The Epstein–Barr Virus-Encoded Glycoprotein gp 110 (BALF 4) Can Serve as a Target for Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

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Antibody-dependent cell-mediated cytotoxicity (ADCC) is thought to play a major role in controlling the spread of the Epstein–Barr virus (EBV) in an infected individual. Recently, the viral membrane protein gp 350/220, which is also expressed at the surface of the virus producing cell, was identified as a target for ADCC reactions. Due to its glycoprotein nature, the EBV protein gp 110 is another possible ADCC target. It is one of the most abundant proteins found during the late phase of viral replication; until now, however, researchers have not been able to localize it on the surface of EBV positive cells. By means of recombinant vaccinia viruses containing the genes for gp 350/220 and gp 110, respectively, we expressed these proteins in lymphoblastoid cells, which were then used as targets in ADCC studies with sera from EBV-positive and -negative individuals. In these experiments we were able to demonstrate the feasibility of our approach for the investigation of EBV-specific ADCC reactions and could confirm the role of gp 350/220 as an ADCC target. Furthermore, we were able to show that gp 110 can also be recognized in an ADCC reaction, proving that at least some gp 110 molecules must be expressed at the cell surface.   © 1994 Academic Press, Inc.

The Epstein–Barr virus is the causal agent of infectious mononucleosis (*1*) and is also associated with three human malignancies, Burkitt's lymphoma (BL) (*2*), nasopharyngeal carcinoma (*3*), and malignant lymphomas of immunosuppressed patients (*4*). Infected individuals become lifelong carriers of EBV with the virus residing in B lymphocytes, predominantly in a latent state (*5*). *In vitro* EBV-infected cells grow out giving rise to immortalized lymphoblastoid cell lines (*6*). However, *in vivo* the uncontrolled outgrowth of EBV-transformed B cells seems to be strictly inhibited by different immunomechanisms. Specific HLA-restricted cytotoxic T lymphocytes directed against EBV-encoded proteins are thought to play an important role in the immunosurveillance of EBV-infected cells (*7–9*). Nevertheless, other control mechanisms may still be active as well. A previous study was able to show that antibodies against EBV-induced membrane antigens were involved in the elimination of EBV-producing cells by antibody-dependent cell-mediated cytotoxicity (ADCC) (*10*). In this reaction effector lymphocytes express Fc receptors and target cell recognition is mediated by the interaction of these receptors with the Fc portion of antibodies bound to the target cell. Cells capable of performing ADCC, previously named K cells, are natural

killer (NK) cells and cytotoxic T lymphocytes expressing Fc receptors for IgG, but macrophages and monocytes are able to mediate this reaction as well. Recently Khyatti and co-workers (*11*) were indeed able to demonstrate that the EBV glycoprotein gp 350/220 can serve as a target antigen for EBV specific ADCC. Gp 350/220 is the major protein of the viral envelope and also a prominent constituent of the plasma membrane of the EBV-producing cell (*12, 13*). At least three other glycoproteins are known to be coded by EBV: gp 78/55, gp 85, and gp 110, which are also possible ADCC targets. Gp 78/55 was recently identified as a molecule present in the viral envelope at a low copy number (*14*); gp85 is another minor constituent of EBV and also present in the plasma membrane of virus producing cells (*15*). Gp 110, on the other hand, is found in large amounts in the infected cell during the late phase of viral replication. This protein, encoded by the open reading frame BALF 4, displays a high degree of colinear homology to glycoprotein B (gB) of the herpes simplex virus (HSV) (*16*).

