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Monoclonal antibody against a 250,000-dalton glycoprotein of Epstein–Barr virus identifies a membrane antigen and a neutralizing antigen

(Burkitt lymphoma/infectious mononucleosis)

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ABSTRACT **An antibody-secreting hybrid cell line was produced by fusion of mouse myeloma cells with splenocytes from mice immunized with virions of the B95-8 strain of Epstein–Barr Virus (EBV). The monoclonal IgG antibody was shown to have anti-EBV activity by the following criteria: (*i*) It reacted with the membranes and the cytoplasm of seven different EBV-producing lines, but with no nonproducing line. (*ii*) The individual cells identified by the murine antibody were shown to be the same cells identified by a human serum having anti-EBV activity. (*iii*) The antibody significantly reduced the infectivity of two independent strains of EBV (namely, P3HR1K and B95-8). The antigen being recognized was characterized by immunoprecipitations of radiolabeled EBV-producer cell lysates. A single glycoprotein with an estimated molecular weight of 250,000 was identified. It is concluded that neutralization of EBV can be achieved by an IgG-class monoclonal antibody directed against a single antigenic site on a 250,000-dalton glycoprotein, which is a constituent of the EBV virion.**

The Epstein–Barr Virus (EBV) is of clinical importance because of its relationship to the human diseases Burkitt lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis. Although there has been progress in analyzing the nucleic acid of the virus, little progress has been made with the polypeptides (1, 2) because of the difficulty in obtaining sufficient material of suitable purity. Most of the current knowledge has been derived by the laboratories of the Henles of Philadelphia and the Kleins of Stockholm, using human sera as immunological probes. They have defined early antigens (EA), neutralizing antigens (NA), membrane antigens (MA), viral capsid antigens (VCA), and a nuclear antigen (EBNA), most of which are clearly complex mixtures with several antigenic determinants. As alternatives to human sera, some workers have developed allo-antisera (3–5). However, these have often been of low titer, and require extensive adsorption. They do not overcome the basic problem, which is the need for purity of either the antibody or the antigen.

In 1975 Köhler and Milstein (6) opened up a different immunological approach with their description of the monoclonal antibody technique. This allows immunization with complex mixtures of antigens, and generates pure populations of antibodies that can distinguish single antigenic sites. The method has been applied to the study of other viruses (e.g., refs. 7–9), and this report shows that it is applicable to EBV. One monoclonal antibody that defines a membrane antigen of EBV has the ability to neutralize two strains of the virus. Immunoprecipitations characterize the antigen as a 250,000-dalton glycoprotein that is a virion constituent.

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MATERIALS AND METHODS

Cell Lines. The producer cell lines used were P3HR1K (Burkitt lymphoma) and QIMR-WIL (leukemia, but probably infectious mononucleosis) of human origin, and B95-8, MCUV, MCUV-CC (infectious mononucleosis), X35BL4, and CC-34 (both Burkitt lymphoma) representing marmoset cell lines established by infection with human virus of the origin indicated. Human nonproducer lines were BJAB, Namalwa, Raji (all of Burkitt lymphoma origin), and MOLT-4 (from a patient with acute lymphatic leukemia). The cells were received as gifts from George Miller of Yale, Elliott Kieff of Chicago, and Dennis Moss and Alan Rickinson of Bristol. Two EBV-negative cotton-top marmoset lines (70N2 and 1022) were given by Lawrence Falk, Jr., of the New England Primate Center. P3X63Ag8 mouse myeloma cells were obtained from Patricia Gearhart of the Carnegie Institution and from the Cell Distribution Center of La Jolla.

Lymphoblastoid cells were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and gentamycin at 20 μg/ml. Cells used for making smears were generally taken from cultures fed daily. The P3X63Ag8 cells were maintained in Dulbecco's modified Eagle's medium with 20% heat-inactivated fetal calf serum, gentamycin at 20 μg/ml, and 8-azaguanine at 20 μg/ml.

Preparation of Virus. B95-8 virions were purified from 5-liter portions of culture supernatant essentially as described by Dolyniuk and colleagues (2).

