the nitrocellulose paper. For molecular weight determinations, standard proteins were also electrophoresed, transferred to nitrocellulose paper, and identified by staining with Coomassie blue.

Hybrid selection, in vitro translation, and immunprecipitation. Cytoplasmic, polyadenylated RNA was purified (16) from B-95-8 cells 3 days after induction with TPA (20 ng/ml) and sodium butyrate (3 mM). DNA fragment-specific RNA was selected by hybridizing the RNA at a concentration of 1 mg/ml for 4 h at 50°C to EBV DNA fragments covalently bound to diazobenzyloxymethyl-paper (32) in 50% recrystallized formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.5–0.8 M NaCl–0.5% SDS–2 mM EDTA (17). Unbound RNA was removed by washing the filters five times for 5 min at 50°C in 50% formamide–40 mM PIPES, pH 6.5–15 mM NaCl–0.5% SDS. EBV-specific RNA was eluted at 70°C with 1.5 ml of 99% formamide–10 mM PIPES, pH 6.5–13.3 μg of tRNA (Boehringer Mannheim Corp., New York, N.Y.) per ml. The eluted RNA was diluted with two volumes of water, adjusted to 0.2 M potassium acetate, and precipitated with ethanol at −20°C. The RNA was translated in vitro in a rabbit reticulocyte lysate (New England Nuclear) with [^{35}S]methionine at 37°C for 60 min. The translation reaction was adjusted to 0.14 M NaCl–20 mM Tris, pH 7.4–1% Nonidet P-40–0.5% deoxycholate–1 mg of ovalbumin per ml and preabsorbed twice with EBV-negative serum and staphylococcal protein A-Sepharose (Pharmacia). EBV antigens were immunoprecipitated by incubating the preabsorbed lysate with 4 μl of antiserum or with 1 μl of H126, a human antiserum with high titers to EBV antigens (VCA, 1:40, 960; EA, 1:10, 240; EBNA, 1:160) at 4°C for 18 h. Antigen-antibody complexes were removed with protein A-Sepharose, washed, eluted with SDS gel sample buffer, and electrophoretically separated on 10% polyacrylamide gels. The gels were stained, destained, impregnated with En^{3}Hance (New England Nuclear), dried, and subjected to fluorography. Molecular weights were assigned by migration of the polypeptides relative to unlabeled molecular weight markers (myosin; 210K; β-galactosidase, 116K; phosphorylase B, 92.5K; bovine serum albumin, 67K; ovalbumin, 45K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 21.5K; lysozyme, 14.4K; Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Production of monoclonal antibodies to EA components. Three clones designated R3, K8, and K9 were identified that were producing antibodies to EBV-induced intracellular antigens. None of these reacted with the EBV genome-negative Ramos or BJAB cells or with the EBV genome-positive, latently infected Raji cell line (Table 1). They all reacted, however, with the virus-producing, TPA-activated P_3HR-

