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Impaired Transporter Associated with Antigen Processing-Dependent Peptide Transport during Productive EBV Infection

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Human herpesviruses, including EBV, persist for life in infected individuals. During the lytic replicative cycle that is required for the production of infectious virus and transmission to another host, many viral Ags are expressed. Especially at this stage, immune evasion strategies are likely to be advantageous to avoid elimination of virus-producing cells. However, little is known about immune escape during productive EBV infection because no fully permissive infection model is available. In this study, we have developed a novel strategy to isolate populations of cells in an EBV lytic cycle based on the expression of a reporter gene under the control of an EBV early lytic cycle promoter. Thus, induction of the viral lytic cycle in transfected EBV+ B lymphoma cells resulted in concomitant reporter expression, allowing us, for the first time, to isolate highly purified cell populations in lytic cycle for biochemical and functional studies. Compared with latently infected B cells, cells supporting EBV lytic cycle displayed down-regulation of surface HLA class I, class II, and CD20, whereas expression levels of other surface markers remained unaffected. Moreover, during lytic cycle peptide transport into the endoplasmic reticulum, was reduced to <30% of levels found in latent infection. Because steady-state levels of TAP proteins were unaffected, these results point toward EBV-induced interference with TAP function as a specific mechanism contributing to the reduced levels of cell surface HLA class I. Our data implicate that EBV lytic cycle genes encode functions to evade T cell recognition, thereby creating a window for the generation of viral progeny. The Journal of Immunology, 2005, 174: 6829–6838.

Immune evasion strategies are exploited by many viruses to allow their successful persistence in the infected immunocompetent host (see reviews in Refs. 1–4). These strategies should delay elimination of the infected cell long enough to enable the virus to replicate, colonize the host, and have the potential for transmission to other individuals. Exemplary in this context are the herpesviruses, large DNA viruses that are further categorized into three subfamilies (α-, β-, and γ-herpesviruses) based on their biological properties and tissue tropisms (5). Immune evasion strategies have been identified for members of all three subfamilies, e.g., HSV (a α-herpesvirus), human CMV (a β-herpesvirus), and EBV (a γ-herpesvirus). In the infected cell, herpesvirus uses general evasive approaches, such as blocking the induction of programmed cell death and shutting down host protein synthesis (5). In addition, herpesviruses specifically perturb recognition by virus-specific T cells via a number of mechanisms, many of which act to thwart the MHC class I and class II processing and presentation pathways. Down-regulation of cell surface MHC class I is a common strategy to escape from class I-restricted immunity. Interestingly, the mechanisms that viruses use to achieve reduced surface expression of MHC class I molecules vary considerably and include: retention of MHC class I molecules in the endoplasmic reticulum (ER); inhibition of TAP-mediated peptide transport; proteosomal degradation of class I H chains; and diversion of class I molecules toward lysosomes (see reviews in Refs. 1–4). The observation that some herpesviruses encode multiple gene products to combine their immune evasive functions underscores the importance of efficiently blocking MHC class I Ag presentation.

EBV is the prototypic γ-herpesvirus and is carried as a persistent and largely asymptomatic infection by over 90% of all adults. The ubiquitous distribution of EBV in all populations is indicative of the success of this virus in establishing a nonpathogenic relationship with its human host. Infection is, however, not always innocuous because EBV is the causative agent of infectious mononucleosis and posttransplant lymphoproliferative disorders, and is associated with a number of malignancies of lymphoid (Burkitt’s lymphoma and Hodgkin’s disease) or epithelial (undifferentiated nasopharyngeal carcinoma) cell origin (6). Upon primary infection in vivo, EBV normally establishes a latent infection in memory B cells that do not proliferate and does not express immunogenic viral proteins (6–8). Consequently, these EBV+ memory B cells are not targeted by T cell surveillance. Under certain circumstances, partial expression of the virus genome leads to synthesis of a limited subset of so-called “latent gene products” with concomitant growth transformation of the EBV-infected B cells (6). In vivo, the potential pathogenicity of these proliferating cells is counteracted by potent CD8+ and CD4+ T cell responses directed toward several latent viral proteins (6, 9, 10). The critical role of these T cells in controlling growth-transformed virus-infected B cells is underlined by the fact that EBV-associated lymphoproliferative disease develops in...
transplant patients with impaired T cell functions and can be resolved by adoptive transfer of EBV-specific T cells (11).