Immunization. A viral pellet (approximately 5×10^{10} particles) was disrupted by the addition of Emulphogene 720 (GAF, New York), a nonionic detergent, and fixed in 1% formaldehyde, and the aldehyde was neutralized by Dulbecco's modified Eagle's medium. The virus was then emulsified in Freund's complete adjuvant, and approximately 5×10^9 to 10^{10} particles were injected subcutaneously in the backs of BALB/c mice. Two weeks later a subcutaneous booster injection of a similar amount of virus was given in incomplete Freund's adjuvant. Fusions were performed up to three months later. A last boost, given approximately 5 days before the fusion, consisted of disrupted and fixed virus in incomplete Freund's adjuvant injected intraperitoneally.

Cell Hybridization. The method used was a minor modification of that of Galfre and colleagues (10). The cells of one spleen were copelleted with 10^7 P3X63Ag8 myeloma cells and fused by the addition of a mixture of 25% polyethylene glycol 1000 and 25% dimethyl sulfoxide (vol/vol) in Dulbecco's modified Eagle's medium. The products of each fusion were divided into 48 13-mm wells and grown in selective medium.

Abbreviations: EBV, Epstein–Barr virus; EA, early antigens; NA, neutralizing antigens; MA, membrane antigens; VCA, viral capsid antigens; EBNA, EBV nuclear antigen.

Before definitive use, a producing culture was twice cloned in soft agar. Producer cells were also grown in oil-induced ascites in BALB/c mice to yield an antibody-containing ascitic fluid.

Screening Assays. The first method used was a radioactivity-binding assay. The antigen was glutaraldehyde-fixed cells of a producer line, B95-8. The fixed antigen was then exposed successively to potential antibody and to radio-iodinated affinity-purified goat IgG directed against murine IgG. After washing, the bound radioactivity was measured in a Beckman Gamma 310 counter.

The reference assay was an indirect immunofluorescence microscopy assay. Cells from a producer cell line were washed, smeared on slides, air-dried, and fixed in acetone at −20°C for 3 min. The labeled conjugates were fluorescein-labeled goat anti-murine IgG and goat anti-human IgG (Cappel Laboratories, Cochranville, PA). A reference human serum, having an immunofluorescence microscopic assay titer against EBV of 1:1000, was used as a positive control. Slides were examined by transmitted or epi-fluorescence on a Zeiss Photomicroscope.

Neutralization Assays. Two assays were used. The first was the inhibition of the induction of EA after superinfection of Raji cells by P3HR1K virus, as described by Coope *et al.* (5). The second assay was the inhibition of transformation of human umbilical cord lymphocytes by MCUV virus, and was carried out on coded serum samples by George Miller of Yale University by the method described (11). Results were scored by the Reed–Muench method. Murine monoclonal antisera having activity against marmoset cell membranes and a nonsurface EBV antigen were included as controls.

RESULTS

Generation of Antisera. Four of four mice immunized with disrupted B95-8 virions showed evidence of anti-EBV antibody when tested by indirect immunofluorescence on smears of producer cells. Fusion of the spleen of one of these mice with myeloma cells gave hybrids with myeloma-like morphology in 44 of 48 wells. Another independent fusion yielded hybrids in 24 of 48 wells.

Supernatant fluids were screened by two different techniques. The radioactivity-binding assay proved insensitive but was able to identify five strongly binding supernatants. An indirect immunofluorescence microscopy method was used for most studies.

An antibody-secreting line (designated 72A1) was selected and twice cloned through soft agar. The property of antibody secretion proved stable over 3 months of culture. Supernatant from cultured cells could be diluted up to 1:5000 and still give positive immunofluorescence. An ascitic fluid taken 8 days after intraperitoneal injection into BALB/c mice gave positive immunofluorescence up to a dilution of 1:10,000.

Staining of Cell Lines. The results of indirect immunofluorescence microscopy are shown in Table 1. All producer lines, whether human or marmoset, showed strongly stained cytoplasm of a subpopulation of cells. The pattern of staining is illustrated in Fig. 1 *A* and *B*. There was a tendency for the rim of the cell to be particularly strongly stained (best seen in different planes of focus), while nuclei usually showed little overlying fluorescence. None of the nonproducer cell lines, whether EBNA-containing or not, and whether human or marmoset, showed any staining.

The results of staining live cells in suspension are also shown in Table 1. This method defines MA. The cell lines that stained as smears also showed membrane staining and, again, only a subpopulation of cells became fluorescent. Cell lines that were negative when tested as smears were also negative as suspensions. The pattern of staining of positive cells is shown in Fig. 1 *C* and *D*. The cells present were identified in a phase-contrast micrograph of the same field as shown in the fluorescence micrograph. The peripheral distribution of the staining can be seen to be slightly clumped ("patched").

