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Production and Characterization of Monoclonal Antibodies Against the Epstein-Barr Virus Membrane Antigen

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Five murine hybridoma lines that produce monoclonal antibodies against Epstein-Barr virus membrane antigen (MA) were established. Immunoprecipitation experiments demonstrated that three of the antibodies precipitated both the 236,000 (236K) MA and the 212K MA. The other two antibodies precipitated the 86K MA. Antibodies against the 236K-212K MA and the 86K MA mediated complement-dependent cytolysis of Epstein-Barr-virus-infected cells. The antibodies against the 86K MA neutralized both the B95-8 and P3HR-1 viruses.

Immunoprecipitation experiments performed in several laboratories, including our own, have established correlations between Epstein-Barr virus (EBV) membrane antigen (MA) and several infected-cell proteins (4, 5, 7, 8, 11, 12, 14, 16, 17). In our laboratory EBV MA was associated with three glycoproteins with molecular weights of 236,000 (236K), 212K, and 141K on the basis of immunoprecipitation experiments performed with human sera and EBV-positive cells radiolabeled by the neuraminidase-galactose oxidase-tritiated borohydride [NGO-NaB($^3H_4$)] technique. A fourth protein at 86K was specifically precipitated when the cells were labeled with a tritiated amino acid mixture. The 86K protein is also a glycoprotein because it binds to lentil lectin-Sepharose (15). Delineation of the humoral and cell-mediated immune responses elicited by the individual components of EBV MA is dependent upon the purification of the individual MA components and the production of monospecific antisera to them.

Since only small amounts of antigen were available for immunization, the establishment of continuous mouse hybridoma lines producing monoclonal antibodies appeared to be the appropriate course of action. We began by purifying the 236K MA to electrophoretic homogeneity by a two-step procedure of lentil lectin-Sepharose chromatography and SDS-acrylamide gel electrophoresis (15). A mouse immunized with the purified 236K MA produced a specific but weak immune response as measured in the indirect membrane fluorescence assay. However, when numerous hybridoma lines from this mouse were screened for the production of EBV MA-specific antibodies, none was detected. This suggested that the purification procedure was reducing the native antigenicity or immunogeneticity of the 236K molecule. Therefore, to minimize the possibility of antigen denaturation and maximize the immunogenic potential of the antigen, we turned to an immunization protocol using intact EBV-positive cells. In this paper we report on the establishment of five mouse hybridoma lines that produce monoclonal antibodies to EBV MA and on the characterization of these antibodies with respect to immunoglobulin subclass, EBV MA component specificity, mediation of complement-dependent cytolysis, and virus-neutralizing ability.

MATERIALS AND METHODS

Cell growth conditions. All of the human and cotton-top marmoset lymphoblastoid cell lines used in these experiments were grown in RPMI 1640 medium containing 10% decomplemented fetal bovine serum supplemented with 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). The cells were grown at 37°C in a humidified atmosphere of 95% air–5% CO_2.

Preparation of mouse hybridoma lines producing EBV-MA-specific monoclonal antibodies. A mouse was immunized with intact B95-8 cells. B95-8, an EBV-transformed cotton-top marmoset cell line, was induced for EBV MA by growth for 3 days in medium containing 20 ng of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) per ml (18). After a 3-day induction period approximately 20% of the cells in the culture were MA(+). The cells were washed three times in Dulbecco PBS (DPBS) and suspended at a concentration of 1×10^7 cells per ml. One-milliliter volumes of cell suspension were layered on top of discontinuous Histopaque (Sigma Chemical Co., St. Louis, Mo.) gradients which were formed by placing 3 ml of 80% Histopaque in a 15-ml conical centrifuge tube followed by 5 ml of 50% and 5 ml of 25% Histopaque. The gradients were centrifuged at $250 \times g$ for 10 min at 10°C. The cells at the 25 to 50% interface were removed for injection. This subpopulation of B95-8 cells was >90% MA(+) and constituted approximately 5% of the cells layered on the gradient.

A BALB/c mouse was immunized with four weekly,

intraperitoneal injections of TPA-induced B95-8 cells. Three days after the final injection, spleen and lymph node cells were fused to BALB/c P3/NS1/1-Ag4 myeloma cells (obtained indirectly with permission from C. Milstein), and selected hybridoma cells were cloned as previously described (9). Ascites fluids were produced in pristane-treated BALB/c mice.

