

Inhibition of Epstein-Barr Virus (EBV) Release from P3HR-1 and B95-8 Cell Lines by Monoclonal Antibodies to EBV Membrane Antigen gp350/220

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Antibody-mediated inhibition of Epstein-Barr virus (EBV) release from the EBV-productive cell lines P3HR-1 and B95-8 was probed with two monoclonal antibodies (MAbs), 72A1 and 2L10, which immunoprecipitated the same EBV membrane antigen (MA) gp350/220 found with the 1B6 MAb with which inhibition of EBV release from P3HR-1 cells was first described. These three MAbs were not equivalent in either MA reactivities or functional effects, reflecting the variable expression of different epitopes of gp350/220. 1B6 recognized MA on P3HR-1 cells, which expressed predominately the gp220 form of MA. 1B6 did not recognize (or barely recognized) a determinant on B95-8 cells. MAbs 2L10 and 72A1 reacted as well with B95-8 cells as they did with P3HR-1 cells. MAbs 1B6 and 2L10 neutralized neither P3HR-1 nor B95-8 virus, but 72A1 neutralized both viruses. MAbs 1B6 and 72A1 inhibited P3HR-1 virus release, as measured by the assay for infectious virus and by DNA hybridization analysis of released virus, but 2L10 had no such activity. 72A1 (but not 1B6) inhibited release of EBV from B95-8 cells. These experiments pointed to the presence of three different epitopes on gp350/220, identified with the respective MAbs and having varying involvement in virus neutralization and virus release inhibition.

We seek to define the functions of Epstein-Barr virus (EBV)-determined membrane antigens (MA) and anti-MA antibodies on the EBV cycle and in immune defense against EBV infections. MA is a general term which applies to several EBV-induced membrane proteins, including, at least, those of molecular weights 350,000 (gp350), 220,000 (gp220, from an alternately spliced mRNA of the gp350 gene), 140,000 (p140), and 85,000 (gp85) (1, 3, 9, 17). On cell surfaces, these MA can be targets for antibody-dependent cellular cytotoxicity (22) or complement-mediated cytotoxicity (30). On viral envelopes, they can be targets for virus-neutralizing antibodies (33). Additionally, a monoclonal antibody (MAb), 1B6, to gp350/220 was found not to neutralize infectious EBV but yet blocked the release of EBV from the EBV-producing cell line P3HR-1 (28).

In the current study, we have further analyzed the effects of MAb 1B6 and two additional anti-gp350/220 MAbs, 72A1 (8) and 2L10 (11), on inhibition of virus release from two EBV-producing cell lines, P3HR-1 (7) and B95-8 (15). P3HR-1 cells produce a virus which is nontransforming and early antigen (EA) inducing (16), and they synthesize predominately gp220 rather than gp350 (3, 19). In contrast, B95-8 cells produce transforming virus (15), and their expression of gp350 is much greater than that of gp220 (3, 19). gp350 and gp220 appear to mediate virus attachment to the CR2 receptor, a form of complement receptor that binds the C3d component of complement on EBV-infectable cells (18, 31). We have found that these MAbs have variable reactivities against MA and variable functional effects in terms of inhibiting virus release from EBV-producer cell lines and of virus neutralization. EBV release inhibition was analyzed not only by functional indicator assays (EA-inducing activity for P3HR-1 EBV and transforming activity for B95-8 EBV)

but also by EBV virion genome hybridization. These results have been interpreted to reflect the roles of various gp350/220 epitopes in virus neutralization and in release of EBV from productive cells.

MATERIALS AND METHODS

Medium and cell lines. RPMI 1640 medium (GIBCO, Grand Island, N.Y.) with streptomycin (100 µg/ml) and penicillin (100 IU/ml) was supplemented with 10% fetal calf serum. Raji, an EBV-nonproducer cell line (21), was the target for a quantitative superinfection assay of EBV from the P3HR-1 cell line (7). Two EBV-producer cell lines, P3HR-1 and B95-8 (15), passaged at 33°C (25) and 37°C, respectively, were used to assay virus production during culture with various MAbs.

MAbs. The derivation and some functional characteristics of MAb 1B6 have been described (28). MAb 72A1 (8) was the gift of G. J. Hoffman, Johns Hopkins University, Baltimore, Md., and D. A. Thorley-Lawson, Tufts University, Boston, Mass. MAb 2L10 (11) was the gift of G. R. Pearson, Georgetown University, Washington, D.C. MAb C1 (32) was the gift of D. A. Thorley-Lawson. All MAbs were of the immunoglobulin G (IgG) class.

Virus preparations and quantitation of infectious virus. P3HR-1 EBV was concentrated from supernatants of P3HR-1 cell cultures after 14 days at 33°C (28). B95-8 EBV was obtained from supernatants of cultures maintained for 7 days at 37°C (15). The procedures for virus preparations in the MAb-mediated EBV release inhibition assays are described in Results. Infectivity of P3HR-1 EBV was assayed by viral induction of EA in Raji target cells and was quantitated as EA-inducing units (EAIU) per milliliter (24). Infectivity of B95-8 EBV was assayed by transformation of human umbilical cord blood lymphocytes (15) and quantitated as 50%

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transforming units (TD₅₀) per milliliter by the Reed-Muench formula (26).

