JOURNAL OF VIROLOGY, Jan. 1985, p. 107–113
0022-538X/85/010107-07$02.00/0

Vol. 53, No. 1

Characterization of a Major Protein with a Molecular Weight of 160,000 Associated with the Viral Capsid of Epstein-Barr Virus

BEN VROMAN, JANOS LUKA, MOSES RODRIGUEZ, AND GARY R. PEARSON†*

Departments of Cell Biology and Neurology, Mayo Foundation, Rochester, Minnesota 55905

Received 14 May 1984/Accepted 25 September 1984

A monoclonal antibody designated V3 was produced against a late protein associated with the Epstein-Barr virus-induced viral capsid antigen complex. The antibody reacted with discrete patches in the nuclei of infected cells as well as with virus particles, as shown by immunofluorescence and ultrastructural immunoperoxidase staining. The molecular weight of the protein precipitated by this monoclonal antibody was ca. 160,000. All anti-viral capsid antigen antibody-positive sera tested in an enzyme-linked immunosorbent assay reacted with this purified protein. The synthesis of the antigen was inhibited by phosphonoacetic acid but was not affected by tunicamycin, indicating that this was a late nonglycosylated viral protein. No differences were noted between the protein isolated from the P3HR-1 and B-95-8 cell lines as determined by immunoprecipitation and peptide mapping. By isoelectric focusing, this protein had a pI on the basic side ranging from 7.5 to 9.0.

Monoclonal antibody (MoAb) technology has been used successfully for identifying some of the polypeptides associated with the major Epstein-Barr virus (EBV)-induced antigens. Such antibodies have now been produced against components of the membrane antigen (8, 15, 23, 30, 34), the diffuse and restricted components of the early antigen complex (21), and against one of the major polypeptides associated with the viral capsid antigen complex (12, 33). These antibodies have been useful for the purification and characterization of these different viral proteins and, more recently, for the development of new assays for measuring antibodies in human sera to specific virus-induced polypeptides (13). In addition, through the use of one of the MoAbs, the major polypeptide of the diffuse component of the early antigen complex was mapped to the *Bam*HI M fragment of the EBV genome (21).

In addition to the proteins identified above with MoAbs, a number of other viral polypeptides have been identified by the use of human sera and have been tentatively classified as belonging to one or more of the major EBV antigen complexes. This includes polypeptides ranging from 26,000 to 165,000 (26K to 165K) in molecular weight (1–6, 9–11, 16, 17, 26, 27, 31, 32; J. Luka, T. Kreofsky, T. C. Spelsberg, and G. R. Pearson, submitted for publication). In general, these polypeptides were classified as early or late viral gene products based on their expression in the presence of inhibitors of viral DNA synthesis. Analysis of purified nucleocapsids by Dolyniuk et al. (3) indicated that a major polypeptide associated with the viral capsid had a molecular weight of 160K. This article reports on the production of a MoAb to the 160K polypeptide and provides confirmatory data that this protein is indeed associated with the viral capsid and with the viral capsid antigen (VCA) complex as previously defined in infected cells by immunofluorescence (19). This antibody has been used to purify and partially characterize this protein.

MATERIALS AND METHODS

Cell lines. The lymphoblastoid cell lines used in these experiments included the EBV genome-positive B-95-8, P3HR-1, and Raji cell lines as well as the EBV genome-negative Ramos and BJAB cell lines. All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) and 2 mM L-glutamine. 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 MoAbs to EBV-induced late intracellular antigens. A BALB/c mouse was given a single intraperitoneal injection of 65×10^6 activated B-95-8 cells. The cells had been activated with 20 ng of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) per ml and 3 mM sodium butyrate for 72 h to induce late antigen synthesis. Such cultures routinely contained 40 to 60% VCA-positive cells as determined by immunofluorescence, using standard human control sera. Spleen cells were removed after 3 days for hybridoma production.

For the production of hybridomas, spleen cells were fused with 50×10^6 P3NS-11-AG4-1(NS-1) murine plasmacytoma cells at a ratio of 1:1. Briefly, the cells were mixed and washed twice in serum-free medium (SFM), and the "dry" cell pellet was suspended in 0.5 ml of 50% polyethylene glycol 4000 (J. T. Baker Chemical Co., Phillipsburg, N.J.) in 0.15 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5) and 10% dimethyl sulfoxide. After 2 min, 0.5 ml of SFM was added, and an additional 0.5 ml of SFM was added after each minute for the next 4 min. At the end of this 6-min period, 10 ml of SFM was added to further dilute the polyethylene glycol 4000, and the cells were pelleted by centrifugation at $160 \times g$ for 3 min. The cells were then diluted with 72 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, 2 mM L-glutamine, 10% National Collection of Type Cultures (NCTC) 135 medium, 10 nM sodium selinite, and 10% heat-inactivated FCS (Hyclone) and distributed into eight 96-well microtiter plates containing 4×10^3 to 6×10^3 peritoneal macrophages per well as feeder cells. Cultures were refed with HAT medium every 3 days, and wells containing growing clones were tested by indirect immunofluorescence for positive reactions on acetone-fixed slides of cells expressing EBV antigens. Wells giving positive results were recloned by limiting dilution and re-

* Corresponding author.
† Present address: Department of Microbiology, School of Medicine, Georgetown University, Washington, DC 20057.

screened for antibody production. Positive clones were grown in Falcon flasks, and 1×10^6 to 3×10^6 cells were injected intraperitoneally into mice which had been primed for 7 days with 0.5 ml of pristane for the production of high-titered ascitic fluid. The immunoglobulin subclass produced by each clone was determined by radial immunodiffusion with antibodies against defined mouse immunoglobulin isotypes.

