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Detection by Monoclonal Antibodies of an Early Membrane Protein Induced by Epstein-Barr Virus

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Two monoclonal antibodies, E8B3 and E8D2, were raised against Epstein-Barr virus (EBV)-producing cells and were shown to immunoprecipitate a protein with an approximate molecular weight of 105,000 (p105). The protein was detectable only in EBV-containing cells which were supporting the virus lytic cycle, and its synthesis increased after cells were induced with phorbol esters. The molecule was radiolabeled and immunoprecipitated from virus-producing cells that had been extrinsically labeled with ^{125}I, and the antibodies E8B3 and E8D2 reacted in immunofluorescence assays with infected cells; the molecule was also associated with virion particles. Synthesis of p105 was not blocked by phosphonoacetic acid and could be induced in Raji cells by superinfection with virus derived from P3HR1 cells. These data support the conclusion that p105 is an EBV-specific early membrane protein.

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and is associated with two human neoplasms, Burkitt's lymphoma and nasopharyngeal carcinoma (8). The virus is derived in cultures from latently infected lymphoblastoid cell lines of human and marmoset origin. A small percentage of these cells spontaneously support the virus lytic cycle, and the percentage can be increased by induction with phorbol esters (29). Several apparently virus-specified proteins have been identified in the membranes of such virus-producing cells and in the envelope of the mature virion. These proteins are probably important to ingress and egress of virus and serve as targets for the immune response. Although analysis of EBV membrane-associated proteins with high-titered human sera (9, 22, 28) or hyperimmune rabbit sera (7) has indicated that both early proteins (made in the absence of viral DNA synthesis) and late proteins (dependent on viral DNA synthesis) are present in the membrane of virus-producing cells, analysis with monoclonal antibodies has as yet only provided evidence for four late proteins. These include three glycoproteins, gp350/300, gp250/200, and gp90/85 and one nonglycosylated protein, p140, all of which are present in both cell and virus membranes (26). We describe here the use of monoclonal antibodies to detect a fifth EBV-specific protein, present in both cell and virus membranes, which is apparently made in the absence of viral DNA synthesis.

MATERIALS AND METHODS

Lymphoblastoid cell lines. Cell lines were grown at 37°C and diluted at least biweekly in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum, 100 IU of penicillin per ml and 100 μg of streptomycin per ml. These lines included four human EBV genome-positive B lymphoblastoid cell lines, the latently infected Raji cell line (20), the EBV-producing cell line P3HR1 (13) and its subclones P3HR1-Cl13 and P3HR1-Cl5 (12), a virus-producing marmoset cell line, MCUV5 (the latter three lines were a gift from George Miller, Yale University, New Haven, Conn.), and an EBV genome-negative human T-cell line, Molt 4 (17). Virus obtained from P3HR1 cells will superinfect Raji cells and induce expression of virus lytic-cycle antigens (3). Virus obtained from MCUV5 cells does not superinfect Raji cells but will transform fresh human B cells and induce them to secrete immunoglobulin (4). In our experiments, 3 to 5% of the P3HR1-Cl13 cells spontaneously express virus lytic-cycle antigens and produce low levels of virus, but after induction with 30 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis, Mo.) per ml, 30 to 50% of the cells express viral antigen and produce large amounts of virus, very little, if any, of which will superinfect Raji. Currently, in our laboratory, P3HR1-Cl5 cells express little or no virus lytic-cycle antigen with or without TPA induction and never produce enough virus to detectably superinfect Raji cells.

Virus production and superinfection. Producer cells were induced with 30 ng of TPA per ml and, after 7 days, virus was collected from the spent culture medium as previously described (23). Briefly, the cells were centrifuged at 4,000 × *g* for 10 min to remove cells, 100 μg of bacitracin per ml was added to the clarified supernatant, and the virus was pelleted by centrifugation at 20,000 × *g* for 90 min. Pellets were suspended in 1/250 original volume of medium containing 100 μg of bacitracin per ml, reclarified by centrifugation three to four times at 400 × *g*, and filtered through a 0.45-μm-pore filter (Millipore Corp., Bedford, Mass.). For some experiments the virus was also further purified by centrifugation over dextran (19). The virus was repelleted and then suspended in buffer containing 0.01 M Tris, 0.15 M NaCl, and 100 μg of bacitracin per ml at pH 7.2 (TNB). It was then layered over a discontinuous gradient of 5, 10, 15, and 30% Dextran T10 (Pharmacia, Inc., Piscataway, N.J.) in TNB and centrifuged at 76,000 × *g* for 1 h in an angle-head rotor. The virus was collected from the 15 to 30% interface, dialyzed against TNB to remove dextran, repelleted, and finally resuspended in fresh TNB. For superinfection, 10^7 Raji cells were resuspended in 0.5 ml of P3HR1 virus for 2 h at 37°C. The cells were then pelleted and suspended at a concentration of 2×10^6 cells per ml in RPMI 1640 containing 2% heat-inactivated fetal calf serum.

