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Epstein-Barr Virus (EBV) Glycoprotein gp350 Expressed on Transfected Cells Resistant to Natural Killer Cell Activity Serves as a Target Antigen for EBV-Specific Antibody-Dependent Cellular Cytotoxicity

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Cell surface-associated viral glycoproteins are thought to play a major role as target antigens in cellular cytotoxicity and antiviral immunosurveillance. One such glycoprotein is the Epstein-Barr virus (EBV)-encoded glycoprotein 350 (gp350), which is expressed on both virion envelope and EBV producer cells and carries the virus attachment protein moiety. Although it is known that some antibodies to gp350 can neutralize the virus, the role of this glycoprotein in EBV-specific cellular cytotoxicity is not yet clear. We describe here a study in which we successfully used a new approach to demonstrate that gp350 is a target antigen for EBV-specific antibody-dependent cellular cytotoxicity (ADCC). Transfection of gp350-negative cells resistant to natural killer (NK) cell activity (i.e., Raji) with a recombinant vector (pZIP-MA) containing the gene encoding the EBV-gp350 and the neomycin resistance gene enabled us to isolate cell clones with a stable and strong expression of gp350 on their surface membranes. ADCC determined by using two clones clearly demonstrated that gp350 is the target of the EBV ADCC. Interestingly, this ADCC was comparable to that obtained against the EBV-superinfected (coated) Raji cells expressing the same percentage of gp350 positivity as the two clones. No cytotoxic activity was detected against either nontransfected (gp350-negative) Raji cells or cells transfected with the vector [pZIP-neo-SV(X)1] lacking the gp350 gene. In addition to demonstrating that gp350 is a target molecule for EBV-specific ADCC, our approach in using NK-resistant transfectants provides a lead for probing the role of cell surface-associated viral antigens in specific cellular killing and immunosurveillance.

Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus which can cause infectious mononucleosis upon primary infection that leads to the establishment of a life-long virus carrier state (15). Another result of primary infection is the presence in serum of antibodies to a number of EBV antigens, some of which (anti-viral-capsid antigen, anti-EBV nuclear antigen, and antimembrane antigen [MA]) are maintained at constant levels throughout life (5). EBV is also associated with various human malignancies: endemic Burkitt's lymphoma (3), undifferentiated nasopharyngeal carcinoma (6), salivary gland tumors (25), and the oligoclonal B-cell lymphomas to which immunosuppressed allograft recipients are particularly prone (1). More recently, the EBV genome was found in T-cell lymphomas from three patients with chronic EBV infection (9). One possibility for preventing, delaying, or modifying the course of the various EBV-associated diseases is vaccination. The antigen which has been the focus for the development of an anti-EBV vaccine is the MA complex of EBV (2, 14). This complex, found on both the envelopes of viral particles (20) and the surfaces of EBV producer cells, is composed of a major envelope glycoprotein designated gp350/300 (gp350) and a related glycoprotein designated gp250/200 (gp250); the molecular weights of these glycoproteins as reported by different laboratories vary somewhat (27). Both glycoproteins are encoded by the same gene, which has been mapped to the *Bam*HI L DNA fragment of the B95-8 genome (8). The mRNA of gp250 is produced by splicing an internal portion of the mRNA of gp350 such that the same open reading frame is maintained (8). gp350 is involved in virion binding to human B cells through its specific interaction with receptor for the complement C3d fragment (26). It has been found that immunization of cotton-top tamarins with native gp350 can protect these animals against subsequent EBV-induced lymphomas (14). Recombinant gp350, which can be produced as a secreted protein (29) or expressed through different viral vectors (11, 14), could be evaluated as an appropriate vaccine for administration to human populations at risk for developing EBV-associated tumors. gp350 is also an efficient activator of the alternative complement pathway, and its expression on the membranes of cells producing EBV is associated with the ability to activate complement (13).