Table 1. Definition of antigenic specificity of monoclonal antibody 72A1

Lymphoblastoid cell lines			Fluorescence microscopy*	
EBV antigen status	Species	Designation	Fixed smears	Live cells
Producer	Human	P3HR1K	+++	+++
		QIMR-WIL	+++	+++
	Marmoset	B95-8	+++	+++
		MCUV	+++	+++
		MCUV-CC	+++	+++
		X35BL4	+++	+++
		CC34	+++	+++
Nonproducer	Human	MOLT-4	0	0
		BJAB	0	0
		Namalwa	0	0
		Raji	0	0
	Marmoset	70N2†	0	0
		1022‡	0	0

* Intensity of staining of individual cells on a scale of 0 to +++. Only a percentage of the cells in a population were stained with fluorescent antibody. The figure varied from line to line, and at different times during culture, but was usually between 1 and 10%.

† 70N2 is a cotton-top marmoset line containing the *Herpesvirus saimiri* genome.

‡ 1022 is a cotton-top marmoset line containing the *Herpesvirus ateles* genome.

To further establish that the cellular staining was due to EBV antigens, a double staining procedure was performed. Smears of P3HR1K cells were stained sequentially with the murine antibody, a reference human anti-EBV serum, fluorescein-conjugated goat anti-murine IgG, and rhodamine-conjugated goat anti-human IgG. This order was necessary: if the human antiserum was added before the murine, the binding of the murine antibody was blocked. A panel of controls was used to exclude cross-reactivity of the reagents. Cell smears were examined with the fluorescein filter combination, labeled cells were identified, and then the filter combination was changed to view rhodamine labeling. It was found that all cells labeled with fluorescein were also labeled with rhodamine. When the pattern of screening was reversed, most of rhodamine-labeled cells were seen to be labeled with fluorescein, although a few were not. In other experiments a similar protocol was used with the exception that the anti-murine IgG antibody was labeled with rhodamine, and the anti-human IgG antibody was labeled with fluorescein; identical results were obtained. When EBV-antigen expression was increased by chemical induction, the similarity of staining by murine and human sera was maintained. In all cases, not only were the same cells of the population stained, but also the patterns of staining of the individual cells by the two fluorochromes were seen to be identical (Fig. 1 *E* and *F*).

When producer cell smears were stained with 72A1 supernatant, and then counterstained with rhodamine-conjugated rabbit anti-murine IgG or IgM (the gift of John Cebra), it was found that only the anti-IgG gave fluorescence.

Neutralization of Viral Infectivity. The neutralization of P3HR1K virus was measured by the inhibition of the induction of EA in Raji cells. The titer was the serum dilution that gave a 50% reduction in EA induction. This proved to be between