Immunofluorescence assays. Culture supernatants were screened for antibodies to early and late EBV intracellular antigens by the use of acetone-fixed smears of TPA- (20 ng/ml) and sodium butyrate (3 mM)-activated virus-producing cell lines, EBV genome-positive nonproducer lines, and, as negative controls, genome-negative cell lines as previously described (20). MoAbs which did not produce fluorescence with smears from EBV genome-negative cell lines, EBV genome-positive nonproducer cell lines, or with virus-producer cell lines activated in the presence of 150 μg of phosphonoacetic acid per ml to inhibit late antigen expression (7) and which did not react with the membranes of viable activated virus-producing cells but did react with acetone-fixed smears of virus-producing cultures were classified as anti-VCA antibodies.

Labeling of cells with radioisotopes. For labeling viral antigens with [^{35}S]methionine, viable B-95-8 or P3HR-1 cells at a concentration of 10^6 cells per ml were incubated in complete medium supplemented with 20 ng of TPA per ml plus 3 mM sodium butyrate. As indicated, some cultures also contained 150 μg of phosphonoacetic acid or 3 μg of tunicamycin per ml. At different times after induction, the cells were centrifuged at $900 \times g$ for 5 min, washed once with methionine-free medium containing 5% dialyzed FCS and 2 mM L-glutamine (MFM), and then suspended in MFM containing 20 ng of TPA per ml plus 3 mM sodium butyrate at a cell concentration of 4×10^6 to 5×10^6 cells per ml. [^{35}S]methionine was then added to each flask to give a final concentration of 50 μCi/ml, and the cultures were incubated at 37°C for a 6-h period. The cells were then pelleted and extracted as described below.

Cell extraction and immune precipitation. Labeled cells were extracted with 0.5% Nonidet P-40 (NP-40) in 20 mM Tris-hydrochloride (pH 7.4), 0.15 M NaCl, 2 mM EDTA, and 1.0 mM phenylmethylsulfonyl fluoride and then were sonicated for 15 s. The supernatants were clarified by centrifugation at $38,000 \times g$ for 60 min in a Sorvall SS34 rotor, and immune precipitates were formed by mixing portions of the labeled extracts with either antibody-positive or antibody-negative ascitic fluid or human sera as described previously (22, 24, 25). After a 1-h incubation at room temperature, immune complexes were removed with protein A-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), boiled for 3 min in sodium dodecyl sulfate (SDS) sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (22, 24, 25). The ^{14}C-labeled proteins used as molecular weight standards were myosin (210K), phosphorylase B (92.5K), bovine serum albumin (67K), ovalbumin (45K), and carbonic anhydrase (30K) (New England Nuclear Corp., Boston, Mass.).

Immunoaffinity antigen purification. MoAbs were purified from ascitic fluid on protein A-Sepharose 4B. Briefly, ascitic fluids were dialyzed overnight against sodium phosphate buffer (pH 8.1) containing 150 mM NaCl at 4°C. Dialyzed ascitic fluid (5 to 15 ml) was mixed on a rotator overnight at 4°C with a 5-ml packed volume of protein A-Sepharose beads. The mixture was poured into a chromatography column made with a 10-ml glass syringe, and the column was washed with phosphate buffer at room temperature until the flowthrough indicated that no more protein was being removed, as determined by spectrophotometric absorbance at the 280-nm wavelength. The bound immunoglobulins were then eluted with 0.58% acetic acid and immediately neutralized with 1 M Tris-hydrochloride (pH 8.0). The peak fractions were pooled, dialyzed against distilled water, and lyophilized. Immunoglobulins were rehydrated to a concentration of 10 to 15 mg/ml in coupling buffer and bound to Affigel-10 according to the manufacturer's specifications (Bio-Rad Laboratories, Richmond, Calif.). The affinity gels were stored in NP-40 extraction buffer (pH 7.4) at 4°C.

Labeled cell extracts were incubated with 50 μl of a 50% suspension of the affinity gel in 0.5% NP-40 extraction buffer for 1 h at room temperature, washed 6× with 0.5% NP-40 extraction buffer, suspended in Laemmli SDS-sample buffer, and boiled for 3 min. The samples were then analyzed by SDS-PAGE as described previously (22, 24, 25).

Peptide mapping. Viral proteins were purified by incubating cell extracts with MoAbs conjugated to Affigel-10 beads for 1 h at room temperature and then washing them 4× with NP-40 extraction buffer without phenylmethylsulfonyl fluoride followed by two more washings with extraction buffer without phenylmethylsulfonyl fluoride or NP-40. The washed beads were next apportioned into six tubes. Three tubes were mixed with trypsin at final concentrations of 0.01, 0.1, and 0.2 μg in 0.020 ml, and two tubes received 1 and 2 μg of V8 protease. One tube was designated as the control and received no enzyme. The tubes were incubated at room temperature for 30 min and then immediately diluted with Laemmli sample buffer and boiled for 3 min. The samples were loaded onto a 5 to 15% exponential SDS-polyacrylamide gel, and after electrophoresis, the gel was prepared for fluorography as previously described (22, 24, 25).

