JOURNAL OF VIROLOGY, Aug. 1982, p. 730–736
0022-538X/82/080730-07$02.00/0

Vol. 43, No. 2

NOTES

Identification and Isolation of the Main Component (gp350-gp220) of Epstein-Barr Virus Responsible for Generating Neutralizing Antibodies In Vivo

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Received 2 July 1981/Accepted 26 March 1982

The majority of hybridomas we have characterized against Epstein-Barr virions react with the major glycoproteins gp350 and gp220 (gp350/220). One of these antibodies, ID4C-1, neutralizes virus infection in vitro. The presence of gp350/220 on the viral envelope could be confirmed directly by immunoelectron microscopy. We used lectin affinity (ricin) and immunoaffinity (ID4C-1) to purify gp350/220 and show that this material is able to induce potent virus-neutralizing antibodies. Absorption of four human and one rabbit anti-Epstein-Barr virus sera with purified gp350/220 suggests that this is the primary component responsible for generating neutralizing antibodies in vivo.

The immunological responses to Epstein-Barr virus (EBV) infection include cell-mediated immunity directed against virus-infected lymphocytes (7, 19, 21) and neutralizing antibodies directed against the viral envelope (10). Although antibody titers against virion antigens can be indicative of the subsequent clinical course of disease, it is unclear what role they play in controlling the infection.

There appears to be general agreement that the major components of the viral envelope from either intact virions or the plasma membranes (membrane antigen [MA]) of producer cell lines consist of at least three glycoproteins (molecular weights, 350,000, 220,000, and 85,000 in our hands) and one non-glycosylated protein (molecular weight, 140,000) (2–5, 8, 9, 12, 16, 23, 24). The exact number of components, however, remains unclear. In addition, although monoclonal antibodies against the two large glycoproteins of the EBV MA, gp350 and gp220 (gp350/220), will neutralize the virus (4, 24), it has not been possible so far to identify which are the main components necessary for the induction of virus-neutralizing antibodies in infected humans.

In this report, we present a quick and simple method for the isolation of gp350/220. In addition, we show that the material is antigenically active, as it will induce neutralizing antibodies in experimental animals and absorb neutralizing antibodies from human sera. The latter observation implies that gp350/220 is the main component against which neutralizing antibodies are generated during in vivo infection.

Purification of gp350/220. Fifteen grams of EBV-producing lymphoblastoid cells (P3HR-1, M-ABA, or tetradecanoyl-phorbol-13-acetate-treated B95-8 cells; provided by the Division of Cancer Cause and Prevention, National Cancer Institute) was lysed by nitrogen cavitation, and crude plasma membranes were prepared by differential centrifugation as described previously (23). The membranes were solubilized for 0.5 h at 4°C in a volume of approximately 15 ml with sodium deoxycholate (Sigma Chemical Co.) at a protein/deoxycholate ratio of 1:3, wt/wt (protein was estimated by measuring the absorbancy at 280 nm). The solution was clarified by centrifugation at 100,000 × *g* for 0.5 h in a Beckman SW41 rotor, and glycoproteins were purified as described previously (20) on a 10-ml column of *Ricinus communis* agglutinin II. The material which eluted was then passed over a 5-ml column of Sepharose 4-B, normal mouse immunoglobulin coupled to Sepharose 4-B (preclearing steps), and finally, ID4C-1 (C1) monoclonal antibody coupled to Sepharose 4-B (specific step). The flow-through material was monitored by measuring the absorbancy at 280 nm. When the peak of unbound material emerged, the last (specific step) column was unhooked and

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washed thoroughly with a high-salt buffer of 10% saturated NaCl in 0.2% deoxycholate–10 mM Tris (pH 8.0). Specifically bound material was eluted with 0.2% deoxycholate–50 mM diethylamine (pH 10.5). The fractions containing protein were pooled and dialyzed against phosphate-buffered saline (PBS). The affinity columns were always pretreated with 0.2% deoxycholate–50 mM diethylamine (pH 10.5) before use and were washed and stored in PBS with 0.2% NaN_3. In this way, the columns could be multiply used.

