JOURNAL OF VIROLOGY, June 1997, p. 4657–4662 Vol. 71, No. 6
0022-538X/97/$04.00+0

Epstein-Barr Virus Uses HLA Class II as a Cofactor for Infection of B Lymphocytes

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Received 21 January 1997/Accepted 17 March 1997

Infection of B lymphocytes by Epstein-Barr virus (EBV) requires attachment of virus via binding of viral glycoprotein gp350 to CD21 on the cell surface. Penetration of the cell membrane additionally involves a complex of three glycoproteins, gH, gL, and gp42. Glycoprotein gp42 binds to HLA-DR. Interference with this interaction with a soluble form of gp42, with a monoclonal antibody (MAb) to gp42, or with a MAb to HLA-DR inhibited virus infection. It was not possible to superinfect cells that failed to express HLA-DR unless expression was restored by transfection or creation of hybrid cell lines with complementing deficiencies in expression of HLA class II. HLA class II molecules thus serve as cofactors for infection of human B cells.

Epstein-Barr virus (EBV) is carried by more human beings than any other persistent virus. The virus is widely known for its ability to immortalize human B lymphocytes in vitro, and infection of B cells in vivo not only establishes a permanent reservoir of virus in the body but also contributes to the pathology of the lymphoproliferative diseases that are associated with infections in some individuals. As it has evolved to its position as one of the most ubiquitous and successful persistent viral parasites of man, EBV has adopted many strategies to exploit the unique environment of the B cell (29), beginning with its first encounter with the cell surface. The major envelope glycoprotein of the virus, gp350, has evolved to include sequences similar to those of the complement fragment C3dg (43). It binds the virus to complement receptor type two (CR2, CD21) (15, 43) and in doing so triggers endocytosis of the virion (55). Penetration of the cell membrane then minimally requires a complex of molecules that includes two less-abundant glycoproteins, gH and gL, which have functional homologs in all herpesviruses studied so far (16–18, 21, 27, 28, 36, 48, 51, 61). Virosomes made from EBV proteins will bind and fuse with receptor-positive cells, but if they are depleted of the EBV gH:gL complex they bind but fail to fuse (22). In keeping with the known biology of EBV, however, recent work has suggested that penetration, like attachment of virus, involves a unique adaptation to the B cell. A third glycoprotein, gp42, which is not known to have a homolog in other human herpesviruses, associates noncovalently with the EBV gH:gL complex (34). A monoclonal antibody (MAb) called F-2-1 that reacts with gp42 has no effect on virus attachment to CR2 but inhibits fusion of the virus with the B cell membrane (39) and neutralizes infection of this cell type. In contrast, the same MAb has no effect on infection of an epithelial cell line engineered to express CR2 (34).

Glycoprotein gp42 is the product of the BZLF2 open reading frame (34), which is predicted to encode a protein of 223 amino acids with an amino-terminal signal peptide but no other hydrophobic domain long enough to be membrane spanning (3). The sequence includes four potential N-linked glycosylation sites, and the native protein which is present in the membrane of EBV-producing cells and in the envelope of the virion is glycosylated (34). Computer-assisted analysis indicated that gp42 has some features characteristic of members of the C-type lectin gene family (52) and stimulated the search for a cellular partner with which it might interact. The extracellular domain of the protein was expressed as a soluble chimeric molecule, designated BZLF2.Fc, in which the putative signal peptide was replaced by the Fc portion of human immunoglobulin G1 (IgG1). The probing of a cDNA expression library made from activated T cells and an analysis of monkey kidney cells transfected with wild-type or mutant HLA-DRβ chains demonstrated that the chimeric protein bound to the β_1 domain of the HLA class II protein HLA-DR (52). For clarity, in this paper we refer to BZLF2.Fc, the soluble form of gp42, as gp42.Fc.

As recent studies with the human immunodeficiency virus (2, 6, 8, 10, 11, 14), adenovirus (58), and herpes simplex virus (41) have so eloquently demonstrated, attachment to and penetration of the cell membrane by a virion may be separate, cooperative events that involve distinct proteins. There is little direct evidence to indicate that other cell surface molecules in addition to CD21 are required for EBV infection. However, postbinding events are not efficient in non-B cells expressing recombinant CD21 (1, 5) and the presence of CD21 on some cell surfaces is not sufficient to ensure internalization (38, 39), suggesting that molecules in addition to CD21 might be involved. Since gp42 had already been shown to be involved in penetration of EBV into B lymphocytes, we set out to determine whether the newly identified interaction between soluble gp42.Fc and HLA class II was relevant to the process. We demonstrate here that HLA class II plays the role of a cofactor in infection of B cells by EBV.