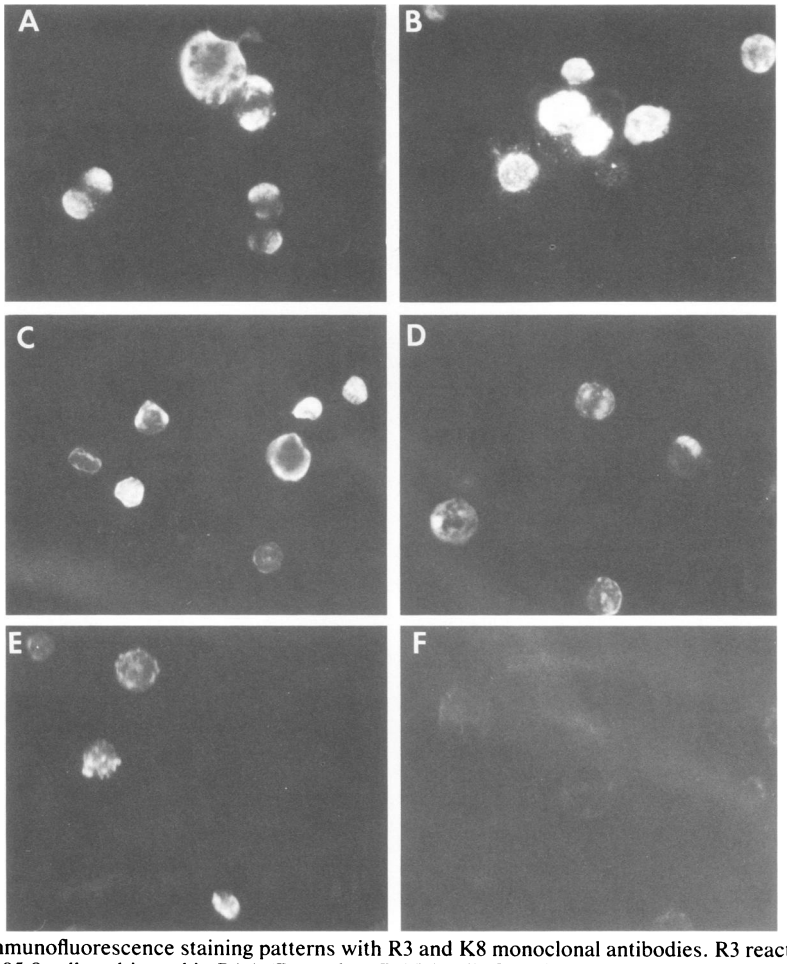

FIG. 1. Immunofluorescence staining patterns with R3 and K8 monoclonal antibodies. R3 reactivity on B-95-8 cells (A), B-95-8 cells cultivated in PAA (B), and on B-95-8 cells fixed in methanol (C); K8 reactivity on B-95-8 cells (D), B-95-8 cells cultured in the presence of PAA (E), and B-95-8 cells fixed in methanol (F) (×425).

1 cell line, establishing that they were directed against antigens produced during the virus replication cycle. Similar results were obtained with the B-95-8 cell line (data not shown). They also reacted with acetone-fixed smears of EA-positive, VCA-negative P_3HR-1 virus-superinfected or TPA-and-sodium butyrate-activated Raji cells and TPA-activated P_3HR-1 cells cultivated in the presence of PAA, thereby establishing that the antigens were related to the EA complex (Table 1). In addition, the R3 antibody reacted with the methanol-fixed EA-positive, VCA-negative cells, suggesting that this antibody was directed against a D component of the EA complex. In contrast, K8 and K9 appeared to be directed against an R component since these antibodies did not react with cells fixed in methanol. None of these antibodies reacted with membrane antigen-positive viable cells, indicating that they were not directed against viral-induced membrane components (data not shown). Immunoglobulin typing of the antibodies produced by these clones showed that the R3 antibody was of the IgG1 class, whereas the K8 and K9 antibodies were both of the IgG2a class.

Photographs showing the immunofluorescence with the R3 and K8 monoclonal antibodies on fixed cells are presented in Fig. 1. The R3 antibody reacted primarily with the nuclei of acetone-fixed TPA-activated B-95-8 cells (Fig. 1A). Reactivity was not inhibited by culturing the cells in medium containing PAA (Fig. 1B) or by fixation of the cells in methanol (Fig. 1C). The staining pattern of the same cells was quite different with the K8 and K9 monoclonal antibodies. The reaction with acetone-fixed cells was largely cytoplasmic and much less intense (Fig. 1D and E). There was no reaction with methanol-fixed cells (Fig. 1F).

Figure 2 shows the kinetics of appearance of

the antigens detected by the R3 and K8 monoclonal antibodies after superinfection of Raji cells with P_3HR-1 virus. The R3 antibody reacted with less than 1% of the infected cells until about 8 h postinfection. At that time, the percentage of antigen-positive cells increased to 13%. Approximately 20% of the cells were positive at 12 h, 35% at 16 h, and 40% at 20 h postinfection. In contrast, the K8 monoclonal antibody reacted with less than 1% of the cells until 12 h postinfection. Approximately 10% of the cells were positive at 12 h, and the percentage increased to 30% at 16 h and approximately 35% at 20 h postinfection.