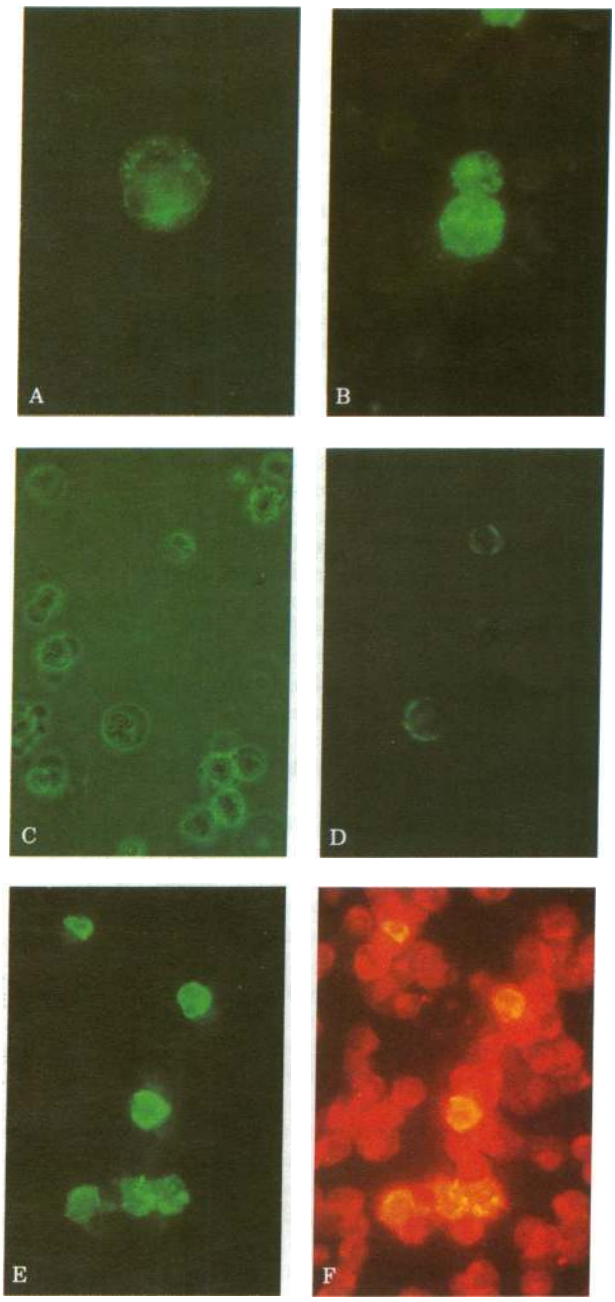

FIG. 1. Immunofluorescence staining. (*A* and *B*) Pattern of smears of EBV-producing cells stained by murine monoclonal antibody. Air-dried acetone-fixed cells were stained by an indirect immunofluorescence technique. (*A*) P3HR1K cell; (*B*) B95-8 cells. (×280.) (*C* and *D*) Membrane staining pattern of live EBV-producing cells by murine monoclonal antibody. Live cells were stained in suspension in silicone-treated 6 × 50 mm glass tubes by an indirect immunofluorescence technique. (*C*) Phase-contrast micrograph to identify cells in a field. (*D*) Fluorescence micrograph of the same field as *C*. Note the uniform ring-like fluorescence pattern, which corresponds with the membranes of two cells identified in *C*. (×180.) (*E* and *F*) Double staining of an EBV-producing cell line by human and murine antisera. An air-dried acetone-fixed smear of P3HR1K cells was sequentially exposed to murine monoclonal antibody 72A1, reference human anti-EBV antibody, fluorescein-conjugated goat anti-murine IgG, and rhodamine-conjugated goat anti-human IgG. Smears were examined on a Zeiss Photomicroscope with: (*E*) a highly selective fluorescein combination 450–490-nm band-pass filter, a 510-nm reflector, and a 520–560-nm band-pass barrier filter; or (*F*) a rhodamine filter combination, a 546-nm band-pass filter, a 580-nm reflector, and a 590-nm low-pass barrier filter. (×112.)

1:8 and 1:16 and was reproducible in three independent experiments. The data from one experiment are illustrated in Fig. 2. Two monoclonal antibodies were used as controls. One reacts with marmoset, but not human, cell surface membranes. The other reacts with EBV-containing cells, but not with their surface membranes. Neither had any effect on the EA induction.

The second assay was of induction of immortalization of umbilical cord lymphocytes by MCUV virus. This was carried out by George Miller of Yale University. Antibody titer is measured by the inhibition of the appearance of colonies after 28 days. The test antibody gave a titer of approximately 1:400 by the Reed–Muench equation, whereas the anti-marmoset antibody described before gave a titer of less than 1:10, and the other control serum gave a titer of 1:20. In neither this assay nor the EA induction was there any evidence of cytotoxicity of the antiserum; the cells with antiserum added grew as well as those in control cultures.

Identification of the Antigen. Immunoprecipitations were carried out on intrinsically radiolabeled cell lysates of B95-8 cells, with MOLT-4 cells (containing no EBV genome) as controls. As control sera, medium containing 20% fetal calf serum and a nonreactive monoclonal antibody were used. In all cases in which sufficient material was loaded on the gel, and in which the gels were stained with Coomassie blue, a unique high molecular weight band (>200,000) was identified in tracks where the test antiserum was present but not in any controls. Immunoprecipitation from radiolabeled methionine and lysine extracts gave unacceptably large numbers of background bands. The precipitates with the test antibody and the [^{3}H]glucosamine-labeled extracts gave a single band not precipitated in the controls, as illustrated in Fig. 3 *Left*. It is clear that the test antibody precipitates a glucosamine-containing polypeptide of molecular weight greater than 200,000 (track B) not precipitated by either the medium control (track A) or another monoclonal antibody with a different specificity (track C). When an independent precipitate was analyzed on a uniform 5% polyacrylamide gel along with protein markers of molecular weights ranging from 12,300 to 220,000, the graph in Fig. 3 *Right* was generated. This established the molecular weight estimate as 250,000. Because of the paucity of markers of the appropriate size, it is not possible to approach the molecular weight better by development of a Ferguson plot (log R_F vs. % gel concentration). It is proposed that the antigen identified be termed gp250, and that the antibody be termed α-gp250.