Typically, about 5 to 10 μg of protein was obtained from each gram of lymphocytes used, as judged by measuring the absorbancy at 280 nm (assuming arbitrarily that a 1-mg/ml solution had an absorbancy at 280 nm of 1). We assessed the efficiency of both the lectin and immunoaffinity steps of our purification by analysis of flow-through and bound material for the presence of gp350/220 by immunoprecipitation with rabbit anti-EBV antiserum (24). The ricin step is relatively efficient, with greater than 85% of the gp350/220 molecules being recovered from the specifically bound material. Similarly, 80% of the antigen was recovered from the C1 column, giving an overall recovery of about 70%.

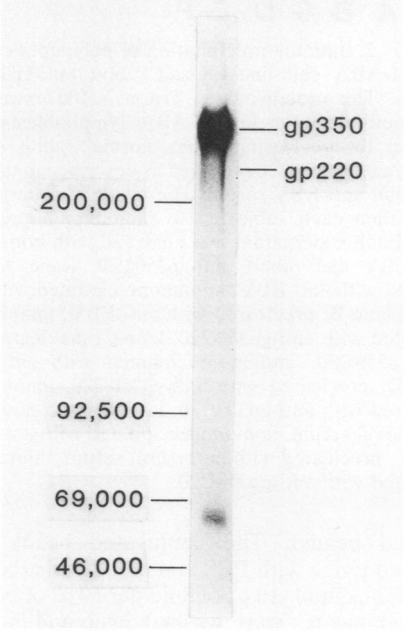

FIG. 1. Autoradiography of ^{125}I-labeled, purified gp350/220. gp350/220 were isolated as described in the text. Twenty microliters of supernatant containing approximately 1 μg of gp350/220 was labeled with ^{125}I by the chloramine-T method. To this was added 200 μl of sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the sample was boiled. Five microliters of the sample was run on the gel. The electrophoresis front was allowed to run off the end of the gel to remove free ^{125}I. Autoradiography was performed for 30 min. Note that there is considerably more gp350 than gp220, since the B95-B cell line was used for this experiment and this line expresses only low amounts of gp220.

TABLE 1. Neutralization of EBV infection in vitro, using a specific anti-gp350/220 rabbit antiserum[a]

Antibody	Time added	Addition of virus	Infectivity assayed by[b]: DNA synthesis[c]	Infectivity assayed by[b]: Outgrowth[d]
α-gp350/220	1 h preinfection	+	118	0/6
	2 h postinfection	+	9,119	6/6
Normal rabbit serum	1 h preinfection	+	5,909	6/6
	2 h postinfection	+	9,177	6/6
None		+	6,707	6/6
		−	485	0/6

[a] A rabbit was immunized four times at 10-day intervals, using 50-μg injections of purified gp350/220. The heat-activated serum was absorbed three times for 1 h at room temperature to remove nonspecific antibodies and was used at a dilution of 1:15.

[b] The assay is based on the original method of Robinson and Miller (13) and has been described in detail elsewhere (22).

[c] [^{3}H]thymidine incorporation.

[d] Number of wells with transformed foci.

Figure 1 shows an autoradiogram of B95-8 cell material obtained from the eluate of a C1 column after iodination and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It should be noted that the material is virtually pure gp350/220 with low amounts of gp220, because this polypeptide is poorly expressed in B95-8 (3). In addition, a minor contaminating band with a molecular weight of 50,000 to 60,000 was seen in some preparations. Although the origin of this protein is unsure, it may be the heavy chain of immunoglobulin from the affinity column, as it was not seen in preparations metabolically labeled with [^{35}S]methionine or ^{3}H-amino acids (data not shown).

Generation of neutralizing antibodies with purified gp350/220. To determine whether the purified gp350/220 was still antigenically active and able to generate neutralizing antibodies in experimental animals, a rabbit was immunized with gp350/220 purified from the P3HR-1 cell line, and the resulting antiserum was tested for its ability to neutralize EBV infection in vitro, as described previously (18). The antiserum specifically neutralized the virus in vitro, with a titer of approximately 1:1,000; control sera had no effect (Table 1). Furthermore, to confirm the specificity of the antiserum for gp350/220, im-

munoprecipitation studies were performed on [^{35}S]methionine-labeled extracts of the M-ABA cell line, as described previously (3, 6). The extracts were precleared by the immune serum, a rabbit antiserum against EBV previously defined as able to precipitate the components of the MA complexes (23), or normal rabbit serum. Each precleared sample was then in turn analyzed with the immune serum, the rabbit anti-EBV serum, or normal rabbit serum. The results (Fig. 2) demonstrated that only the gp350/220 molecules were immunoprecipitated with the immune serum. The identity of these polypeptides was confirmed because the rabbit anti-EBV serum was unable to immunoprecipitate gp350/220 after preclearing with the immune serum, and conversely, the rabbit anti-EBV serum precleared the two polypeptides recognized by the immune serum. Immunoprecipitation with unabsorbed rabbit anti-gp350/220 revealed only the two glycoproteins, even in overexposed autoradiograms (data not shown), indicating that the purified gp350/220 was devoid of detectable amounts of antigenically active contaminating material.