GB is present on the outer surface of the virion and on the surface of HSV-infected cells. EBV gp 110, in contrast, has not yet been able to be localized on the plasma membrane of the EBV-infected cell, although it is one of the most abundant viral proteins found in high amounts in cytoplasmic vesicles as well as in the inner and outer nuclear membrane by immunoelectron microscopy. Nevertheless, it cannot be ruled out that a small amount of the protein may reach the surface of the virus-producing cell (*17*). As antibodies to gB are thought to play a major role in the immunomediated destruction of HSV-infected

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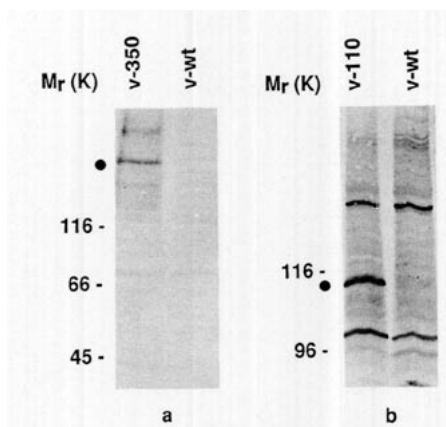
FIG. 1. Expression of gp 350/220 (a) and gp 110 (b) after infection of CV1 cells. Cells were infected with wild-type vaccinia virus (v-wt) or the recombinant viruses containing the gp 350 gene (v-350) or the gp 110 gene (v-110) at 10–20 pfu per cell and incubated at 37° for 24 hr in serum-free MEM. Infected cells were harvested and boiled in electrophoresis buffer for 5 min. SDS–polyacrylamide gel electrophoresis and Western blotting were performed as described previously (16). For the detection of expressed proteins the monoclonal antibody 11D7 specific for gp 350/220 (17) and an EBV positive human serum with high antibody levels against gp 110 were used.

cells (18), we were interested in whether gp 110 could also be an ADCC target possibly involved in the defense against EBV.

In order to study EBV-specific ADCC mechanisms we first cloned the genes coding for gp 350/220 and gp 110 in vaccinia virus. The coding sequences of both proteins were inserted into vaccinia viruses under the control of the early/late 7.5 K promoter using the protocol of Mackett and co-workers (19). Coding regions of the reading frames BLLF1 and BALF4 were cloned in the correct orientation in the plasmid construct pAvB (20) adjacent to the 7.5 K promoter, flanked on either side by vaccinia virus thymidine kinase (TK) sequences. CV1 cells infected with wild-type vaccinia strain WR were transfected with the plasmid construct by the $CaPO_4$ method (21). Homogenous recombination gave rise to TK-negative recombinants which were selected by 5-bromdeoxyuridine in TK-negative B143 cells (22). Cells infected with these recombinant viruses were assayed for foreign gene expression by Western blotting. The virus was cloned twice prior to large-scale preparations of virus stocks by serial passaging on CV1 cells. Infection of various cell lines with the recombinant vaccinia viruses resulted in an efficient expression of the two proteins (Fig. 1).

ADCC was studied in a standard chromium release assay (23). As targets, EBV-transformed lymphoblastoid cell lines (LCLs) were used after infection with the recombinant vaccinia viruses. Effector cells were peripheral blood lymphocytes freshly prepared from healthy donors. EBV positive and negative sera were drawn from children not vaccinated against smallpox to avoid the possible interference of anti-vaccinia antibodies. In a first series of experiments we optimized the assay conditions using cells expressing gp 350/220 (infected with the vaccinia virus recombinant v-350 containing the gp 350 gene). Figure 2 shows a representative experiment with both EBV-negative and -positive sera demonstrating a clear specific cytotoxic reaction against gp 350 expressing cells mediated by a serum containing antibodies against EBV. Seven EBV-positive sera tested under these conditions revealed specific lysis rates against gp 350 (after subtraction of lysis rates obtained with vaccinia wild-type infected cells) between 2.5 and 28.5% (mean, 14.5%) (data not shown).