Two-dimensional gel electrophoresis. The purified protein isolated from the P3HR-1 cell line with a V3 affinity column was subjected to two-dimensional gel electrophoresis by the procedure described by O'Farrell (18) with a pH gradient between 3.5 and 10.

ELISA procedure. Viral antigens were affinity purified as described above. Antigen bound to the affinity beads was washed 5× with NP-40 extraction buffer and eluted with a minimal volume of 3 M $MgCl_2$ as previously described (13). This solution was stored at −20°C until used to coat enzyme-linked immunosorbent assay (ELISA) plates.

For coating wells on ELISA plates, the affinity-purified antigen was dialyzed against 5 mM Tris-hydrochloride (pH 7.4) and lypholized. The antigen was then diluted in 0.5 M Na_2CO_3 buffer (pH 9.5), 100-μl portions were added to each well of the polystyrene microtiter plates (Linbro; Flow Laboratories, Inc., Rockville, Md.), and the plates were incubated overnight at 4°C. After this incubation period, the plates were washed twice with Tris-hydrochloride (pH 7.4) containing 0.05% Tween 20, 150 mM NaCl, and 100 mg of ovalbumin per liter (designated TBS-T; Sigma Chemical Co., St. Louis, Mo.) and dried for 20 min at room temperature. The plates were stored dry at 4°C until used.

For a determination of the optimal antigen concentration to use in the ELISA, microplates were coated with twofold dilutions of antigen, starting with a protein concentration corresponding to 1 μg/ml. The plates were then screened with standard anti-VCA positive and negative human control sera. The last dilution of antigen yielding an absorbance ratio of 5 with a 1:40 dilution of the positive control sera was used as the optimal antigen concentration in the ELISA. The

absorbance ratio was calculated by dividing the optical density figure for the antibody-positive serum by the optical density figure for the antibody-negative serum.

The ELISA was performed as previously described, using alkaline phosphatase-labeled protein A and dinitrophenyl phosphate (Sigma) in 1 M diethanolamine buffer as the substrate (13). Absorbance values were recorded with a Microplate Reader, MR600 (Dynatech Laboratories, Inc., Alexandria, Va.), at 410 nm. The antibody titer for each serum was defined as the last dilution of serum to give an absorbance ratio of 2.

Preparation of cells for immunoelectron microscopy. P3HR-1 cells (40×10^6) were activated as described above and harvested after 72 h of incubation. The cells were washed 3 times with phosphate-buffered saline (PBS), suspended for fixation in 4% paraformaldehyde in 0.1 M phosphate buffer, and incubated for 5 min at room temperature. The cells were carefully centrifuged, washed 2× in PBS, and then suspended in PBS plus 3% FCS for 30 min at room temperature to block nonspecific binding sites. Samples of the fixed cells were then incubated for 30 min at 37°C with conditioned media containing the V3 MoAbs. The cells were washed (3×) with PBS containing 3% FCS as well as receiving two additional washes with PBS without FCS before immunoperoxidase staining.

Ultrastructural immunoperoxidase staining (28). Cells pretreated with the MoAb were incubated with a 1:100 dilution in PBS of rabbit anti-mouse immunoglobulin G (IgG) (heavy- and light-chain specific) bound to peroxidase (Cappel Laboratories, Cochranville, Pa.) for 1 h at 37°C. The cells were thoroughly washed and incubated at room temperature with Hanker-Yates reagent, *p*-phenylenediamine pyrocatechol (Polysciences, Inc., Warrington, Pa.) in the presence of 0.01% hydrogen peroxide for 15 min. The reaction was monitored under a light microscope. When a dark brown precipitate formed in the nuclei of infected cells, the reaction was stopped by washing several times in PBS. The labeled cells were then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h. Cells were carefully washed in PBS and treated with 1% osmium in 0.1 M phosphate buffer for 30 min, after which the cells were washed and dehydrated in graded alcohol (10, 30, 50, 75, 95, 95, 100, and 100%). After dehydration, cells were transferred to a 1:1 solution of Spurr in absolute alcohol for 1 h and then to a 3:1 solution of Spurr in absolute alcohol for 1 h. After this treatment, the cells were centrifuged, the supernatant was removed, and the cell pellet was infiltrated with pure Spurr solution. Blocks were hardened at 65°C in an oven for 24 to 48 h and trimmed, and sections were cut with an LKB ultramicrotome. Sections were routinely viewed without counterstaining with uranyl acetate or lead citrate. Selected sections were counterstained, however, for greater resolution of cellular details.

RESULTS

Detection of a MoAb to a late viral protein. A MoAb designated V3 was identified which reacted with an intracellular antigen expressed only in virus-producing cells. This was determined by immunofluorescence against a spectrum of cell lines. The MoAb reacted with the virus-producing P3HR-1 and B-95-8 cell lines after cultivation in the presence of TPA and sodium butyrate to activate the virus replication cycle. The V3 antibody did not react with these same cell lines cultivated in the presence of phosphonoacetic acid, however, indicating that the relevant antigen was a late viral gene product. This antibody also did not react with the membranes of infected cells, with genome-positive non-producer cells, or with genome-negative cell lines, providing further evidence that V3 was directed against a late intracellular viral gene product. The immunoglobulin isotype of the antibody was determined to be IgG2a.

By immunofluorescence, this antibody was seen to react primarily with the nuclei of infected cells (Fig. 1A). When cells were stained by the immunoperoxidase technique and then examined by electron microscopy, staining was noted in discrete patches in the nuclei bordering along the nuclear membrane and on virus particles (Fig. 1B and D). Occasional staining was also noted in the cytoplasm of some infected cells. These results demonstrated that the protein was associated mainly with the viral capsid as well as with free viral protein in the cell nucleus.