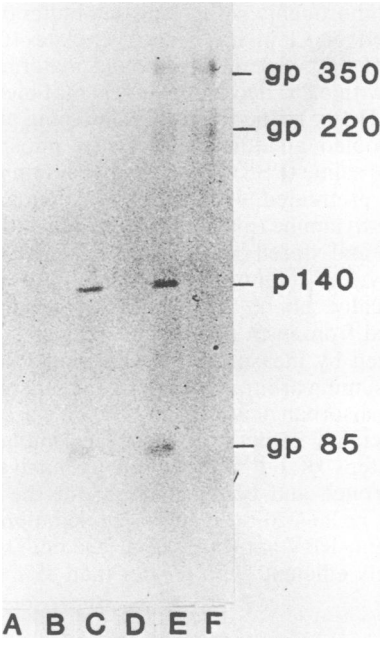

FIG. 2. Immunoprecipitation of polypeptides from the M-ABA cell lines, using rabbit anti-gp350/220 serum. The proteins from Triton X-100 extracts of [^{35}S]methionine-labeled M-ABA lymphoblasts were subject to preclearing, using normal rabbit serum, rabbit anti-gp350/220 prepared as described in the text, or rabbit anti-EBV serum. The resulting supernatants were then each subjected to radioimmunoprecipitation. Each supernatant was analyzed with both rabbit anti-EBV and rabbit anti-gp350/220. Lane A, Precleared with anti-EBV, immunoprecipitated with anti-EBV; lane B, precleared with anti-EBV, immunoprecipitated with anti-gp350/220; lane C, precleared with anti-gp350/220, immunoprecipitated with anti-EBV; lane D, precleared with anti-gp350/220, immunoprecipitated with anti-gp350/220; lane E, precleared with normal rat serum, immunoprecipitated with anti-EBV; lane F, precleared with normal rat serum, immunoprecipitated with anti-gp350/220.

Immunoelectron microscope demonstration of gp350/220 on the surfaces of virions. The presence of gp350/220 on the surfaces of intact virions was confirmed directly by two methods. In the first method, live cells of the B95-8 or P3HR-1 strain were incubated for 1 h at 4°C with either 10× concentrated culture supernatant or ascites fluid from the C1 hybridoma cell line. The cells were then washed with PBS and incubated for 1 h at 4°C with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Miles-Yeda). After several washes, the cells were prepared for electron microscopy. A typical result is shown in Fig. 3, where the dense stain can be seen specifically associated with the plasma membrane of a cell which contains virus particles. The specificity of the reaction was confirmed in the following two ways: (i) irrelevant monoclonal antibody did not react with either the viral envelope or the membrane of producer cells, and (ii) 400 cells were assessed, 200 with a positive immunochemical reaction and 200 without. In every case, C1 antibody only stained the plasma membrane of cells containing internal virions (Fig. 3). Similarly, of 15 enveloped particles which we observed, all were stained.

In the second method, C1 antibody was coupled to protein A-Sepharose via a bridging antibody of rabbit anti-mouse immunoglobulin. Protein A-Sepharose (Pharmacia Fine Chemicals, Inc.) was rehydrated, washed in PBS, and then incubated at room temperature with a hyperimmune rabbit anti-mouse immunoglobulin antiserum (approximately 50 μl of serum per 1 μl of packed beads). The conjugated beads were washed twice with PBS and then incubated with C1 ascites fluid (10 μl of fluid per 50 μl of beads). The conjugates were washed again and incubated with 15 μl of virus in either PBS or 0.5% Triton X-100–PBS (50 μl of conjugate was incubated with concentrated enveloped virus from approximately 100 ml of supernatant from the B95-8 cell line purified by the method of Dolyniuk et al. [2]). The conjugates were washed six times with PBS. At this stage, care was taken to pellet the beads gently by either standing or centrifugation at 50 × *g* for 1 min. The beads were then prepared for electron microscopy. Enveloped but not detergent-treated virions (20 observed) adhered to the C1 beads but not to beads coated with control antibodies (Fig. 3B).