Neutralization assay. The P3HR-1 EBV neutralization assay quantitated inhibition, by a given dilution of antibody, of the ability of a viral sample to induce EA expression upon infection of Raji cells (20, 29). Mixtures of 0.2 ml of a virus suspension (2×10^5 EAIU/ml) and 0.2 ml of a dilution of heat-inactivated antibody were incubated for 60 min at 37°C. Raji cells (0.1 ml; 10^7 cells per ml) were added for 90 min at 37°C to such virus-antibody mixtures or to a virus-medium mixture, washed, and suspended at 10^6 cells per ml. The infected cells were incubated at 37°C for 2 days. Acetone-fixed smears of those cells were prepared for immunofluorescent measurement of EA induction (6). A neutralization index was calculated by the formula of Pearson et al. (20).

The neutralization assay for B95-8 EBV was modified from the method of Miller et al. (14). Twofold serial dilutions of MAb were prepared in culture medium. These solutions (0.2 ml) were mixed with 0.2 ml of a virus suspension containing 200 TD₅₀/ml and was held at 37°C for 1 h. The MAb-virus mixture (0.1 ml) was added to human umbilical cord blood lymphocytes (0.1 ml at 3×10^6 cells per ml) in microtiter wells (plate no. 3596; Costar, Cambridge, Mass.). Virus neutralization endpoints were read 42 days after inoculation, and neutralization titers reflected the final dilution of MAb with some neutralizing activity.

Immunofluorescence assays. An indirect membrane immunofluorescence (MIF) technique was used to assay MA (27). Approximately 10^6 cells were pelleted and resuspended for 30 min at 37°C in appropriately diluted MAb. After being washed with phosphate-buffered saline (PBS), the cells were reacted for 30 min at 37°C with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Organon-Teknika-Cappel, Malvern, Pa.). After being washed with PBS, the cells were suspended in 50% glycerol in PBS and placed under a cover slip for examination with a fluorescence microscope. EA was detected with a serum (anti-EA, 1:320; anti-VCA, 1:320) from a patient with nasopharyngeal carcinoma. Viral capsid antigen (VCA) was detected with a normal human serum (anti-VCA, 1:160; anti-EA, less than 1:10). Cells were spotted on a glass slide, dried, and fixed in acetone for 10 min at room temperature. The fixed cells were incubated with a drop of diluted antibody at 37°C for 30 min. After being washed in PBS, the slides were treated with FITC-conjugated anti-human IgG (Organon-Teknika-Cappel) at 37°C for 30 min. The slides were washed in PBS, mounted in glycerol-PBS (1:1) solution, and examined under a fluorescence microscope.

Determination of MA forms. P3HR-1 or B95-8 cells, logarithmically growing at 37°C, were cultured with 12-*O*-tetradecanol-phorbol-13-acetate (TPA; 20 ng/ml) (Sigma Chemical Co., St. Louis, Mo.) and *n*-butyrate (4 mM; Sigma) for 48 h (28). These cells (10^8) were [³⁵S]methionine-labeled for 18 h, harvested by centrifugation, washed three times with cold PBS, and suspended in 2 ml of ice-cold extraction buffer (10 mM Tris hydrochloride buffer [pH 7.2], 0.15 M NaCl, 0.02% NaN₃, 0.5% [wt/vol] Triton X-100, 10 mM *N*-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride). After vortexing and sonication on ice, the undissolved material was sedimented at $110,000 \times g$ for 30 min. The supernatant fluid was divided into 200- μ l portions and stored at -80°C. For immunoprecipitations, a portion was incubated (three times for 15 min each on ice) with Affi-Gel 10 (100 μ l; Bio-Rad Laboratories, Richmond, Calif.) coupled with goat anti-mouse immunoglobulins antiserum (Organon-Teknika-Cappel), and cleared by sedimentation of the beads at $12,000$

$\times g$ for 90 s. The cleared supernatant was used for immunoprecipitations. Immunoabsorbents were prepared by coupling affinity-purified goat anti-mouse IgG antibody (Organon-Teknika-Cappel) to Affi-Gel 10, followed by incubation (3 h, 4°C) with MAb (10 μ l) plus 990 μ l of 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.5. After being washed once with 0.5% Triton X-100 buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.4], 0.02% sodium azide) and three times with 0.05% Triton X-100 buffer, antigen preparations were added and rocked overnight at 4°C. The following day, after being washed five times with 0.05% Triton X-100 buffer, antigen was eluted by suspending the beads in 100 μ l of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 10% [wt/vol] glycerol, 5% 2-mercaptoethanol, 2.3% SDS). These samples and radiolabeled molecular weight standards were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the gels were autoradiographed.

