INHIBITION OF EPSTEIN-BARR VIRUS (EBV) RELEASE FROM THE P3HR-1 BURKITT'S LYMPHOMA CELL LINE BY A MONOCLONAL ANTIBODY AGAINST A 200,000 DALTON EBV MEMBRANE ANTIGEN

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Our focus has been the characterization of antibody-mediated defense mechanisms against Epstein-Barr virus (EBV)[1] through the development of monoclonal antibodies (mAb) against EBV membrane antigens (MA). EBV has been studied extensively because it has a central role in the pathogenesis of infectious mononucleosis (IM), two human tumors (Burkitt's lymphoma [BL] and nasopharyngeal carcinoma), and the X-linked lymphoproliferative syndrome (XLP), and because the virus persists in certain chronically or recurrently infected states (1). EBV genome–carrying lymphoblastoid cell lines are termed "producer" or "nonproducer" cells, depending upon whether they do or do not shed infectious virus and spontaneously express viral capsid antigen (VCA). EBV-related antigens on such cells can be classed as early or late on the basis of sensitivity to inhibitors of viral DNA synthesis such as cytosine arabinoside or phosphonoacetic acid (PAA) (2, 3). Late antigens are expressed only in cells actively producing the virus, and frequently appear in the virion itself (4, 5). EBV induces two kinds of cell surface neoantigens on transformed cells. Lymphocyte-determined membrane antigen (LYDMA) is expressed on all EBV-transformed cells (6), but EBV-determined membrane antigens are expressed only on producer cells (7). LYDMA is defined with cytotoxic T cells from patients with IM. In vivo, such cytotoxic T cells probably destroy the EBV-transformed cells that are found in the peripheral blood of acute IM patients (6, 8). On the other hand, MA on producer cells could also be relevant to in vivo immune defense against EBV because MA, which are also components of the EBV envelope (5, 9), are target antigens for

This work was supported by grants CA-25873 and CA-37645 from the National Institutes of Health, Bethesda, MD.

[1] *Abbreviations used in this paper:* AVRF, anti–virus release factor; BL, Burkitt's lymphoma; C, complement, CIF, cytoplasmic immunofluorescence; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EA, early antigen; EAIU, early antigen–inducing unit; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IM, infectious mononucleosis; LYDMA, lymphocyte-determined MA; MA, membrane antigen; mAb, monoclonal antibody; MIF, membrane immunofluorescence; NI, neutralization index; P3-T/B, P3HR-1 cells cultured in TPA and *n*-butyrate; PAA, phosphonoacetic acid; PBS, phosphate-buffered saline; TPA, 12-*o*-tetradecanoyl phorbol-13-acetate; VCA, viral capsid antigen; XLP, X-linked lymphoproliferative syndrome.

virus-neutralizing antibody (10), antibody-dependent cell-mediated cytotoxicity (11, 12), and complement (C)-dependent cytolysis (13). MA appear to consist of at least four distinct polypeptides: 350/300, 250/200, 160/140, and 85/80 kilodaltons (kD) (5, 9, 13–19). Our report describes an anti-MA mAb that recognizes a 200 kD late MA, and has no neutralizing activity without C. However, this mAb inhibits release of virus from P3HR-1 producer cells. The mAb thus permits analysis of the mechanism of EBV release, and probably defines another mechanism for immune defense against EBV infections.

Materials and Methods

Medium and Cell Lines. RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) with 100 μg/ml streptomycin and 100 IU/ml penicillin was supplemented with 2% fetal calf serum and 8% newborn calf serum. Raji, an EBV-nonproducer BL cell line (20), was a target for superinfection with EBV derived from the P3HR-1 cell line (21). CEM was a T cell line (22).

Preparation of Microsomal Membrane Immunogen. P3HR-1 cells (10^8) were treated with 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) (20 ng/ml) and *n*-butyrate (4 mM) for 2 d at 37°C (hereafter termed P3-T/B cells), and washed with phosphate-buffered saline (PBS). Such cells expressed high levels of MA (50–70%, in contrast to 0.1–2% for MA^+ control P3HR-1 cells). The cell pellets were lysed in 0.01 M Tris-HCl buffer, pH 8.1, by vortex agitation for 1 min. This suspension was centrifuged at 600 *g* for 5 min. The pellet was reextracted, and the two supernatants from each extraction were pooled and centrifuged at 110,000 *g* for 1 h. The resulting microsomal membrane pellet was suspended at ~4 mg/ml by Dounce homogenization in 4 ml of 0.15 M NaCl, and was stored in aliquots at −70°C.

mAb Production. BALB/c mice were immunized subcutaneously in footpads with antigen emulsified in complete Freund's adjuvant, and were boosted with three intraperitoneal injections of saline-suspended immunogen (0.3 ml of a 1:10 dilution) given at 3-wk intervals. Fusions were made according to the EMBO protocol (23). Briefly, 4×10^7 spleen cells and 2×10^7 SP 2/O myeloma cells were fused in the presence of polyethylene glycol. The cells were distributed into 48 wells (1 ml/well) with feeder mouse thymocytes in Dulbecco's modified Eagle's medium (DMEM) containing hypoxanthine, aminopterin, thymidine, penicillin, streptomycin, glutamine, 2-mercaptoethanol, 2% fetal calf serum, and 8% newborn calf serum. After 14 d of feeding in this medium, cells were transferred to DMEM containing hypoxanthine, thymidine, and basic supplements. Supernatants of these cultures were assayed for anti-MA by enzyme-linked immunosorbent assay (ELISA) and immunofluorescent techniques. Cloning of selected cultures was performed by limiting dilution of cells. Ascites fluid was raised from intraperitoneal culture of hybridomas in pristane-primed BALB/c mice, and heat-inactivated.