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MATERIALS AND METHODS

Cells. Raji (45), Akata (54), P3HR1-Cl13 (24), BLS-1 (50), SJO (4), .174 (7), BLS-1×SJO, BLS-1×.174, .174.DR4, and SJO.DR4 (31) cells were grown in

RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) supplemented with 15% heat-inactivated fetal bovine serum (Gibco/BRL Life Technologies, Grand Island, N.Y.). Peripheral blood mononuclear cells (PBMC) were collected from three adult donors and depleted of T cells by erythrocyte rosetting as described previously (39).

Antibodies. Five purified MAbs were used: F-2-1 (MAb to gp42) (34), 72A1 (MAb to gp350) (25), Alva 42 (MAb to the β chain of HLA-DR) (20, 52), and two American Type Culture Collection antibodies, HB55 (also known as L423) (33) and HB180 (57) (MAbs to the αβ heterodimer of HLA-DR).

Virus. Akata strain virus was obtained by induction of Akata cells with goat anti-human immunoglobulin as described previously (34) and harvested from unconcentrated clarified culture medium that had been passed through a 1.2-μm-pore-size filter. P3HR1 strain virus was obtained by induction of P3HR1-Cl13 cells with 30 ng of 12-*o*-tetradecanoylphorbol-13-acetate per ml, concentrated 100-fold by high-speed centrifugation, and resuspended in fresh medium as described previously (22). For binding assays, Akata virus was radiolabeled by inducing cells with anti-human immunoglobulin and adding 6 μCi of [^{3}H]thymidine (specific activity, 43 Ci per mmol; Amersham Corp., Arlington Heights, Ill.) per ml for 4 to 72 h postinduction. Labeled virus was concentrated 100-fold from clarified culture supernatants by centrifugation and filtered through a 0.8-μm-pore-size filter.

Fc constructs. The soluble form of gp42, gp42.Fc, previously designated BZLF2.Fc, was made as described previously (51). The unrelated heterologous recombinant Fc chimeric protein, Cont.Fc, was constructed in a similar manner by fusing amino acids 19 to 258 of a vaccinia virus open reading frame (Copenhagen strain, p35) to the Fc portion of human IgG1 (60). Both proteins were purified to homogeneity by affinity chromatography on protein A-agarose (13). Purity was confirmed by gel electrophoresis and silver staining of the gel, and protein concentrations were determined by an enzyme-linked immunosorbent assay specific for the constant domain of human IgG1 as described previously (60). The purified proteins were also screened for low endotoxin levels by the *Limulus* amebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, Md.).

Transformation of lymphocytes. Six hundred thousand PBMC were preincubated for 30 min on ice with gp42.Fc, Cont.Fc protein, or antibody in 180 μl and then with 120 μl of stock Akata strain virus for 60 min. RPMI medium (540 μl) containing 10% heat-inactivated fetal calf serum was added, and 140 μl containing 10^5 cells was plated into each of five wells of a 96-well plate. Cells were fed by replacing 50% of the medium with fresh medium at weekly intervals, and transformation was judged by outgrowth of cells over 5 weeks of culture.

Superinfection of cell lines. To test for superinfection and inhibition of superinfection of Raji cells, 1.5×10^6 cells were preincubated with 80 μg of gp42.Fc, 80 μg of Cont.Fc protein, or 300 μg of MAb in 260 μl of RPMI or with medium alone for 30 min (Fc proteins) or 1 h (MAbs) at 37°C. Stock P3HR1 virus (100 μl) was added for an additional 90 min of incubation. In some experiments the sequence of addition of virus and gp42.Fc was reversed. Four milliliters of RPMI supplemented with serum was added, and cells were incubated for 3 days. To test for superinfection of cell lines which expressed or failed to express HLA class II, 10^6 cells were incubated for 2 h in 100 μl of stock P3HR1 virus or RPMI. Four milliliters of RPMI with serum was added, and cells were incubated for 3 days. Superinfection was visualized by Western blotting. Cells were harvested by centrifugation, resuspended in 100 μl of radioimmunoprecipitation buffer, and treated in a sonic bath for 30 s. One hundred microliters of 2× sodium dodecyl sulfate reducing sample buffer was added; samples were boiled for 5 min; proteins were fractionated on 9% polyacrylamide gels, transferred to a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, N.H.), and immunoblotted with a 1/100 dilution of virus early antigen (EA-D) MAb (Capricorn Products, Inc., Scarborough, Maine); and antibody binding was visualized either with alkaline phosphate-conjugated rabbit anti-mouse antibody (HyClone, Logan, Utah) and the substrate 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (BCP/NBT; Sigma) as described previously (34) or with the enhanced chemiluminescence system (Amersham).