Identification and characterization of the protein detected with the V3 MoAb. For a determination of the size of the protein reactive with V3 MoAb, P3HR-1 cells were labeled with [^{35}S]methionine at different times after activation and extracted with 0.5% NP-40, and the extracts were subjected to immunoprecipitation and SDS-PAGE analysis. Results are shown in Fig. 2. Immunoprecipitates were formed at various times after activation with a human antibody-positive serum (Fig. 2, lanes A to D), the V3 MoAb (lanes E to H), a second new candidate MoAb (I to L), a MoAb designated L2 previously shown to be directed against a late glycoprotein with a molecular weight of ca. 125K (12) (M to P), and with an antibody-negative human serum (R to V). The V3 MoAb reacted optimally with a protein with a molecular weight of ca. 160K. Optimal time for precipitation was ca. 42 h postactivation (Fig. 2, lane G). A number of other minor proteins were also present at this time interval. However, these were also routinely precipitated in the presence of the antibody-negative sera. In addition, most of these bands were eliminated when 0.05% SDS was included in the extraction buffer, indicating that they were not specifically reacting with this MoAb. The 160K protein, among others, was also precipitated by the human antibody-positive serum (Fig. 2, lanes A to D). The second candidate anti-VCA MoAb also gave a weak reaction with the 160K protein at 48 h postactivation (Fig. 2, lane L). In contrast, the L2 antibody precipitated the 125K protein also optimally at ca. 42 h postactivation (Fig. 2, lane O).

The size of the protein recognized by the V3 antibody was confirmed by affinity chromatography. Extracts of P3HR-1 cells labeled with [^{35}S]methionine were passaged over antibody affinity columns coupled with the V3 or L2 MoAbs. The eluates from these columns were then analyzed by SDS-PAGE. Results are shown in Fig. 3. The major protein eluted from the V3 affinity column had a molecular weight of ca. 160K, as opposed to 125K for the protein eluted from the L2 affinity column. A similar experiment performed with a labeled extract from B-95-8 cells also yielded a protein of ca. 160K from the V3 column (unpublished data).

To further establish the EBV specificity of the 160K protein, affinity-purified protein was employed as the target antigen in the ELISA for different human sera. All sera tested from individuals positive for antibodies to VCA as determined by immunofluorescence were positive in the ELISA. These included 10 sera from healthy, anti-VCA antibody-positive individuals and 38 sera from patients with infectious mononucleosis. In contrast, none of five sera tested from anti-VCA antibody-negative individuals were positive in this assay. Representative titrations are shown in Table 1.

For further examination of the nature of this protein, P3HR-1 cells were again labeled with [^{35}S]methionine after

