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Immunology

Identification of Epstein–Barr virus strain differences with monoclonal antibody to a membrane glycoprotein

(immunoprecipitation/antigen/electrophoresis/immunofluorescence)

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ABSTRACT **Two monoclonal antibodies directed against Epstein–Barr virus (EBV)-induced membrane antigens (MA) were isolated in this study. One of the monoclonal antibodies, designated 2F5.6, was an IgG2 which, as detected by membrane and fixed cell immunofluorescence, reacted with MA-positive lymphoblastoid cell lines that produced transforming EBV but not with the MA-positive P_3HR-1 cell line that produced the lytic, nontransforming strain of this virus. This antibody precipitated the M_r 320,000/350,000 glycoprotein from B-95 virus infected cultures and the M_r 300,000 and 220,000/250,000 glycoproteins from Raji cells superinfected with P_3HR-1 virus but did not precipitate any of these EBV-specific glycoproteins from the P_3HR-1 cell line. In contrast, the second monoclonal antibody, IgM designated B10.3, reacted with all virus-producing cell lines including the P_3HR-1 cell line. The identity of the glycoprotein that serves as the target for this antibody is still unknown. Neither antibody had neutralizing activity against the B-95 or P_3HR-1 strain of EBV. These results indicated that the 2F5.6 monoclonal antibody was directed against an antigenic determinant on the major membrane glycoprotein which is common to transforming strains of EBV but absent from the lytic P_3HR-1 strain whereas the B10.3 monoclonal antibody was directed against a group-specific EBV-induced membrane determinant.**

The Epstein–Barr virus (EBV) has been studied extensively because of its possible involvement in the etiology of nasopharyngeal carcinoma and African Burkitt lymphoma. Such studies have led to the identification of EBV isolates with different biological activities (1). The two prototype viruses that have been utilized extensively in these investigations are the P_3HR-1 virus produced by a subline derived from a cell line established from an African Burkitt lymphoma biopsy sample (Jijoye) (2) and the B-95 virus isolated from a patient with infectious mononucleosis and propagated in marmoset cells (3). Although the viruses produced by both of these cell lines are morphologically similar and can be neutralized by sera from EBV-infected individuals, the biological activity and biochemical composition of these viruses show some significant differences.

The P_3HR-1 virus is cytolytic for B lymphocytes, the target cell for EBV, and has apparently lost its ability to immortalize or transform B lymphocytes (4, 5). Therefore, it is categorized as a lytic nontransforming virus. This virus does not induce the expression of the EBV-induced nuclear antigen (EBNA) upon infection of B lymphocytes nor does it stimulate DNA synthesis, two characteristics of transforming strains of EBV (6). Upon superinfection of EBV-genome-positive nonproducer cell lines with P_3HR-1 virus, the synthesis of the early antigen (EA) complex is initiated followed by a shutdown of cell synthesis of macromolecules and the eventual death of the infected cell (7, 8).

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In contrast, transforming strains of EBV, including the B-95 virus, generally do not initiate synthesis of the EA complex which signals the eventual death of the cell but they are highly efficient in transforming or immortalizing B lymphocytes after infection (4, 5). Such transformed cells generally do not produce large amounts of virus but every cell expresses EBNA (9).

Biochemically these two prototypes differ somewhat in the electrophoretic gel patterns obtained after digestion of their DNAs with the same endonuclease restriction enzymes even though the DNAs of the two strains are approximately 90% homologous (10, 11). The P_3HR-1 DNA appears to have many more fragments than does the B-95 virus, and some of the fragments are present in submolar amounts, suggesting a heterogeneity in the P_3HR-1 virus DNA composition (10, 11). More recently, it was reported that, although the DNA of the P_3HR-1 virus showed many areas of similarity with B-95 virus, the molecules were rearranged with respect to the B-95 genome (12).

There is little information in the literature to indicate that these two virus strains differ immunologically. Analysis of extracellular virus particles produced by these two cell lines failed to reveal any differences in the major polypeptides present in them (13). More recently, however, analysis of EBV-induced membrane glycoproteins has revealed differences between cells infected with the P_3HR-1 and B-95 viruses (14). Whether these differences were true strain differences or species differences due to the fact that the viruses were cultivated in cells from different species, as suggested by other authors (15), was unclear.

In this report, immunological evidence obtained with monoclonal antibodies is presented which suggests that at least one of the major high molecular weight glycoproteins expressed in the membranes of EBV-infected cells contains both group-specific and strain-specific antigenic determinants.