Immune precipitation of ^{35}S-labeled cell extracts from TPA-activated B-95-8 cells. To identify the EBV-specific polypeptide reactive with these monoclonal antibodies, immune precipitates formed after the reaction of each of these monoclonal antibodies with ^{35}S-labeled extracts prepared from TPA-activated B-95-8 cells cultivated in the presence or absence of PAA were analyzed by exponential SDS-polyacrylamide gel electrophoresis (Fig. 3). The R3 monoclonal antibody precipitated two polypeptides, with sizes of approximately 50K and 52K from both the PAA-treated and non-PAA-treated B-95-8 cell extracts (Fig. 3A and B, lane e). The K8 and K9 monoclonal antibodies both precipitated a protein with a molecular weight of approximately 85K from both extracts (Fig. 3A and B, lanes f and g). A human anti-EA (both D and R) positive control serum precipitated the 50K, 52K, and 85K polypeptides from both extracts (Fig. 3A and B, lane a), confirming that these were major EA components. This serum also precipitated 140K, 150K, and 320K polypeptides from the TPA-activated B-95-8 extract (Fig. 3A, lane a)

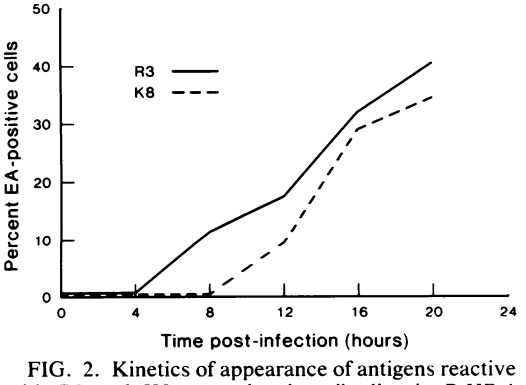

FIG. 2. Kinetics of appearance of antigens reactive with R3 and K8 monoclonal antibodies in P_3HR-1 virus-superinfected Raji cells as determined by immunofluorescence. Cells were reacted with each monoclonal antibody at 4-h intervals up to 20 h postinfection. Both monoclonal antibodies had titers of approximately 5,000 by immunofluorescence.

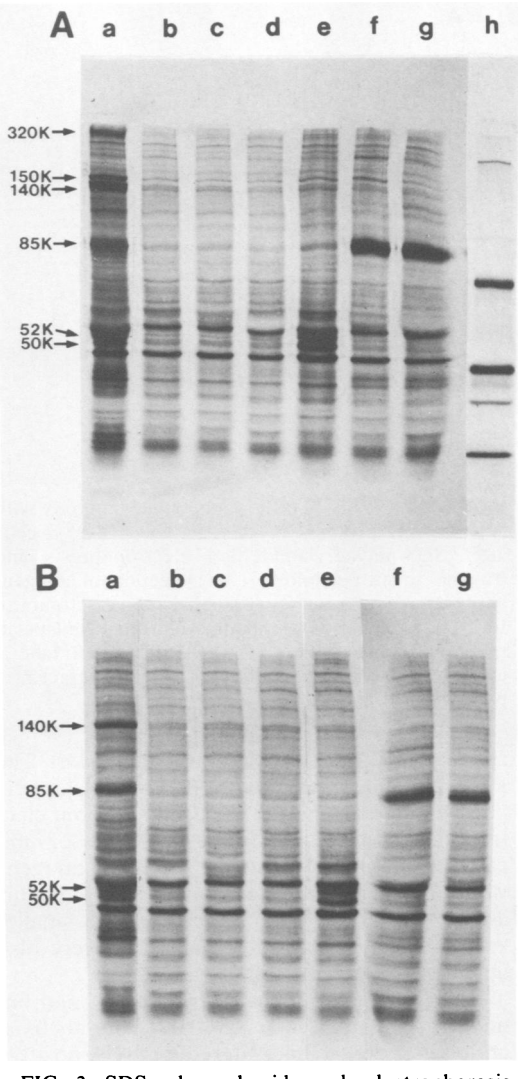

FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of proteins precipitated by R3, K8, and K9 monoclonal antibodies from [^{35}S]methionine-labeled TPA-activated B-95-8 cells (A) or activated B-95-8 cells cultured in the presence of PAA (B). Lane a, anti-VCA-positive, anti-EA-positive human control serum; lanes b and c, antibody-negative human sera; lane d, antibody-negative mouse ascites; lane e, R3 monoclonal antibody; lane f, K8 monoclonal antibody; lane g, K9 monoclonal antibody; lane h, standards.