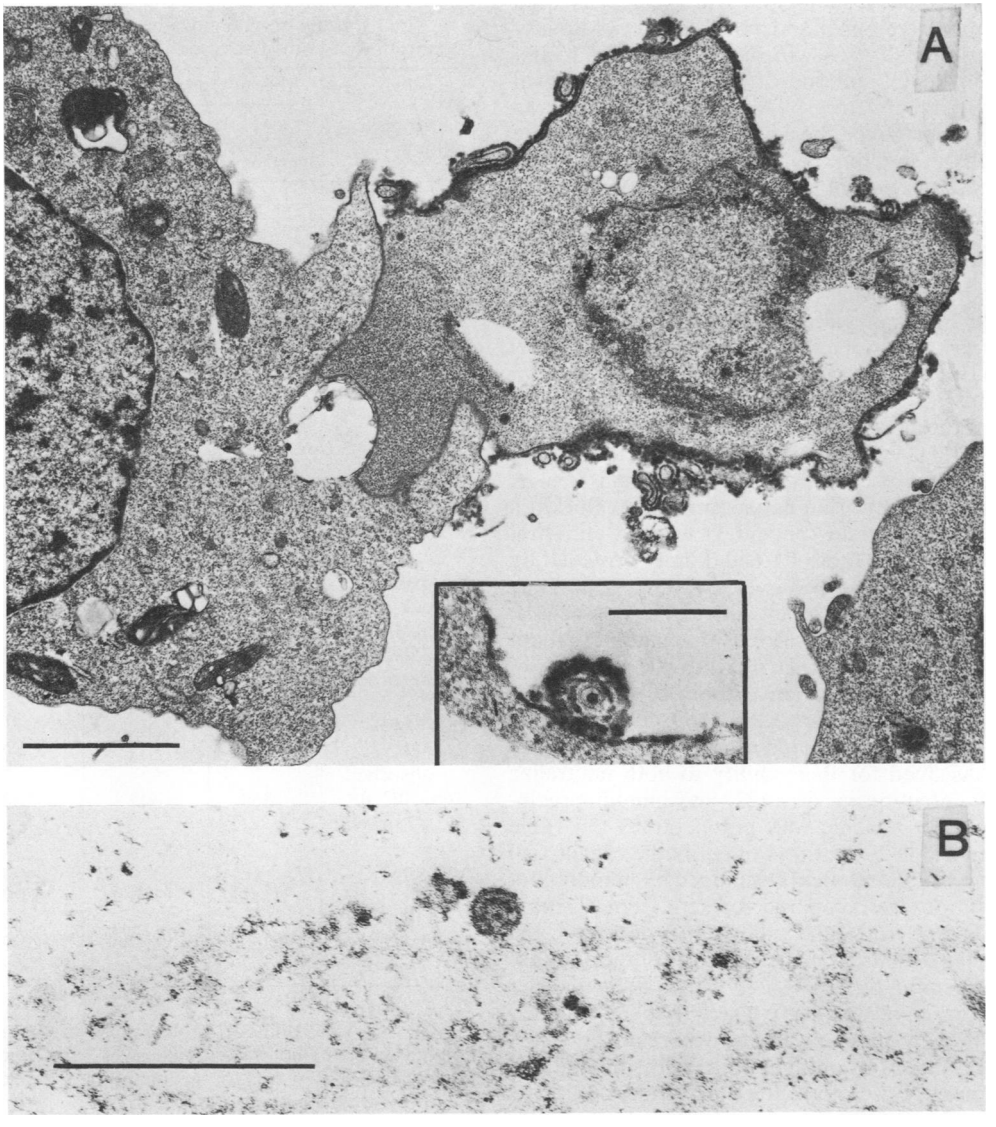

FIG. 3. (A) Electron micrographs of a producer cell in which the gp350/220 antigen is revealed by using the monoclonal antibody C1, followed by a peroxidase-conjugated rabbit anti-mouse immunoglobulin. The cell in the upper right portion of the figure has virus particles in its nucleus and a dense peroxidase reaction product over its surface, whereas the nonproducer cells to the left and lower right have no indication of a positive reaction. Bar, 2 μm. The insert shows a virion and a portion of plasma membrane from a different cell. Both the viral envelope and the cell membrane are stained by the immunochemical reaction. Bar in inset, 0.5 μm. (B) Electron micrograph of an Epstein-Barr virion on the surface of Sepharose-protein A rabbit anti-mouse C1 bead. The bead is represented by the darker region in the lower part of the electron micrograph. Virions were found sparsely but regularly around the perimeter of the C1 beads because the electron micrographs were of ultrathin sections. No virions were found on beads with control antibodies or on C1 beads incubated with virions in the presence of 0.5% Triton X-100 or sodium deoxycholate. Bar, 0.5 μm. Cells and beads were fixed as described by Schneeberger-Keeley and Karnovsky (14), washed once in a 0.12 M cacodylate buffer, suspended in 2% agar at 4°C, centrifuged, and then the agar pellet was fixed in the same fixative. The pellet was postfixed with 1% O_sO_4 in 0.1 M cacodylate (pH 7.2), dehydrated with acetone, and embedded in Epon-Araldite. Ultrathin sections, some stained with lead citrate and uranyl acetate, were viewed at 80 kV with a JEOL-100B transmission electron microscope.

Thus, it was concluded that, in antibody excess staining, C1 antibody will specifically bind to viral envelopes, and in virus excess affinity experiments, virus will bind specifically to immobilized C1 antibody. Furthermore, removal of the viral envelope by treatment with Triton X-100 also removed the antigen, which allowed the virions to combine with the antibody. This confirms the nature of gp350/220 as a component of the viral envelope.