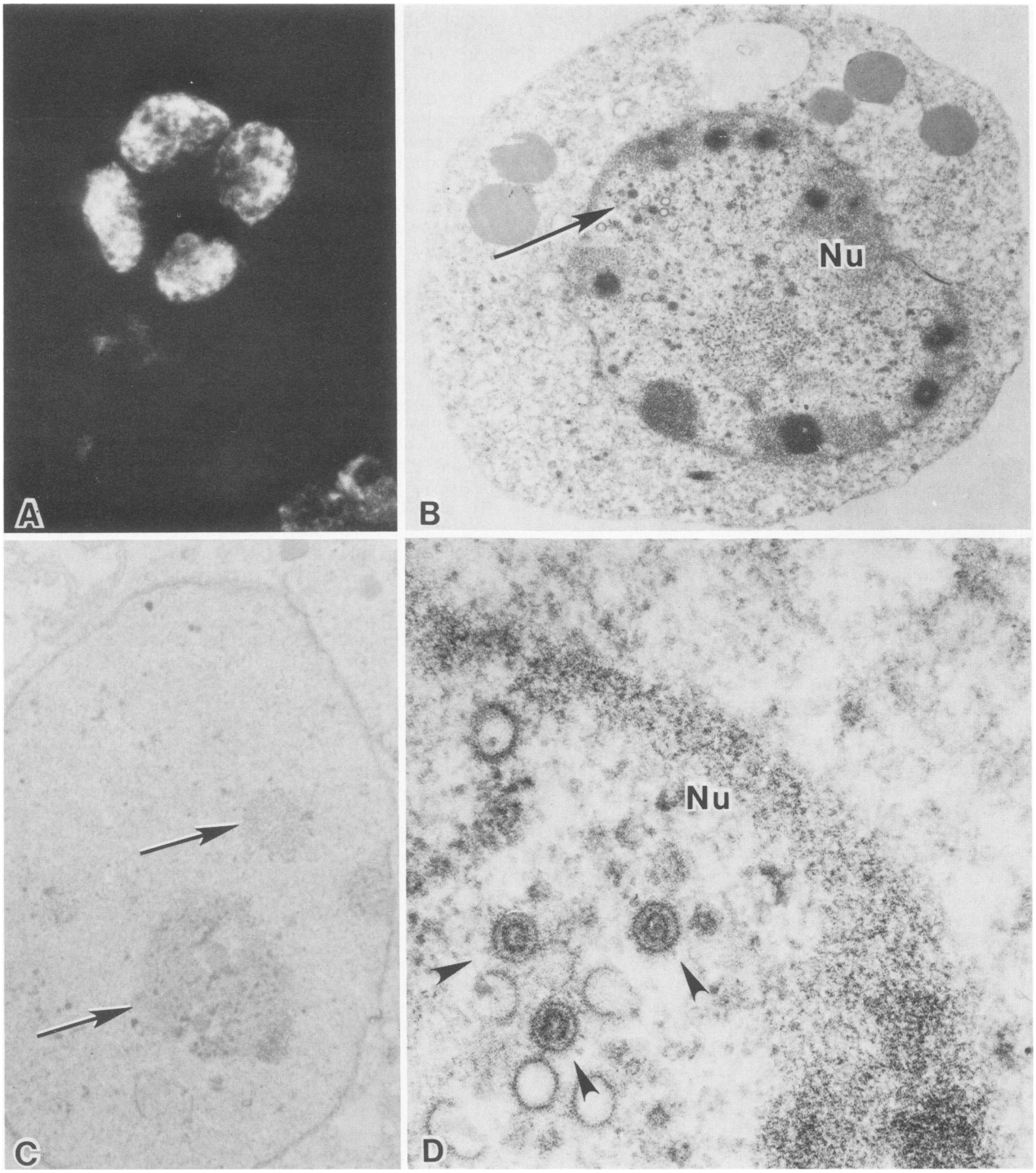

FIG. 1. (A) Immunofluorescence staining of P3HR-1 cells with V3 MoAb. Note nuclear staining. Magnification, ×425. (B) Ultrastructural localization of the V3 antigen in P3HR-1 cells by immunoperoxidase staining. Note dark immunoperoxidase staining within nucleus (Nu) of cell. Arrow points to peroxidase-labeled virus particles. Some less intense staining was also noted in the cytoplasm of this cell. Magnification, ×16,200. (C) Negative control for immunoperoxidase staining by omitting antibody during the first step. Arrows point to viral inclusion within the nucleus of an infected cell. Magnification, ×15,000. (D) Immunoperoxidase staining of virus particles (arrows). This is a higher magnification of the cell shown in B (×90,000).

activation in the presence or absence of phosphonoacetic acid or tunicamycin for 48 h (Fig. 4). The cells were then extracted, and immune precipitates were formed with a human antibody-positive serum (Fig. 4, lanes A, G, M), an antibody-negative human serum (lanes B, H, N), two antibody-negative mouse ascitic fluids (C, D, I, J, N, O), an ascitic fluid containing the V3 MoAb (E, K, R), and, for comparisons, an ascitic fluid containing the L2 MoAb (F, L, T). The results showed that the synthesis of both the V3 (160K) and L2 (125K) antigens was inhibited by phospho-

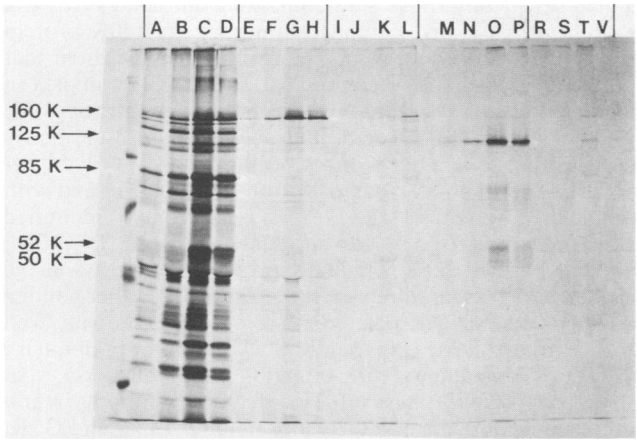

FIG. 2. Identification of the V3-precipitated, ^{35}S-labeled polypeptide at different times after activation of P3HR-1 cells with TPA and sodium butyrate. Immunoprecipitates were prepared at 24, 36, 42, and 48 h postactivation with a human antibody-positive serum (lanes A to D); V3 MoAb (lanes E to H); a second candidate anti-VCA MoAb (lanes I to L); the previously described L2 MoAb which reacts with a glycoprotein with a molecular weight of ca. 125K (lanes M to P); and a human antibody-negative serum (lanes R to V).

noacetic acid (lanes M to T). In addition, tunicamycin treatment prevented the precipitation of the 125K glycoprotein by L2 but had no effect on the 160K protein precipitated by the V3 antibody (Fig. 4, lanes G to L). These results provided further evidence that the V3 protein was a late viral gene product, as previously noted for the L2 antigen (12),