Immunofluorescence. An indirect membrane immunofluorescence technique was used to assay for EBV-MA (24). ~10^6 cells were pelleted, resuspended for 30 min at 37°C in 2 drops (~0.05 ml) of either 1:20 diluted anti-MA–reactive normal human sera 53 or 70 (anti-MA titers 1:160), or appropriately diluted mAb. After washing with PBS, the cells were incubated at 37°C for 30 min with two drops of fluorescein isothiocyanate (FITC)-labeled rabbit antibody against human IgG (gamma-specific), or $F(ab')_2$ sheep anti–mouse IgG showing no crossreaction with human IgG. After washing with PBS, the cells were suspended in one drop of 50% glycerol in PBS. A drop of this cell suspension was placed under a coverslip for examination with a fluorescence microscope.

The indirect immunofluorescence test for intracellular antigens was carried out according to established procedures (25). Early antigen (EA) was detected with a serum from a patient (103) with nasopharyngeal carcinoma (anti-EA titer 1:320). VCA was detected with two human sera, 53 or 70 (anti-VCA titer 1:160; anti-EA titer <1:10 in each case). Cells were washed in PBS, spotted on a glass slide, dried, and fixed in acetone for 10 min at room temperature. The fixed cells were incubated at 37°C with a drop of diluted

antibody for 30 min. After being washed in PBS, the slides were mixed with the FITC-conjugated anti-human or anti-mouse IgG at 37°C for 30 min. The slides were washed in PBS, mounted in 1:1 glycerol/PBS, and examined with a fluorescence microscope.

Virus Production. P3HR-1 EBV was prepared from supernatants of P3HR-1 cell cultures after 14 d at 33°C (26). The supernatants were centrifuged at 900 *g* for 20 min to remove cellular debris, then centrifuged again at 26,000 *g* for 90 min. The virus-containing pellets were resuspended in medium at a 100-fold concentration relative to the original culture medium. The concentrated virus suspension was filtered through a membrane filter (0.45 μM pore size) and stored in aliquots at −70°C. The virus titer of this preparation was 5×10^6 early antigen inducing units (EAIU)/ml (27).

Assay of Viral Infectivity. Infectious virus was assayed by viral induction of EA. Raji cells were mixed with virus suspensions to give a final concentration of 10^6 cells/ml, then incubated at 37°C for 90 min. After washing, the cells were suspended in fresh medium and incubated at 37°C in a 5% CO_2 incubator for 2 d. Infectivity of virus was determined by analysis for EAIU, where EAIU/ml = (number of target Raji cells/ml) × (fraction of EA^+ cells)/(reciprocal dilution of the virus test solution) (27). For assay of cell-associated virus, pelleted cells were washed and resuspended in fresh medium, sonicated for 60 s in an ice bath, and freed of debris by centrifugation at 900 *g* for 10 min. The supernatant was assayed for virus.

Neutralization Assay. The neutralization assay quantitated the abrogation of virus-induced EA expression (10, 28). Mixtures of 0.2 ml of virus suspension (diluted 1:5) and 0.2 ml of heat-inactivated antibody dilutions were incubated overnight at 4°C. Raji cells (0.1 ml at 10^7 cells/ml) were then exposed for 90 min at 37°C to such virus–antibody mixtures or to a virus–medium mixture, then washed and resuspended at 10^6 cells/ml. For the assay of C-dependent neutralizing antibody, 0.05 ml of a freshly frozen seronegative human serum (serum 60, in a 1:2 dilution, as a source of C) was added to 0.4 ml of the virus–serum mixture (after incubation overnight at 4°C) for a 60 min incubation at 37°C. RPMI 1640 with 25 mM Hepes was used as a diluent of either C or virus suspensions. In order to avoid lysis of target Raji cells by cell-induced activation of the properdin pathway (29), 0.05 ml of 100 mM EDTA (final concentration 10 mM) was added to the mixtures and incubated at 37°C for 10 min before addition of Raji cells. The infected cultures were incubated at 37°C for 2 d. Acetone-fixed smears were prepared for the immunofluorescence test of EA induction (25). A neutralization index (NI) was calculated according to the formula described by Pearson et al. (10): NI = ([percent EA^+ virus–medium mixture] − [percent EA^+ virus–antibody mixture])/(percent EA^+ virus–medium mixture).

Determination of MA Molecular Weight. A microsomal membrane pellet was prepared from 10^8 [^{35}S]methionine-labeled P3HR-1 cells, which were harvested 48 h after treatment with TPA and *n*-butyrate. The microsomal membrane pellet was resuspended in ice-cold 1% Triton X-100 in PBS (2 ml), and membrane proteins were solubilized by shaking gently for 30 min at 4°C. Undissolved material was sedimented at 110,000 *g* for 1 h. The supernatant was incubated with 50 μl of normal rabbit serum overnight at 4°C, and cleared with 0.2 ml of a 10% suspension of formalin-fixed *S. aureus* (Cowan strain), by incubation for 10 min at 4°C and sedimentation of the bacteria at 12,000 *g* for 15 min. The cleared supernatant (divided into 100 μl aliquots) was used for immunoprecipitation with antibodies (50 μl of serum 86 [anti-MA, titer 1:1,280], from a chronic IM patient, or 5 μl of ascites) at 4°C for 1 h. This was followed by addition of an *S. aureus* suspension (0.4 ml) for 10 min at 4°C, then centrifugation PBS washing, and elution of antigen. Antigen elution was done by resuspending the bacteria in 100 μl of 4% SDS in 6 M aqueous urea, and heating at 100°C for 10 min. This preparation was centrifuged at 12,000 *g* for 15 min, and the supernatant was collected. 20 μl of 60 mM dithiothreitol (DTT) in Laemmli sample buffer were added to each supernatant for incubation at 37°C for 10 min (30). The DTT-reduced proteins were alkylated by the addition of 20 μl of 75 mM iodoacetamide. These samples and radiolabeled molecular weight standards were subjected to SDS-PAGE. Gels were autoradiographed.

