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Neutralization of Lymphocyte Immortalization by Different Strains of Epstein-Barr Virus with a Murine Monoclonal Antibody

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A murine monoclonal antibody was raised against the B95-8 strain of Epstein-Barr virus (EBV), which was isolated from a case of mononucleosis after blood transfusion (Hoffman et al. Proc. Natl. Acad. Sci. U.S.A. 77:2979, 1980). We provide evidence that neutralization of immortalization by this monoclonal antibody is virus specific, since its potency was inversely related to the dose of challenge virus. Furthermore, the monoclonal antibody recognized antigens on viruses grown in human as well as in marmoset cells. We show that this monoclonal antibody neutralized three other transforming strains of EBV originating, respectively, from American patients with mononucleosis and fatal polyclonal lymphoma and from an African child with Burkitt lymphoma. However the antibody did not neutralize or detect antigens by immunofluorescence in the W91 strain of EBV. The hybridoma antibody did neutralize other EBV strains derived from the same Burkitt lymphoma cell line (Nyevu), as was the case with the W91 strain. This monoclonal antibody provides clear evidence of antigenic differences on the surface of EBVs and will ultimately prove useful in defining the antigenic site on EBV which elicits neutralizing antibody.

A murine monoclonal immunoglobulin G antibody, designated 72A1, has been raised against virions of the B95-8 strain of Epstein-Barr virus (EBV), using the hybridoma method (5). 72A1 reacts in immunofluorescence tests with the cytoplasm and plasma membrane of human and marmoset lymphoid cells which actively synthesize EB virions; it does not react with human lymphoid cells such as Raji which contain EBV DNA but do not produce virus, nor does the antibody recognize antigens present in human or marmoset lymphoid cells which lack EBV DNA. When lymphoid cells producing EB virions are studied by two-color immunofluorescence with the 72A1 monoclonal antibody and with EBV-antibody-positive human sera, the same cells which contain antigens recognized by the monoclonal antibody are stained by the human antibodies. Antibody-positive human sera, however, invariably react with more cells than does 72A1. Human sera with antibody to membrane antigens completely block immunofluorescence by the monoclonal antibody, but the converse is not true. These findings imply that the human sera contain antibodies to a greater diversity of antigens than is recognized by the monoclonal antibody.

72A1 identifies a high-molecular-weight (approximately 250,000) glycoprotein in lysates of B95-8 cells which have been metabolically labeled with [^{3}H]glucosamine.

The present report describes the virus-neutralizing properties of the 72A1 monoclonal antibody. We demonstrate that 72A1 neutralizes several strains of EBV of diverse origin other than the virus strain which was used to raise the antibody. The results provide evidence that antibody to one glycoprotein is sufficient to neutralize infectivity and lymphocyte immortalization by EBV (5, 13).

MATERIALS AND METHODS

Virus strains. These studies employed strains of EBV isolated from four different patients. The Hawley strain is derived from blood leukocytes of an elderly woman with posttransfusion mononucleosis. The first passage in cotton-top marmoset (CTM) cells is B95-8; a further passage in CTM cells is called MCUV (7). The Nyevu strain originates from the tumor of an African child with Burkitt lymphoma (6). Two independent second-passage isolates of this strain were studied: one called W91 in the cells of CTM no. 727 and another called CC34-5 in the cells of CTM no. 752. A third passage of the Nyevu isolate was produced by transforming lymphocytes from marmoset cells with the CC34-5 virus. This passage is called FF346-7. The T. S. strain was recovered from blood leukocytes of a

4-year-old girl with fatal diffuse polyclonal lymphoma after mononucleosis (11). The first passage in marmoset cells is RA37-13. The fourth strain comes from saliva of a college student with infectious mononucleosis. The first passage in marmoset cells is FF41 (3).