and the 140K protein from the extract of cells cultivated in the presence of PAA (Fig. 3B, lane a), indicating that the 140K protein was also "early." None of these proteins were precipitated by two human sera negative for antibodies to EBV (Fig. 3A and B, lanes b and c) or by antibody-negative mouse ascitic fluid (Fig. 3A and B, lane d). In addition, none of these proteins were precipitated from Raji cells in the

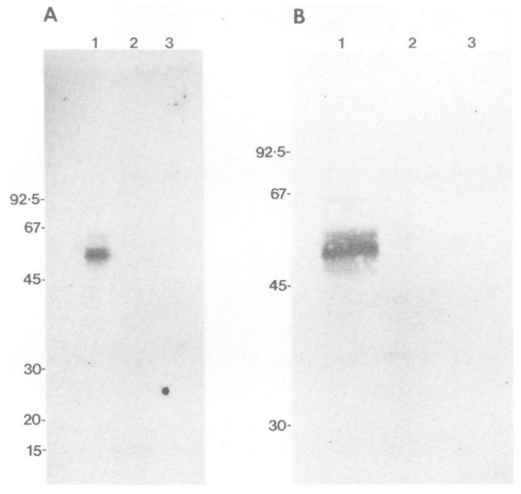

FIG. 4. Reactivity of R3 monoclonal antibody with 50K to 52K proteins in TPA-activated B-95-8 cells after SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose. (A) Detection of antigen-antibody reaction with ^{125}I-labeled rabbit anti-mouse IgG. (B) Detection with alkaline phosphatase-labeled rabbit anti-mouse IgG. Lane 1, B-95-8 extract; lane 2, extract prepared from Raji cells; lane 3, extract prepared from Ramos cells.

absence of superinfection (data not shown). The R3 antibody reacted with the 50K and 52K polypeptides even after SDS denaturation, electrophoresis, and transfer to nitrocellulose, indicating that the determinants on these antigens were at least partially resistant to SDS denaturation (Fig. 4). Two minor bands, one smaller than 50K and one larger than 52K, were also detected by this method.

Hybrid selection, in vitro translation, and immunoprecipitation. The K8 and K9 monoclonal antibodies and a high-titered anti-EA-positive human serum did not immunoprecipitate an 85K polypeptide from in vitro translation products of B-95-8 polyadenylated RNA. A smaller polypeptide, however, was precipitated by these antibodies. The 85K protein could, therefore, be a post-translational modification of this smaller polypeptide. Because of the uncertain relationship of the smaller polypeptide to the 85K protein, the DNA encoding for the smaller polypeptide was not mapped. The R3 antibody did, however, recognize polypeptides of 44K and 47K translated in vitro from B-95-8 polyadenylated RNA (Fig. 5, lane marked B-95 RNA, R3 imm.). To identify the EBV DNA fragments which encode for these polypeptides, RNA from productively infected cells was hybrid selected with EBV DNA fragments and translated in vitro, and the translation products were immunoprecipitated with the R3 antibody. Polypeptides of this size had previously been mapped to the EBV DNA *Bam*HI M and I fragments (17). These fragments were therefore used for hybrid selection. Figure 5 shows the results of in vitro translations of total polyadenylated RNA, tRNA (a negative control), and the RNAs eluted from EBV DNA *Bam*HI M or I filters. The interpretation of the results was complicated by the fact that a 47K protein was translated by reticulocyte extract to which only tRNA was added. This polypeptide was endogenous to the reticulocyte lysate since it was not immunoprecipitable with negative serum (see lane marked B-95-8 RNA, NRS imm.) or with the R3 serum (see lane marked *Bam*HI I, R3 imm.). The R3 antibody, however, specifically immunoprecipitated 47K