Virus binding assay. The ability of MAb to inhibit binding of radiolabeled virus to Raji cells was determined as described previously (39) by preincubating virus with equal volumes of MAb at a concentration of 100 μg per ml or with phosphate-buffered saline for 1 h on ice. The virus was then incubated for an additional hour with cells that had been briefly fixed with 0.1% paraformaldehyde, cells were washed five times, and the acid-precipitable radioactivity remaining attached to cells was counted.

Flow cytometry. Flow cytometric analyses of gp42.Fc binding to major histocompatibility complex class II-expressing Raji cells was performed with gp42.Fc or Cont.Fc (300 ng/ml) and a biotinylated goat anti-human MAb to Fc.

RESULTS

Soluble gp42.Fc which binds HLA class II also inhibits EBV infection. The effects of gp42.Fc or an unrelated heterologous, recombinant Fc chimeric protein (Cont.Fc), in which the viral protein extracellular domain was derived from a vaccinia virus protein, were first examined on transformation of freshly isolated T-cell-depleted PBMC by EBV. In repeated experiments with different lymphocyte donors, gp42.Fc, but not the heterologous chimeric protein, inhibited the ability of the Akata strain of EBV to transform B cells (Table 1). EBV virions have been reported to incorporate HLA class II proteins (30), and thus we could not be certain whether gp42.Fc might be inhibiting infection by binding to HLA class II in the virus or on the surface of the cell. However, the P3HR1 strain of EBV is produced from cells that only express HLA-DQ and gp42.Fc does not bind HLA-DQ (52a). We therefore also examined the effects of gp42.Fc on B-cell infection by P3HR1 strain virus. P3HR1 virus is a nontransforming strain but can superinfect EBV-transformed cell lines (23). Superinfection of cells such as the Raji cell line, which expresses CD21 and HLA-DR, results in induction of virus early antigen (EA-D) which can be detected by Western blot analysis with a MAb that recognizes up to four differentially phosphorylated forms of the BMRF1 protein (19, 44, 47). Soluble gp42.Fc but not the heterologous Fc protein inhibited superinfection by P3HR1 virus (Fig. 1A), suggesting that inhibition resulted from protein binding to the cell surface rather than to the virion particle. Inhibition was seen only if gp42.Fc were added before virus and not if it were added 2 h later (Fig. 1B).

TABLE 1. Effects of gp42.Fc and Cont.Fc protein on the ability of Akata strain EBV to transform T-cell-depleted PBMC

Expt. no.	No. of transformed wells/total no. of wells infected					
	gp42.Fc (μg/ml)			Cont.Fc (μg/ml)		
	50	15	5	50	15	5
1	0/5	4/5	5/5	4/5	5/5	5/5
2	0/5	2/5	5/5	5/5	5/5	5/5
3	2/5	1/5	5/5	5/5	5/5	5/5
4	0/4	4/4	4/4	4/4	4/4	4/4
5	0/5	1/5	5/5	4/5	5/5	5/5
6	0/5	3/5	5/5	5/5	5/5	5/5

Antibodies that block the interaction of gp42 with cells expressing HLA class II also inhibit EBV infection. We next determined whether the neutralizing activity of antibody F-2-1 might result from an effect on the binding of gp42 in the virion to the cell surface. Flow cytometric analysis indicated that F-2-1 inhibited binding of gp42.Fc to Raji cells (Fig. 2A and B). Since this effect was presumed to be mediated by interaction of F-2-1 with an epitope on gp42.Fc close to or identical with the