Virus stocks. EBV to be used in the neutralization studies was prepared from supernatant fluids of continuous lines of marmoset cells harboring different strains of virus. In one experiment a virus stock was also prepared directly from cultured peripheral blood lymphocytes of the patient with diffuse polyclonal malignant lymphoma. Supernatants were filtered through 0.450-μm filter and stored at −70°C. The lymphocyte-immortalizing titer of each virus stock was determined by endpoint dilution in a microtiter system. Eight microwells containing mixed human umbilical cord lymphocytes were inoculated with each virus dilution, and endpoints of virus titer were calculated on the basis of the presence or absence of morphological transformation of the lymphocytes after 8 weeks.

Hybridoma antibodies. Hybridoma antibodies were raised by immunizing mice with partially purified, concentrated, detergent- and formaldehyde-treated virions of the B95-8 EBV strain. Three monoclonal antibodies were studied for their EBV-neutralizing properties. They were 72A1, which reacts with plasma membranes and cytoplasm of cells producing virions, 72A2, which weakly stains the cytoplasm of virus producer cells in immunofluorescence tests, and 71C4, which is reactive with the surfaces of all of the cells in marmoset lymphoblastoid lines. All of the hydridomas were propagated intraperitoneally in mice, and the ascites fluid served as the source of the antibody.

Virus neutralization. The hybridoma was diluted in RPMI 1640 medium without serum, and 0.1 ml was mixed with 0.1 ml of a virus dilution. The titer of the virus stock had been determined previously by endpoint dilution and was usually, but not always, remeasured in the neutralization experiment. Serial twofold dilutions of the hybridoma, from 1:10 to 1:640, were challenged with 200 to 15,800 50% transforming units (TD_{50}). The hybridoma-virus mixture was held at 37°C for 1 h and then 1.8 ml of human umbilical cord blood mononuclear cells was added. The cells and virus were mixed together and distributed to eight replicate microwells. The endpoints of neutralization were read 25 to 35 days after inoculation, and the titers were calculated by the Reed-Muench formula.

Immunofluorescence. Indirect immunofluorescence was used to test the ability of human sera to detect antigen either on the cytoplasmic membrane of living cells or inside acetone-fixed cells of lymphoid lines productive of EBV. The second reagent was either fluorescein isothiocyanate-conjugated antihuman immunoglobulin or antimouse immunoglobulin purchased from a commercial source.

RESULTS

Specificity of neutralization reaction. In an initial experiment (Table 1), we compared the neutralizing activity of three hybridomas against the MCUV passage of B95-8 virus. The highest titer of activity was present in the 72A1 hybridoma, which reacts with an antigen present on the surfaces of cells synthesizing virus; a low level of antibody was found in the 72A2 ascites fluid, which detects a cytoplasmic antigen; no neutralization was achieved by 71C4, which is directed against a marmoset-specific cell surface antigen. Since all of the monoclonals had similar titers by immunofluorescent assays, these results indicated that the neutralizing activity was not likely to be due to antilymphocyte rather than antiviral activity.

TABLE 1. Three hybridomas challenged with the MCUV strain of EBV

Final dilution	Hybridoma[a]		
	72A2	72A1	71C4
1:10	1/8[b]	0/8	6/8
1:20	5/8	0/8	5/8
1:40	6/8	3/7	3/8
1:80	7/8	0/8	4/8
1:160	5/8	0/5	4/8
1:320	6/8	2/8	3/8
1:640	6/8	5/8	4/8

[a] Calculated titer for 72A2, 1:34; for 72A1, 1:437; and for 71C4, <1:10.

[b] Number of wells with morphological transformation/number of wells inoculated; readings were taken 25 days after inoculation.

Further support for the specificity of the neutralization reaction was obtained in three experiments (Table 2) in which the titer of neutralizing capacity of the 72A1 hybridoma was assayed as a function of the challenge dose of virus. As expected for a virus-specific antibody, the neutralizing titer of the hybridoma antibody was higher when the challenge dose of virus was lower.