Assay of EBV DNA by agarose gel electrophoresis. The form and quantity of EBV DNA were determined by agarose gel electrophoresis and genome hybridization of the blotted electrophoretic patterns (5). Briefly, 10^6 cells or virus pellets were suspended in 0.1 ml of sample buffer which contained 15% Ficoll and 0.01% bromophenol blue in 89 mM Tris-borate buffer, pH 8.0, and loaded in vertical 0.75% agarose gels. Lysis buffer (0.1 ml), containing 5% Ficoll, 1% SDS, 1 mg of pronase per ml, and 0.05% xylene cyanol green in 89 mM Tris-borate buffer, was layered over the sample. The electrophoresis ran at 20 V for 2 h and then at 80 V for 18 h at 4°C. The gels were stained with ethidium bromide, and DNA in the gel was partially dephosphorylated with 0.25 M HCl, denatured in 0.5 M NaOH in 1.5 M NaCl, neutralized, and blotted to a nitrocellulose filter in transfer buffer (0.5 M Tris hydrochloride buffer, 1.5 M NaCl [pH 7.0]) under vacuum (12). The papers were baked at 80°C for 1 h, treated with prehybridization buffer, and hybridized with cloned EBV DNA pBamHI-W plasmid (a generous gift of E. Kieff, Harvard University, Cambridge, Mass.) which was labeled by nick repair with [³²P]dCTP. Each filter was washed five times in 250 ml of 0.1 \times sodium citrate buffer (0.0015 M sodium citrate, 0.015 M NaCl [pH 7.5])–0.1% SDS and exposed to Kodak XAR film at -70°C.

RESULTS

Immunoprecipitation of gp350/220. Original publications on MAbs 1B6, 72A1, 2L10, and C1 reported their target MA molecules to be 200, 250, 200/250, and 350/220 kilodaltons, respectively (8, 11, 28, 32). In our experiments, these MAbs all recognized the same set of molecules, among which a 220-kilodalton protein predominated in P3HR-1 cells cultured with TPA and *n*-butyrate (P3-T/B) (Fig. 1A). Also in P3-T/B cells, minor components of about 350 kilodaltons and 160 kilodaltons were found. MA immunoprecipitations from B95-8 cells were demonstrated with MAb 72A1 but not MAb 1B6 (Fig. 1B). MAb 72A1 immunoprecipitated principally a 350-kilodalton MA molecule, and a 220-kilodalton MA was present as a minor component.

Comparison of anti-MA MAb reactivities on P3HR-1 and B95-8 cells. The MIF reactivities of MAbs 1B6, 72A1, and 2L10 were compared on P3-T/B cells and on B95-8 cells (Table 1). Similar percentages (40 to 50%) of MA⁺ P3-T/B cells were identified with each of these MAbs. With these cells, no variations among the three MAbs were found in terms of immunofluorescence patterns or intensities of reac-

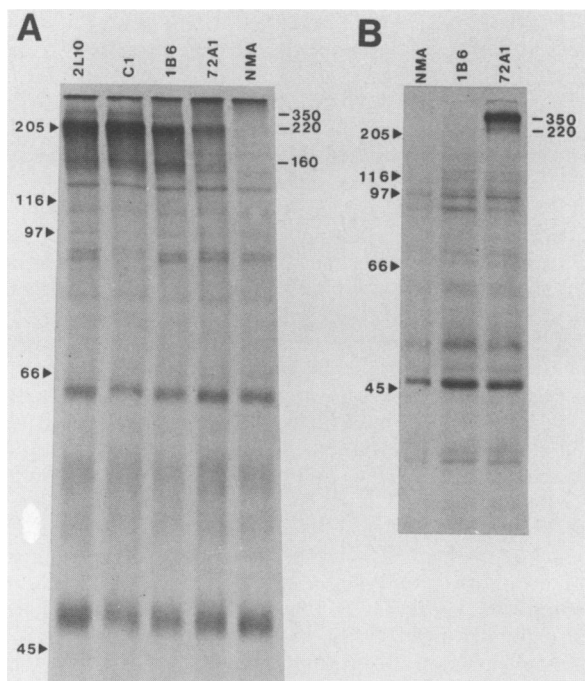


FIG. 1. SDS-PAGE of immunoprecipitates with MAbs 1B6, 72A1, 2L10, or C1 or normal mouse ascites (NMA), and [³⁵S]methionine-labeled cell extracts from TPA- and *n*-butyrate-treated P3HR-1 (P3-T/B) (A) or B95-8 (B) cells. Positions of standards (in kilodaltons) are indicated with arrows. gp350, gp220, and putative precursor p160 are indicated at the right.

tions. However, with B95-8 cells, in which gp350 was predominantly expressed, 72A1 and 2L10 showed comparably strong MIF staining of about 10% of cells, while 1B6 occasionally stained faintly only about 1 to 2% of the cells or (usually) no cells. We concluded on the basis of these MIF and immunoprecipitation experiments that each of these three MAbs recognized gp350/220 but at two or more different epitopes.