Results

Development of mAb against EBV MA. Hybridoma fusions were obtained by standard methods (23), and culture supernatants were evaluated by ELISA, testing for binding to P3-T/B microsomal membranes and low nonspecific binding to CEM (T cell line) microsomal membranes. Among 17 cultures that were reactive with P3-T/B but not CEM microsomal membranes, 7 showed membrane immunofluorescence (MIF) specific for a subset of P3-T/B cells. Among these, which were subcloned twice and transferred to ascites, was clone 1B6, which is the subject of this report. The ascitic preparation of this mAb showed (*a*) MIF on P3-T/B cells (Fig. 1*A*), detectable to a 1:320,000 dilution; (*b*) cytoplasmic immunofluorescence (CIF) on acetone-fixed P3-T/B cells (Fig. 1*B*), detectable to a 1:160,000 dilution; and (*c*) ELISA reactivity to a 1:2,560 dilution on P3-T/B membranes, while it was unreactive to CEM membranes at a dilution of 1:40 (results not shown). The antibody is of IgG2a isotype, as determined by Ouchterlony gel diffusion tests.

EBV Specificity of 1B6. The correspondence between MIF and CIF patterns of 1B6 and two standard anti-MA$^+$ sera was tested under various conditions of EBV antigen induction in P3HR-1 cells. Both TPA and *n*-butyrate can, alone, induce EBV antigens (31, 32), and they can act synergistically (33). For comparison, VCA and MA were detected with EBV$^+$ sera from healthy individuals (53 and 70 1:20 dilution). In each instance of control or activated cell populations, 1B6 reactivity closely paralleled that demonstrated with serum 53 (Fig. 2) and with a second control serum, 70 (not shown, being indistinguishable from serum 53). In addition to these studies with activated P3HR-1 cells that had been cultured at 37°C, levels of MA and VCA were assessed in 33°C-passaged P3HR-1 cells that spontaneously express EBV antigens (21, 26). Again, the 1B6 mAb reactivity paralleled MA and VCA expression, as detected with sera 53 and 70. 1B6 also showed MIF to subpopulations of cells of other EBV-producing cell lines: B95-8 (34), QIMR-WIL (35), and Jijoye (36) cells, indicating crossreaction to EBV MA expressed by other strains of EBV. The intensity of MIF staining was much less on those cells than on P3HR-1 cells.

Effect of PAA on Expression of MA. The effect of PAA on EBV MA induction by *n*-butyrate or by *n*-butyrate and TPA was followed by 1B6 or anti-MA$^+$ human serum 70 (Table I). *n*-Butyrate or *n*-butyrate plus TPA clearly induced MA within 48 h in P3HR-1 cultures. However, the addition of PAA (200 μg/ml) completely blocked the induction of the 1B6-defined MA, indicating that the target antigen was a late MA component.

Molecular Mass of MA Recognized by 1B6. Immunoprecipitates formed by the reaction of 1B6 antibody or EBV$^+$ serum (86, from a chronic IM patient) with TPA plus *n*-butyrate–activated, [^{35}S]methionine-labeled P3HR-1 cells were analyzed by SDS-PAGE (Fig. 3). A 200 kD protein was immunoprecipitated with 1B6 mAb and with human serum 86.

Virus Neutralization. The 1B6 antibody was tested for P3HR-1 EBV neutralization in the presence and absence of C (Fig. 4). Without C, no neutralizing activity (NI = 0) was observed at any mAb concentration, from 1:160 to 1:10. A control EBV seropositive human serum (53) demonstrated neutralizing activity in a dose-dependent fashion. With C, the level of neutralization with the sero-