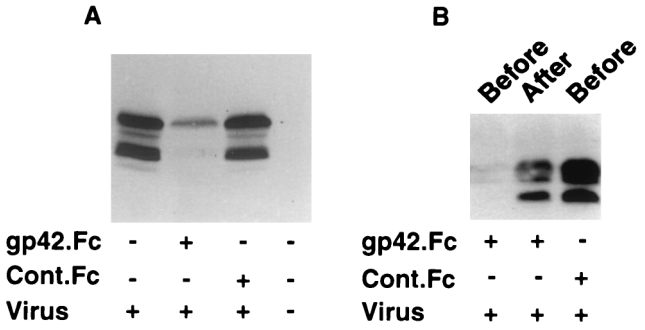

FIG. 1. Effects of gp42.Fc on superinfection of Raji cells by P3HR1 strain EBV. Cells were harvested 72 h after superinfection, and equal numbers were examined by Western blotting with a MAb for expression of EA-D. (A) Effects of preincubating Raji cells with (+) or without (−) gp42.Fc or Cont.Fc. (B) Comparison of effects of adding (+) or not adding (−) gp42.Fc before or after addition of virus.

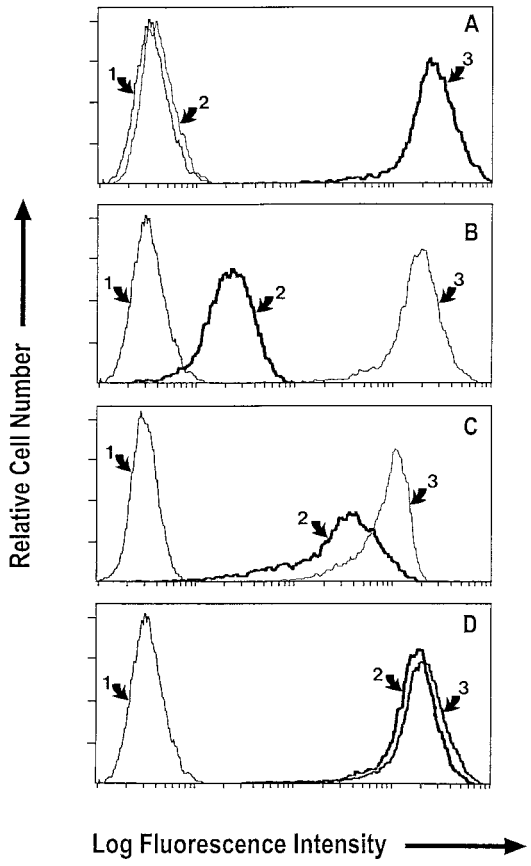

FIG. 2. Flow cytometric analyses of the effects of MAbs on binding of gp42.Fc to Raji cells. (A) Binding of gp42.Fc (arrow 3) and Cont.Fc (arrow 2) protein in the absence of antibody; (B) binding of gp42.Fc in the presence of either MAb F-2-1 to gp42 (arrow 2) or isotype control antibody (arrow 3); (C) binding of gp42.Fc in the presence of either MAb Alva 42 to the β chain of HLA-DR (arrow 2) or isotype control antibody (arrow 3); (D) binding of gp42.Fc in the presence of MAb HB180 (arrow 2) or HB55 (arrow 3) to the αβ heterodimer. The MAbs used in panel D are of the same isotype and serve as controls for one another. Binding of the labeled second-step reagent alone to cells is indicated by arrows 1 on the far left of each panel.

binding site for HLA-DRβ, we also investigated the possibility that antibody to HLA class II protein might have a similar effect. Three MAbs which recognize three different epitopes on HLA-DR were tested for their ability to inhibit binding of gp42.Fc to Raji cells. Only one of these MAbs, Alva 42, interfered significantly with gp42.Fc binding (Fig. 2C), while HB55 and HB180 had little or no effect (Fig. 2D). Interestingly, the Alva 42 MAb, but not MAb HB55, also inhibited transformation of T-cell-depleted PBMC with Akata strain virus (Table 2), suggesting that a threshold of inhibition of gp42 binding is required to interfere with the infection process. In addition, Alva 42, and to a slight extent MAb HB180, inhibited superinfection of Raji cells with P3HR1 strain virus (Fig. 3). A previous unconfirmed report (46) had suggested that MAb HB55 blocked binding of EBV to Raji cells. However, using Akata strain virus radiolabeled with [^{3}H]thymidine, we found no evidence for the ability of HB55, the other anti-HLA antibodies, or antibody to gp42 to inhibit significantly the attachment of virus to Raji cells (Table 3); in contrast, virus binding could be reduced by MAb 72A1, a neutralizing antibody to gp350 previously shown to inhibit virus binding in a similar assay (39).