Several monoclonal antibodies (MAbs) directed against gp350 from B95-8 cells and gp250 from P3HR-1 cells (18) significantly reduced the infectivity of these two strains of virus in vitro, thereby indicating that neutralizing antibodies were directed against these glycoproteins (7, 24, 27). Similarly, neutralizing antibodies have been detected with MAb to gp85, a third membrane glycoprotein which is antigenically distinct from gp350 and gp250 (7, 23). Moreover, gp350 seems to be responsible for the induction of cytotoxic antibodies (22, 27). It has been assumed that increased sensitivity to antibody-dependent cellular cytotoxicity (ADCC) following induction of the lytic cycle in producer B95-8 cells pretreated with 12-*O*-tetradecanoylphorbol-13-acetate and Raji cells superinfected with P3HR-1 is a result of the recognition of EBV-associated MAs by an ADCC mechanism (10, 17, 19, 22). Although in the past gp350 has

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been considered a possible target for ADCC against EBV-infected cells, direct evidence for the role of this glycoprotein in ADCC has not been provided. By using the model of a gp350-negative, natural killer (NK) activity-resistant (17) target cell line (Raji) (21) expressing recombinant EBV gp350 after transfection, we provide here unequivocal evidence for the role of this viral glycoprotein in EBV-specific ADCC.

Transfection of gp350 gene and its expression in Raji cells. A previous study suggested that antibodies directed against EBV-induced MAs may play a role in elimination of EBV-producing cells by an ADCC mechanism (22). In that study, however, the target antigen for ADCC was not identified. It was also not clear from that report (22) which gp350-specific MAb was able to block ADCC. Therefore, to determine the role of gp350 in EBV ADCC activity, we transfected Raji cells with a plasmid containing the complete gp350 gene. The plasmids pZIP-neo-SV(X)1 and pZIP-MA, which is pZIP-neo-SV(X)1 containing the EBV gp350 DNA fragment inserted into the *Bam*HI site of the expression vector, were a gift from E. Kieff (Harvard Medical School and Brigham and Women's Hospital, Boston, Mass.). In these plasmids, the murine leukemia virus long terminal repeat promoter drives the expression of both the gp350 gene and the neomycin (G418) phosphotransferase gene, which allows positive selection (29). Neomycin-resistant clones of Raji cells transfected with pZIP-MA and pZIP-neo-SV(X)1 plasmids, referred to as Raji-pZIP-MA and Raji-pZIP-neo, respectively, were selected by plating at limiting dilution. In general, 84 to 97% of cells expressed gp350 in the two gp350-positive clones as determined by flow cytometric analysis with a fluorescence-activated cell sorter (FACS; Becton-Dickinson, Immunocytometry FACScan System). In contrast, both nontransfected Raji cells and Raji cells transfected with the control vector (Raji-pZIP-neo) used as negative controls under the same experimental conditions were negative in the membrane immunofluorescence (MIF) assay (Fig. 1 and Table 1). The B95-8 cell line, which generally expresses gp350 in 10 to 30% of cells, was used as a positive control. gp350 antigen was expressed on transfected cells as large bright patches covering most of the plasma membrane (Fig. 2). Thus, the distribution was similar to that observed in both EBV-coated Raji and B95-8 cells (16). The transfected cells have a strong expression of cell surface gp350, as demonstrated by MIF, in terms of both percentage of positive cells and intensity of immunofluorescence. This expression was found to be highly stable when tested at different times. The two clones were expanded for ADCC studies and were both negative for Fc-receptor expression when tested using a rabbit immunoglobulin G (IgG)-coated sheep erythrocyte rosette test (data not shown).

ADCC assay on transfected Raji cells. ADCC was successfully demonstrated against Raji cells transfected with pZIP-MA and expressing gp350 (Table 2). ADCC on two clones of Raji-pZIP-MA was comparable and always reproducible. This ADCC with pZIP-MA-transfected cells as targets was similar to that obtained when EBV-superinfected Raji cultures containing the same percentage of MA-positive cells were used as targets. This observation indicates that gp350 is indeed the EBV-induced MA that can serve as a target in EBV-ADCC. Nontransfected Raji cells and Raji transfected with pZIP-neo-SV(X)1 control vector used in the same assay were not susceptible to ADCC. A variety of target cells could be used for ADCC. However, the choice of Raji cells for transfection is important because of the fact that these cells are resistant to NK activity but sensitive to ADCC (17). Other target cells with any degree of susceptibility to NK cytotoxicity would make it difficult to discriminate between the overlapping of NK and ADCC activities. For these reasons, and for optimal interpretation of results, Raji cells have proven very useful. It is important to note that neither NK sensitivity of Raji cells nor spontaneous sodium [^{51}Cr]chromate (^{51}Cr) release changed after transfection of these cells with either pZIP-MA or its control vector [pZIP-neo-SV(X)1]. The percentages of spontaneous release