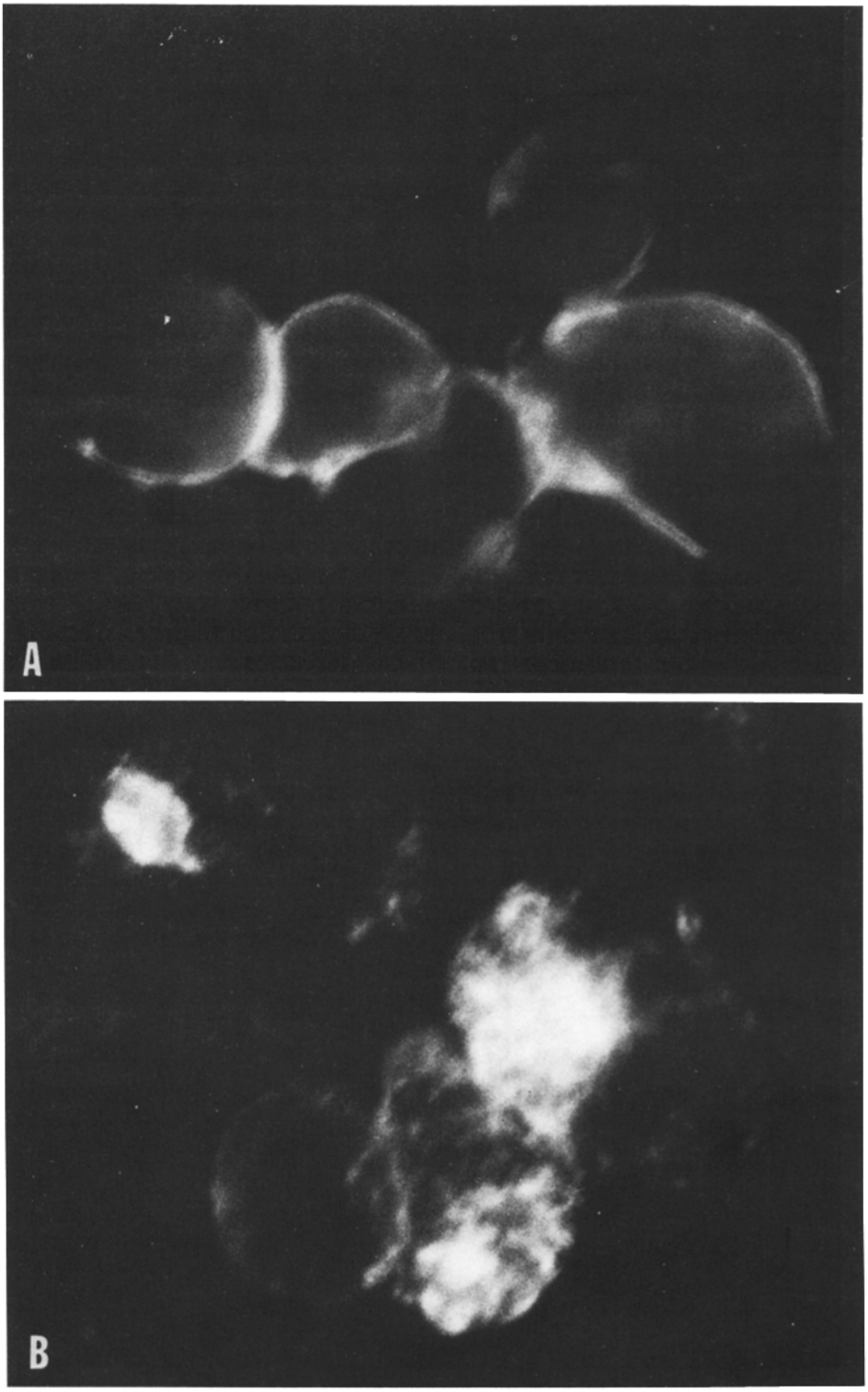

FIGURE 1. Reactivity of 1B6 mAb with P3-T/B cells as MIF in unfixed cells (*A*) or as CIF in acetone-fixed cells (*B*).

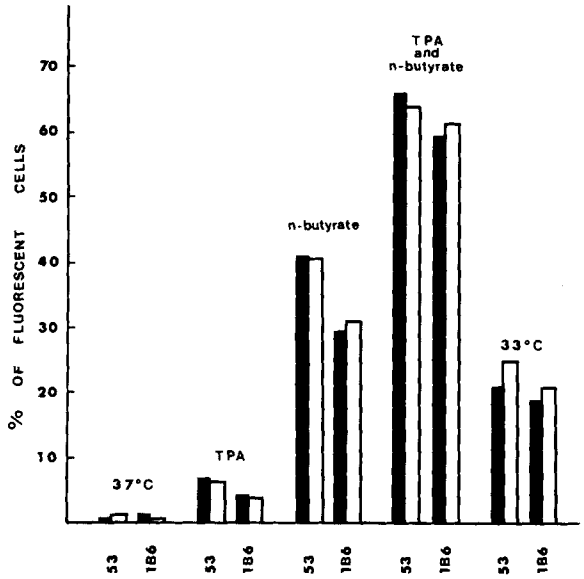

FIGURE 2. Reactivity of P3HR-1 cells with an EBV-seropositive human serum, 53 (1:20) or 1B6 mAb (1:160) by indirect immunofluorescence (■, MIF; □, CIF). P3HR-1 cells were cultured in various conditions: *37°C*, at 37°C for 2 d; *TPA*, with TPA (20 ng/ml) at 37°C for 2 d; *n-butyrate*, with *n*-butyrate (4 mM) at 37°C for 2 d; *TPA and n-butyrate*, with TPA (20 ng/ml) and *n*-butyrate (4 mM) at 37°C for 2 d; *33°C*, at 33°C for 10 d.

TABLE I

Effect of PAA on Expression of 1B6 Antibody-recognized MA

	Antibody	MA^+ cells (%)		
		0 h	48 h	
			Without PAA	With PAA
Control	70	1.0	3.1	0.3
	1B6	0.9	1.7	0.1
n-Butyrate	70	1.0	18.7	1.2
	1B6	0.9	11.9	0.6
n-Butyrate and TPA	70	1.0	56.3	4.2
	1B6	0.9	51.2	0.9

P3HR-1 cells were treated with *n*-butyrate (4 mM) alone, or together with TPA (20 ng/ml), and were cultured in medium with or without PAA (200 μg/ml). The MA^+ cells were quantitated by MIF at 48 h using 1B6 mAb or anti-MA^+ human serum, 70.

positive serum increased. Neutralization with 1B6 was observed, with NI = 0.6 at 1:10, and then a plateau of NI = 0.3 at 1:20–1:160 dilutions of mAb. A slight neutralizing activity was observed until 1:640.