TABLE 2. Effects of MAbs to HLA-DR on transformation of T-cell-depleted PBMC by Akata strain EBV

Expt. no.	No. of transformed wells/total no. of wells infected					
	Alva 42 (μg/ml)			HB55 (μg/ml)		
	30	10	3	30	10	3
1	0/4	3/4	4/4	4/4	4/4	4/4
2	0/5	5/5	5/5	5/5	4/5	5/5
3	0/5	4/5	5/5	4/5	5/5	5/5

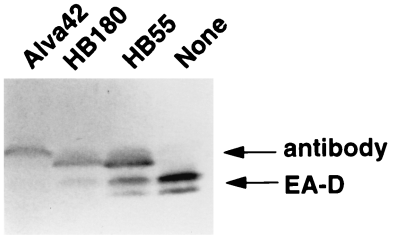

FIG. 3. Effect of antibodies to HLA class II on superinfection of Raji cells by P3HR1 strain virus. Cells were harvested 72 h after infection, and equal numbers were analyzed by Western blotting with a MAb for expression of EA-D. The top band in each of the first three lanes is MAb heavy chain.

Cells that lack HLA class II can be superinfected only if HLA class II expression is restored. Type II bare-lymphocyte syndrome (BLS) is a rare severe primary immunodeficiency that is caused by lack of expression of HLA class II (9, 35). Cells from patients with BLS might be expected to be relatively resistant to infection with EBV. Although the relative ease with which B-cell lines can be derived from such patients is uncertain, it has, however, proven possible to derive so-called BLS lines from BLS patients by cell transformation with EBV. BLS cell lines and their derivatives then offer a unique opportunity to explore further whether HLA class II is relevant to efficient infection of B cells. Four complementation groups, I to IV, have been defined for BLS. The gene mutated in complementation group II (also referred to as group A) encodes an essential class II gene transactivator, CIITA (53, 62). None of the cell lines we were able to obtain in this complementation group were completely lacking in expression of HLA class II as judged by flow cytometric analysis. Complementation groups I and IV (also referred to as groups B and C) represent deficiencies in one or the other of the two subunits of RFX, a protein which binds to the *cis*-acting transcriptional control sequence called the X box (12). The BLS-1 cell line, which falls in complementation group I (25, 50), and the SJO

TABLE 3. Effects of MAbs to HLA-DR or to EBV on the ability of [^{3}H]thymidine-labeled EBV to bind to Raji cells

MAb[a] (100 μg/ml)	Radioactivity[b]
HB180	1,307
HB55	1,164
Alva 42	963
F-2-1	1,155
72A1	212
None	1,339

[a] Antibodies HB180, HB55, and Alva 42 react with HLA-DR; F-2-1 reacts with gp42; and 72A1 reacts with gp350, the virus attachment protein.
[b] Acid-precipitable radioactivity (in counts per minute) bound to cells.

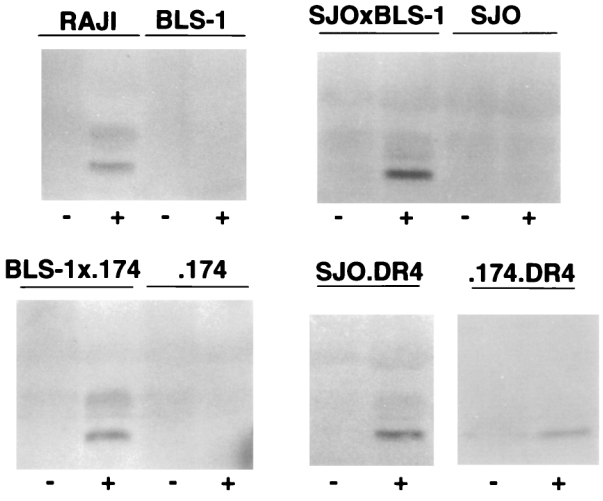

FIG. 4. Superinfection of B cells lines which express HLA class II (Raji, SJO×BLS-1, BLS-1×.174, SJO.DR4, .174.DR4) or fail to express HLA class II (BLS-1, SJO, .174). Cells were incubated with P3HR1 strain virus (+) or with medium (−) as indicated. Cells were harvested at 72 h, and equal numbers were examined by Western blotting with a MAb for expression of EA-D. The blots shown are representative of at least three separate experiments.