Using the same assay format different sera were tested against cells infected with the vaccinia constructs
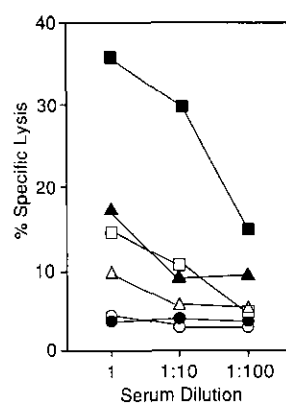

FIG. 2. Antibody dependent cell mediated cytotoxicity (ADCC) against EBV membrane antigen gp350/220. Target cells were cells of a lymphoblastoid cell line (LCL) established from a healthy EBV positive donor according to von Knebel-Doeberitz et al. (30). In brief, mononuclear cells were prepared from 20 ml of heparinized blood by the Ficoll–Hypaque technique according to Böyum (31) and suspended in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU of penicillin per milliliter, and 100 µg of streptomycin per milliliter (B cell culture medium). Cells were infected with the B95-8 strain of EBV by adding 0.5 ml of supernatant of B95-8 cells (32) to 10 ml of cells at a final concentration of 10^6 per milliliter. Ciclosporin A (Sandoz AG, Basel, Switzerland) was added at a final concentration of 2 µg per milliliter to prevent cytolysis of outgrowing EBV transformed cells by cytotoxic T cells. Outgrowth of transformed cells was seen after 4–5 weeks. LCLs were routinely maintained by subculture in B-cell culture medium twice a week. The cells were positive for EBV but did not express EBV membrane antigens gp 350/. 220 or gp110. These cells were infected with vaccinia wild type virus (v-wt) or the recombinant virus containing the gp 350/220 gene (v-350) at a virus concentration of 10–20 pfu per cell and incubated at 37° for 24 hr; 5 × 10^5 cells, uninfected or infected with v-wt or v-350, were labeled with 250 µCi $Na_2^{51}CrO_4$, and 5 × 10^3 labelled cells per well were incubated in round bottom microtiter plates for 4 hr at an effector/ target ratio of 50:1, in the presence of 10 µl serum (undiluted and diluted 1:10 and 1:100) of EBV negative and EBV positive children. Effector cells were freshly prepared peripheral blood lymphocytes (PBLs) from a healthy EBV positive donor. All tests were performed in triplicate; the percentage specific lysis was calculated as (cpm in sample − cpm spontaneously released)/(cpm cells lysed in 1% SDS − cpm spontaneously released) × 100. In all cell lines studied the spontaneous release was below 20%. Target cells: (●, ○) uninfected LCL; (▲, △) LCL infected with v-wt; (■, □) LCL infected with v-350; closed symbols, cells incubated with EBV positive serum; open symbols, cells incubated with EBV negative serum.

TABLE 1

ADCC Against LCLs Expressing EBV Membrane Antigen gp 350/220 or EBV gp 110 Using EBV Negative and Positive Sera

Serum[b]		Specific lysis[a]					
		LCL/v-wt[c]		LCL/v-350[c]		LCL/v-110[c]	
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
1 (neg.)	Undiluted	3.4	0.0	1.1	0.4	4.0	2.3
	1:10	0.0	0.0	0.0	1.2	0.0	0.0
	1:100	0.0	0.0	0.0	0.0	0.0	0.0
2 (pos.)	Undiluted	5.3	8.7	21.7	27.0	—	10.0
	1:10	0.0	0.0	15.7	17.9	—	2.9
	1:100	0.0	0.0	3.3	4.9	—	1.3
3 (pos.)	Undiluted	4.8	6.7	21.1	—	8.8	14.6
	1:10	0.5	0.0	9.1	—	2.7	3.3
	1:100	1.1	0.0	0.0	—	0.0	0.0
4 (pos.)	Undiluted	8.4	4.1	27.5	—	21.3	21.6
	1:10	0.0	0.0	7.2	—	3.6	8.6
	1:100	0.0	0.0	0.2	—	0.0	2.4
5 (pos.)	Undiluted	—	—	—	—	—	—
	1:10	15.5	—	44.0	—	36.5	—
	1:100	0.0	—	29.1	—	36.7	—

[a] Specific lysis rates obtained with vaccinia infected target cells in a ^{51}Chromium release assay (see legend to Fig. 2) after subtraction of background reactivity observed with uninfected LCLs.

[b] Sera from EBV positive and negative children. All sera were heated at 56° for 3 min.