Neutralization of P3HR-1 or B95-8 EBV with MAb. Neutralization assays were performed with MAbs 1B6, 72A1, and 2L10 and, as a control, with a human serum which had neutralizing antibodies (Fig. 2). With P3HR-1 EBV, neutralizing activity was observed with 72A1 to a 1:320 dilution but not at 1:640, and neutralization was seen with the human serum to a 1:320 endpoint. No neutralization of P3HR-1 EBV was observed at any dilutions of 1B6 or 2L10 tested. With B95-8 EBV, neutralizing activity was observed with 72A1 at a 1:320 dilution but not at a 1:640 dilution (Table 2). No neutralization of B95-8 EBV was observed with 1B6 or 2L10. The findings of neutralization of P3HR-1 and B95-8

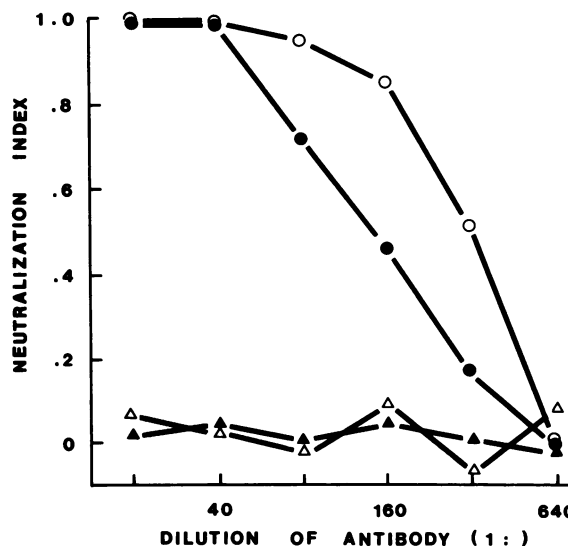


FIG. 2. P3HR-1 EBV neutralization with MAbs 1B6 (▲), 72A1 (○), and 2L10 (△) and with EBV-seropositive human serum (●).

EBV by 72A1, while 1B6 and 2L10 did not neutralize these viruses, pointed to the existence of functionally distinct epitopes on gp350/220.

MAb effects on EBV production by P3HR-1 cells. P3HR-1 cells (4×10^5 cells per ml) were cultured with various dilutions (1:100, 1:400, 1:1,600, and 1:6,400) of each of the MAbs at 33°C for 15 days (Fig. 3). The cell numbers (1.5×10^6 cells per ml) and viability (80%) of all cultures at 15 days were comparable. The EA-inducing titers of P3HR-1 EBV in the culture supernatants were assayed (Fig. 3A). A control culture without MAb had 3.7×10^5 EAIU/ml. All cultures with 2L10 had viral titers similar to the control culture. The 1B6- or 72A1-containing cultures demonstrated an MAb dose-dependent decrease in virus titers even to 1:6,400 dilution of either MAb. The percentages of EA⁺ and VCA⁺ P3HR-1 cells were enumerated in all 15-day cultures (Fig. 3B). 2L10-treated cultures showed a slightly higher frequency of EA⁺ or VCA⁺ cells than did the control culture, but 1B6- or 72A1-treated cultures showed the same frequency of EA⁺ or VCA⁺ cells as did the control culture. The mechanism of 1B6 restriction of EBV titers in culture supernatants was shown previously to reflect an inhibition of release of virus, which accumulated intracellularly in treated cells and did not reflect virus neutralization, virus aggregation, or inhibition of virus replication (28). A possible alternative mechanism was also evaluated for the neutralizing effect of 72A1.

P3HR-1 EBV-neutralizing activity of MAb 72A1. In contrast to 1B6, the 72A1 MAb was found to have neutralizing

TABLE 1. Summary of MAb characteristics

MAb	MA reactivity ^a		MA antibody titer		Neutralizing activity (titer)		Virus release-inhibiting activity	
	P3HR-1 cells	B95-8 cells	P3HR-1 cells	B95-8 cells	P3HR-1 EBV	B95-8 EBV	P3HR-1 cells	B95-8 cells
1B6	++++	±	1:163,840	ND ^b	<1:20	<1:20	+	-
72A1	++++	++++	1:5,120	1:10,240	1:320	1:320	+	+
2L10	++++	++++	1:81,920	1:20,480	<1:20	<1:20	-	ND

^a Maximal intensity of MIF determined at antibody excess. P3-T/B cells were used for the MIF assays.

^b ND, Not done.