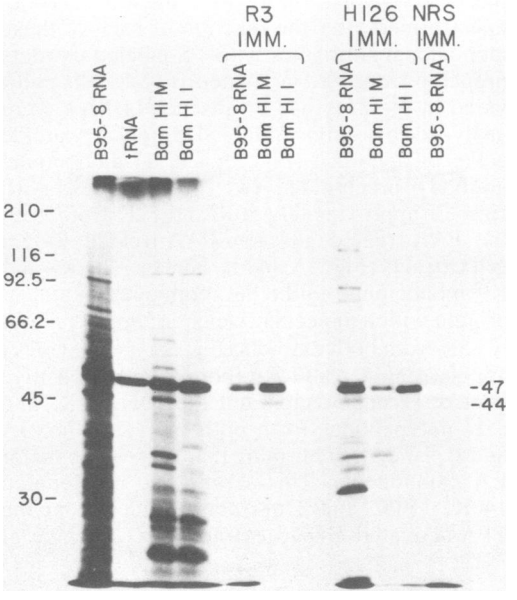

FIG. 5. Mapping the R3 antigen. Autoradiograms of 10% polyacrylamide gels of polypeptides translated from RNA and immunoprecipitated with various antisera. The RNA added to the in vitro translation is indicated above the lane: B-95-8 RNA is total cytoplasmic, polyadenylated RNA from B-95-8 cells; tRNA is tRNA added instead of mRNA to show products endogenous to the lysate; and *Bam*HI I and *Bam*HI M are RNAs hybrid selected by the EBV DNA *Bam*HI M or I DNA fragments. Immunoprecipitates (IMM.) are indicated by brackets, and the antisera are shown above. R3 is a monoclonal antibody specific for a D component of early antigen; H126 is a human antiserum with high titers to many EBV antigens; and NRS is normal rabbit serum used as an EBV-negative control. In vitro translation products were immunoprecipitated with R3 after preabsorption with NRS, and with H126 after immunoprecipitation with R3. Numbers to the left (in kilodaltons) indicate the position of molecular weight markers; numbers to the right indicate the molecular weights of polypeptides encoded by the *Bam*HI M and I fragments.

and 44K polypeptides translated from *Bam*HI M-selected RNA (see lane marked *Bam*HI M, R3 imm.). The 47K *Bam*HI M-encoded polypeptide was more abundant than the 44K polypeptide. The polypeptides in the in vitro translations which did not react with the R3 antiserum were immunoprecipitated with H126, a human serum with high antibody titers to EA and VCA (see lane marked *Bam*HI M, H126 imm.). Immunoprecipitation of the *Bam*HI M polypeptides with H126 showed that some of the R3 antigen remained after immunoprecipitation with R3 serum and that *Bam*HI M also encoded for an antigen of 37K. Polypeptides of 47K and 28K encoded by *Bam*HI I were not immunoprecipitated with the R3 antiserum but were immunoprecipitated by H126 (see lane marked *Bam*HI I, H126).

Since the polypeptides precipitated from labeled cells by R3 antibody seemed to be larger than those precipitated from in vitro translations, both immunoprecipitates were run on the same gel for direct comparison (Fig. 6). The major in vitro translation products were smaller than the in vivo proteins, which ranged in size from 50K 55K. With longer autoragiographic exposure, minor polypeptides similar in size to the in vitro translation product could be detected in the gel of the in vivo immunoprecipitate.

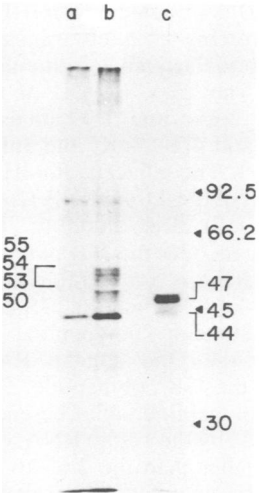

FIG. 6. Comparison of the R3 immune precipitate of polypeptides made in vivo with that made in vitro. Extract of B-95-8 cells labeled with [^{35}S]methionine in vivo immunoprecipitated with normal rabbit serum (lane a) or R3 (lane b) or an in vitro translation of *Bam*HI M-specific RNA immunoprecipitated with R3 (lane c). The bracket indicates a series of R3 polypeptides ranging in molecular weight from 50 to 55 kilodalton immunoprecipitated from the cell extract labeled in vivo (lane b). The 47- and 44-kilodalton R3 polypeptides encoded by *Bam*HI M are shown in lane c. Triangles indicate the position of molecular weight markers in the 10% polyacrylamide gel.