Antibodies to gp350/220 are the main component of human neutralizing antibodies. Having demonstrated that purified gp350/220 could generate EBV-neutralizing antibodies in rabbits, it was critical to establish whether in vivo infection gives rise to neutralizing antibodies predominantly against gp350/220. To determine this, 400 μg of purified gp350/220 was coupled to Sepharose 4-B (11), and the resulting material was used as an affinity column to absorb human sera. A 0.1-ml amount of an antiserum from a Burkitt's lymphoma patient (patient 1) with an anti-viral capsid antigen titer of 1:640 (kindly provided by G. Klein) was passed over a column containing either gp350/220 or mock-coupled Sepharose 4-B. Sera from a healthy seropositive patient (patient 2; viral capsid antigen titer, 1:640) and a seronegative person (patient 3; both courtesy of W. Henle) were similarly absorbed. The resulting sera were then collected in a final volume of 1 ml and assayed for their ability to both neutralize EBV infection in vitro (22) and immunoprecipitate EBV-specific MA polypeptides (3; Table 2). In Table 2, the titers of antibodies against all of the MA components as judged by immunoprecipitation are compared for sera from all three patients before and after absorption on the gp350/220 column. Only the titers of the antibodies specific for gp350/220 were affected after absorption of the positive sera (patients 1 and 2) on the gp350/220 column, whereas the gp85, p160, and p140 polypeptides were precipitated normally. For both of the positive sera (patients 1 and 2), there was a concomitant loss of neutralizing antibodies along with the loss of antibodies to gp350/220.

TABLE 2. Titers of virus-neutralizing antibodies and antibodies precipitating [^{35}S]methionine-labeled, virus-specific polypeptides before and after absorption of human sera with purified gp350/220[a]

Serum[b]	Titer of serum from following patient:		
	1 (Burkitt's lymphoma)	2 (Healthy seropositive)	3 (Healthy seronegative)
Anti-viral capsid antigen	1:1,280	1:640	<1:10
Neutralizing antibodies			
Mock absorbed	1:60	1:20	<1:5
gp350/220 absorbed	<1:10	1:5	<1:5
Anti-gp350/220			
Mock absorbed	1:700	1:60	<1:20
gp350/220 absorbed	<1:30	<1:20	<1:20
Anti-p160			
Mock absorbed	1:3,240	ND[c]	<1:20
gp350/220 absorbed	1:3,240	ND	<1:20
Anti-p140			
Mock absorbed	1:360	ND	<1:20
gp350/220 absorbed	1:360	ND	<1:20
Anti-gp85			
Mock absorbed	1:1,080	1:180	<1:20
gp350/220 absorbed	1:360	1:180	<1:20

[a] See text.
[b] All sera were diluted 10-fold by passage over the columns. For the neutralization assays, serial twofold dilutions were used, and for the immunoprecipitations, serial threefold dilutions were used.
[c] ND, Too weak to assess titers.