line, which falls in complementation group IV (4), express only HLA-DQα. A hybrid of the two, BLS-1×SJO, has restored HLA-DR expression (31). The cell line .174 expresses CIITA and RFX but has a deletion of genes coding for HLA class II (7). BLS-1×.174, a hybrid of BLS-1 and .174, has restored expression of HLA class II (31). For two additional lines, .174.DR4 and SJO.DR4, the expression of HLA-DR was restored by virtue of retrovirus-mediated gene transfer of cDNA encoding DR4w4 (31a, 32). Each of the cell lines bound EBV as judged by indirect immunofluorescence with an antiviral MAb (data not shown), and since, like Raji cells, each line already carried endogenous EBV genomes, each was examined for its ability be infected with EBV in a superinfection assay using the P3HR1 strain virus. An increase in the expression of EA-D, indicating successful superinfection, correlated exactly with expression of HLA-DR (Fig. 4).

DISCUSSION

Entry of animal viruses into cells is a complex process that is still incompletely understood. In several cases, it appears to require of the cell not only a receptor to interact with the virus attachment protein and tether the virus to the cell surface but also at least one additional molecule to interact with the virus and facilitate penetration. The findings reported here indicate that HLA class II is required in addition to the CD21 receptor for efficient infection of B cells by EBV. The fact that EBV-transformed cell lines have been derived from individuals suffering from BLS indicates that HLA class II is probably not the only cell surface molecule that EBV can use to facilitate penetration of lymphocytes. This is perhaps not surprising in view of the identification of more than one cofactor for fusion of the human immunodeficiency virus (2, 6, 8, 10, 11, 14), the probable use of multiple mediators for entry of herpes simplex virus (reviewed in reference 41), and our previous observation that MAb F-2-1 failed to affect infection of an epithelial cell line that does not constitutively express HLA class II (34). However, the aggregate of evidence thus far suggests that gp42 is critical to penetration of the resting B-cell membrane by EBV and that an interaction with HLA class II is important to this event. Although the extent of infection of the BLS-derived cells was variable and did not approach the levels achievable with Raji cells, which have a high episome copy number and are unusually responsive to induction (29), superinfection was consistently and exclusively associated with the restoration of expression of HLA class II.

Like the human immunodeficiency virus, EBV enters cells in a pH-independent manner (40). Recent studies have shown that binding of the human immunodeficiency virus attachment protein gp120 to CD4 induces a conformational change that is important to virus entry and creates a new recognition site for one of the chemokine receptors used by some isolates of this virus to facilitate penetration (56, 59). Preliminary analysis by immunoprecipitation (unpublished data) indicates that when gp42 is complexed with gH and gL in the virion, it is not associated with HLA class II. This suggests that, as previously speculated (37), a conformational change, similar to that occurring in gp120, may result from the binding of EBV to its receptor and, further, that such a change may be required in order to allow an interaction to take place between gp42 and the HLA β chain. It is possible that HLA class II molecules act simply as secondary receptors for virus. The initial binding of EBV to CD21 via gp350 puts the virus at a distance of approximately 50 nm from the cell surface (42). Interaction with a second molecule may then be important to catalyze events that require closer proximity of virus and cell membranes. However, since some studies have implicated major histocompatibility complex class II proteins in cell signaling (49), it is also formally possible that the role of HLA class II may be more indirect. In this regard, the use of molecules with potential for cell signaling as postbinding cofactors by adenovirus (58), herpes simplex virus (41), and the human immunodeficiency virus is provocative (2, 6, 8, 10, 11, 14). The use of HLA class II by EBV represents yet another example of the extraordinary adaptation of EBV to its unique biologic niche in the human B cell. The interaction of gp42 with the polymorphic region of HLA-DR also suggests that it may be informative to attempt to determine whether allelic differences affect the efficiency of infection and hence potentially influence the outcome of an encounter with this most ubiquitous of persistent human viruses.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI20662 (L.M.H.-F.) and AI08903 (S.K.) from the National Institutes of Allergy and Infectious Diseases and by a Marion Merrell Dow SEP postdoctoral fellowship (Q.L.).

We thank R. DeMars for the gift of the .174 cell line.

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