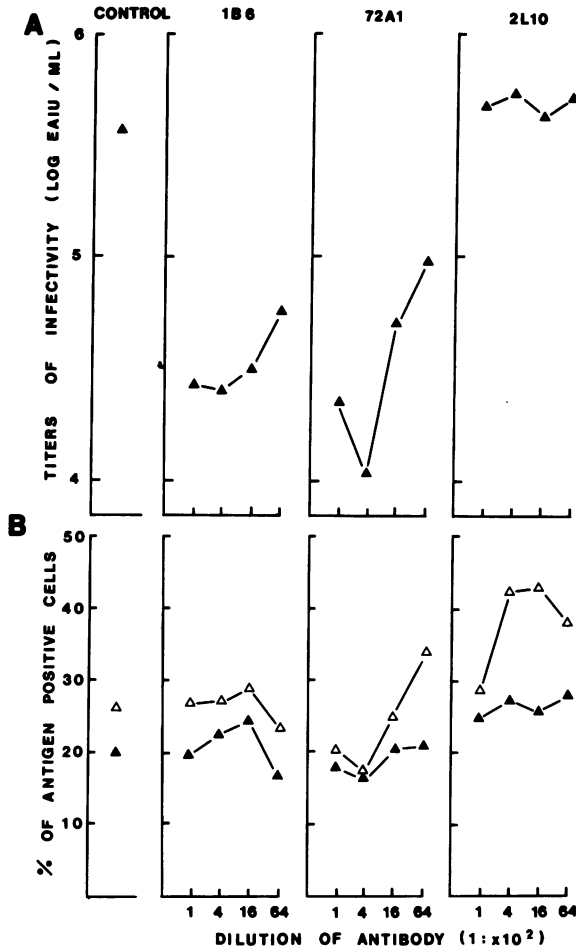


FIG. 3. Effect of MABs on EBV production by P3HR-1 cells. P3HR-1 cells were cultured with various dilutions of MABs 1B6, 72A1, and 2L10 or without MAB (control) at 33°C for 15 days. (A) Infectious EBV titers in culture supernatants. (B) Frequency of EBV antigen-positive cells (Δ , EA; \blacktriangle , VCA).

TABLE 2. Neutralization of B95-8 EBV with MABs

Final MAb dilution	No. transformed/3 wells inoculated ^a		
	1B6	72A1	2L10
1:20	3	0	3
1:40	3	0	3
1:80	3	0	3
1:160	3	2	3
1:320	3	2	3
1:640	3	3	3
1:1,280	3	3	3

^a Number of wells transformed at 42 days per three wells inoculated per MAB.

activity for P3HR-1 EBV (8, 14). However, this neutralizing activity was observed only to a maximal dilution of 1:320 (Fig. 2), while supernatant production of virus was inhibited even at MAB dilutions of 1:1,600 and 1:6,400 (Fig. 3A). We further confirmed that the inhibition of virus production at high MAB dilutions was not an effect of virus neutralization, by assay of the EBV virion DNA in the culture supernatants.

Assay for EBV DNA by agarose gel electrophoresis and hybridization. The structure and quantity of intracellular EBV genomes were assayed to test whether 72A1 might alter intracellular replication of EBV in P3HR-1 cells at 33°C for 15 days. Extrachromosomal EBV DNA can be found in virus-containing cells in two forms: episomal circular (C) DNA and virion-associated linear (L) DNA (5). No differences were observed in quantities of L-DNA between control and 72A1-treated cells (Fig. 4A). The mechanism for decreased titers of infectious extracellular EBV cannot be explained by inhibition of intracellular virus replication with 72A1.

Next, the amount of virion DNA in culture supernatants was assayed quantitatively to determine whether the low amount of virus was due to inactivation of viral infectivity or inhibition of virus release (Fig. 3). A significantly lower amount of EBV DNA was observed in preparations from cultures treated with 1B6 or 72A1 but not with 2L10 than in the control culture (Fig. 4B). These hybridization experiments correlated with the titers of infectious virus (Fig. 3A).

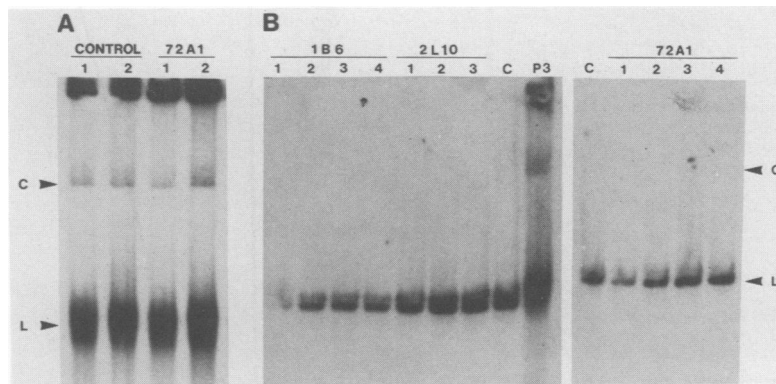


FIG. 4. EBV DNA analyzed by agarose gel electrophoresis. (A) Intracellular EBV DNA from P3HR-1 cells cultured with MAB 72A1 or control ascites for 15 days at 33°C. Cells (10^6) were loaded in the well of an agarose gel, lysed, subjected to electrophoresis, and blotted to nitrocellulose filters for EBV DNA hybridization. Lanes 1 and 2 show EBV DNA bands from cells cultured with 1:100 and 1:400 dilutions of MAB or control ascites, respectively. C, Circular EBV DNA. L, Linear EBV DNA. (B) Virion-derived L-DNA in supernatants of the MAB-treated P3HR-1 cells presented in Fig. 3. The supernatants were centrifuged at $5,000 \times g$ for 10 min to remove cellular debris, and the supernatants (3 ml) were centrifuged at $53,000 \times g$ for 60 min. The virus pellet was suspended in 200 μ l of sample buffer, and 100 μ l of that suspension was loaded into a well. Lanes 1, 2, 3, and 4 show L-DNA from culture fluids at 1:100, 1:400, 1:1,600, and 1:6,400 dilutions of MAB, respectively. Lane C, Control without MAB. Lane P3, P3HR-1 cells at 33°C to show the migration of C- and L-DNA.