This experiment was also performed in a different way with four additional human sera (provided by W. Henle) and our own rabbit antiserum against the intact virion. The human sera included one from a Burkitt's lymphoma patient, one from a nasopharyngeal carcinoma patient, and our standard seropositive (patient 2) and seronegative (patient 3) sera. The sera were first titered for neutralizing antibodies and then diluted to the lowest dilutions which would give complete neutralization in an outgrowth assay. To each serum 70 μg of gp350/220, purified in sodium deoxycholate as described above, or an equivalent volume of sodium deoxycholate alone was then added. The detergent was removed by extensive dialysis against 10 mM Tris (pH 7.2) and then PBS, and the resulting mixture was tested for the presence of neutralizing antibodies in the outgrowth assay (Table 3). In every case, the control-treated sera were able to neutralize infectivity. However, the neutralizing antibodies for each serum were removed by preincubation with purified gp350/220.

We have shown previously that experimental infection of a mouse with EBV allowed us to generate hybridomas against the virus, one of which, C1 antibody, would neutralize the virus (24). It is noteworthy that, of 10 anti-EBV

TABLE 3. Removal of neutralizing antibodies by preincubation with gp350/220

Serum	Neutralizing antibody titers	No. of wells with transformed foci/total no. of wells	
		With sodium deoxycholate alone	With gp350/220
77-1090-BL	1:16	0/6	6/6
78-50 NPC	1:32–1:64	3/6	6/6
Patient 2	1:8	1/6	6/6
Patient 3	ND[a]	6/6	6/6
Rabbit α-EBV	1:20	3/6	6/6

[a] ND, Seronegative; no detectable neutralizing antibodies.

monoclonal antibodies that we developed, 6 immunoprecipitated gp350/220. We have been able to use C1 antibody to purify gp350/220 by immunoaffinity chromatography. The purified material was obtained in high yield, suggesting that immunoaffinity with monoclonal antibodies may be applicable for isolating other EBV-associated antigens. The isolated gp350/220 was antigenically active, as it was a potent stimulator of neutralizing antibodies in rabbits and could be used to specifically absorb human anti-EBV sera. Such sera depleted of anti-gp350/220 antibodies could no longer neutralize the virus in vitro. Our experiments do not discount a role for neutralizing antibodies against other components, such as those recently reported for gp85 (17); however, they do lead to the conclusion that gp350/220 is the major antigen responsible for stimulating neutralizing antibodies in vivo.

It was interesting to note that the six monoclonal antibodies immunoprecipitated both gp350 and gp220. Recent studies in our laboratory indicated that this is owing to immunological cross-reactivity rather than to complex formation, as four of the antibodies will immunoprecipitate both polypeptides in the presence of sodium dodecyl sulfate at concentrations where the polypeptides migrate independently on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Previous experiments have shown a correlation between antibody titers to MA and viral envelope antigens (1, 15, 18, 20). Furthermore, MA components copurify with virions (3, 6, 7). However, there has been a lingering doubt about the purity of EBV preparations and the possibility that some proteins may be copurified contaminants. These experiments have been further complicated by a lack of monospecific sera for identifying viral antigens. The electron microscope analysis with the monoclonal antibody C1 directly demonstrates by two independent methods, for the first time, the presence of gp350/220 on the envelopes of virions. Combined with the evidence that specific anti-gp350/220 antibodies neutralize the virus (4, 24), it can be concluded that gp350/220 is a part of the viral envelope. The final proof that gp350/220 are viral proteins will be the demonstration that they are virally encoded by analyzing the in vitro translation products of virus-specific messenger RNAs with specific reagents such as our C1 antibody and rabbit anti-gp350/220.

The diseases which are induced by EBV in cotton-top marmosets share many features in common with the human diseases Burkitt's lymphoma and fatal infectious mononucleosis. It will be interesting now to see whether immunization with purified gp350/220 will protect marmosets from subsequent infection. Such experiments will allow us to better understand the role of neutralizing antibodies in preventing EBV-associated diseases.

This study was supported by Public Health Service grants AI 15310 and CA 28737 from the National Institutes of Health and by a grant from the University of California Cancer Research Coordinating Committee.

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