72A1 hybridoma antibody neutralizes several different EBV strains. The 72A1 antibody neutralizes, in addition to the virus strain (B95-8, MCUV) which elicited the antibody, a virus, FF41, which was derived from the saliva of a different patient with infectious mononucleosis

TABLE 2. Effect of challenge dose on neutralizing antibody titer of the 72A1 hybridoma

Marmoset strain[a]	EBV challenge dose (TD_{50})	72A1 hybridoma neutralizing titer
B95-8	10,000	1:66
	1,000	1:100
MCUV	15,800	1:30
	1,580	1:245
FF41	4,000	1:60
	400	1:426

[a] B95-8 and MCUV are different marmoset lines harboring the same strain from a patient with mononucleosis after blood transfusion. FF41 is a marmoset line with a mononucleosis strain from the saliva of a patient.

TABLE 3. Neutralization of an EBV isolated from a fatal polyclonal lymphoma by the hybridoma 72A1

Source of virus	Titer of virus stock (TD_{50}/0.1 ml)	Challenge dose (TD_{50})	72A1 hybridoma neutralizing titer
Cultured blood lymphocytes	3,000	1,000	1:87
Marmoset line RA37-13	2,000	2,000	1:160

(Table 2) (3). We also examined the ability of 72A1 to neutralize a transforming strain of EBV isolated from a fatal lymphoproliferative syndrome which occurred during primary infection with EBV (Table 3) (11). In this instance we tested two sources of this virus strain: one was a marmoset line transformed by a virus obtained from the blood leukocytes; the other was a virus harvested directly from the supernatant of the patient's leukocyte culture, which synthesized large amounts of EBV. Both sources of this virus strain were effectively neutralized by the 72A1 antibody. This finding indicates that the antigen recognized by 72A1 is found on a virus replicated in both human cells and marmoset cells and is not limited to a virus which has been derived from marmoset cells transformed in vitro.

We were also interested to learn whether 72A1 recognized antigens found on EBV originating in an African patient with Burkitt lymphoma. In these studies we examined the ability of the antibody to neutralize the Nyevu strain, propagated in various marmoset passages (Table 4). We obtained evidence that 72A1 was not able to neutralize all EBV variants; it was unable to neutralize a passage of the Nyevu strain designated as W91. This inability to neutralize W91 was seen on several repeated trials, even with low challenge doses of virus. However other marmoset cell passages of the Nyevu strain, such as CC34-5 and FF346-7, were effectively neutralized by the 72A1 hybridoma.

TABLE 4. Neutralization of the Nyevu Burkitt lymphoma EBV strain with the 72A1 hybridoma

Marmoset strain	EBV challenge dose (TD_{50})	72A1 hybridoma neutralizing titer
W91[a]	1,000	<20
	250	<20
CC34-5[a]	2,000	1:36
	200	1:490
FF346-7[b]	300	1:338

[a] Both W91 and CC34-5 are independent passages of Nyevu virus by transformation of lymphocytes from different marmosets (W91 to CTM-727; CC34-5 to CTM-752).

[b] A further passage in marmoset cells of CC34-5 virus.

We found that the 72A1 hybridoma did not react with antigens present within fixed cell smears or on the surface of the W91 line (Table 5). The 72A1 hybridoma was able to recognize an antigen in the CC34-5 line, which harbors the same strain of virus, and in a variety of other EBV producer lines tested (data not shown). The presence of antigen inside and on the surfaces of W91 cells was detectable with human sera (Table 5) and with several other mouse monoclonal antibodies (unpublished data). These results raise the possibility that W91 virus carries a mutation in the gene for the glycoprotein which is responsible for eliciting neutralizing antibody.

DISCUSSION

These results permit several conclusions about the reaction in which the ability of EBV to immortalize lymphocytes is neutralized by a murine monoclonal antibody. It is likely that the antibody recognizes a viral rather than a cellular antigen. The antibody neutralizes several different strains of transforming viruses released both from human and marmoset cells. A hybridoma which does react with a marmoset cell surface antigen does not neutralize EBV. Neutralizing potency correlates with the dose of challenge virus; further, only that hybridoma which reacts strongly with the cytoplasm and plasma membranes has significant neutralizing activity.