DISCUSSION

Previous studies on the composition of EA complex have utilized human sera which reacted with a broad spectrum of EBV antigens produced in permissive EBV infections. It has, therefore, not been possible to conclusively associate a specific polypeptide component with the antigens detected in fixed cells by immunofluorescence. Human sera with high antibody titers to EA(R) precipitated several polypeptides, including the 85K polypeptide detected with the K8 and K9 monoclonal antibody, whereas sera with anti-EA(D) reactivity precipitated many of the same proteins but also new polypeptides with molecular weights between 50K and 55K (2, 3, 6–8, 18–20, 22, 23, 31, 33, 34). These data suggested that the 85K protein was a component of EA(R) and that the 50K to 55K polypeptides were components of EA(D). The results from the investigations with the K8 and K9 monoclonal antibodies conclusively demonstrate that the 85K protein has the characteristics of EA(R), which have been defined by immunofluorescence (14). It is a methanol-sensitive antigen restricted to the cytoplasm. In contrast, the R3 monoclonal antibody detects major proteins with molecular weights of 50K and 52K which are methanol resistant and largely intranuclear. These are distinguishing characteristics of EA(D). Other early EBV proteins might also share these properties and, therefore, also be part of the EA(D) and EA(R) antigen complexes.

The 50K to 52K proteins appeared in the nucleus of cells within hours of induction of a permissive EBV infection, whereas the 85K protein appeared somewhat later in the cytoplasm. This is consistent with earlier immunofluorescence studies, which indicated that EA(D) was synthesized before EA(R) (14). The nuclear site of the 50K to 52K polypeptides suggests that it might be involved in an early stage of viral DNA or RNA synthesis. The difference in size between the intracellular 50K to 52K protein and the primary in vitro translation products reported in this paper and the inability to detect an 85K in vitro translation product with monoclonal antibodies which recognize this protein suggests that both proteins undergo post-translational modification. This is supported by findings by B. Kallin and co-workers which indicate that the 50K to 52K polypeptides are phosphorylated in vivo (personal communication). In addition, preliminary results in our laboratory suggest that the 85K protein is ADP ribosylated.

The fact that the R3 antibody recognizes a shared determinant present on the 47K and 44K in vitro translation products encoded by the

same EBV DNA fragment (*Bam*HI M) indicates that both proteins are likely translated from the same nucleotide sequence in the identical reading frame. The EBV *Bam*HI M fragment encodes 4.0-, 2.65-, and 1.9-kilobase (kb) early RNAs (16) and 47K, 44K, and 36K early polypeptides (17). The adjacent *Bam*HI Z fragment encodes part of the 4.0-kb RNA (16) and the 36K polypeptide (17). It is, therefore, likely that the 4.0-kb RNA encodes the 36K polypeptide. Since the 2.65-kb RNA is 5- to 10-fold more abundant than the 1.9-kb RNA and the 47K polypeptide is the most abundant polypeptide encoded by *Bam*HI M, then the 2.65-kb RNA probably encodes for the 47K polypeptide. The less abundant 44K polypeptide could be encoded by the 2.65-kb RNA, by the 1.9-kb RNA, or by an RNA 60 bases smaller than the 2.65-kb RNA, which would not be detected as a separate RNA in Northern blot analysis. Variable initiation or termination of a single RNA has been observed with herpes simplex virus and adenovirus (4, 9, 27).