MATERIALS AND METHODS

Cell Lines. The cell lines used in this study included the virus-producing P_3HR-1 cell line, a B-95 virus-induced cotton-top marmoset tumor cell line (16) received from Harvey Rabin (Frederick Cancer Research Center) and designated 1605L, the B-95-8 cell line (3), and Raji cell line, the EBV-negative BJAB and Ramos cell lines, and the mouse plasmacytoma cell line P_3NS-1 1-AG4-1 (NS-1). All cell lines were grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (56°C,

Abbreviations: EBV, Epstein–Barr virus; MA, membrane antigen; MF, membrane immunofluorescence; EA, EBV-induced early antigens; EBNA, EBV-induced nuclear antigen; P_i/NaCl, phosphate-buffered saline; TPA, phorbol 12-myristate 13-acetate.

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30 min) and gentamycin at 50 μg/ml. For the NS-1 cell line, this medium was supplemented with 4.5 g of glucose per liter. The cell cultures were passaged every 3–4 days and reseeded at a concentration of 5×10^5 cells per ml.

Immunization. Mice were immunized once with 1×10^7 1605L cells activated 3 days earlier with the tumor-promoting agent phorbol 12-myristate 13-acetate TPA (40 ng/ml; Sigma) as described (14). Such cultures generally contained 30–50% membrane antigen (MA)-positive cells. Viable cells for immunization were mixed with an equal volume of complete Freund's adjuvant and injected intraperitoneally. Spleens for the production of hybridomas were isolated 4 days after the initial immunization.

Production of Monoclonal Antibodies. Hybridomas were established according to the protocol of Kennett *et al.* (17). Spleen cells (10^8 cells per ml) were mixed with 10^7 NS-1 myeloma cells and the mixture was pelleted at $900 \times g$ for 5 min. The supernatant was discarded and the cells were washed once with S-0 medium (RPMI-1640 medium supplemented with 4.5 g of glucose per liter and 10 ml of 200 mM glutamine). All medium was then drained from the tubes and the pellets were gently resuspended in 0.2 ml of 30% polyethylene glycol 1500 (J. T. Baker, Phillipsburg, NJ). The cells were exposed to polyethylene glycol for 8 min and centrifuged at $900 \times g$ for 3 min of this 8 min period, after which 5 ml of S-0 medium was added and the cells were recentrifuged at $900 \times g$ for 5 min. The pellet was then resuspended in 30 ml of HAT medium (0.1 mM hypoxanthine/0.4 μM aminopterin/16 μM thymidine) in S-20 medium (S-0 medium plus 20% fetal calf serum and 10% NCTC 135) at a concentration of $1–2 \times 10^6$ cells per ml. The cells were distributed into each of the wells in 96-well microtiter plates and fed every 3 days with HAT medium. Wells with visible clones were marked and the supernatant fluids from such wells were assayed for antibodies to EBV-induced MAs. Positive cultures were further cloned twice in soft agar and the clones were rescreened. Positive clones were again picked and grown in Falcon flasks. Growing cultures were then injected into mice primed with 0.3 ml of pristane for the production of antibody-containing ascites fluid.

Membrane Immunofluorescence (MF) Assay. Culture medium from microtiter wells containing growing clones were screened for antibodies to EBV-induced MA by MF (18) using fluorescein-conjugated goat or rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA). Uninfected Raji cells and Raji cells superinfected with P_3HR-1 virus were initially used as targets in the screening assay. Culture fluids positive for antibodies to EBV-induced MA expressed in the superinfected Raji cells but negative for antibodies to uninfected Raji cells were also tested against BJAB and TPA-activated 1605L and B-95-8 cells to confirm EBV specificity. Clones producing EBV-specific antibody were then injected into mice, and ascites fluids from such mice were also screened by MF for antibodies to EBV-induced MA.