A membrane immunofluorescence (MF) assay employing TPA-induced P3HR-1, an EBV-transformed human cell line, was used to screen for hybridomas producing antibody to EBV MA. Hybridomas were selected which produced antibodies giving confluent membrane fluorescence on a percentage of the P3HR-1 cells similar to that obtained with a control MA(+) human serum. The MF assay was also used to determine the concentration of antibody in each ascites fluid, and the membrane fluorescence titers (MFT) of the ascites fluids are given below. The monoclonal antibodies in the ascites fluids were typed in double-diffusion precipitin assays with rabbit anti-mouse immunoglobulin sera (Miles Laboratories, Elkhart, Ind.).

Determination of the EBV MA component specificities of the monoclonal antibodies. B95-8 cells were either treated with 20 ng of TPA per ml for 3 days to induce MA synthesis or treated with 100 μg of phosphonoacetic acid (PAA) per ml for 3 days to inhibit MA synthesis. Both groups of B95-8 cells were radiolabeled by the NGO-NaB(^{3}H)$_4$ technique, as previously described (3, 13).

P3HR-1 cells were either treated with 20 ng of TPA per ml for 3 days to induce MA synthesis or passaged daily for 3 days to reduce the number of cells producing virus capsid antigen and MA. After harvest and washing three times with PBS, both groups of P3HR-1 cells were radiolabeled by the IODO-GEN procedure (2, 6). Briefly, 1-ml volumes of cell suspension (1×10^7 cells per ml of Dulbecco PBS) were placed in glass scintillation vials, the bottoms of which had each been coated with 100 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (IODO-GEN, Pierce Chemical Co., Rockford, Ill.) Carrier-free [^{125}I]Na (New England Nuclear Corp., Boston, Mass.) was diluted in 2 ml of Dulbecco PBS, and 0.1 ml (250 μCi) was added to each vial. After incubation for 10 min at room temperature, the cells were transferred to a tube and washed three times.

For immunoprecipitation, 1×10^7 intact radiolabeled cells were incubated for 30 min at 37°C with 0.2 ml of decomplemented ascites fluid. MA(+) human serum 7 (MFT = 1:40), absorbed with MLC-1 (a cotton-top marmoset T cell line) to remove nonspecific fluorescence, and MA(−) human serum 1 (14) were included in each set of ascites fluids tested, as controls. The cells were lysed, and the antigen-antibody complexes were isolated, dissociated, and subjected to electrophoresis on 8% SDS-acrylamide gels as previously described (13).

Antibody-mediated complement-dependent cytolysis assay. B95-8 cells were induced for 3 days with 20 ng of TPA per ml. The induced B95-8 cells were centrifuged on discontinuous Histopaque gradients. To obtain