Inhibition of EBV Release. Having found that antibody against an MA inhibits expression of EA in EBV-superinfected Raji cells (37), and knowing that antibody against a membrane-expressed influenza virus neuraminidase inhibits release of

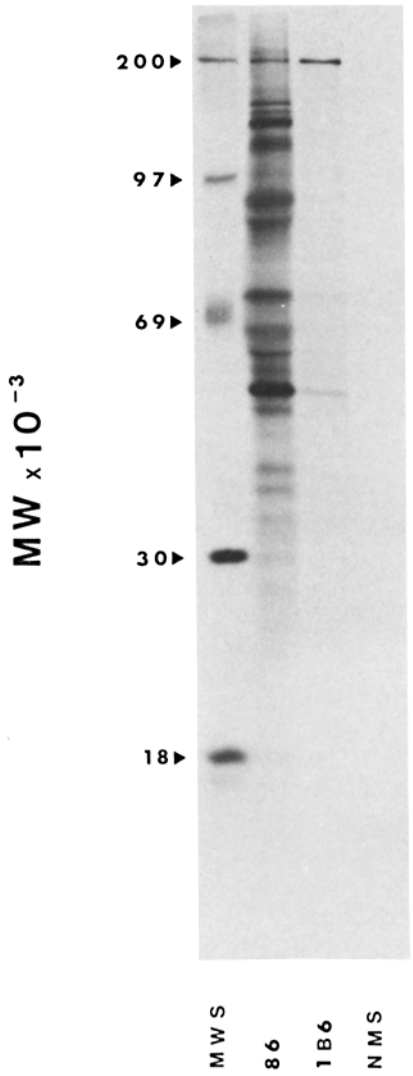

FIGURE 3. Immunoprecipitation of [^{35}S]methionine-labeled cell extracts from P3-T/B cells, comparing 1B6 mAb and serum 86 from a patient with chronic IM. *NMS*, normal mouse serum. *MWS*, molecular weight standards.

influenza virus (38), we tested 1B6 for its effect on expression of viral antigens and release of infectious virus in 33°C cultured P3HR-1 cells. P3HR-1 cells were cultured with either 1B6 antibody (1:100 and 1:1000), or in control ascites without anti-EBV antibodies (also at 1:100 and 1:1000). Cell growth and the frequency of VCA^+ cells were not affected by 1B6 (Fig. 5*A*). However, the titer of infectious virus in culture fluids was decreased at days 13 and 20 after cultivation with 1B6 (Fig. 5*C*). While titers of cell-associated infectious virus were unchanged or even enhanced at 13 and 20 d in cultures containing antibody (Fig. 5*B*), the titers of infectious virus were 10-fold (or more) reduced from those in the control culture fluids (Fig. 5*C*). The effect of varying concentrations

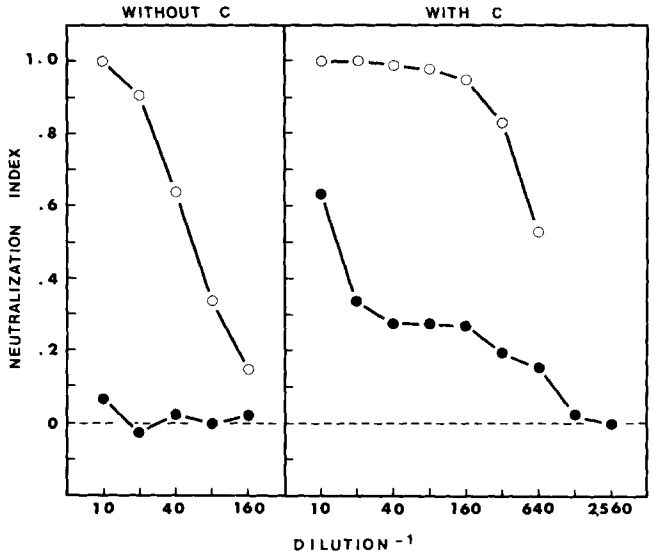

FIGURE 4. P3HR-1 EBV neutralization with 1B6 mAb (●) and with seropositive human serum 53 (○) was tested with or without the addition of complement from a seronegative healthy donor (60).

of 1B6 on EBV in culture supernatant was tested as a function of time after cultivation at 33°C (Fig. 6). Inhibition of the virus' appearance in culture medium was dependent upon dose of 1B6 mAb, and on the duration of culture. 50% inhibition of release of virus into the supernatant was observed at 1B6 ascitic titers of 1:400,000, 1:100,000, and 1:6,400 on days 10, 16, and 20, respectively.

The effect of 1B6 antibody on virus release was also tested with TPA plus *n*-butyrate–activated P3HR-1 cells at 37°C (Fig. 7). P3HR-1 cells were cultured with TPA (20 ng/ml) and *n*-butyrate (4 mM) in medium containing either 1B6 (1:1,000), or control ascites (1:1,000) for 6 d. The culture fluids and cell-associated infectious virus were assayed by the EAIU assay. In these cultures, maximal induction of EA occurred in 60% of the cells at day 2, and 1B6 antibody–recognized MA expression was seen in 35–50% of the cells at 2 and 3 d after incubation with TPA and *n*-butyrate (Fig. 7*A*). Cell viability decreased to 50–60% at day 3. 1B6 inhibited the appearance of virus in the culture medium; 2.5×10^3–4.0×10^3 EAIU/ml vs. 1.5×10^4–6.0×10^4 EAIU/ml at days 1–6 (Fig. 7*C*). Cell-associated virus increased in both control and treated cultures to day 3 (Fig. 7*B*). After 3 d, levels of cell-associated virus in control cultures decreased, but not in the 1B6 antibody-treated cultures. Net levels of virus production in cells and culture fluids of antibody-treated and control cultures were comparable. Thus, inhibition of virus release was found not only in 33°C-cultivated P3HR-1 cells, but also in the 37°C-cultured P3HR-1 cells that had been treated with TPA plus *n*-butyrate.