Membrane Solubilization and Immune Precipitation. MA-positive cells radiolabeled with ^{125}I as described (14, 19) were mixed with 1% Triton X-100 in phosphate-buffered saline (P_i/NaCl) containing 2 mM phenylmethylsulfonyl fluoride (Pierce) at a concentration of 1 ml of solvent per 2×10^7 cells. After 30 min at 25°C, nuclei and particulate matter were removed by centrifugation at $10,000 \times g$ for 1 hr. The supernatant fluid was then decanted and exhaustively dialyzed against 0.1% Triton X-100/P_i/NaCl for 2 days. For radioimmunoprecipitation, aliquots of the extracts containing 0.4 to 1.0×10^6 cpm of ^{125}I were added to 0.1 ml of antibody-negative or antibody-positive ascites in the presence of P_i/NaCl containing magnesium and calcium and supplemented with 1% bovine serum albumin. The mixtures were then incubated overnight at 4°C. Immune complexes were removed by precipitation with protein A-Sepharose 4B (Pharmacia) as outlined in detail elsewhere (14, 19). The complexes were then analyzed for the presence of EBV-specific membrane components by polyacrylamide gel electrophoresis as described (14, 19). The ^{14}C-labeled proteins used as molecular weight standards were myosin (210,000), phosphorylase *b* (92,500), human serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lactoglobulin A (18,310) (New England Nuclear).

RESULTS

Isolation of EBV-Specific Monoclonal Antibodies. Approximately 60 clones were screened by MF for antibodies to EBV-induced MA. Of these, two clones, designated B10.3 and 2F5.6, were found to be producing antibodies specific to these antigens. The immunoglobulin produced by clone 2F5.6 was identified, by double radial immunodiffusion, to be of the IgG2b class; that produced by clone B10.3 was identified as an IgM antibody.

The MF results are shown in Table 1. The 2F5.6 ascites reacted at high titers against the virus-positive 1605L marmoset cell line, P_3HR-1 virus-superinfected Raji cells, and the B-95-8 virus-producing cell line which expressed 40–50% MA-positive cells after 72 hr of exposure to TPA. No reactivity was noted against uninfected Raji cells, BJAB cells, or, interestingly, the TPA-activated virus-producing P_3HR-1 cell line even though this culture contained 30–40% MF-positive cells as determined with standard human sera. The B10.3 ascites contained antibodies reactive with all virus-producing cell lines including TPA-activated P_3HR-1 cells but not with the two EBV-negative cell lines. The B476 ascites, used as a negative control, did not react with any of the cell lines.

These ascites were also tested for EBV-specific antibodies against acetone-fixed smears of two virus-producing cell lines (TPA-activated P_3HR-1 and TPA-activated B-95-8 cells) and against acetone-fixed smears of P_3HR-1 virus-infected Raji cells cultivated in the presence of phosphonoacetic acid which inhibits the synthesis of late antigens (viral capsid antigens, MAs) but not EBV-induced EAs (20). Interestingly, a similar pattern of reactivity was noted with these targets (Table 2). The 2F5.6 ascites reacted at high titer with the TPA-activated B-95-8 culture but not with the TPA-activated P_3HR-1 culture or with the phosphonoacetic acid-treated, EA-positive P_3HR-1 superinfected Raji culture. In contrast, the B10.3 ascites reacted with smears from both virus-producer cultures but not with the phosphonoacetic acid-treated, EA-positive superinfected Raji cells. Again, the negative ascites did not react with any of the target cell lines.

Table 1. Antibody titers of monoclonal antibodies to EBV-induced MA against viable uninfected and infected cell lines as determined by MF

Ascites fluid	Antibody titers*					
	Raji	BJAB	1605L	P_3HR-1-Raji†	P_3HR-1‡	B-95-8‡
2F5.6§	<10	<10	>640	640	<10	>1280
B10.3¶	<10	<10	>640	640	>640	>1280
B476	<10	<10	<10	<10	<10	<10

* Reciprocal of antibody dilution showing positive fluorescence.
† P_3HR-1 virus superinfected Raji cells.
‡ TPA-activated cultures.
§ Immunoglobulin class identified as IgG2. Antibody did not neutralize infectivity of P_3HR-1 or B-95 viruses.
¶ Immunoglobulin class identified as IgM. Antibody did not neutralize infectivity of P_3HR-1 or B-95 viruses.

Table 2. Antibody titers of monoclonal antibodies to EBV-induced MA against acetone-fixed EBV-infected cell preparations

Ascites	Antibody titers*		
	P_3HR-1†	B-95-8†	P_3HR-1-Raji‡
2F5.6	<10	>1280	<10
B10.3	640	>1280	<10
B476	<10	<10	<10

* Reciprocal of antibody dilution showing positive fluorescence.
† TPA-activated cultures.
‡ Cultivated in the presence of phosphonoacetic acid and expressing EA only.