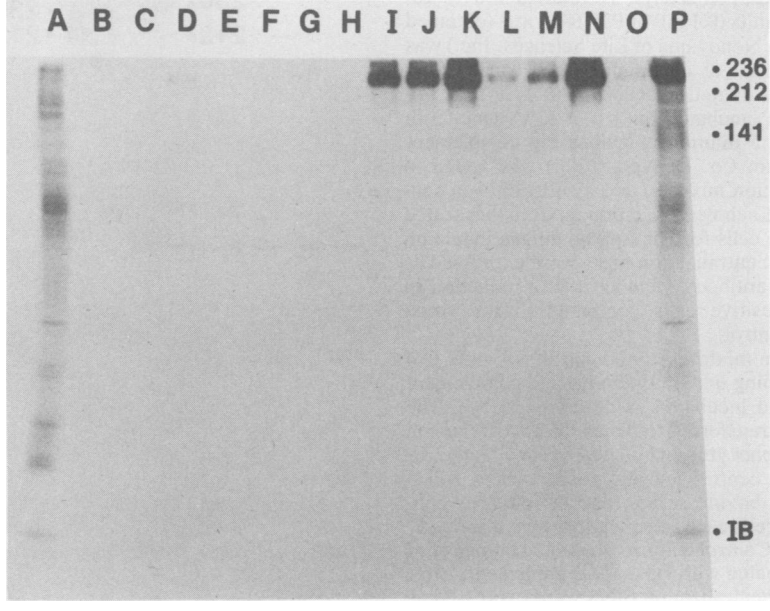

FIG. 1. Fluorogram of the NGO-NaB(^{3}H)$_4$-labeled surface glycoproteins immunoprecipitated from B95-8 cells. PAA-treated B95-8 cells (1% MA+ cells), tracks A through H. TPA-induced B95-8 cells (10% MA+ cells), tracks I through P. The total labeled surface glycoprotein patterns, tracks A and P. Immunoprecipitates obtained with ascites fluids or human sera controls: A-2-5 (MFT = 1:512), tracks B and I; B-1-1 (MFT = 1:2,048), tracks C and J; C-3-2 (MFT = 1:5,460), tracks D and K; F-2-1 (MFT = 1:2,048), tracks E and L; G-3-1 (MFT = 1:16,384), tracks F and M; EBV-positive human serum 7 (MFT = 1:40), tracks G and N; EBV-negative human serum 1, tracks H and O. The numbers in the margin indicate the molecular weight (in thousands). IB indicates the ion boundary.

enough cells for the assay, all of the cells in the 25 and 50% Histopaque layers were used. These cells, which were 65% MA(+), were washed in DPBS and suspended at 2×10^6 cells per ml of RPMI 1640–10% fetal bovine serum. A 125-μCi amount of ^{51}Cr (sodium chromate, 200 to 500 Ci/g of chromium, New England Nuclear) was added per ml of cell suspension, and the cells were incubated at 37°C in a 95% air–5% CO_2 atmosphere overnight. The next day the cells were washed three times with RPMI 1640 and suspended in RPMI 1640–10% fetal bovine serum at 2×10^5 cells per ml. The cytolysis reactions were set up in quadruplicate in a round-bottomed microtiter plate. A 50-μl amount of ^{51}Cr-radiolabeled cell suspension was mixed with 50 μl of diluted decomplemented ascites fluid or MA(+) human serum 11 (MFT = 1:320), and 50 μl of a 1:16 dilution of normal rabbit serum was added as a source of complement. The reaction mixtures were incubated for 4 h at 37°C, and the supernatants were collected with a Flow Titertek harvesting system. Maximum ^{51}Cr release (max release) was obtained by lysing 50 μl of cells with 100 μl of 1% Triton X-100. The ^{51}Cr release due to complement alone (C′ release) was measured in the absence of antibody. The percent nonspecific release was calculated as (C′ release)/(max release) × 100. The percent specific cytolysis was calculated as (test release - C′ release)/(max release - C′ release) × 100. The titers were expressed as the highest dilution of antibody giving ^{51}Cr release greater than the ^{51}Cr release obtained with C′ alone at the 0.05 level of significance, as determined by Student's *t* test.

Virus neutralization assays. A total of 8.6×10^4 superinfecting units (0.1 ml) of P3HR-1 virus (obtained through Meihan Nonoyama of Life Sciences, Inc.) was mixed with 0.2 ml of decomplemented ascites fluid fourfold serially diluted in RPMI 1640–10% fetal bovine serum and incubated for 1 h at 37°C. One-tenth milliliter of a 1:5 dilution of guinea pig complement (Colorado Serum Co, Denver, Colo.) was added to each neutralization mixture, and incubation was continued for 1 h. Each neutralization mixture was tested on 1×10^6 Raji cells for EBV early antigen induction after 72 h. The neutralization titers were expressed as the dilution of antibody yielding a 50% reduction in early-antigen-positive cells compared to the virus-complement control.

Neutralization mixtures containing B95-8 virus (3.4×10^3 transforming units), antibody, and complement were set up and incubated as described above. The mixtures were transferred to pellets of 2×10^6 human cord blood lymphocytes and incubated for 1 h at 37°C. The cells were centrifuged and suspended in RPMI 1640–10% fetal bovine serum, and six cultures containing 2×10^5 cells each were set up for each antibody dilution tested. Control cultures contained human cord lymphocytes treated with virus and complement. After six weeks, the neutralization titers were expressed as the dilution of antibody which prevented transformation in at least half of the cultures.