The R3, K8, and K9 monoclonal antibodies will facilitate purification of the 85K and 50K to 52K proteins and studies of their intracellular interactions and function. They should also be useful for the development of immunological assays for detecting antibody responses against these specific polypeptides. Measurement of antibody to specific EA polypeptides might prove to be more discriminating between individuals with and without EBV-associated diseases than the current assays and therefore could be of importance in the diagnosis of primary and premalignant EBV infections.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grants CA20679, CA19264, and CA17281 from the National Cancer Institute; grant MV32G from the American Cancer Society; and a grant from Biotech Research Laboratories, Inc., Rockville, Md. E.K. is a faculty research awardee of the American Cancer Society. M.H. is supported by U.S. Public Health Service grant 07182-04.

LITERATURE CITED

1. **Bayliss, G. J., and M. Nonoyama.** 1978. Mechanisms of infection with Epstein-Barr virus. 3. The synthesis of proteins in superinfected Raji cells. Virology **87**:204–207.
2. **Bayliss, G. L., and H. Wolf.** 1981. The regulated expression of Epstein-Barr virus. 3. Proteins specified by EBV during the lytic cycle. J. Gen. Virol. **56**:105–118.
3. **Bodemer, W. W., W. C. Summers, and J. C. Niederman.** 1980. Detection of virus-specific antigens in EB-(P_3HR-1) virus-superinfected Raji cells by immunoprecipitation. Virology **103**:340–349.
4. **Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, P. J. van den Elsen, A. J. van der Eb, and H. van Ormondt.** 1981. The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell **27**:121–131.
5. **Burnette, W. N.** 1981. "Western blotting:" electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
6. **Feighny, R. J., M. P. Farrell, and J. S. Pagano.** 1980. Polypeptide synthesis and phosphorylation in Epstein-Barr virus-infected cells. J. Virol. **34**:455–463.
7. **Feighny, R. J., B. E. Henry, and J. S. Pagano.** 1981. Epstein-Barr virus polypeptides: effect of inhibition of viral DNA replication on their synthesis. J. Virol. **37**:61–71.
8. **Feighny, R. J., B. E. Henry, and J. S. Pagano.** 1981. Epstein-Barr virus polypeptides identification of early proteins and their synthesis and glycosylation. J. Virol. **39**:651–655.
9. **Frink, R., K. Anderson, and E. Wagner.** 1981. HSV-1 *Hin*dIII fragment L encodes spliced and complementary mRNA species. J. Virol. **39**:559–572.
10. **Gerber, P.** 1972. Activation of Epstein-Barr virus by 5'-bromodeoxyuridine in virus-free human cells. Proc. Natl. Acad. Sci. U.S.A. **69**:83–85.
11. **Gergely, L., G. Klein, and I. Ernberg.** 1971. Appearance of Epstein-Barr virus-associated antigens in infected Raji cells. Virology **45**:10–21.
12. **Granlund, D. J., and G. R. Pearson.** 1977. Membrane antigen expression in Epstein-Barr virus-infected Raji cells in the presence of phosphonoacetic acid. Virology **83**:217–220.
13. **Hampar, B., J. Derge, L. Martos, M. Tagemets, S. Chang, and M. Chakrabarty.** 1973. Identification of a critical period during the S phase for activation of the Epstein-Barr virus by 5-iododeoxyuridine. Nature (London) New Biol. **244**:214–217.
14. **Henle, G., W. Henle, and G. Klein.** 1971. Demonstration of two distinct components in the early antigen complex of Epstein-Barr virus-infected cells. Int. J. Cancer **8**:272–278.
15. **Henle, W., G. Henle, B. A. Zajac, G. Pearson, R. Waubke, and M. Scriba.** 1970. Differential reactivity of human serums with early antigens induced by Epstein-Barr virus. Science **169**:188–190.
16. **Hummel, M., and E. Kieff.** 1982. Epstein-Barr virus RNA. VIII. Viral RNA in permissively infected B95-8 cells. J. Virol. **43**:262–272.
17. **Hummel, M., and E. Kieff.** 1982. Mapping of polypeptides encoded by the Epstein-Barr virus genome in productive infection. Proc. Natl. Acad. Sci. U.S.A. **79**:5698–5702.
18. **Kallin, B., J. Luka, and G. Klein.** 1979. Immunochemical characterization of Epstein-Barr virus (EBV)-associated early and late antigens in n-butyrate treated P_3HR-1 cells. J. Virol. **32**:710–716.
19. **Kawanishi, M., and Y. Ito.** 1982. Similarity of Epstein-Barr virus early polypeptides induced by various tumor promoters. Cancer Lett. **16**:19–23.
20. **Kawanishi, M., K. Sugawara, and Y. Ito.** 1981. Epstein-Barr virus polypeptides: a comparative study with superinfected Raji, IUdR-treated and n-butyrate-treated P_3HR-1 cells. Virology **109**:72–81.
21. **Michelson, S., F. Horodniceanu, M. Kress, and M. Tardy-Panit.** 1979. Human cytomegalovirus-induced immediate early antigens: analysis in sodium dodecyl sulfate polyacrylamide gel electrophoresis after immunoprecipitation. J. Virol. **32**:259–267.
22. **Mueller-Lantzsch, N., B. Georg, N. Yamamoto, and H. zur Hausen.** 1980. Epstein-Barr virus induced proteins. 3. Analysis of proteins from P_3HR-1 EBV superinfected NC37 cells by immunoprecipitation. Virology **102**:231–233.
23. **Mueller-Lantzsch, N., N. Yamamoto, and H. zur Hausen.** 1979. Analysis of early and late Epstein-Barr virus associated polypeptides by immunoprecipitation. Virology **97**:378–387.
24. **Nyormoi, O., D. A. Thorley-Lawson, J. Elkington, and J. L. Strominger.** 1976. Differential effect of phosphonoacetic acid on the expression of Epstein-Barr viral antigens and virus production. Proc. Natl. Acad. Sci. U.S.A.