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Production of monoclonal antibodies. P3HR1-Cl13 cells were induced with 30 ng of TPA per ml, and after 3 days cells were fractionated on a discontinuous gradient of Ficoll-Hypaque by the procedure of Strnad and co-workers (25). More than 80% of the cells at the 25 to 50% interface expressed late viral antigens in the membrane as judged by reactivity with two monoclonal antibodies to epitopes shared by EBV gp350/300 and gp250/200. These antibodies were A-2-5 (a gift from Bruce Strnad, BBL Research Center, Baltimore, Md.) and D4.16 (one of our own antibodies). Antibody binding was visualized with fluorescein isothiocyanate-conjugated F $(ab')_2$ sheep anti-mouse immunoglobulin (Cooper Biomedical, Inc., Malvern, Pa.). Cells from the 25 to 50% interface were collected and used for immunization. Four- to six-week-old BALB/c mice received 3 weekly intraperitoneal injections of 2×10^7 fractionated cells, and 2 weeks after the last injection they were boosted intravenously with virus concentrated from supernatant media of TPA-induced P3HR1-Cl13 cells. Spleen cells were harvested 3 days later, fused with SP2/0 Ag.14 myeloma cells, and plated in HAT medium as previously described (2). Culture supernatants were initially tested in an indirect immunofluorescence assay for reactivity with the membranes of cell briefly fixed with 0.1% paraformaldehyde (23). Supernatant fluids that reacted with TPA-induced P3H41-Cl13 but not Raji cells, were further tested for the ability to react with virion envelopes in an indirect immunofluorescence assay in which EBV was first bound to receptor-positive Raji cells, and then Raji cells with and without virus were incubated sequentially with culture supernatants and fluorescein-conjugated sheep anti-mouse immunoglobulin G. Clones growing in supernatants that reacted with Raji cells plus virus but not with Raji cells alone were further monocloned, retested, and frozen. Concentrated culture supernatants were used to determine the isotype of immunoglobulin produced, and high-titered ascitic fluids were raised by injecting the clones intraperitoneally into pristane-primed BALB/c mice (2).

Neutralization. Neutralization of virus infectivity by antibody was expressed as the ability to inhibit virus-induced immunoglobulin synthesis by fresh human B lymphocytes as previously described (14). Briefly, leukocytes were obtained from heparinized human blood by flotation on Ficoll-Hypaque and depleted of T cells by forming rosettes with sheep erythrocytes. A total of 200,000 cells were incubated with or without virus that had been preincubated for 1 h at 37°C with various concentrations of known neutralizing antibody (G-3-1, a monoclonal antibody which reacts with gp85 and which was a gift from Bruce Strnad) and test antibody. Cells and virus were plated in a total volume of 100 μl in 96-well round-bottomed tissue culture plates. After 6 days in culture, 100 μl of medium was added to each well. On day 12, the immunoglobulin concentrations in the media were measured by a double-sandwich micro-enzyme-linked immunosorbent assay (27) with appropriate concentrations of rabbit anti-human immunoglobulin, peroxidase-conjugated rabbit anti-human immunoglobulin, and the substrate hydrogen peroxide with 5-aminosalicylic acid. The colorimetric change was measured at 492 nm.

Radiolabeling and drug treatment. Fractionated and unfractionated cells and virus were labeled extrinsically with ^{125}I (Amersham Corp., Arlington Heights, Ill.) by using tetrachlorodiphenylglycouril (Iodogen; Pierce Chemical Co., Rockford, Ill.) (11). Cells (10^7) were suspended in phosphate-buffered saline and labeled with 0.5 mCi of ^{125}I. Labeled cells were washed five times before extraction or lysis for immunoprecipitation. The virus was pelleted at $100,000 \times g$ for 1 h, resuspended in 200 to 400 μl of phosphate-buffered saline, reclarified twice by centrifugation at $800 \times g$ for 10 min, lysed, and centrifuged to remove nucleocapsids as described below for immunoprecipitation. Cells were labeled intrinsically with $[^{35}S]$methionine (specific activity of 1,380 Ci/mmol; Amersham Corp.) as previously described (15). Briefly, after 2 days with or without induction with 30 ng of TPA per ml, cells were washed and suspended at a concentration of 10^7 cells per ml in Hanks balanced salt solution supplemented with nonessential amino acids but with 1/10 normal concentration of methionine, with 5% dialyzed heat-inactivated fetal calf serum, and again, with or without TPA. After 1 h, 25 μCi of $[^{35}S]$methionine per ml was added, and 24 h later cells were harvested.