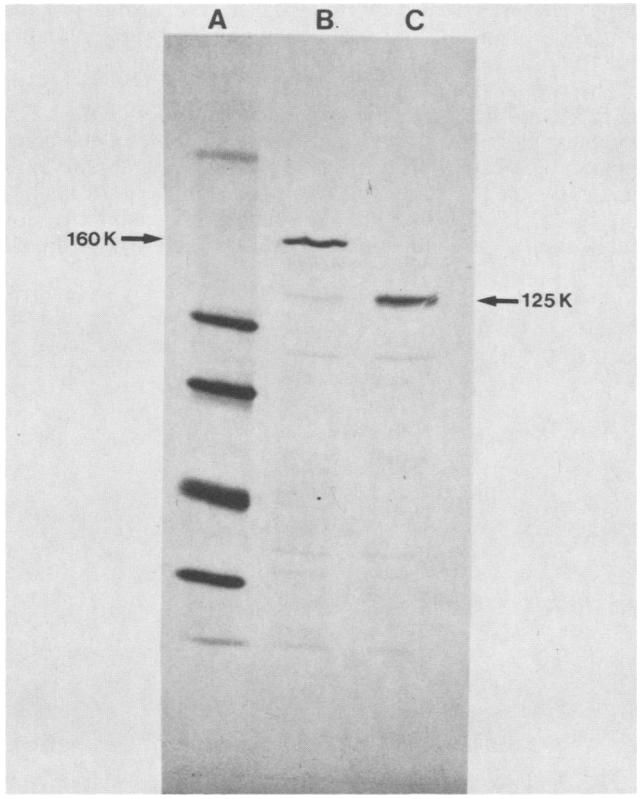

FIG. 3. SDS-PAGE analysis of the polypeptides purified from ^{35}S-labeled P3HR-1 cells, using affinity columns prepared with (B) V3 MoAb and (C) L2 MoAb. ^{14}C-standards are shown in lane A.

TABLE 1. Titration of human sera in ELISA against the 160K protein purified from the V3 antibody affinity column

Serum	IF[a] titer	Dilution	OD	ELISA titer[b]
1	<10	10	0.069	<10
		40	0.028	
		160	0.009	
		640	0.001	
2	<10	10	0.041	<10
		40	0.002	
		160	0.005	
		640	0.006	
3	<10	10	0.072	<10
		40	0.023	
		160	0.011	
		640	0.000	
4	80	10	0.508	80
		40	0.250	
		160	0.084	
		640	0.022	
5	640	10	0.686	>640
		40	0.489	
		160	0.333	
		640	0.211	
6	320	10	0.599	160
		40	0.320	
		160	0.154	
		640	0.062	

[a] IF, Immunofluorescence.

[b] The last dilution giving an optical density (OD) reading of 0.150 was taken as the ELISA titer. This reading was ca. 2× higher than the highest OD reading for the negative sera.

but unlike the L2 protein, the 160K protein precipitated with the V3 MoAb was not glycosylated. Efforts to label the 160K protein with [^{3}H]glucosamine, [^{3}H]mannose, and [^{3}H]fucose were also unsuccessful, providing further evidence that this protein was not glycosylated (unpublished data).

These findings indicated that the viral protein detected with the V3 MoAb was nonglycosylated and had approximately the same molecular weight in both the P3HR-1 and B-95-8 cell lines. To further compare this protein as it was isolated from both cell lines, peptide maps were prepared from the affinity-purified proteins by using trypsin or protease V8. Results are shown in Fig. 5. The peptide profiles generated by these treatments appeared to be similar for both cell lines, with the exception of lanes C and I. In these lanes the profiles appeared different. However, this was due to incomplete proteolysis of the larger amount of protein in lane I in comparison with C. When larger concentrations of enzyme were used (lanes D and H), the profiles were identical. Trypsin treatment was the most effective in degrading this protein, yielding a major peptide from both cell lines with a molecular weight of ca. 46K after treatment with the highest enzyme concentration.

The purified protein from the P3HR-1 cells was also subjected to two-dimensional gel electrophoresis (Fig. 6). This protein had a pI on the basic side ranging from 7.5 to 9.0.

DISCUSSION

A MoAb was produced which was directed against a late EBV-induced polypeptide with a molecular weight of ca. 160K. This is the approximate size of a major protein associated with the viral capsid, as previously determined

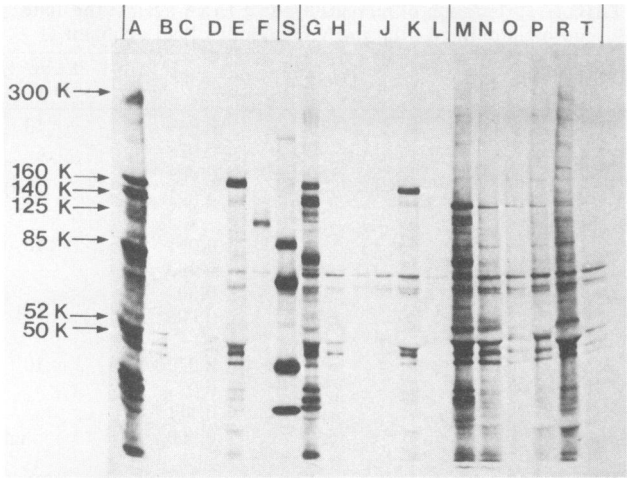

FIG. 4. SDS-PAGE analysis of immunoprecipitates formed between ^{35}S-labeled P3HR-1 cells cultivated in the presence of tunicamycin (lanes G to L), phosphonoacetic acid (lanes M to T) or untreated (lanes A to F) and the following sera: lanes A, G, M, anti-VCA antibody-positive human serum; lanes B, H, N, anti-VCA antibody-negative human serum; lanes C, D, I, J, O, P, two different antibody-negative mouse ascitic fluids; lanes E, K, R, mouse ascitic fluid containing the V3 MoAb; and lanes F, L, T, mouse ascitic fluid containing the L2 MoAb. ^{14}C-standards are shown in lane S.

through analysis of purified EBV nucleocapsids (3). Confirmatory data showing that this protein was associated with the capsid of viral particles was provided by the immunoelectron microscopy findings presented in this paper. The MoAb designated V3 reacted with viral particles in the nuclei of infected cells, as shown by the immunoperoxidase method. In addition, staining was noted in discrete patches in the nuclei with both the ultrastructural immunoperoxidase and immunofluorescence assays. These nuclear areas presumably contained the soluble form of the 160K protein.