Reversibility of EBV Release-inhibition. The reversibility of the inhibition of release of EBV by 1B6 antibody was tested in P3-T/8 cells (Fig. 8). Such cells were cultured for 48 h with 1B6 mAb, or with control ascites fluid (each at 1:1,000 dilution). The cells were washed twice and suspended at 8×10^5

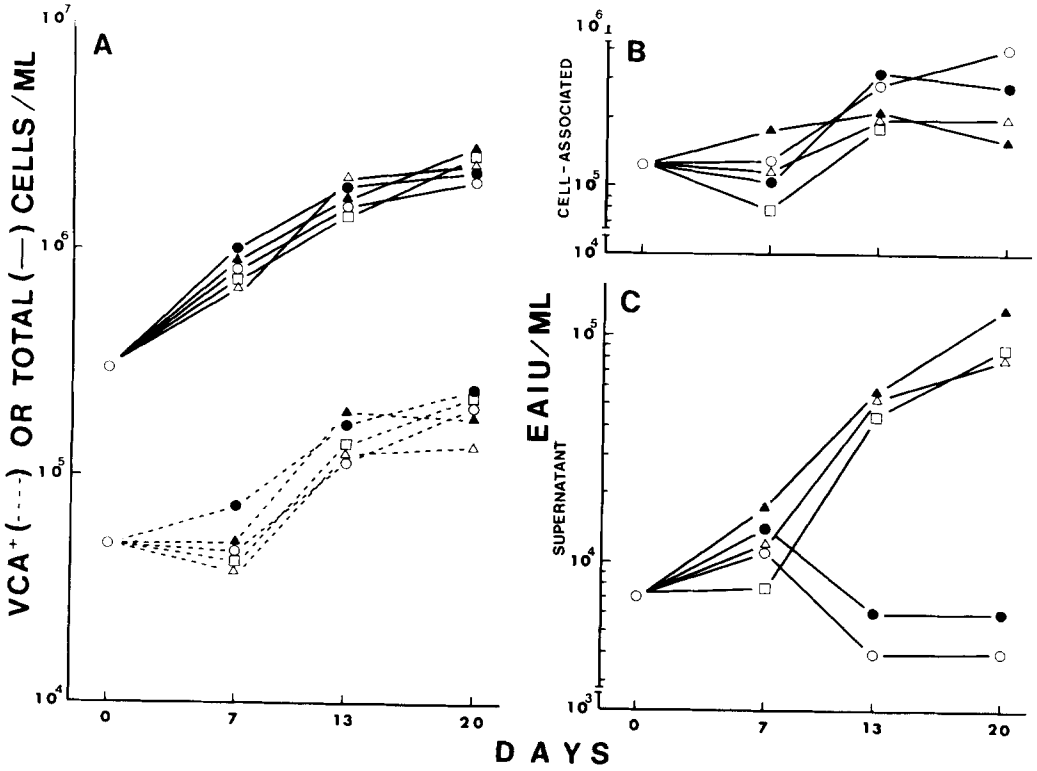

FIGURE 5. Effect of 1B6 mAb on expression of VCA$^+$ cells and production of infectious virus in P3HR-1 cells grown at 33°C. Growth of cells and expression of VCA$^+$ cells (*A*). Infectious EBV titers in sonicated cells (*B*) and in culture supernatants (*C*) were determined. 1B6 mAb (○, 1:100; ●, 1:1,000); control mouse ascites (△, 1:100; ▲, 1:1,000); medium only (□).

cells/ml in fresh medium, with or without 1B6 antibody. When cells were resuspended in 1B6 mAb, virus release was inhibited in both cultures pretreated with 1B6 mAb or control ascites fluid for 48 h. However, release of virus was observed in parallel from both cultures resuspended without 1B6 mAb. In the initial samples of cultures resuspended in control ascites medium, 5×10^2 EAIU/ml were observed in the supernatant of the ascites-pretreated control culture, and $<10^2$ EAIU/ml were observed in supernatant of the 1B6-pretreated cultures. At 8 h, the control culture medium demonstrated threefold greater virus titers than did the culture medium from 1B6-pretreated cultures.

Discussion

In this study we describe an mAb against an EBV-induced, 200 kD, PAA-sensitive (late) MA that functions in the release of EBV.The EBV specificity of this determinant was demonstrated by MIF and CIF. 1B6 antibody reactivity paralleled human serum staining for VCA and MA in P3HR-1 cells cultured at 37°C, or 33°C, or in 37°C-cultured and TPA-, *n*-butyrate–, or TPA plus *n*-butyrate–treated cells. Other EBV-producing cells (B95-8, Jijoye, and QIMR-

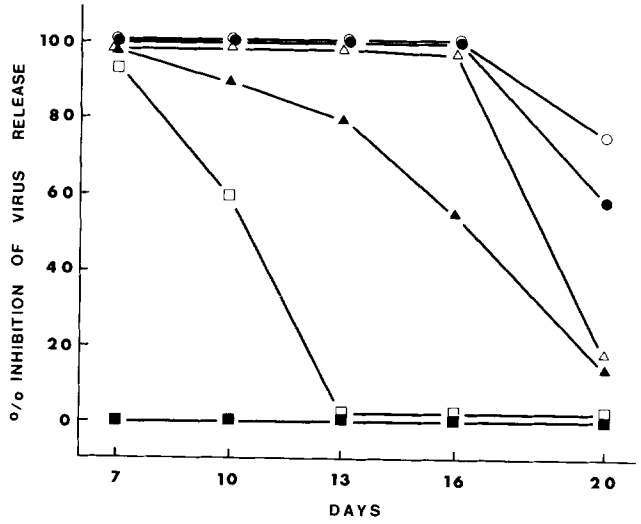

FIGURE 6. Dose response of 1B6 effect on virus release inhibition. P3HR-1 cells were cultured in medium containing various concentrations of 1B6 (○, 1:1.6 × 10^3; ●, 1:6.4 × 10^3; △, 1:2.5 × 10^4; ▲, 1:1.0 × 10^5; □, 1:4.0 × 10^5); or ■, control ascites (1:1,000) at 33°C, and assayed for infectious virus in culture fluids.