In experiments which used phosphonoacetic acid (PAA) (Sigma), cells were washed, suspended in medium containing 200 μg of PAA per ml, incubated for 2 days, washed again, and either labeled immediately with $[^{35}S]$methionine for 24 h or reincubated for 2 days in medium containing PAA. At this time the reincubated cells were also labeled with $[^{35}S]$methionine for 24 h. In experiments which used tunicamycin (Sigma), cells were preincubated in medium containing 5 μg of tunicamycin per ml for 3 h before the addition of the label, and tunicamycin at this concentration was added with the label. In superinfection experiments, cells were labeled with $[^{35}S]$methionine (10 μCi/ml) for 24 h, beginning at 6 h after the initial adsorption period.

Total extracts of labeled cells for analysis by gel electrophoresis were made by suspending cells in sample buffer (0.37 M Tris hydrochloride [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, 10% sodium dodecyl sulfate [SDS]) and boiling for 3 min before electrophoresis. For immunoprecipitation, harvested cells and virus were treated as described below.

Immunoprecipitation and gel electrophoresis. Immunoprecipitation was carried out as previously described (15). Briefly, cells or virus were solubilized with lysing buffer (0.05 M Tris hydrochloride, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1,000 U of Aprotinin per ml, 0.1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at $100,000 \times g$ for 1 h. Supernatants were mixed with antibody and protein A-Sepharose CL4B beads (Sigma). The precipitates were washed, dissociated by boiling, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (2) in 9% acrylamide cross-linked with 0.28% *N,N'*-diallylytartardiamide (Sigma) or 7% acrylamide cross-linked with 0.28% *N,N*-methylenebisacrylamide (Bio-Rad Laboratories, Richmond, Calif.). Molecular weight markers (Sigma) were electrophoresed in parallel tracts. Gels were stained, destained, infused with 2,5-diphenyloxazole (Sigma), dried on filter paper, and placed in contact with XAR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for fluorography (6).

RESULTS

Characterization of antibodies by immunofluorescence. In the initial screening, culture supernatants from two hybridomas, E8B3 and E8D2, showed a very distinct surface fluorescence staining pattern with TPA-induced P3HR1-Cl13 cells. In contrast to the ring-type fluorescence seen with antibodies against gp350/300 and gp250/200 (A-2-5 and D4.16), the staining consisted mainly of patches and partial rings aggregated towards one margin of the cell. Staining of virus bound to Raji cells was weaker than that seen on

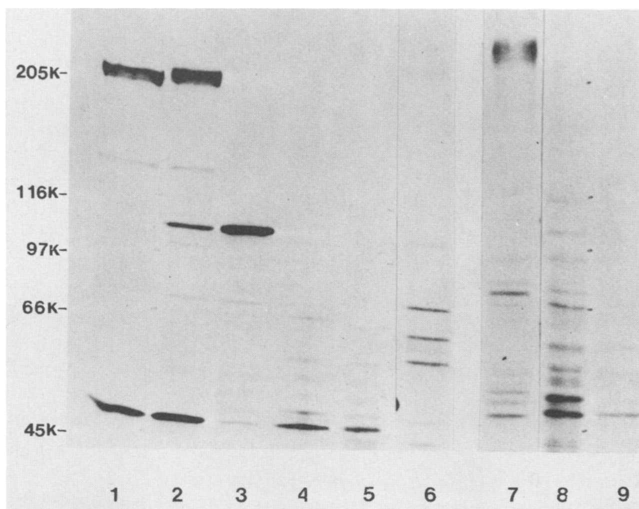

FIG. 1. SDS-PAGE analysis on 9% acrylamide of [^{35}S]methionine-labeled peptides immunoprecipitated by protein A-Sepharose alone (lane 1), by E8D2 (lanes 2 to 6), and by D4.16 (lanes 7 to 9) from lymphoblastoid cell lines. Peptides in lanes 3 to 9 were preabsorbed with protein A-Sepharose. Cells are P3HR1-Cl13 (lanes 1 and 2), TPA-induced P3HR1-Cl13 (lanes 3 and 7), Raji (lanes 4 and 8), Molt 4 (lanes 5 and 9), and TPA-induced P3HR1-Cl5 (lane 6).

producing cell membranes, but nevertheless definite. Both the hybridomas secreted antibodies of the immunoglobulin G2a subclasses and all the antibody-secreting monoclones of the hybridomas continued to produce immunoglobulin G2a antibodies that showed the same characteristic pattern of fluorescence. Representative monoclones were used for further characterization.