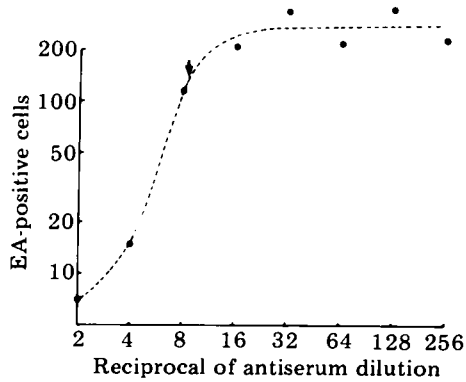

FIG. 2. Neutralization of the P3HR1K strain of EBV by monoclonal antibody 72A1. Infectious supernatant from P3HR1K cells was mixed with dilutions of antibody and then added to Raji cells. Three days later, EA induction was measured by immunofluorescence. Arrow indicates titer.

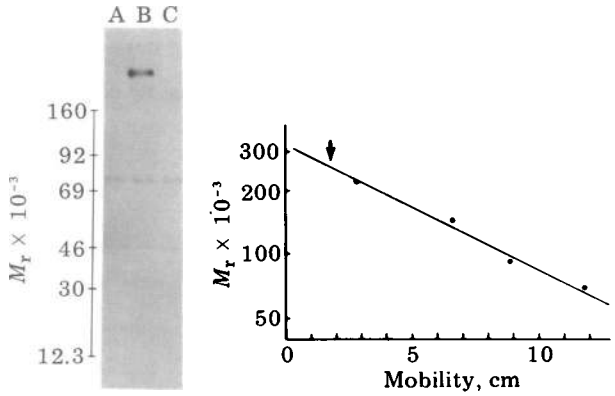

FIG. 3. Polyacrylamide gel electrophoretic analysis of immunoprecipitated [^{3}H]glucosamine-labeled B95-8 cell lysate. B95-8 cells were intrinsically labeled with [1,6-^{3}H]glucosamine (New England Nuclear) at 5 μCi/ml (1 Ci = 3.7 × 10^{10} becquerels) in RPMI 1640 medium containing 10% dialyzed fetal calf serum. After a labeling period of 24 hr, the cells were pelleted, washed, and lysed in buffer containing 0.02 M Tris·HCl (pH 8.0), 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% (vol/vol) glycerol, and 1% Nonidet-P40, with phenylmethylsulfonyl fluoride (200 μg/ml) added as a protease inhibitor. The cell density was 1–2 × 10^7 per ml. After incubation for 20 min on ice with Vortex mixing, the extracts were clarified by centrifugation at 10,000 × *g* at 4°C for 45 min. Immunoprecipitations were performed at 37°C. Lysate (200–300 μl) was made to 0.5 mg/ml in bovine serum albumin and incubated for 1 hr with 10 μl of antiserum. Samples of a 100 mg/ml suspension of *Staphyloccus aureus* (Cowan) protein A coupled to Sepharose 4B (Pharmacia) were added and the mixture was incubated for 15 min. Immunoprecipitates were washed at 4°C in lysing buffer containing 0.5 M NaCl, then resuspended in 0.025 M Tris/glycine (pH 8.3). Analyses of reduced and denatured samples were carried out by sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis with 2.5% stacking gels, using the discontinuous buffer system of Laemmli (12). After electrophoresis, gels were fixed in 8% acetic acid, 30% methanol (vol/vol), and processed for fluorography (13). (*Left*) Fluorogram of a 5–15% gel. Track A, tissue culture medium; track B, medium plus antibody 72A1; track C, medium containing another monoclonal antibody having no anti-EBV activity. The molecular weight estimates are derived from radiolabeled marker proteins analyzed on the same gel. The minor bands migrating at molecular weights 46,000 and 75,000 are seen in all tracks. A unique band of high molecular weight is present in the 72A1 track (B). (*Right*) Molecular weight standardization curve on a uniform 5% gel. The mobility of markers was measured on an autoradiogram of the gel. The immunoprecipitated specific band (indicated by the arrow) migrated as a polypeptide of molecular weight 250,000.