Immunoprecipitation of EBV-Specific Membrane Proteins. To determine which of the major membrane glycoproteins expressed in B-95-8 cells were being detected with these two monoclonal antibodies, immunoprecipitation experiments were set up against the ^{125}I-labeled Triton X-100 extract prepared from TPA-activated B-95-8 cultures. The 2F5.6 ascites precipitated a glycoprotein with M_r 320,000/350,000 (Fig. 1, lane D) identical in M_r to the major glycoprotein precipitated by an antibody-positive human reference serum (Fig. 1, lane E). This is one of the two major membrane glycoproteins previously identified in the membranes of these cells (14). In contrast, no proteins were precipitated from this extract by the B10.3 ascites (Fig. 1, lane C). This was repeated numerous times, with similar results, against ^{125}I-labeled extracts from this cell line and extracts labeled with tritiated sodium borohydride. The low M_r proteins apparent in this gel were nonspecifically precipitated by both antibody-positive and antibody-negative sera as observed (14, 19) and are not EBV-specific.

Because of the differential reactivity of the 2F5.6 antibody with membrane components expressed in different virus-producing cell lines as shown in Tables 1 and 2, immunoprecipitations were also set up with ^{125}I-labeled extracts prepared from MA-positive, P_3HR-1 virus-superinfected Raji cells and TPA-activated P_3HR-1 cells. The 2F5.6 antibody precipitated the M_r 300,000 and 220,000/250,000 EBV-specific glycoproteins from the extracts prepared from the MA-positive, P_3HR-1 superinfected Raji culture (Fig. 2, lane B; Fig. 3). These are the two high M_r glycoproteins previously identified in these cells with human sera (14, 19) as shown in Fig. 2, lane E. In contrast, neither of these glycoproteins was precipitated from ^{125}I-labeled extracts prepared from MA-positive, TPA-activated P_3HR-1 cells (Fig. 2, lane A; Fig. 3) even though both of these proteins were precipitated by a human serum containing antibodies to EBV antigens (Fig. 2, lane D).

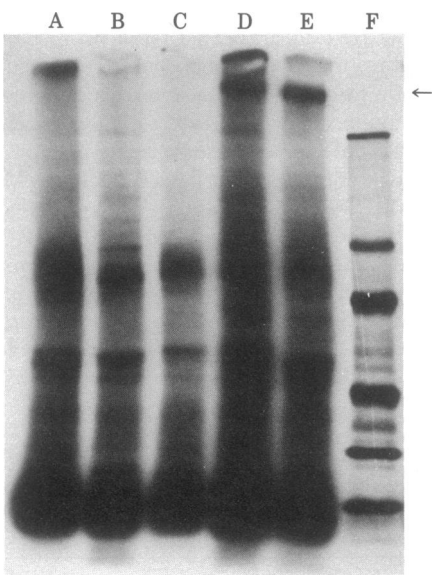

FIG. 1. Autoradiograph of the $NaDodSO_4$/polyacrylamide gel electrophoresis of EBV-induced ^{125}I-labeled surface proteins precipitated from TPA-activated B-95-8 cells with monoclonal antibodies. Lanes: A, anti-EBV antibody-negative mouse ascites; B, anti-EBV antibody-negative human serum; C, B10.3 monoclonal antibody-positive mouse ascites; D, 2F5.6 monoclonal antibody-positive mouse ascites; E, anti-EBV antibody-positive human serum; F, M_r standards. Extracts contained 900,000 cpm of ^{125}I. Note high M_r (320,000) glycoprotein (arrow) precipitated by 2F5.6 monoclonal (lane D) and human reference serum (lane E) but not by anti-EBV-negative ascites (lane A) or serum (lane B).