Since the antibodies were made with cells from mice immunized with TPA-induced P3HR1-Cl13 cells, we considered the possibility that they were directed against a TPA-induced host-membrane component that remained attached to release virus, rather than a virus-induced protein. However, further immunofluorescence characterization of the antibodies indicated that this was unlikely. First, the antibodies reacted not only with TPA-induced P3HR1-Cl13 cells, but also with the 3 to 10% of noninduced P3HR1-Cl13 cells that spontaneously produced virus. Second, when TPA-induced P3HR1-Cl13 cells were fractionated by flotation on Ficoll-Hypaque, more than 80% of cells at the 25 to 50% interface fluoresced both with E8B3 and E8D2 and with the reference antibodies A-2-5 and D4.16. Third, although E8B3 and E8D2 reacted neither with other human nonproducer B cell lines such as Raji and P3HR1-Cl5 nor with the human T-cell line, Molt 4, they did react with MCUV5 marmoset cells when they produced virus with or without TPA induction. Thus, reactivity correlated with expression of virus antigens irrespective of whether or not this expression had been induced by TPA. This was taken as preliminary evidence for reactivity of E8B3 and E8D2 with virus-induced proteins, a conclusion which was further substantiated by the immunoprecipitation experiments described below.

Identification of reactive molecule. To identify the molecule recognized by E8B3 and E8D2, we used the antibodies to immunoprecipitate extracts of TPA-induced and noninduced EBV producer and nonproducer cells which had been intrinsically labeled with [^{35}S]methionine. Initially, it was noticed that a molecule with an approximate molecular weight of 205,000 was precipitated, apparently from all cell lines, by protein A-Sepharose beads in the absence of any antibody (Fig. 1, lane 1). This nonspecific precipitation interfered with patterns of specific immunoprecipitation (Fig. 1, lanes 1 and 2) and, hence, in all subsequent experiments, the lysates were preincubated for 1 h with protein A-Sepharose beads, the beads were removed by centrifugation, and the remaining supernatants were mixed with test antibodies and additional protein A-Sepharose. Identical immunoprecipitation patterns were obtained with both E8B3 and E8D2 antibodies, so data obtained with just one of them, E8D2, are presented. Examination of autoradiograms indicated that a peptide with an approximate molecular weight of 105,000 (p105) was specifically immunoprecipitated by E8D2 from both TPA-induced and noninduced P3HR1-Cl13 cells (Fig. 1, lanes 2 and 3) but that larger amounts of the peptide could be immunoprecipitated after TPA induction. The peptide was not immunoprecipitated from Raji, Molt 4, or the non-virus-producing P3HR1-Cl5 cells (Fig. 1, lanes 4 to 6). Normal mouse serum and EBV-unrelated antibodies also failed to immunoprecipitate it from any cell line. The coexpression of p105 and other lytic-cycle virus antigens was confirmed by immunoprecipitation of cells with antibody D4.16 which recognizes gp350/300 and gp250/200. This antibody immunoprecipitated a predominant diffused molecule with an approximate molecular weight of 250,000 and a less prominent molecule of 350,000 from TPA-induced P3HR1-Cl13 cells and nothing from Raji and Molt 4 cells (Fig. 1, lanes 7 to 9).

Lysates of P3HR1-Cl13 cells that had been TPA induced and labeled with [^{3}H]glucosamine were repeatedly immunoprecipitated with antibody E8D2 without producing any evidence of sugar label in the p105 molecule (Fig. 2, lane 3), although gp350 was labeled (Fig. 2, lane 4). Moreover, treatment of TPA-induced P3HR1-Cl13 cells with tunica-