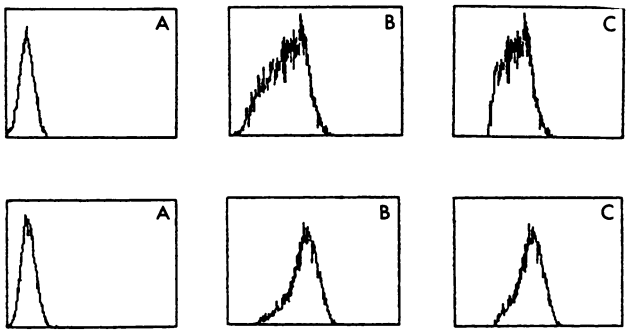

FIG. 1. FACS analysis of two clones of transfected Raji cells treated with gp350-specific antibody. Raji cells were transfected by electroporation as described previously (28). The optimal voltage for DNA transfer to Raji cells ranged from 400 to 600 V at a capacitance of 25 μF. About 30% of the cells survived electroporation, as determined by a trypan blue dye exclusion test 24 h posttransfection. For selection of transfected cells, RPMI medium supplemented with 10% fetal bovine serum and antibodies (RPMI-10) (16) containing 1,500 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml was used 48 h after electroporation. Resistant colonies emerged 15 days after G418 selection. These colonies were used for cloning by plating at limiting dilution. (A) Background fluorescence of Raji cells transfected with vector only (Raji-pZIP-neo) and treated with MAb 2L10 and fluorescein-conjugated goat anti-mouse IgG; (B) distribution of fluorescence intensity of the clones; (C) overlay histogram (i.e., panel B minus panel A). A total of 10^4 cells per sample were examined by FACS. Abscissa, Fluorescence intensity; ordinate, number of cells.

TABLE 1. Expression of gp350 in clones of pZIP-MA-transfected Raji cells

Type of cell	% Fluorescent[a]
Raji	0
Raji-pZIP-neo	0
Raji-pZIP-MA, clone 1	84
Raji-pZIP-MA, clone 2	97
Raji + P3HR-1[b]	92
Raji + B95-8[b]	98
B95-8	30

[a] gp350-positive cells were identified with gp350-specific MAb 2L10 following MIF staining of live cells. The MIF assay was carried out as follows. Cells were first incubated with MAb 2L10 (1:500) specific for gp350 (gift of G. Pearson, Georgetown University) at 4°C for 45 min. After three washings with cold phosphate-buffered saline, they were incubated at 4°C for 45 min with fluorescein-conjugated goat anti-mouse IgG (lot no. FAM121; Ortho). Cells were washed with cold phosphate-buffered saline and analyzed for fluorescence by FACS. Negative controls consisted of Raji and Raji-pZIP-neo stained with MAb 2L10 and a fluorescein-conjugated secondary antibody. An unrelated mouse antibody (OKT3; Ortho Diagnostic Systems) was also tested by MIF assay as a negative control, and the B95-8 cell line, which generally contained 10 to 30% EBV producer, MA-positive cells, was used as a positive control. Values are means (rounded to the nearest whole number) of two separate determinations.

[b] Percentage of gp350-positive cells determined 1 h following virus adsorption on Raji cells.