One could also propose that 72A1 might not only neutralize the EBV but also lead, directly or indirectly, to degradation of the DNA of the MAb-reacted virus over a 10-day period of incubation. To test this possibility, 0.1 ml of EBV (2.7×10^5 EAIU/ml) and 0.1 ml of MAb dilutions were incubated at 33°C for 10 days, and EBV virion DNA was assayed by gel electrophoresis and hybridization (Fig. 5A). Although infectious titers of EBV decreased to an unquantifiable level ($<10^3$ EAIU/ml) during the incubation and the degree of hybridization was lessened by the incubation relative to that in the unincubated control, perhaps due to a degradative process, the amount of EBV genome detected by hybridization was uniform during the period of incubation with 1B6 or 72A1 (Fig. 5A). These data supported the view that EBV release from P3HR-1 cells was inhibited by 1B6 and 72A1 but not by 2L10.

Inhibition of EBV release from B95-8 cells by 72A1 but not 1B6. B95-8 EBV cells were cultured with 1B6 or 72A1 (1:1,000 or 1:10,000 dilution) for 5 days at 37°C without effect on cell growth (data not shown). The transforming titers of infectious virus in the supernatants were significantly decreased by 72A1 at a 1:1,000 dilution and slightly decreased at a 1:10,000 dilution relative to control cultures (Table 3). No effect was seen in the 1B6-treated cultures. A more detailed analysis of the effect of 72A1 dilution on inhibition of virus release was performed (Fig. 6). In this experiment, 72A1 inhibited virus release significantly at a dilution of 1:

TABLE 3. Inhibition of B95-8 EBV production with MABs^a

MAB	MAB dilution	TD ₅₀ /ml
Control (ascites)	1:1,000	10 ^{4.3}
	1:10,000	10 ^{4.0}
1B6	1:1,000	10 ^{4.3}
	1:10,000	10 ^{4.0}
72A1	1:1,000	≤10 ^{0.8}
	1:10,000	10 ^{3.5}

^a B95-8 cells were cultured with medium containing MAB (1:1,000 or 1:10,000) for 5 days at 37°C, and the infectivity of supernatants was assayed by the transformation assay (14).

6,400. Since the neutralizing activity of 72A1 was 1:320 (Table 2), one can conclude that the effect of greater dilutions of 72A1 on extracellular virus titers did not reflect virus neutralization.

Intracellular viral DNA was assayed by agarose gel electrophoresis and hybridization. We saw no effect of 72A1 on the production of intracellular viral genomes (Fig. 7A). Antibody-treated cultures were tested for the amount of virion DNA in the culture supernatant (Fig. 7B). MAB 72A1 treatment decreased significantly the amount of hybridized EBV DNA at 1:1,000 and slightly at 1:10,000 dilutions of MAB. No effect was seen with 1B6 compared with control

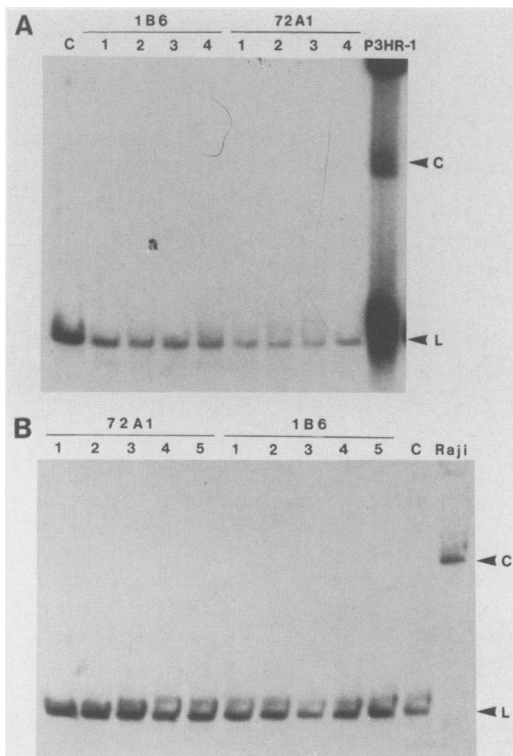


FIG. 5. Effect of incubation of virus and MAB on EBV virion. P3HR-1 (A) or B95-8 (B) EBV was incubated at 33°C for 10 days or at 37°C for 5 days, respectively, with various dilutions of MAB 1B6 or 72A1. Virion DNA was assayed by hybridization after agarose gel electrophoresis. Lanes C, EBV stored at -70°C without subsequent incubation. Lanes 1, 2, 3, 4, and 5, L-DNA incubated with 1:100, 1:400, 1:1,600, 1:6,400, and 1:25,600 dilutions of MAB, respectively. P3HR-1 or Raji cells were used to show the positions of C- and L-DNA.