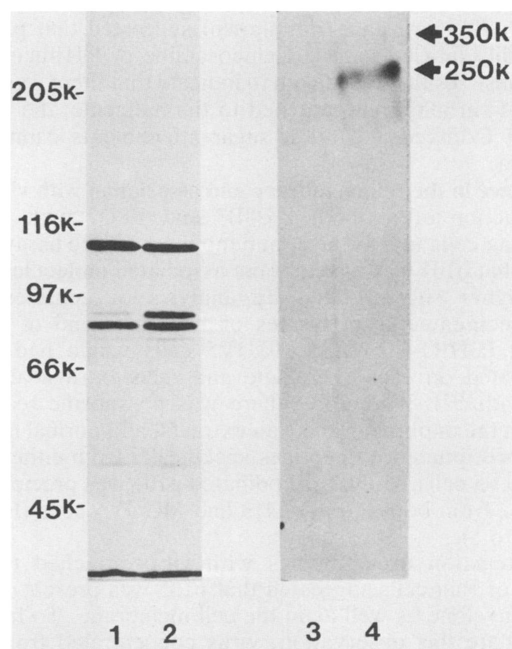

FIG. 2. SDS-PAGE analysis on 9% acrylamide of [^{35}S]methionine-labeled (lanes 1 and 2) and [^{3}H]glucosamine-labeled (lanes 3 and 4) peptides immunoprecipitated by E8D2 (lanes 1 to 3) and D4.16 (lane 4) from TPA-induced P3HR1-Cl13 cells (lanes 1, 3, and 4) and TPA-induced P3HR1-Cl13 cells treated with 5 μg of tunicamycin per ml (lane 2).

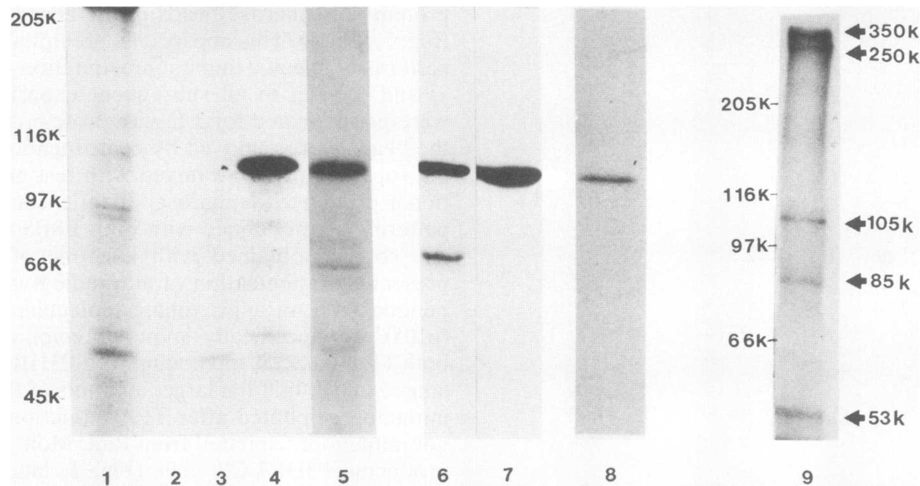

FIG. 3. SDS-PAGE analysis on 9% acrylamide of ^{125}I-labeled peptides immunoprecipitated by normal mouse serum (lanes 1 and 2), or E8D2 (lanes 3 to 8) from labeled cells (lanes 1 to 5), labeled virus (lanes 6 and 7), or a labeled complex of virus bound to receptor positive Raji cells (lane 8). Cells are Raji (lanes 1, 3, and 8), TPA-induced MCUV5 (lanes 2 and 4), and TPA-induced P3HR1-Cl13 (lane 5). Virus is derived from TPA-induced P3HR1-Cl13 cells (lanes 6 and 8) and TPA-induced MCUV5 cells (lane 7). SDS-PAGE on 7% acrylamide of dextran-purified virus from P3HR1-Cl13 labeled with ^{125}I (lane 9).

mycin before and during labeling, a procedure which inhibits cotranslational N-linked glycosylation of EBV glycoproteins and which we have shown in parallel experiments does alter the mobility of gp350 (15), did not alter the mobility of the 105,000-molecular-weight (105K) peptide (Fig. 2, lanes 1 and 2). A molecule with an approximate molecular weight of 90,000 (90K) became more prominent in the tunicamycin-treated cells (Fig. 2, lane 2), but was not consistently immunoprecipitated under these conditions; were the 90K peptide to be a precursor to p105, we might expect the mature protein to be more easily labeled with [^{3}H]glucosamine. Preliminary data (not shown) suggested that p105 is not readily labeled with [^{3}H]glucosamine or [^{3}H]fucose either. These results were taken to indicate that there was little N-linked carbohydrate attached to the molecule; the possibility of O-linked and other sugar attachments cannot be excluded.

Presence in the cell membrane and association with virions. The reaction of antibodies E8B3 and E8D2 with virus-producing cells in a surface immunofluorescence assay suggested that p105 was a membrane-associated molecule. This was further substantiated by analysis of peptides immunoprecipitated from lysates of Raji cells and of TPA-induced P3HR1-Cl13 and MCUV5 cells which had been fractionated on Ficoll-Hypaque and then extrinsically labeled with ^{125}I. As before, there was no specific reaction between the antibodies and Raji extracts, and normal mouse serum precipitated no peptides specifically from either Raji or MCUV5 cells. However, iodinated p105 was precipitated by E8D2 from both P3HR1-Cl13 and MCUV5 cells (Fig. 3, lanes 1 to 5).