WIL) expressed the recognized determinant, but not as abundantly as did P3HR-1. Since 1B6 recognized a membrane antigen specific to EBV-producing cells, its target antigen was thought to be an EBV MA. Since PAA inhibited expression of this MA, it was, furthermore, a late MA, requiring the functioning of EBV-DNA polymerase for its expression. The antibody detected a 200 kD protein immunoprecipitated from [^{35}S]methionine-labeled, detergent-solubilized microsomal membranes of TPA plus *n*-butyrate-treated P3HR-1 cells. A molecule of similar mass has already been defined by others (13–16, 18) to be a principal component of EBV MA.

Some MA components (gp350, gp220, and gp85) are incorporated into the EBV envelope and are targets for neutralizing antibodies (10, 39). However, 1B6 antibody had no neutralizing activity without C (Fig. 4), and did not block neutralizing activity of seropositive human sera (data not shown). This finding contrasts with the report (14, 18) of C-independent neutralization of EBV with other anti-gp220 mAb. We have previously reported (28) the assay of C-dependent, EBV-neutralizing antibody in human sera. 1B6 antibody gave a unique pattern of neutralization with C. Although this pattern was observed with different preparations of virus and C, it might not truly represent susceptibility of a subset of virus to C-dependent neutralization with 1B6. It is, therefore, an object of further study.

The 1B6 antibody blocked release of EBV in culture fluids with two methods for the production of P3HR-1 virus, but did not inhibit synthesis of VCA or EA, or expression of intracellular infectious virus. Spontaneous release of virus from 33°C cultured cells, or release of virus from 37°C cultured, TPA plus *n*-butyrate–activated cells was blocked by cultivation with 1B6. In the 33°C cultures, the titer of infectious virus in the culture medium dropped appreciably

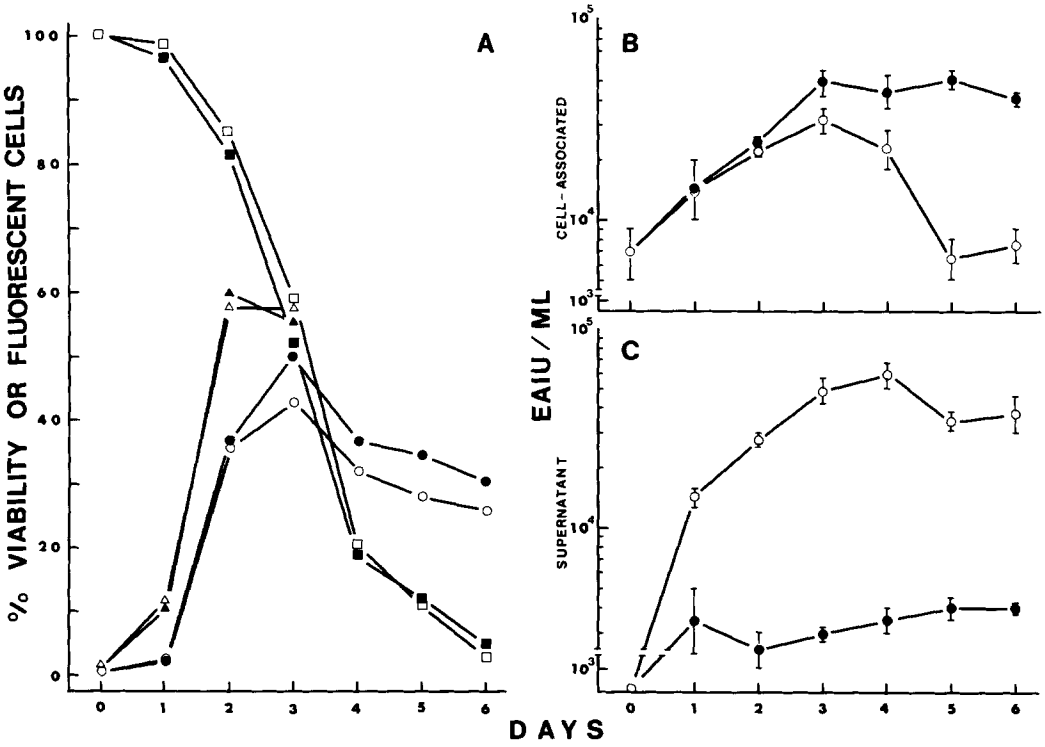

FIGURE 7. Effect of 1B6 on production of infectious virus of P3-T/B. P3-T/B cells were cultured with 1B6 mAb (1:1,000) or control ascites (1:1,000) at 37°C. (*A*) Expression of EA^+ cells (▲, 1B6; △, ascites), MA^+ cells (●, 1B6; ○, ascites), and viability (■, 1B6; □, ascites). (*B*) Titers of virus present in washed and sonicated cell pellets (●, 1B6; ○, ascites). (*C*) Titers of virus released into culture medium (●, 1B6; ○, ascites).

at 13 d. Throughout this process, intracellular infectious virus was still produced, in greater even amounts inside the viable, but release-blocked P3HR-1 cells. In the case of TPA plus *n*-butyrate–induced virus production, 1 or 2 d of culture of such cells with 1B6 was sufficient to demonstrate inhibition of virus release into culture medium. Again, intracellular virus was abundant in these release-inhibited cells. The effect of blocking virus release was exquisitely sensitive to the mAb; 50% inhibition of the virus release was observed (Fig. 6) to a dilution of $1:4 \times 10^5$ at 10 d or $1:10^5$ at 16 d.