RESULTS

EBV specificity of the monoclonal antibodies. Ninety-six microwell hybridoma cultures were set up from the mouse immunized with TPA-induced B95-8 cells. When the culture fluids were tested in the indirect MF assay, several exhibited fluorescence on a percentage of P3HR-1 cells similar to that observed with the MA(+) human serum control. Five cloned hybridoma lines were established and used to produce high MFT ascites fluids in BALB/c mice. The antibodies exhibited EBV specificity in the indirect MF assay since they reacted with the appropriate percentages of both B95-8 and P3HR-1 cells. They did not react either with Raji, an EBV nonproducer MA(−) human cell line, or with 70-N-2, a herpesvirus saimiri-positive cotton-top marmoset T cell line which was virus capsid antigen-negative at the time it was tested.

Determination of the EBV MA component specificities of the monoclonal antibodies. The EBV MA component specificity of each antibody was determined by immunoprecipitation. Precipitation experiments were first conducted with PAA-treated or TPA-induced B95-8 cells labeled by the NGO-NaB(^3H)$_4$ procedure. Tracks A and P of Fig. 1 show the surface glycoprotein labeling patterns of PAA-treated B95-8 (1% MA+) and TPA-induced B95-8 (10% MA+) cells, respectively. An MA(+) human

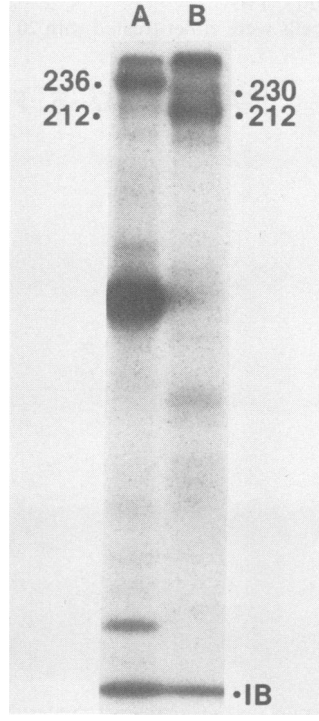

FIG. 2. Autoradiogram of ^{125}I-labeled surface proteins immunoprecipitated by monoclonal antibody B-1-1 (MFT = 1:1,024) from TPA-induced B95-8 cells (7% MA+ cells), track A, and TPA-induced P3HR-1 cells (14% MA+ cells), track B. The numbers in the margins indicate the molecular weight (in thousands). IB indicates the ion boundary.

serum control (track N) precipitated glycoproteins at 236K, 212K, and 141K from TPA-induced B95-8 cells. The MA(−) human serum (track O) nonspecifically bound only a very small amount of the 236K antigen, as previously described in our original study (14). Tracks I, J, and K show that the A-2-5, B-1-1, and C-3-2 monoclonal antibodies precipitated both the 236K and 212K MA. The other two monoclonal antibodies in tracks L (F-2-1) and M (G-3-1) bound the 236K antigen in amounts comparable to that bound by the MA(−) human serum. Tracks B through H show that antigens were not precipitated from PAA-treated B95-8 cells. The 86K EBV MA was previously identified in immunoprecipitation experiments employing cells labeled with a tritiated amino acid mixture. This antigen radiolabels readily with amino acids, ^{125}I, or glucosamine, but it is not radiolabeled by the NGO-NaB(^{3}H)$_4$ procedure. To determine whether the F-2-1 and G-3-1 monoclonal antibodies were specific for the 86K antigen, immunoprecipitation experiments were initially conducted with B95-8 cells labeled with ^{125}I by the IODO-GEN procedure. However, when B95-8 cells were surface labeled with ^{125}I, a protein in the vicinity of 86K was nonspecifically precipitated by protein A-Sepharose and obscured precipitation of the 86K antigen. This nonspecifically precipitated protein was not present in P3HR-1 cells. Figure 2 shows a comparative immunoprecipitation experiment performed with the B-1-1 monoclonal antibody and TPA-induced B95-8 (track A) or P3HR-1 cells (track B), which illustrates this point. This experiment also demonstrated that the slower component of the 236K-212K antigen had a slightly lower molecular weight in P3HR-1 cells (i.e., 230K) than it did in B95-8 cells. A similar observation has been made in several other laboratories (8, 12, 16).