The reaction of antibodies with virus attached to the surface of Raji cells suggested that p105 was present in the virion envelope as well as in the cell membrane. To further substantiate this observation, virus concentrated from the spent culture media of P3HR1-Cl13 and MCUV5 cells was labeled with ^{125}I, solubilized with lysing buffer, centrifuged at 100,000 $\times$ g to remove nucleocapsids, and immunoprecipitated. The peptide p105 was immunoprecipitated from both virus lysates (Fig. 3, lanes 6 and 7). Finally, experiments were done in which the virus was allowed to absorb to Raji cells for 1 h, and the cells were washed extensively to remove unbound virus. The entire cell-virus complex was labeled with ^{125}I, solubilized, and immunoprecipitated. Again, the only peptide specifically immunoprecipitated was p105 (Fig. 3, lane 8). A protein with similar mobility was one of the only five detectable in SDS-PAGE analysis of dextran-purified virus that had been iodinated (Fig. 3, lane 9).

Presence in superinfected Raji cells. Many previous studies have shown that Raji cells superinfected with P3HR1 virus are induced to synthesize a number of virus-specified peptides (3, 5, 10, 18, 21). To determine whether p105 is one of these peptides, Raji cells were mock superinfected or superinfected with virus derived from TPA-induced P3HR1 cells, P3HR1-Cl13 cells, or P3HR1-Cl5 cells, labeled with [^{35}S]methionine, and analyzed by SDS-PAGE. There was considerably more virus present in the preparations derived from P3HR1-Cl13 cells than in those from P3HR1 cells, but little if any virus capable of inducing synthesis of virus-specific peptides. Very little virus at all was present in preparations made from P3HR1-Cl5 cells. More than four new peptides could be seen in autoradiograms of whole-cell extracts of Raji cells superinfected with P3HR1 virus (Fig. 4, lane 4), none of which could be seen in mock-superinfected cells or cells superinfected with P3HR1-Cl13 or P3HR1-Cl5 virus (Fig. 4, lanes 1 to 3). These four peptides are among those commonly seen under these conditions of superinfection (10, 23) and included a prominent peptide with a molecular weight of 105,000. Immunoprecipitation of the lysates of mock-infected Raji cells and the Raji cells superinfected with P3HR1 indicated that this peptide was recognized by antibody E8D2 (Fig. 5).

PAA resistance. When TPA-induced P3HR1-Cl13 cells were fractionated by flotation on Ficoll-Hypaque, the cells collected from the 25 to 50% interface reacted with E8D2, E8B3, A-2-5, and D4.16 in the same proportions. However, a small percentage of cells (approximately 3%) that sedimented through the 50% Ficoll-Hypaque reacted with E8D2 and E8B3 but not with A-2-5 and D4.16. This suggested that the membrane antigen recognized by E8D2 and E8B3, rather than being a late antigen such as those recognized by A-2-5

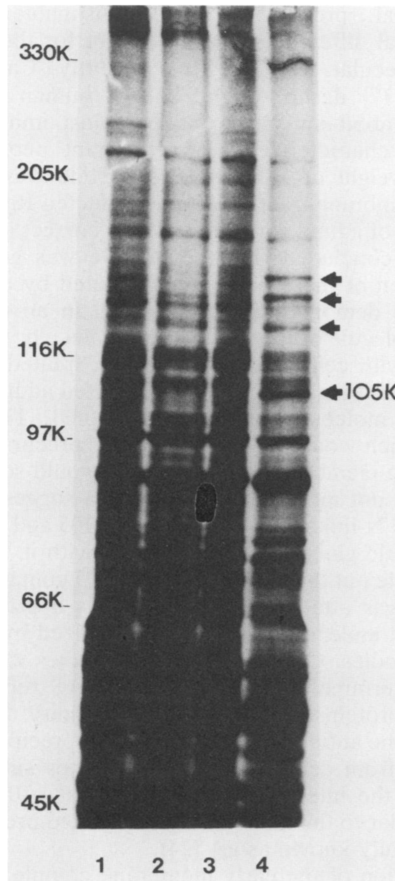

FIG. 4. SDS-PAGE analysis on 9% acrylamide of [^{35}S]methionine-labeled peptides in Raji cells after mock infection (lane 1), superinfection with concentrated medium from TPA-induced P3HR1-Cl5 cells (lane 2), superinfection with virus concentrated from TPA-induced P3HR1-Cl13 cells (lane 3), and superinfection with virus concentrated from TPA-induced P3HR1 cells (lane 4). Arrows on the right indicate four peptides characteristic of a successful superinfection.