73:1745–1748.

25. **Pearson, G. R.** 1980. Epstein-Barr virus: immunology, p. 739–767. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
26. **Pearson, G. R., G. Henle, and W. Henle.** 1971. Production of antigens associated with Epstein-Barr virus in experimentally infected lymphoblastoid cell lines. J. Natl. Cancer Inst. **46**:1243–1250.
27. **Preston, C., and D. McGeoch.** 1981. Identification and mapping of two polypeptides encoded within the HSV-1 TK gene sequences. J. Virol. **38**:595–605.
28. **Qualtiere, L. F., R. Chase, B. Vroman, and G. R. Pearson.** 1982. Identification of Epstein-Barr virus strain differences with monoclonal antibody to a membrane glycoprotein. Proc. Natl. Acad. Sci. U.S.A. **79**:616–620.
29. **Qualtiere, L. F., and G. R. Pearson.** 1980. Radioimmune precipitation study comparing the Epstein-Barr virus membrane antigens expressed on P_3HR-1 virus-superinfected Raji cells to those expressed on a B-95 virus-transformed producer culture activated with tumor-promoting agent (TPA). Virology **102**:360–369.
30. **Reedman, B., and G. Klein.** 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and nonproducer lymphoblastoid cell lines. Int. J. Cancer **11**:499–520.
31. **Roubal, J., B. Kallin, J. Luka, and G. Klein.** 1981. Early DNA-binding polypeptides of Epstein-Barr virus. Virology **113**:285–292.
32. **Stark, G., and J. Williams.** 1979. Quantitative analysis and specific labeled RNAs using DNA covalently linked to diazobenzyl-oxymethyl-paper. Nucleic Acids Res. **6**:195–203.
33. **Sugawara, K., M. Kawanishi, and Y. Ito.** 1982. Epstein-Barr virus-related DNA-binding proteins induced by n-butyrate in P_3HR-1 cells. Virology **116**:354–358.
34. **Sugawara, K., and T. Osato.** 1973. Two distinct antigenic components in an Epstein-Barr virus related early product induced by halogenated pyrimidines in nonproducing human lymphoblastoid cells. Nature (London) New Biol. **243**:209–210.
35. **Summers, W. C., and G. Klein.** 1976. Inhibition of EBV DNA synthesis and late gene expression by phosphonoacetic acid. J. Virol. **18**:151–155.
36. **zur Hausen, H., E. Hecker, E. O'Neill, and U. Freese.** 1978. Persisting oncogenic herpes virus induced by tumor promoter TPA. Nature (London) **272**:373–375.