[c] LCLs used as target cells infected with vaccinia wild type virus (v-wt), v-350 and v-110.

containing either the gp 350/220 or the gp 110 gene; wild-type vaccinia virus (v-wt)-infected cells as well as uninfected LCLs served as controls (Table 1). All of the EBV-positive sera tested showed a clear reactivity against LCLs expressing the gp 350/220. Toward gp 110, three out of the four positive sera showed reactivity: sera 4 and 5 were similarly reactive as in ADCC tests against gp 350/220. Serum 3 was marginally reactive in two experiments, whereas no clear reactivity was seen with serum 2.

These experiments (a) demonstrate the feasibility of our approach for the investigation of ADCC reactions against EBV proteins, (b) confirm the role of the EBV membrane protein gp 350/220 as an ADCC target, and (c) show for the first time that gp 110 can also serve as a target for EBV-specific ADCC.

Until now ADCC reactions against EBV have been studied using as targets either EBV producer cells (B95.8) pretreated by 12-O-tetradecanoylphorbol-13-acetate to induce the lytic cycle, or Raji cells superinfected with P3HR-1 virus (10, 24, 25, 26). Although these assays were able to demonstrate ADCC reactivity, they were difficult to standardize due to the varying number of cells expressing EBV proteins at their surface. Khyatti et al. (11) used a Raji cell line transfected with a vector containing the gene encoding for the EBV gp 350/220. This assay has the advantage of being able to measure reactions against well-defined antigens under standardized conditions. Our approach using LCLs infected by recombinant

vaccinia viruses containing genes coding for viral glycoproteins has been successfully used for the demonstration of ADCC reactions directed against HIV env proteins (27). This system allows for the surface expression of specific proteins in high density in over 90% of the cells. Thus, assays using vaccinia-infected cells as targets should show higher sensitivity than test systems using target cells where only a small fraction expresses the antigen under investigation. In fact, using gp 350/220 as a target, we obtained specific lysis rates (after subtraction of lysis of vaccinia wild-type infected cells) between 16.3 and 28.5% for four different EBV positive sera used in the experiments described here, whereas all specific lysis rates obtained with other systems to date centered at most around 10%, usually less (11).

The essential finding of our study is that the EBV-encoded glycoprotein gp 110 can function as a target for an ADCC reaction. Gp 110, a homolog of HSV gB, which plays a major role in the immune destruction of HSV infected cells, was shown to be one of the most abundant proteins expressed during the late lytic cycle (16). However, according to immunoelectron microscopy studies by Gong and Kieff (17) this protein after glycosylation largely remains confined to the endoplasmatic reticulum (ER) and the nuclear membrane and has not yet been detected on the surface of the cell or the viral envelope. The authors conclude therefore that gp 110 must lack a necessary signal for Golgi transport or possess a signal for ER retention or nuclear membrane localisation. Our

results, however, clearly show that (first) at least in the vaccinia system described here newly synthesized and glycosilated gp 110 molecules are able to reach the plasma membrane, and (second) that these molecules can be recognized at the surface of the cell by specific antibodies. This, of course, does not necessarily prove that the same mechanism is active in EBV-infected cells, as vaccinia virus infection may alter glycoprotein transport (although there is no evidence for it so far). On the other hand the findings by Gong and Kieff (17) mentioned above are not necessarily contradictory to such a mechanism. There is some evidence from their study that at least a small amount of Golgi processing of gp 110 takes place and therefore some molecules may be introduced into the cell's outer membrane. This might well suffice for the cell being destroyed by an ADCC type mechanism, as it is known that only a few molecules have to be recognized for an effective cell killing. The failure to detect these components so far may be due to their low density at the cell surface, but may also be due to a specific conformation of the membrane-bound molecule not recognized by antibodies raised against recombinant antigens in solution. In future studies antibodies recognizing surface-bound gp 110 should therefore be characterized to evaluate their role as well as the role of gp 110 in EBV-specific immunological defense mechanisms. It may well be that in addition to gp 350/220 this molecule also plays some part in the complex multistep process which so efficiently controls the latent infection with a potentially oncogenic virus.

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