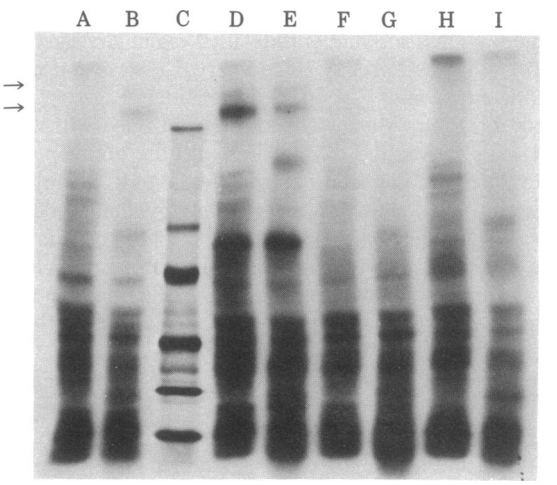

FIG. 2. Autoradiograph of the $NaDodSO_4$/polyacrylamide gel electrophoresis of EBV-induced ^{125}I-labeled surface proteins precipitated from TPA-activated P_3HR-1 cells or P_3HR-1 virus-superinfected Raji cells with monoclonal antibodies. Lanes: A, TPA-P_3HR-1 extract plus 2F5.6 monoclonal antibody; B, P_3HR-1 virus-superinfected Raji cell extract plus 2F5.6 monoclonal antibody; C, M_r standards; D, TPA-P_3HR-1 extract plus antibody-positive human (anti-VCA titer = 640; anti-MA = 320; anti-EA = 10); E, P_3HR-1 virus-superinfected Raji cell extract plus antibody-positive human serum; F, TPA-P_3HR-1 extract plus antibody-negative human serum; G, P_3HR-1 virus-superinfected Raji cell extract plus antibody-negative human serum; H, TPA-P_3HR-1 extract plus anti-EBV antibody-negative mouse ascites; I, P_3HR-1 virus-superinfected Raji cell extract plus anti-EBV antibody-negative mouse ascites. Each extract contained 430,000 cpm of ^{125}I. Note high M_r EBV-specified glycoproteins precipitated by 2F5.6 ascites from P_3HR-1 superinfected Raji cells (lane B) which are identical to those precipitated by human reference serum (lane E). In contrast, these same two high M_r glycoproteins present in TPA-activated P_3HR-1 cells (lane D) were not precipitated by the 2F5.6 monoclonal antibody from this cell line (lane A). Arrows show M_r 300,000 and 250,000.

DISCUSSION

Two monoclonal antibodies against EBV-induced MA components expressed in EBV-infected cells were isolated in this study. One of these, designated 2F5.6, precipitated the M_r 320,000/350,000 glycoprotein previously identified in B-95 virus-infected cells by using human sera (14). This antibody did not neutralize the infectivity of P_3HR-1 virus as determined by inhibition of EA induction in superinfected Raji cells or of B-95 virus as determined by inhibition of EBNA induction in superinfected Ramos cells. These experiments were run in both the presence and absence of an exogenous source of complement. Thus, this antibody appeared to recognize a different determinant on this molecule than that recognized by the monoclonal antibodies reported by Hoffman *et al.* (21) and by Thorley-Lawson and Gerlinger (22) which appeared to recog-

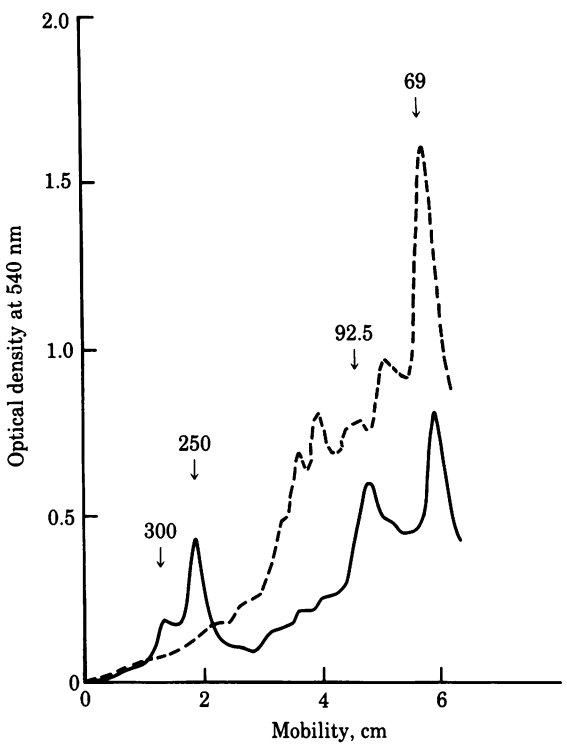

FIG. 3. Densitometric tracing of the high M_r regions in lanes A and B of Fig. 2, showing that the 2F5.6 monoclonal antibody specifically precipitated the M_r 300,000 and 220,000/250,000 glycoproteins from P_3HR-1 virus-superinfected Raji cells (——) but not from the MA-positive P_3HR-1 cell line (----). M_rs are shown × 10^{-3}.

nize the same high M_r glycoprotein but which also neutralized EBV infectivity.