and D4.16, might be an early antigen expressed in the induced cell membrane before the lytic cycle has progressed far enough to cause a detectable decrease in cell density. To test this possibility, P3HR1-Cl13 cells were labeled with [^{35}S]methionine in the presence or absence of PAA, which inhibits synthesis of EBV DNA and late viral proteins. The p105 peptide was immunoprecipitable from both TPA-induced and noninduced P3HR1-Cl13 cells in the presence or absence of PAA (Fig. 6). Neither gp350/300 nor gp250/200 molecules, both late antigens recognized by A-2-5 and D4.16, could be immunoprecipitated from the same PAA-treated cells (data not shown).

Effect of E8D2 and E8B3 on virus infectivity. Evidence thus far indicated that p105 is an early EBV-induced protein present not only in the producing cell membrane but also in the envelope of the mature virion. We therefore determined whether either E8D2 or E8B3 antibody was capable of interfering with the ability of MCUV5 virus to induce immunoglobulin synthesis by fresh human B cells, a property associated with and assumed to be a function of virus gene expression. Interestingly, rather than inhibiting the induction of immunoglobulin synthesis, like the known neutralizing antibody G-3-1, both antibodies enhanced this property of the virus (Table 1).

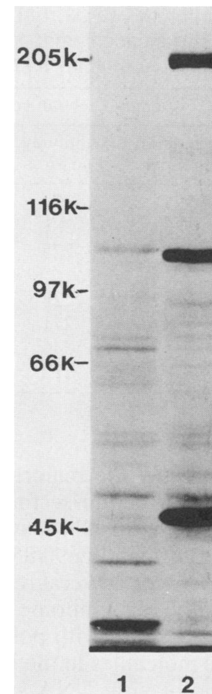

FIG. 5. SDS-PAGE analysis on 9% acrylamide of [^{35}S]methionine-labeled peptides immunoprecipitated by E8D2 from mock-infected Raji cells (lane 1) and Raji cells superinfected with P3HR1 virus (lane 2).

DISCUSSION

Identification, isolation, biochemical, and functional characterization of virus-specified membrane proteins are fundamental to a rational analysis of virus-cell interactions and important to the design and development of antiviral drugs. Several laboratories have studied EBV-induced membrane

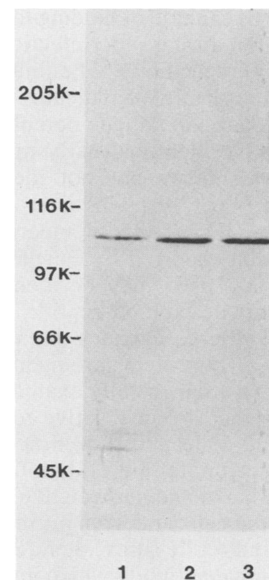

FIG. 6. SDS-PAGE analysis on 9% acrylamide of [^{35}S]methionine-labeled peptides immunoprecipitated by E8D2 from P3HR1-Cl13 cells (lane 1), P3HR1-Cl13 cells treated with PAA (lane 2), and TPA-induced P3HR1-Cl13 cells treated with PAA (lane 3).

TABLE 1. Effect of antibodies E8D2, E8B3, and G-3-1 on the ability of MCUV5 virus to induce immunoglobulin synthesis in fresh T-depleted human leukocytes

Expt	Antibody (concn)	Immunoglobulin concn (ng/ml) with:			
		No antibody, no virus	Antibody, no virus	Virus, no antibody	Antibody, virus
1	E8D2 (1/5)	375	374	4,296	8,022
	E8D2 (1/10)	375	388	4,296	7,569
	G-3-1 (1/100)	375	725	4,296	555
2	E8B3 (1/5)	238	224	5,026	8,291
	E8B3 (1/10)	238	271	5,026	7,243
	E8B3 (1/50)	238	246	5,026	5,775
	G-3-1 (1/100)	238	222	5,026	515

proteins and, by consensus, the majority of protein in the virus membrane is said to comprise four late peptides, all sensitive to inhibitors of viral DNA synthesis. These are gp350/300, gp250/200, p140, and gp90/85 (reviewed in reference 25). There are, however, several reasons for supposing that there are virus-specified membrane proteins in addition to these four. First, early work with polyclonal human sera indicates that there are molecules in the infected cell that are insensitive to inhibition of viral DNA synthesis (9, 22, 28). Second, several groups have described the immunoprecipitation of additional molecules (26) which have not yet been satisfactorily identified as either precursors for or breakdown products of the four late proteins. Third, discriminant analysis of the hypothetical proteins deduced from the EBV DNA sequence (1), by the program of Klein and colleagues (16), indicates that there are at least 33 open reading frames which potentially code for transmembrane proteins. Finally, analogy with other herpesviruses of both human and animal origins might suggest that the existence of more than four membrane proteins could reasonably be anticipated (24).