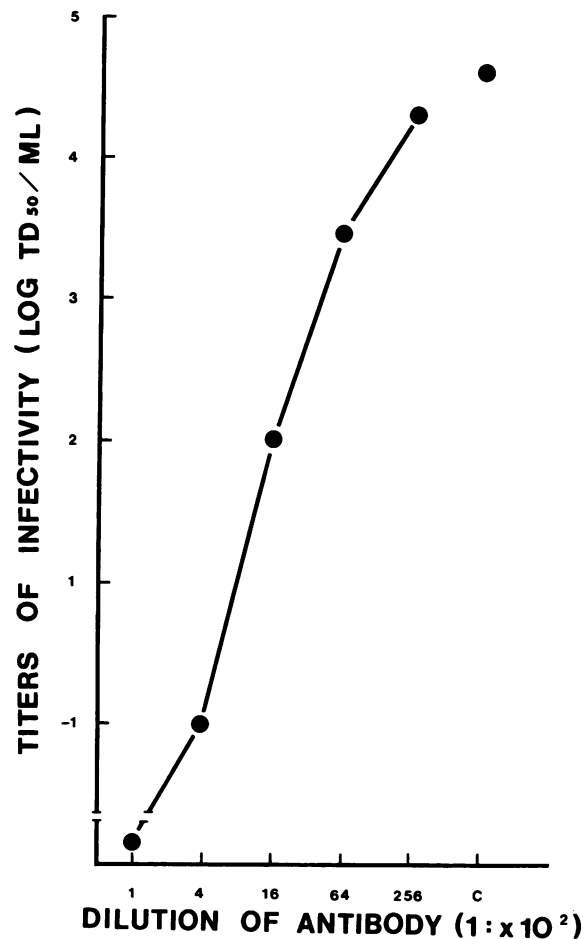


FIG. 6. Effect of MAB 72A1 on infectious EBV production by B95-8 cells. The cells were cultured with various dilutions of 72A1 at 37°C for 5 days. Infectious EBV was assayed in the supernatants by the transformation assay (14). C, Control culture without MAB.

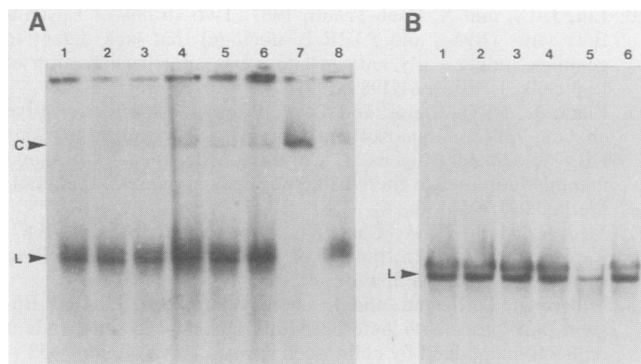


FIG. 7. Effects of MAbs on EBV production by B95-8 cells. B95-8 cells were cultured with various dilutions of MAb 1B6 or 72A1 or without MAb (control) at 37°C for 5 days. The form and quantity of EBV genomes were assayed by EBV genome hybridization with (A) cells and (B) culture supernatants. The cells were cultured with medium containing control ascites at 1:1,000 (lanes 1) or 1:10,000 (lanes 2); 1B6 at 1:1,000 (lanes 3) or 1:10,000 (lanes 4); or 72A1 at 1:1,000 (lanes 5) or 1:10,000 (lanes 6). Lane 7, Raji cells with only C-DNA. Lane 8, P3HR-1 cells with both L- and C-DNA forms.

cultures. As in the case of P3HR-1 EBV, a possible effect of 72A1 in degrading EBV genome was tested (Fig. 5B). B95-8 EBV DNA was not decreased during a 5-day incubation at 37°C with 72A1, as was also the case in similar studies with 1B6. These data supported the view that EBV release from B95-8 cells was inhibited by 72A1 but not by 1B6.

DISCUSSION

Our objective has been to describe mechanisms by which antibodies to MA control EBV infections. Our focus has been on EBV release-inhibiting antibodies which might play a role in EBV infections to block the spread of virus (28). We first described the EBV release-inhibiting phenomenon through the use of MAb 1B6, which did not neutralize EBV. Having established the basic biological process with infectious-virus assays of EBV release inhibition, we turned to the EBV genomic hybridization assay to quantitate EBV virion DNA to rule out mechanisms of neutralization at release. These biological and genomic analytical methods have now been applied to a comparative study of other MAbs to MA to look for: (i) various functional epitopes on the MA, (ii) differences in MAb effects on different EBV-producer cell lines and viruses, and (iii) distinctions among antibodies in virus release inhibition and neutralization.

Three MAbs, 1B6, 72A1, and 2L10, were defined with immunoprecipitation assays to recognize the same gp350/220 MA, although the original reports on 1B6 (28), 72A1 (8), and 2L10 (11) MAbs identified MA molecules of 200, 250, and 200/250 kilodaltons, respectively. SDS-PAGE of these immunoprecipitates from P3HR-1 cells showed a strong band at 220 kilodaltons and fainter bands at 350 and 160 kilodaltons. It is known that the 350-kilodalton form of gp350/220 is weakly expressed on P3HR-1 cells (1, 3) and that a p160 is a nonglycosylated precursor of gp220 (4). These molecules were also identified in immunoprecipitates with C1, which had been characterized to recognize gp350/220 (32). With B95-8 cells, 72A1 immunoprecipitated a protein of 350 kilodaltons as the major band and a protein of 220 kilodaltons as a minor band; however, 1B6 appeared not to immunoprecipitate any protein. We concluded that these four MAbs recognized the same gp350/220 MA.