Figure 3 shows the results of an immunoprecipitation experiment performed with uninduced P3HR-1 cells (2% MA+ cells) and TPA-induced P3HR-1 cells (12% MA+ cells) labeled with ^{125}I by the IODO-GEN procedure. Track M shows that the MA(+) human serum precipitated antigens at 230K, 212K, and 86K from TPA-induced P3HR-1 cells. These antigens were not precipitated by the MA(−) human serum in track N. Tracks K and L clearly indicate that the specificities of the F-2-1 and G-3-1 antibodies were directed against the 86K MA. Tracks I and J confirm the fact that the B-1-1 and C-3-2 antibodies were directed against the 230K-212K antigen. The specificity of the A-2-5 antibody for the 230K-212K antigen was not determined in this experiment. In several other experiments, two faint antigen bands were precipitated when ^{125}I- or ^{3}H-amino acid-labeled cells were used

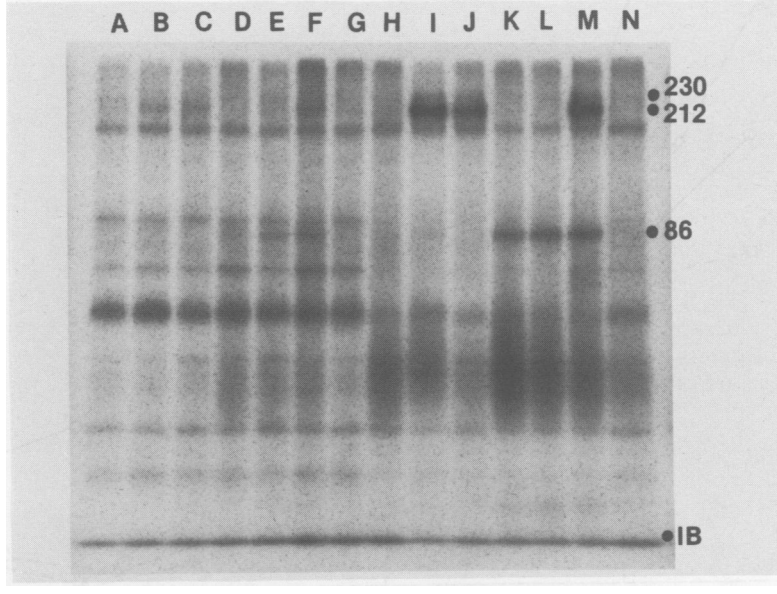

FIG. 3. Autoradiogram of ^{125}I-labeled surface proteins immunoprecipitated from P3HR-1 cells. Daily passaged P3HR-1 cells (2% MA+ cells), tracks A through G. TPA-induced P3HR-1 cells (12% MA+ cells), tracks H through N. Immunoprecipitates obtained with ascites fluids or human sera controls: A-2-5 (MFT = 1:1,024), tracks A and H; B-1-1 (MFT = 1:2,048), tracks B and I; C-3-2 (MFT = 1:5,460), tracks C and J; F-2-1 (MFT = 1:2,048), tracks D and K; G-3-1 (MFT = 1:16,384), tracks E and L; EBV-positive human serum 7 (MFT = 1:40), tracks F and M; EBV-negative human serum 1, tracks G and N. The numbers in the margins indicate the molecular weight (in thousands). IB indicates the ion boundary.

(data not shown). However, the specificity of the A-2-5 antibody is determined most readily in experiments with borohydride labeled cells, probably because the NGO-NaB(^{3}H)$_4$ labeling procedure incorporates a large proportion of the radioactivity into the 230K-212K antigen, thereby making it a more sensitive labeling procedure for detecting antibodies with low avidities for this antigen. However, it is also possible that the site on the 230K-212K antigen recognized by the A-2-5 antibody contains a tyrosine residue and that the avidity of the A-2-5 antibody for the antigen is adversely affected by iodination. Tracks A through G show the precipitates obtained with uninduced P3HR-1 cells. Small amounts of the 212 and 86K antigens were precipitated from uninduced cells by some antibodies, reflecting the higher percentage of MA(+) cells in the uninduced population used for this experiment.

Characterization of the potential biological activity of the monoclonal antibodies. The EBV-MA-specific monoclonal antibodies were also tested in two assays which measured their potential biological activity. The antibodies were first tested in a complement-dependent cytolysis assay (Fig. 4). The C-3-2, F-2-1, and G-3-1 antibodies all belong to subclass IgG2a, and they mediate the complement-dependent cytolysis of TPA-induced B95-8 cells exhibiting titers of 1:230, 1:1,030, and 1:59,000, respectively, in this assay. Since the A-2-5 and B-1-1 antibodies belong to subclass IgGl, and this subclass does not bind complement (10), it is not surprising that neither antibody lysed TPA-induced B95-8 (data not shown).