Further evidence that this antibody recognized a different determinant on this glycoprotein came from the interesting observation that the 2F5.6 monoclonal antibody did not react with TPA-activated P_3HR-1 cells in an immunofluorescence assay nor did it precipitate the M_r 220,000/250,000 glycoprotein from this cell line. This glycoprotein was precipitated from this cell line by the monoclonal antibody isolated by Thorley-Lawson and Gerlinger (22) and is the major glycoprotein expressed in these cells and in Raji cells superinfected with P_3HR-1 virus. The 2F5.6 antibody did precipitate the M_r 220,000/250,000 glycoprotein from these latter cells, however, which reportedly produce transforming virus (23, 24). These findings suggest, therefore, that the activated resident genome in Raji cells must participate in the synthesis of MA in these superinfected cells.

Because the P_3HR-1 cells produce lytic but nontransforming virus whereas the other virus-infected cells used in this study (1605L, B-95-8, and EBV-superinfected Raji cells) all produce transforming virus, these results raise the interesting possibility that the 2F5.6 monoclonal antibody is detecting a strain-specific antigen common to some transforming strains of EBV and that the M_r 320,000/350,000 glycoprotein, therefore, expresses both group-specific and strain-specific determinants. It is necessary to examine cell lines producing other strains of transforming virus, however, before a definitive conclusion can be reached on this point. One such line, designated AG876, was recently tested by immunofluorescence and was also positive with the 2F5.6 antibody. These results also provide further evidence supporting a previous conclusion that there were possible antigenic differences between the P_3HR-1 and B-95 strains of EBV (14). Whether the determinant expressed in B-95 infected cells that is absent in the P_3HR-1 cells is present on the carbohydrate or protein moiety of the M_r 320,000/350,000 molecule remains to be determined.

In addition, these results confirm our previous speculation that the M_r 300,000 and 220,000/250,000 glycoproteins expressed in P_3HR-1 virus-superinfected Raji cells and M_r 320,000/350,000 glycoprotein produced in B-95 virus-infected cells are related biochemically and antigenically (14). The precipitation of the three antigens by the same monoclonal antibody in this study clearly demonstrates immunological crossreactivity and, therefore, biochemical similarities. The molecular weight differences of these three antigens noted on NaDodSO$_4$ gels therefore must represent distinct differences in the synthesis, glycosylation, or processing of the high M_r antigen(s) as controlled by the P_3HR-1 and B-95 genomes.

The second monoclonal antibody isolated in this study, designated B10.3, showed a different specificity. By immunofluorescence, this antibody reacted with the MAs expressed in all the cell lines tested including the P_3HR-1 cell culture. This was noted with both viable cells and acetone-fixed preparations. The fact that this antibody as well as the 2F5.6 antibody reacted with acetone-fixed smears of virus-producing cell lines indicated that these components were also expressed on cytoplasmic membranous components of the cells, supporting a similar conclusion drawn by Hoffman *et al.* (21) and by Thorley-Lawson and Gerlinger (22). The fact that neither of the monoclonal antibodies reacted with fixed cells from P_3HR-1 virus-superinfected Raji cells grown in the presence of phosphonoacetic acid and which express only EAs indicated that the membrane determinants detected by these antibodies were late products of the viral genome.

It has not been possible to identify definitively the membrane protein in EBV-infected cells that serves as the target antigen for the B10.3 antibody. The reasons for this are not clear. It is probable that this is due to the fact that this is an IgM antibody which does not bind to protein A. However, attempts to use a second antibody directed against this monoclonal antibody to precipitate immune complexes have also been unsuccessful. One possible explanation is that the B10.3 IgM is a low-avidity antibody whose binding is disrupted during the radioimmunoprecipitation procedure. Another might be that the antigen recognized by this antibody might not be protein in nature and, therefore, not labeled or precipitated by the methods used in this study. However, based on the specificity of the immunofluorescence reactions discussed above, it is clear that the EBV-specific determinant recognized by this antibody differs from that identified by the 2F5.6 monoclonal and is group-specific but not strain-specific.

In addition to the above tests, both antibodies were also tested in the antibody-dependent cellular cytotoxicity assay against P_3HR-1-superinfected Raji cells (25). Neither antibody mediated cytotoxicity in these preliminary experiments. Whether this was due to antibody avidity or to the fact that these antigenic determinants are not the targets remains to be determined.

In summary, two monoclonal antibodies directed against EBV-membrane antigens were isolated in this study. One of these appeared to be directed against a determinant on the M_r 320,000/350,000 glycoprotein which is shared by transforming strains of EBV but not by the lytic P_3HR-1 strain. The second antibody was directed against a group-specific determinant.

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