MAbs 1B6, 72A1 and 2L10 appeared in immunoprecipitation, immunofluorescence, neutralization, and virus release inhibition studies to recognize different epitopes on gp350/220. As summarized in Table 1, the three MAbs reacted equally, in terms of MIF staining patterns and intensities, to P3HR-1 cells, which expressed mainly the gp220 form (Fig. 1A). However, on B95-8 cells, which expressed predominantly gp350 (Fig. 1B), 1B6 stained faintly sometimes or not at all, while 72A1 and 2L10 reacted strongly and as well as they did on P3HR-1 cells. In EBV neutralization assays, 72A1 neutralized P3HR-1 EBV, while 1B6 and 2L10 did not. Likewise, 72A1 neutralized B95-8 EBV, while no neutralization was observed with 1B6 or 2L10. 72A1 mediated EBV release inhibition of both P3HR-1 and B95-8 EBV. 1B6 mediated P3HR-1 EBV release inhibition but not B95-8 EBV release inhibition. 2L10 was negative in P3HR-1 EBV release inhibition assays. These results supported the conclusion that specific functional epitopes on gp350/220 mediated virus neutralization and/or inhibition of EBV release.

The negligible 1B6 MIF staining of B95-8 cells, its lack of immunoprecipitation of B95-8 gp350/220, and its failure to inhibit release of or neutralize B95-8 EBV were consistent with the view that 1B6 recognized an epitope expressed on the gp220 of P3HR-1 cells but not of B95-8 cells. One might expect that the 1B6-recognized epitope has some conformation- or glycosylation-dependent component that is absent or masked in B95-8 cells, since the primary amino acid sequence of gp220 is included in gp350 and genomic MA sequences for P3HR-1 and B95-8 gp350/220 appear to be identical (1, 9), and because 1B6 gave a weakly positive MIF reaction with some preparations of B95-8 cells.

Demonstration of EBV release inhibition by 72A1, in the face of its neutralizing capacity, depended on its significantly higher titer for EBV release inhibition than for EBV neutralization. 72A1 neutralized both P3HR-1 and B95-8 EBVs to a dilution of 1:320. If one were considering a potential neutralizing activity of 72A1 in cultures tested for inhibition of EBV release, potential neutralizing titers must be decreased by absorption of a large portion of the neutralizing MAb to MA⁺ cells in the assay. Thus, even though 72A1 had a neutralizing activity for P3HR-1 and B95-8 EBVs, we concluded that 72A1 also had EBV release-inhibiting activity for each EBV-producer cell line. This view was strengthened by hybridization assays for virion DNA in culture supernatants. MAb dose-dependent decreases in levels of virion DNA were found in supernatants of 72A1-treated P3HR-1 and B95-8 cell cultures (Fig. 4B and 7B). This assay for EBV DNA further confirmed the view of antibody-mediated EBV release inhibition which had been first demonstrated with the infectious virus assay (28).

72A1 release inhibition of B95-8 EBV was more sharply titrated than was its release inhibition of P3HR-1 EBV. In the case of P3HR-1 cells, 72A1 (and 1B6) decreased the supernatant virus titer from 1/10 to 1/50 of control levels but never completely eliminated infectious virus from the supernatants, even at high MAb concentrations (Fig. 3A). On the other hand, 72A1 completely eliminated infectious virus from supernatants of B95-8 cells at an MAb dilution of 1:100 and showed a dose-dependent inhibition of virus release to a dilution of 1:25,600 (Fig. 6). These patterns of release inhibition as a function of MAb dilution were confirmed in direct genomic assays (Fig. 4B and 7B). The differences between P3HR-1 and B95-8 cells might reflect differences in the viruses of those cell lines (2, 23). P3HR-1 cells contain unique, defective virions which are maintained by cell-to-cell spread and can activate the latent EBV genome (13).

However, B95-8 cells were observed to lack such defective virion molecules, which are associated with the P3HR-1 heterologous DNA (10). In addition, since different culture conditions were used for antibody treatments of the two cell lines (15 days at 33°C for P3HR-1 and 5 days at 37°C for B95-8), destruction of antibody activity or of EBV-containing cells could contribute to this apparent difference in the mechanism of antibody-mediated EBV release inhibition. Additionally, functional differences in release inhibition might exist for the two principal MA forms, gp350 on B95-8 cells and gp220 on P3HR-1 cells.

Since gp350/220 mediates attachment of EBV to CR2 for internalization of EBV (18, 31), one might ask whether gp350/220 also plays a role in trafficking newly synthesized virus for release. Structural differences in gp350/220, for example, those induced by proteolysis or glycosylation, might determine the function of those glycoproteins in virus trafficking and release. The findings that gp350/220 is the target for MAbs which inhibit EBV release and that multiple epitopes on gp350/220 have variable roles in EBV release inhibition and neutralization increase our understanding of the molecular basis for MA functions.

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