The 160K protein was purified on antibody-affinity columns and used as the target antigen in the ELISA with human sera. Only those sera containing antibodies to VCA reacted in this assay, demonstrating that the 160K protein was a virus-induced antigen. The results also indicated that this protein was a major component of the VCA complex in infected cells, as defined by immunofluorescence (19). Whether this is the major protein identified with antibody-positive human sera has still not been resolved. A glycoprotein reported to be associated with the viral capsid and with a molecular weight of ca. 125K was previously identified with MoAbs prepared by two different groups (12, 33). When both the purified 125K and 160K proteins were used in the ELISA, sera from antibody-positive donors, whether nondiseased or with EBV-associated cancers, reacted equally well with both of these polypeptides. However, preliminary analysis from patients with infectious mononucleosis suggests that, after a primary EBV infection, antibody is first produced against the 125K glycoprotein (J. Luka and G. R. Pearson, manuscript in preparation). This would indicate that the 125K glycoprotein and not the 160K protein is the strongest immunogen associated with the viral capsid.

Comparison of the 125K and 160K proteins revealed some major differences. It was found that, in contrast to the 125K protein, the 160K protein was not glycosylated. This was shown through the use of tunicamycin to inhibit glycosylation and through the use of radioactive sugars. Thus, this protein did not appear to be modified by glycosylation, although it still has not been determined whether the protein is modified in other ways such as by phosphorylation. In addition, the size of the V3-recognized protein was approximately the same in cells infected with the P3HR-1 or B-95-8 strains of EBV. In contrast, the 125K glycoprotein was routinely shown to be larger in P3HR-1 virus-infected cells in comparison with cells infected with the B-95-8 strain of virus. This was shown by both SDS-PAGE analysis of the purified protein and by peptide mapping, and is presumably due to differences in glycosylation (12).

The results from the current studies conclusively identified a second EBV-induced protein associated with the VCA complex. Previous studies had identified some of the components associated with the EBV-induced membrane antigen, early antigen, and nuclear antigen complexes (8, 12, 14, 19, 21–25, 27, 29, 30, 33, 34; Luka et al., submitted for publication). Continued use of MoAb technology should

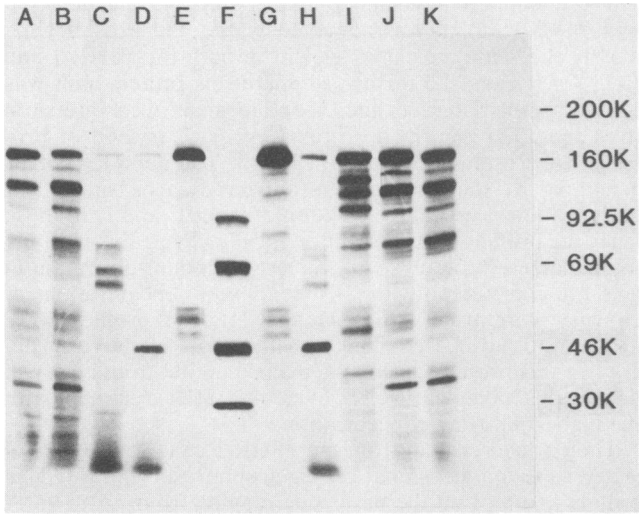

FIG. 5. Peptide maps of the affinity-purified V3 polypeptide from ^{35}S-labeled B-95-8 (lanes A to E) and P3HR-1 (lanes G to K) cells after treatment with trypsin or protease V8. Lanes: E and G, untreated; A and K, treated with 1 μg of protease V8; B and J, 2 μg of protease V8; C and I, 0.1 μg of trypsin; D and H, 0.2 μg of trypsin. ^{14}C-labeled strandards are shown in lane F.

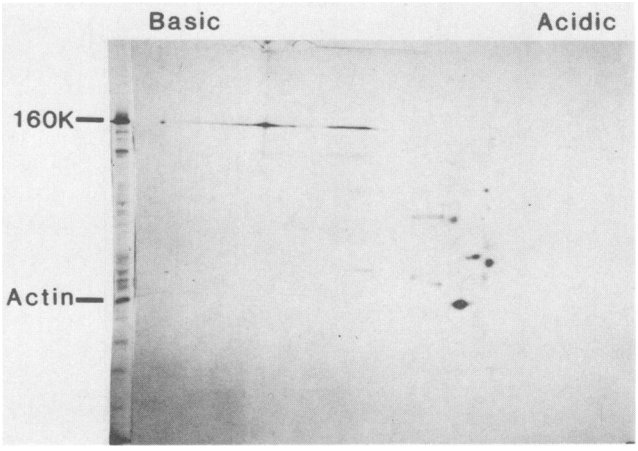

FIG. 6. Two-dimensional gel electrophoresis of V3 antigen purified from ^{35}S-labeled P3HR-1 cells by affinity chromatography. SDS-PAGE analysis of V3 protein eluted from antibody column is shown on the left for reference.

allow for the classification of other virus-associated proteins into one or another of these antigens complexes and for the development of a common nomenclature for the description of these EBV proteins.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA 20679 from the National Cancer Institute. M.R. is the recipient of Public Health Service Teacher Investigator Award NS-00849-01 from the National Institute of Neurological and Communicative Disorders and Stroke, and is a Rappaport Scholar of the Mayo Foundation.