The EBV-MA-specific antibodies were also tested for the ability to neutralize both the B95-8 and P3HR-1 viruses. The neutralization data can be summarized as follows. The three monoclonal antibodies directed against the 236K-212K antigen (i.e., A-2-5, B-1-1, and C-3-2) did not neutralize either virus even in the presence of complement. The F-2-1 and G-3-1 antibodies directed against the 86K MA neutralized both viruses most effectively in the presence of complement. The F-2-1 antibody (MFT = 1:4,096) had a neutralization titer of 1:10,240 against both viruses in the presence of complement, and the G-3-1 antibody (MFT = 1:32,768) had neutralization titers of 1:2,560 and 1:640 against the B95-8 and P3HR-1 viruses, respectively. The

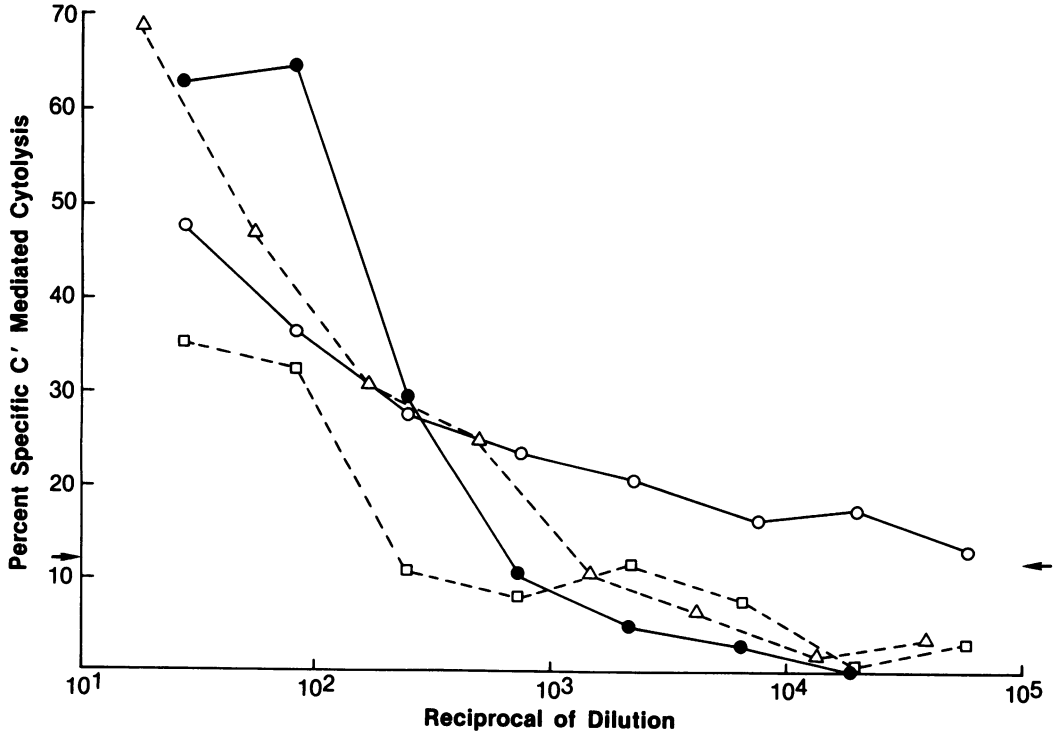

FIG. 4. The antibody-mediated, complement-dependent cytolysis of TPA-induced B95-8 cells (65% MA+ cells). Symbols: ●, EBV-positive human serum 11 (MFT = 1:320); □, C-3-2 (MFT = 1:16,384); △, F-2-1 (MFT = 1:1,024); ○, G-3-1 (MFT = 1:16,384). The arrows indicate the percent specific complement-dependent cytolysis above which the cytolysis is significant at the 0.05 level.