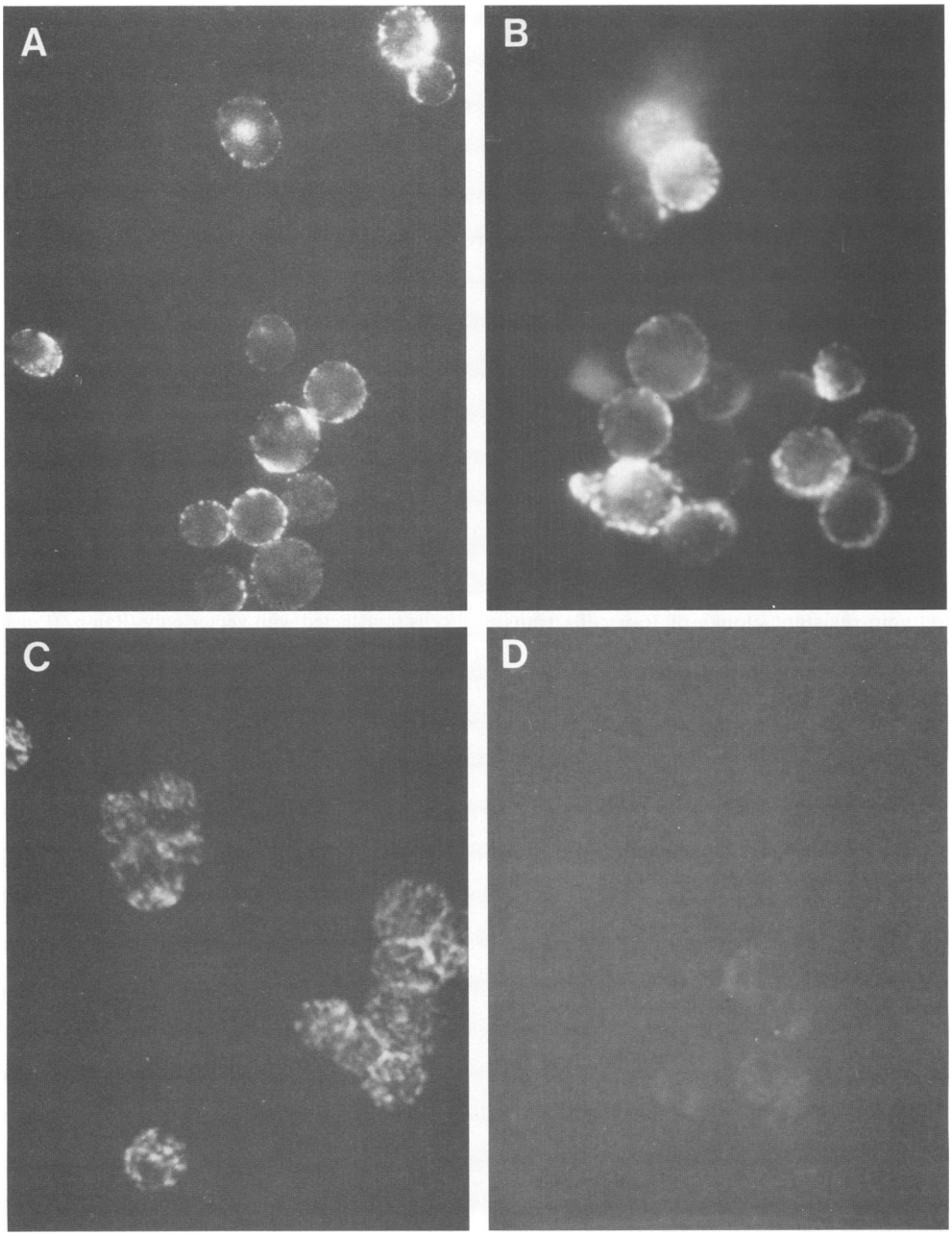

FIG. 2. MIF assay of gp350-expressing Raji cells. gp350-expressing cloned Raji cells (A [clone 1] and B [clone 2]), P3HR-1 virus-superinfected Raji cells (C), and cells transfected with vector only (D) were prepared for MIF assay with a gp350-specific MAb (2L10) and fluorescein-conjugated goat anti-mouse IgG.

by control Raji, Raji-pZIP-neo, and Raji-pZIP-MA were 0.3, 0.08, and 0, respectively. However, the percent NK activity against EBV-superinfected Raji cells showed a significant increase over that of uninfected cells (Table 2). If ratios of ADCC/NK activity are considered, then the difference becomes more dramatic and emphasizes the utility of Raji-pZIP-MA transfectants in discriminating between these two cytotoxic mechanisms.

Blocking of ADCC by MAbs to gp350. Fifteen MAbs (Table 3) were pooled, and different dilutions (i.e., up to 10^{-4}) of this pool tested in the ADCC assay were found to lack the ability to mediate ADCC (data not shown); furthermore, these MAbs were unable to block ADCC activity mediated by a positive human reference serum (Table 4). In the competitive experiments between the MAbs and the human polyclonal antiserum in which the ^{51}Cr-labeled targets were directly incubated in microplates with MAbs at various concentrations and the EBV ADCC-positive human serum, the MAbs failed to compete even minimally with the human EBV antibody-positive serum. Preincubation of target cells with MAbs was performed both at room temperature and at 4°C to avoid antigen-antibody internalization, but in both cases there was no inhibition of ADCC. Whether this was due to weak antibody avidity or to the possibility that the