Our results indicate that p105 is one of these additional virus-specified membrane proteins. The correlation between its expression and entry of virus into the lytic cycle, its association with virus capable of binding to receptor positive cells, and its induction during superinfection of Raji cells are all suggestive of viral specificity. The failure of PAA treatment to inhibit synthesis of p105 indicates that the peptide is part of the early membrane antigen complex.

Several groups have identified early membrane proteins with approximately similar, but not identical, molecular weights. The pattern of virus-induced proteins that we obtained by SDS-PAGE analysis of superinfected Raji cells is very similar to that reported by Feighny and colleagues (10) and suggests that p105 may be identical to the early protein these workers designated peptide 11. However, this report, like many others, includes no information as to whether any of the early proteins are membrane associated. Only three groups have specifically examined EBV-induced membrane proteins that are insensitive to DNA inhibitors. Mueller-Lantzch and colleagues (18) first used human antisera to immunoprecipitate an arabinofuranosylcytosine-resistant protein with a molecular weight of 80,000 from both the surface of infected cells and from purified virus particles. Thorley-Lawson and colleagues then cited unpublished work with a monoclonal antibody which immunoprecipitated a PAA-resistant membrane protein with a molecular weight of 95,000 (26). They suggested that this molecule, which was expressed on the surface of producing cells and copurified with virions, might be the same as that described by Mueller-Lantzch et al., presumably on the reasonable assumption that technical differences might account for the discrepancies in molecular weights. Most recently, Casareale and co-workers (7) described the use of human sera and a polyclonal rabbit antiserum made against immunopurified protein, to characterize a PAA-resistant peptide with a molecular weight of 105,000. The peptide was associated with the membrane fraction of superinfected Raji cells and, on the basis of a faint sugar level in the correct region of the gel, it was concluded that the peptide was glycosylated. Interpretation of these data is complicated by the fact that experiments demonstrating sugar label in an immunoprecipitated molecule of the appropriate molecular weight were done only with cells that had not been treated with PAA. Also, the rabbit serum immunoprecipitated additional molecules with molecular weights of 79,000, 110,000, and 190,000, which would be appropriate for precursor and final forms of late membrane proteins, so it would seem that the reagent was not monospecific. Our data suggest that there may be little N-linked glycosylation of p105 and that there is relatively little glucosamine associated with it. We cannot, however, rule out the possibility that p105 contains O-linked or other sugar attachments or that it is a precursor to a glycosylated molecule that is not recognized by our monoclonal antibodies. Cross testing of antibodies will be necessary to determine whether any of these reported early membrane proteins are identical. Preliminary data indicate that one of the antibodies (E8B3) immunoprecipitates glycoprotein gB from cells infected with herpes simplex virus. This raises the interesting possibility that p105 may be a protein similar to the minimally glycosylated precursor form of gB, formally known as gA (24).

The function of the early membrane complex of EBV is unknown, although it has been speculated that it may be useful to the host as a marker on cells in the early stages of the lytic cycle (26). The presence of p105 in the virion envelope might suggest that this peptide is additionally important to entry or release of virus from infected cells. Neither of the two monoclonal antibodies E8B3 or E8D2 neutralized virus infectivity in the absence of complement. At this point, we do not know whether the two antibodies recognize the same epitope on p105, so the biologic significance of the observation is not clear. However, it is noteworthy that the polyclonal rabbit antiserum, used by Casareale and colleagues to identify what may be the same molecule, also did not neutralize infectivity (7). Interestingly, both of our monoclonal antibodies did apparently enhance the ability of virus to induce immunoglobulin synthesis in fresh human B cells. The mechanism of this enhancement is obscure, but it seems possible that the antibodies have either enhanced uptake of virus in some way, perhaps via an Fc receptor, or enhanced a postentry step of virus infectivity. Preliminary experiments with EBV labeled intrinsically with [^{3}H]thymidine suggest that both antibodies can enhance binding of virus to receptor-positive cells. Further work will be necessary to establish whether this is a property of all nonneutralizing antibodies that bind to EBV membrane proteins, only those of the immunoglobulin G2a subclass, or only those that bind to p105.

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