LITERATURE CITED

1. **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.
2. **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.
3. **Dolyniuk, M., E. Wolff, and E. Kieff.** 1976. Proteins of Epstein-Barr virus. II. Electrophoretic analysis of the polypeptides of the nucleocapsid and the glucosamine- and polysaccharide-containing components of enveloped virus. J. Virol. **18**:289–297.
4. **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.
5. **Feighny, R. J., B. E. Henry II, and J. S. Pagano.** 1981. Epstein-Barr virus polypeptides: effect of inhibition of viral DNA replication on their synthesis. J. Virol. **37**:61–71.
6. **Feighny, R. J., B. E. Henry II, and J. S. Pagano.** 1981. Epstein-Barr virus polypeptides: identification of early proteins and their synthesis and glycosylation. J. Virol. **39**:651–655.
7. **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.
8. **Hoffman, G. F., S. G. Lazarowitz, and S. D. Hayward.** 1980. Monoclonal antibody against a 250,000 glycoprotein of Epstein-Barr virus identified a membrane antigen and a neutralizing antigen. Proc. Natl. Acad. Sci. U.S.A. **77**:2979–2983.
9. **Kallin, B., J. Luka, and G. Klein.** 1979. Immunochemical characterization of Epstein-Barr virus-associated early and late antigens in *n*-butyrate-treated P3HR-1 cells. J. Virol. **32**:710–716.
10. **Kawanishi, M., and Y. Ito.** 1982. Similarity of Epstein-Barr virus early polypeptides induced by various tumor promoters. Cancer Lett. **16**:19–23.
11. **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.
12. **Kishishita, M., J. Luka, B. Vroman, J. F. Poduslo, and G. R. Pearson.** 1984. Production of monoclonal antibody to a late intracellular Epstein-Barr virus-induced antigen. Virology **133**:363–375.
13. **Luka, J., R. C. Chase, and G. R. Pearson.** 1984. A sensitive enzyme linked immunosorbent assay (ELISA) against the major EBV-associated antigens. I. Correlation between ELISA and immunofluorescence titers using purified antigens. J. Immunol. Methods **67**:145–156.
14. **Morgan, A. J., J. R. North, and M. A. Epstein.** 1983. Purification and properties of the gp340 component of Epstein-Barr virus membrane antigen in an immunogenic form. J. Gen. Virol. **64**:455–460.
15. **Mueller-Lantzsch, N., B. Georg-Fries, H. Herbst, H. zur Hausen, and D. G. Braun.** 1981. Epstein-Barr virus strain- and group-specific determinants detected by monoclonal antibodies. Int. J. Cancer **28**:321–327.
16. **Mueller-Lantzsch, N., B. George-Fries, 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.
17. **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.
18. **O'Farrell, P. M.** 1975. High resolution of two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
19. **Pearson, G. R.** 1980. Epstein-Barr virus: immunology, p. 713. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
20. **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.
21. **Pearson, G. R., B. Vroman, B. Chase, T. Sculley, M. Hummel, and E. Kieff.** 1983. Identification of polypeptide components of the Epstein-Barr virus early antigen complex with monoclonal antibodies. J. Virol. **47**:193–201.
22. **Qualtiere, L. F., R. Chase, and G. R. Pearson.** 1982. Purification and biologic characterization of a major Epstein-Barr virus-induced membrane glycoprotein. J. Immunol. **129**:814–818.
23. **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.
24. **Qualtiere, L. F., and G. R. Pearson.** 1979. Epstein-Barr virus-induced membrane antigens: immunochemical characterization of Triton X-100 solubilized viral membrane antigens from EBV-superinfected Raji cells. Int. J. Cancer **23**:808–817.
25. **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 cells in B-95 virus-transformed producer cultures activated with tumor-promoting agent (TPA). Virology **102**:360–369.
26. **Roubal, J., B. Kallin, J. Luka, and G. Klein.** 1981. Early DNA-binding polypeptides of Epstein-Barr virus. Virology **113**:285–292.
27. **Sculley, T. B., T. Kreofsky, G. R. Pearson, and T. C. Spelsberg.** 1983. Partial purification of the Epstein-Barr virus nuclear antigen. J. Biol. Chem. **258**:3974–3982.
28. **Sternberger, L. A.** 1979. Immunocytochemistry, p. 104–169. John Wiley & Sons, Inc., New York.
29. **Strnad, B. C., T. C. Schuster, R. F. Hopkins III, R. H. Neubauer, and H. Rabin.** 1981. Identification of an Epstein-Barr virus nuclear antigen by fluoroimmunoelectrophoresis and radioimmunoelectrophoresis. J. Virol. **38**:996–1004.
30. **Strnad, B. C., T. Schuster, R. Klein, R. F. Hopkins III, T. Witmer, R. H. Neubauer, and H. Rabin.** 1982. Production and characterization of monoclonal antibodies against the Epstein-Barr virus membrane antigen. J. Virol. **41**:258–264.
31. **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.
32. **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.
33. **Takada, K., S. Fujiwara, S. Yano, and T. Osato.** 1983. Monoclonal antibody specific for capsid antigen of Epstein-Barr virus. Med. Microbiol. Immunol. **171**:225–231.
34. **Thorley-Lawson, D. A., and K. Geilinger.** 1980. Monoclonal antibodies against the major glycoprotein (gp350/220) of Epstein-Barr virus neutralize infectivity. Proc. Natl. Acad. Sci. U.S.A. **77**:5307–5311.