TABLE 2. ADCC against Raji with EBV, Raji-pZIP-MA, and Raji-pZIP-neo target cells

Serum dilution	ADCC[a]					
	Raji	Raji-pZIP-neo	Raji + B95-8	Raji + P3HR-1	Raji-pZIP-MA clones	
					1	2
10^{-1}	0	0	7.2 ± 1.15	8.8 ± 0.9	9.5 ± 0.6	8.6 ± 0.46
10^{-2}	0	0	10.3 ± 0.8	6.1 ± 0.74	3.8 ± 1.15	5.7 ± 0.6
10^{-3}	0	0	3.9 ± 0.6	3.1 ± 0.85	2.4 ± 0.55	2.5 ± 1.2
10^{-4}	0	0	2.0 ± 0.55	2.5 ± 0.8	0.6 ± 0.36	1.8 ± 0.65
NK[b]	0.3 ± 0.26	0.08 ± 0.10	4.5 ± 0.8	2.2 ± 0.45	1.3 ± 0.35	0

[a] Results are means from three experiments and show EBV ADCC expressed as a percentage of EBV-specific ^{51}Cr release from clones of Raji cells transfected with a vector containing the gp350 gene (pZIP-MA) or from Raji cells superinfected (coated) with EBV from transforming (B95-8) or nontransforming (P3HR-1) strains as positive controls. ^{51}Cr-labeled Raji and Raji-pZIP-neo cells were used as gp350-negative controls. P3HR-1 and B95-8 infectious EBV preparations were produced as described elsewhere (16, 17). For the ADCC assay, cells were used as targets in an 18-h ^{51}Cr release microassay (17). ^{51}Cr (100 μCi) (New England Nuclear, Boston, Mass.) was added to pellets (10^6) of different target cells. After an additional hour of incubation at 37°C with occasional shaking, cells were adjusted to 10^5 cells per ml and 100 μl of this cell suspension was added to each well of a V-shaped 96-well microplate (Flow Laboratories). A 50-μl portion of the mononuclear cell fraction containing the effector population adjusted to 4×10^6 cells per ml was added to each well to achieve an effector/target cell ratio of 20:1. Known EBV ADCC-positive and -negative (control) human sera were heat inactivated (56°C for 30 min) and serially diluted in RPMI-10. Fifty microliters of each dilution was added to triplicate wells in the microplate. The microplate was incubated at 37°C in a 5% CO_2 atmosphere for 18 h. After incubation, the plate was centrifuged at $180 \times g$ for 5 min. The specific percent ^{51}Cr release was measured from 100 μl of the cell-free supernatant sample in an automatic gamma counter (LKB) and calculated as follows: % ^{51}Cr release = [(test release − spontaneous release)/(maximum release − spontaneous release)] × 100. The percent spontaneous release of ^{51}Cr by target cells was determined from cells incubated in the presence of an appropriate serum dilution but without effector cells, and maximum ^{51}Cr release by targets was determined by lysis with detergent. To determine ADCC, the percent ^{51}Cr release for effectors incubated in the presence of a negative-control serum was subtracted from the percent ^{51}Cr release for effectors incubated in a corresponding dilution of an antibody (ADCC)-positive serum. Data were analyzed by Student's t test, and only ADCC with $P < 0.05$ is considered statistically significant.

[b] Expressed as percentage of ^{51}Cr release from the same targets in the absence of serum.

antigenic determinants blocked by MAbs do not represent ADCC epitopes remains to be determined. An alternative explanation may be that antigen-antibody reactions are reversible. Therefore, even though a MAb initially blocks a putative ADCC epitope, it may be temporarily replaced by a human antibody, thus allowing the latter to mediate the ADCC reaction following its attachment to the effector cell. However, Qualtiere et al. had reported that an unspecified MAb to gp350 blocked ADCC activity (22). This difference in results is difficult to explain at present.