DISCUSSION

This paper presents an example of the application of the Milstein technique to the study of EBV. There was a satisfactory rate of generation of anti-EBV antibodies, even with the insensitive screening method used. The antibody αgp250, of the IgG class, was readily obtained from tissue culture supernatant with a titer measured by immunofluorescence microscopy at 1:1000 to 1:5000, and a batch of ascitic fluids had a titer between 1:5000 and 1:10,000.

The anti-EBV specificity of the antibody has been defined in two ways. The first was its ability to stain a subpopulation of the cells of virus-producing cell lines. α-gp250 stained both human and marmoset producers, but not nonproducers of either species. In addition, the marmoset cell lines containing *Herpesvirus ateles* and *Herpesvirus saimiri* showed no immunological crossreactivity. The second method compared the staining of cells by α-gp250 with the staining by a human antiserum with anti-EBV activity. It was noted that whereas all cells that stained with the murine antibody stained with the human serum, the converse was not always true; there was sometimes a small number of cells stained only by human serum, reflecting the detection of additional antigens. The patterns of cytoplasmic staining of individual cells by the two reagents were similar. The results indicate clearly that the antigen is an EBV-associated product. When the neutralization evidence is added, it seems fair to conclude that it is a virion constituent, and a virally encoded peptide.

The staining patterns of fixed smears show generalized cytoplasmic staining with relative sparing of the nuclear region. Such a pattern is similar to that classically defined as either the restricted EA or VCA. The staining of living cells defined the antibody as being directed against membrane antigens. It is therefore apparent that appearance of stained smears alone is not sufficient to distinguish different antibody activities. Further analysis of the complexity of the cytoplasmic staining will be possible by means of additional monoclonal antibodies.

Two different outcomes result from infection with different strains of EBV (14). In one, late viral antigens are expressed and cell death results. Such infections are termed lytic, and P3HR1K is the model strain. In the second, only early viral functions are expressed. Infected lymphocytes are converted to lymphoblastoid cell lines: such conversion is termed immortalization, and B95-8 and MCUV strains are representative. Examples of each type have been tested, and neutralization has been demonstrated. The differences in titer are explained by the different sensitivities of the two tests (5). In addition, the source of infectious material may well have played a role. The supernatant fluids used as sources of virus probably contained different amounts of soluble blocking antigens as well as infectious virions (5).

Studies with intrinsic radiolabeling and immunoprecipitation showed that the antigen is a glycoprotein with an apparent molecular weight of 250,000. Such a species was not found by Dolyniuk *et al.* (15) or Mueller-Lantzsch (16), but their gel systems were not suitable to resolve this size class. Qualtiere and Pearson (17) radio-iodinated EBV producer-cell surfaces, and immunoprecipitated them with human anti-MA sera. They described three glycoproteins, having molecular weights of 280,000, 250,000, and 90,000, in addition to a nonglycosylated molecular weight 170,000 protein. Strnad and colleagues (18) have also reported three membrane antigen glycoproteins. These have estimated molecular weights of 236,000, 212,000, and 141,000, although an optimal analytical gel system allowing certainty of their assignments was not used. The present report confirms the existence of the 250,000-dalton species, and has demonstrated its significance in the MA complex. Thorley-Lawson and Edson (4, 19) have described a 350,000-dalton protein immunoprecipitated by a heterologous neutralizing antiserum. It is not evident how this species is related to gp250.

The relationship of MA and NA has received considerable attention (3, 5, 20). It is evident that the MA and NA titers of human sera are generally concordant, but there are cases in which the neutralizing titer is high while the membrane antigen titer is low, suggesting that there are non-membrane-associated NA. However, in the studies of both Miller and colleagues (5) and Thorley-Lawson (3), producer cell membranes were able to adsorb all the neutralizing activity of heterologous antisera. This report shows that antigen gp250 is an MA and a NA. In addition, it shows that an IgG-class antibody against a single antigenic determinant on gp250 is capable of neutralizing EBV. Further studies with monoclonal antibodies will be required to show if other antigens, or any other antigenic sites on gp250, are required for infectivity of EBV.

The availability of monoclonal antibodies makes possible the purification of individual antigens by affinity chromatographic techniques. Examples of the potential application of this

methodology include analysis of the complexity of human neutralizing antisera. It is of interest to know what proportion of the neutralizing titer can be adsorbed by immobilized gp250. Conversely, the significance of gp250 in neutralization can be tested by immunizing animals and testing for protective immunity against EBV infection.

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