TABLE 1. Properties of EBV MA-specific monoclonal antibodies

Monoclonal antibody	Range of ascites MFT[a]	IgG subclass	EBV MA component specificity	Mediation of complement-dependent cytolysis	Neutralization of virus
A-2-5	1:512 to 1:8,192	IgG1	236K-212K	–	–
B-1-1	1:1,024 to 1:4,096	IgG1	236K-212K	–	–
C-3-2	1:2,048 to 1:16,384	IgG2a	236K-212K	+	–
F-2-1	1:1,024 to 1:4,098	IgG2a	86K	+	+
G-3-1	1:16,384 to 1:32,768	IgG2a	86K	+	+

[a] Based on MF assays performed on two or three individual ascites preparations.

properties of the monoclonal antibodies are summarized in Table 1.

DISCUSSION

Previous immunoprecipitation experiments have established correlations between EBV MA and several infected-cell proteins. Table 2 summarizes the results of the groups that have reported on the immunoprecipitation of EBV MA. The reported molecular weight estimates for the MA vary, depending on the molecular weights of the standards employed, the molecular weights assigned to those standards, and the electrophoretic conditions employed (i.e., the gel concentration). In our laboratory, we have assigned molecular weights of 236 and 212K to the two MA with the lowest mobilities. Both antigens from B95-8 cells have mobilities slower than porcine thyroglobulin, to which we assign a molecular weight of 210K, as described by Andersson et al. (1). The molecular weights reported by the other groups of investigators for the two slowest MA range from 250 to 350K and from 220 to 270K, respectively. However, regardless of the molecular weight assigned to these two antigens, all groups agree that there is more of the 236K antigen than of the 212K antigen in B95-8 cells, and that in P3HR-1 cells, the relative amounts of these two antigens are reversed. A third major membrane antigen has been identified between 75 and 95K. This antigen radiolabels readily with glucosamine, amino acids, and iodine, but it is not labeled by the NGO-NaB(^{3}H)$_4$ technique. We have assigned a molecular weight of 86K to this antigen.

We have now established five murine hybridoma lines producing monoclonal antibodies to EBV MA. Three antibodies precipitate both the 236 and the 212K MA. Thorley-Lawson and Geilinger (17) have also reported on an independently derived monoclonal antibody which precipitates these two high-molecular-weight EBV MA. The most probable interpretation of the data is that the proteins have several antigenic sites in common, but the possibility that one of the proteins is aggregated with the other cannot definitely be excluded at the present time.

The results of the complement-dependent cytolysis assay indicate that both the 236K-212K MA and the 86K MA can serve as antibody-binding sites for complement-dependent cell lysis. The antibodies directed against the 86K MA had comparable titers in the MF and complement-dependent cytolysis assays, whereas the antibody directed against the 236K-212K MA exhibited a substantially higher titer in the MF assay than in the cytolysis assay. Although another IgG2a antibody with specificity for the 236K-212K MA is not available to confirm this observation, the data suggest that there may be a subtle difference in the spatial arrangement of the two antigens on the cell surface. However,

TABLE 2. Summary of immunoprecipitation experiments identifying EBV MA

Laboratory	References	Mol wt of EBV MA (antigens designated 1–6 from highest to lowest mol wt)					
		1	2	3	4	5	6
A	(11, 12)	280–320	250	170	120–130	90	
B	(14)	230–236	212		141	86	
C	(16, 17)	350	220		140	75–85	56
D	(5)	275	236	165	134	90+95	
E	(7)	>250	250		140	80	
F	(4)	250					
G	(8)	320–340	240–270	160		85	
Mol wt range		230–350	212–270	160–170	120–141	75–95	56

all three IgG2a antibodies produced full-ring fluorescence on both B95-8 and P3HR-1 cells.

The neutralization data indicate that our monoclonal antibodies directed against the 236K-212K MA did not neutralize EBV; however, the two antibodies against the 86K MA neutralized both the B95-8 and P3HR-1 viruses. Other groups of investigators have reported that two independently derived monoclonal antibodies against the 236K-212K MA neutralize EBV (4, 17). Taken together, the data indicate that both the 236K-212K MA and 86K MA have antigenic sites which stimulate the production of neutralizing antibody. If a role for EBV in the etiology of Burkitt's lymphoma and nasopharyngeal carcinoma is confirmed, the data suggest that a multivalent subunit vaccine might be more effective than a vaccine consisting of a single subunit.

ACKNOWLEDGMENTS

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