One of the main clinical applications of EBV-specific ADCC is in the investigation of the role of gp350-specific IgG in EBV-associated diseases. The fact that the ADCC assay is known to be more sensitive than the more conventional MIF test in determining antibody titers to EBV-induced MA (19) emphasizes the usefulness of transfected Raji cell clones (Raji-pZIP-MA) for diagnostic purposes. Furthermore, as the progressive disease observed more frequently in patients with low ADCC antibody titers (20) is likely the result of IgA antibodies blocking the IgG-mediated cellular cytotoxic reaction (12), ADCC performed on these cells could also serve to determine indirectly the presence of gp350-specific blocking IgA antibodies in patients with nasopharyngeal carcinoma. An important point worth mentioning here is that, throughout the program of vaccine development in the tamarin model, it was noted that protection against virus-induced disease did not always correlate with levels of neutralizing antibodies (4, 14). This raises the possibility of a role for gp350-induced cell-mediated responses in rendering immunized animals resistant to a lymphomagenic dose of the virus. ADCC activity could also be a potentially important

TABLE 3. Characteristics of gp350-specific MAbs used in a pool for competition with ADCC mediated by human antibody-positive serum

gp350-specific MAb (source-reference)	Immunoglobulin subclass	EBV neutralization[a]
2L10 (23)	G2b	+
2F5.6 (24)	G2b	−
B10-3 (24)	M	NT
F16-3E3 (24)	G1	−
F16-11B7 (24)	G1	−
F16-IC10 (24)	G1	−
F29-89 (24)	G1	NT
F29-1G7 (24)	G2a	+
F30-3C2 (24)	G1	+
F30-5C8 (24)	G2a	+
F34-5D3 (24)	G1	+
F34-ID8 (24)	G2b	+
F34-IF2 (24)	G1	+
F34-2B11-D10 (24)	G1	NT
F34-5H7 (24)	G2a	NT

[a] In the absence and/or presence of complement. NT, Not tested.

TABLE 4. Effect of a pool of gp350-specific MAbs on ADCC activity mediated by human gp350 antibody (ADCC)-positive serum[a]

Serum dilution	ADCC (%)			
	Raji + P3HR-1		Raji-pZIP-MA clone 2	
	RPMI-10	MAbs	RPMI-10	MAbs
10^{-1}	8.9 ± 0.84	8.5 ± 1.1	8.6 ± 0.55	8.9 ± 0.7
10^{-2}	4.8 ± 0.6	5.6 ± 0.35	3.6 ± 0.85	4.2 ± 0.25
10^{-3}	2.4 ± 0.45	0	0	0
NK[b]	2.2 ± 0.45	1.1 ± 0.35	0.9 ± 0.4	0

[a] Prior to the ADCC test, ^{51}Cr-labeled target cells were preincubated for 30 min at room temperature or for 1 h at 4°C with RPMI-10 medium or with a pool of 15 gp350-specific MAbs (i.e., containing 100 μl of each MAb) directed against different epitopes of gp350 (see Table 3 for details) before being used in an ADCC assay. ADCC was calculated as described in Table 2, footnote *a*, and ADCC activities in the presence and absence of the anti-gp350 MAbs were compared. Higher dilutions (i.e., up to 10^{-4}) of this MAb pool were also tested but were found to have no effect on this ADCC.

[b] NK activity expressed as percent ^{51}Cr release was measured on the target cells in the absence of gp350-specific antibodies.

resistance mechanism. The ADCC assay measures a cytotoxic reaction mediated through the interaction of two components: (i) IgG antibody directed against MA (anti-gp350) and (ii) Fc receptor-positive lymphoid effector cells which bind the Fc(IgG). Furthermore, it is known that the cellular immune competence of these effector cells varies from individual to individual; thus we speculate that both K-cell function and gp350 antibodies are important for immunosurveillance against EBV-infected and EBV-producer cells. The ADCC assay may thus be used more appropriately for diagnosis by testing the patient's K-cell function with an EBV antibody-positive reference serum and determining titers of the patient's serum for ADCC antibodies by using normal effectors. The transfected cells reported here (Raji-pZIP-MA) could help in the standardization of the ADCC assay by eliminating virus preparation and the consequent use of different virus batches with different titers, thus reducing variability.

In summary, the findings reported in this paper, together with those previously published, show conclusively that gp350 is a target antigen for EBV-specific ADCC. Given the interest in gp350 as a potential subunit vaccine and keeping in mind that ADCC has been considered to play a major role in vivo in controlling the spread of viruses in the infected host (and thereby likely also preventing virus-induced cancers), it will be important to identify which epitopes within the gp350 sequence can elicit antibodies reactive in ADCC. This question is important to the overall strategy of future vaccine design. Moreover, our paper emphasizes the usefulness of the present (Raji cells or a similar) NK-resistant model to probe the role of cell surface antigens in specific mechanisms of cellular cytotoxicity and immunosurveillance.

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