This phenomenon of anti-MA–inhibition of virus release has not been observed previously with clinical sera, probably because virus-neutralizing antibodies masked the effect. That is, inhibition of release of EBV with clinical sera that also contained neutralizing antibodies was not observed because any virus released in such cultures would promptly have been neutralized. Previous reports have shown inhibition of EBV release by anti–virus release factor (AVRF) present in bovine serum (26, 40) and in EBV seronegative human serum (41). AVRF neither neutralized infectivity of EBV, nor inhibited intracellular virus production at concentrations that inhibited the release of virus into culture fluids. The

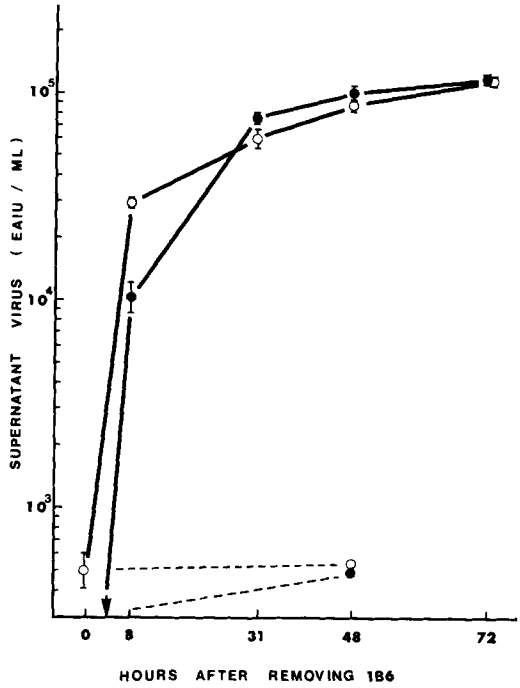

FIGURE 8. Reversibility of virus release-inhibition. P3-T/B cultures treated with 1B6 (●, 1:1,000) or with control ascites (○, 1:1,000) for 48 h were washed and resuspended in either fresh medium (——) or 1B6 (1:1,000) containing medium (– – –).

pattern of virus release inhibition by AVRF was thus comparable to that seen with 1B6 antibody, and might involve common mechanisms.

Since inhibition of virus release by 1B6 is promptly reversed upon removal of the antibody, we can suggest that the blocking of virus release might occur at a final stage in virus release after viral envelopment. We should now be able to study the mechanism of virus release in more detail, using 1B6. Since it appears that only some mAb against gp250/200 exhibit virus neutralization (14, 18), we can also test whether other nonneutralizing anti-gp250/200 mAb also inhibit virus release. In the face of disparity between virus neutralization and virus release-inhibition by various mAb directed against the 250/200 kD protein of MA, one could hypothesize either that two gp250/200 molecules mediate the respective functions, or that different functional domains exist on one molecule.

While we have characterized anti-MA-mediated inhibition of EBV release only in the P3HR-1 strain, this process will probably be generalized to other strains of EBV. Thorley-Lawson and Geilinger (18) have demonstrated shared MA epitopes between gp350, the major glycoprotein in B95-8, and gp220, the major glycoprotein in P3HR-1, suggesting that these molecules are structurally related. We have demonstrated immunofluorescence crossreactivity of the 1B6 antibody-recognized determinant of p200 MA on P3HR-1 and epitopes expressed on B95-8, Jijoye, and QIMR-WIL cells. It will be important to characterize the generality of this EBV release-suppression phenomenon to other laboratory and wild type strains of EBV. Also, since patients raise antibodies

against p200 (Fig. 3), one can also expect to quantitate patients' antibodies against the epitope identified by 1B6 antibody. For example, one could determine, in the course of IM, the appearance of the antibodies that inhibit EBV release or neutralize EBV relative to antibodies against other EBV antigens. Whether such EBV release–inhibiting antibodies occur in chronic or recurrent EBV infections, or whether they are deficient in XLP, could also be explored. We will seek to elucidate the detailed mechanism of inhibition of virus release and the clinical significance of this process.

Summary

In raising murine hybridoma antibodies against Epstein-Barr virus (EBV)-induced membrane antigens (MA), we found one antibody that blocked the release of infectious EBV from cultured P3HR-1 cells. This monoclonal antibody (mAb) recognized a 200 kD, phosphonoacetic acid–sensitive (late) MA, and did not directly neutralize virus without complement. When this mAb was added to 33°C-cultured, spontaneously EBV-producing P3HR-1 cells, the intracellular expression of viral capsid antigen and infectious virus was not inhibited, but the appearance of infectious virus in the culture medium was significantly reduced. The duration of this suppression was dependent upon the concentration of the mAb, an effect being observed to a $1:4 \times 10^5$ titer of the ascites mAb preparation. A more acute effect of suppression of EBV release was observed in a second model of 12-*o*-tetradecanoyl phorbol-13-acetate and *n*-butyrate induction of EBV in 37°C-cultured P3HR-1 cells. Again, intracellular infectious virus production was not inhibited, but the level of infectious virus in the culture medium was significantly reduced as early as 1 and 2 d of culture with antibody. This effect was reversed within 31 h after replacement of mAb-containing medium with fresh medium. This description of antibody-mediated inhibition of EBV release might lead to the characterization of another form of immune defense for the control of EBV infections.

We gratefully acknowledge the excellent secretarial assistance of Ms. Marion Bonin and Ms. Lena DeSantis in the preparation of this manuscript.

Received for publication 7 January 1985

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