The 72A1 antibody appears to immunoprecipitate a single glycoprotein from extracts of virus producer B95-8 cells (4, 5). Consequently it seems likely that antibody to a single glycoprotein is sufficient to neutralize the infectivity of EBV for primary human lymphocytes. Virus neutralization is a property of antibody to the viral membrane antigen, present both on the envelopes of virions and on the cytoplasmic membranes of cells producing virions (2, 9). Membrane antigens appear to consist of at least four distinct polypeptides with molecular weights of approximately 320,000, 240,000, 160,000, and 85,000 (8, 10). The two largest and smallest polypeptides are glycosylated. Only the

TABLE 5. Immunofluorescence tests on different cell lines with human sera and hybridomas

Cell line	Antiserum		
	WAA[a]	LH[b]	72A1
MCUV	14/122[c]	Negative	11/100
W91 (Nyevu)	4/250	Negative	Negative
CC34-5 (Nyevu)	10/115	Negative	11/255

[a] Human serum with EBV-neutralizing antibody.

[b] Human serum without EBV antibodies.

[c] Number of positive cells/number of cells tested.

two largest glycoproteins are recognized by a rabbit antiserum to EBV which possesses virus-neutralizing capacity (8). There is evidence from analysis of the glycoproteins in the P3J-HR-1 line with another monoclonal antibody (C1) (12, 13) as well as with 72A1 (4) that the two large glycoproteins are antigenically related. Ultimately, however, more than one component of the membrane antigen complex may be found to elicit neutralizing activity.

The demonstration that EBV neutralization can be effected by antibody to one putatively viral encoded glycoprotein has obvious implications for the production of a subunit vaccine.

Nothing is yet known about the coding region on the large EBV genome for the membrane antigens and viral envelope glycoproteins which are recognized by the 72A1 antibody. However the genomes of the B95-8 and FF41 viruses have been compared in detail (3). FF41 has an additional stretch of DNA of approximately 8 megadaltons present in a large fragment, *Eco*RI-C, which is partially deleted in B95-8 DNA. Since both viruses are neutralized by the 72A1 antibody, it seems plausible that the deleted region in B95-8 DNA does not code for the neutralizing antigen.

The results with a more specific reagent, the murine monoclonal antibody, generally support conclusions previously reached on the basis of experiments with less well-defined human sera and with alloantibodies raised in rabbits (1, 8, 12). Thus, EBV strains of different geographical origins obtained from patients with both benign and malignant lymphoproliferative disease share cross-reactive neutralizing antigens. These cross-reactions are sufficiently extensive to make it unlikely that there are distinct EBV serotypes which account for differences in disease patterns.

However, the findings reported with the W91 virus, a subvariant which seems to escape neutralization by the 72A1 antibody, are noteworthy. These results provide the first clear-cut evidence for a serological difference among EBV strains with respect to antigenic sites responsible for virus neutralization. This distinction has been possible because of the discriminating power of a monoclonal antibody. These findings appear to be valid since they were obtained in three different neutralization experiments, with different batches of indicator cells. In each of these experiments other EBVs were neutralized. Furthermore, the failure of the monoclonal antibody to neutralize W91 virus is correlated with its inability to detect antigen in W91 cells by indirect immunofluorescence. Since different passages of EBV from the Nyevu strain are neutralized and contain antigens in immunofluorescence tests, W91 probably has acquired a mutation in the gene encoding this antigen while being propagated in the laboratory. We plan to learn whether this mutation in W91 virus is stable on further passage of the virus and to study the cell line which carries it. If this proves to be the case, then the W91 virus may ultimately be useful in an analysis of the antigenic site recognized by 72A1 and other virus-neutralizing antibodies.

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