Although EBV has the ability to replicate its genome via cell division of latently infected B cells, transmission to another host requires production of infectious virions through the lytic phase. Following activation of the lytic cycle, >80 EBV proteins are synthesized in a strictly regulated order: first, immediate-early genes are expressed, which transactivate the early genes, and then the late proteins are produced (12). Especially during the replicative phase, therefore, EBV-infected cells express a large number of viral Ags that may be presented to the immune system. Because cells in lytic cycle can remain viable for several days (Refs. 13, 14 and our study), they are potentially very susceptible to immune surveillance.

EBV-specific T cell immunity against both latent and lytic Ags is present in peripheral blood of healthy individuals (6), yet the virus persists for life in all infected individuals. These observations point to highly effective escape mechanisms that allow EBV to elude the host immune system and thereby permit assembly and spread of viral progeny. During latency, down-regulated expression of viral target Ags by the infected cell serves as a major factor contributing to effective avoidance of elimination by T cells (6–8, 15). In contrast, during productive EBV infection, when >80 virus-encoded proteins are expressed, little is known about interference with antiviral immune responses. This poor understanding is largely due to the absence of an amenable model to study EBV-producing cells because in vitro infection of B cells with EBV normally results in latent rather than lytic cycle gene expression. By analogy with other herpesviruses, we would predict that viral immune evasion molecules are expressed during the lytic phase of EBV infection. Indeed, immune evasive functions have been ascribed to a number of EBV lytic cycle proteins, including an IL-10 homologue (16), a putative viral soluble receptor for CSF-1 (17), and an inhibitor of IFN-γ receptor expression (18). In addition, the late BZLF2 gene product, gp42, interferes with CD4+ T cell responses by associating with HLA class II, causing steric hindrance of TCR recognition of peptide-HLA complexes (19). Thus, some potential immunoevasins have been identified for EBV, but the picture is by no means complete and their contribution to avoidance of immune detection during natural infection remains to be evaluated.

The aim of this study was to identify the mechanisms operating in B cells supporting EBV lytic cycle that may lead to T cell escape. Study of productive EBV infection is technically more difficult than for, for instance, α- and β-herpesviruses because of the lack of a fully permissive infection model. EBV-infected B cells usually show little or no virus production in vitro. Some EBV-1+ cells can be activated by stimulation with phorbol esters or by ligation of the BCR; but even in those lines, only a subpopulation of cells enters lytic cycle (12, 20). To overcome this, we generated a reporter plasmid that, when stably transfected into EBV-1+ B cells, expressed an immunosorbable surface marker for productively infected cells. This novel strategy allowed us to isolate populations of cells in EBV lytic cycle and to perform biochemical and functional analyses. In productively EBV-infected B cells, surface HLA class I and class II expression were shown to be reduced, and impairment of TAP-mediated peptide transport was identified as a mechanism contributing to impairment of Ag presentation via HLA class I.

**Materials and Methods**

**Reporter plasmid construction**

As a nonvirus-encoded reporter protein, we have used the rat CD2-GFP fusion protein, comprising the extracellular and transmembrane domains of rat CD2 fused to the N terminus of enhanced GFP (EGFP). The rat CD2-GFP protein was constitutively expressed from plasmid pCD2-EFGP (21), created by in-frame insertion of codons (1–233) of rat CD2 into the KpnI site of vector pEGFP-N1 (BD Clontech) and containing a CMV early promoter for constitutive expression. To achieve inducible reporter protein expression regulated by EBV lytic cycle entry, we designed a reporter plasmid, pHEBO-BMRF1p-rCD2-GFP, to express the rat CD2-GFP chimeric gene from the 5′ regulatory sequences of the BMRF1 early gene of EBV (see Fig. 1A). To this end, the pHEBO-Zp-luciferase reporter (22), kindly provided by Prof. P. J. Farrell, Ludwig Institute (Imperial College, London, U.K.), was digested with HindIII and SalI to remove the luciferase and promoter sequences, to be replaced with a rat CD2-GFP cassette that was PCR amplified from the pCD2-EFGP plasmid with 5′ HindIII and 3′ SalI restriction sites. A HindIII fragment containing a 5′ regulatory sequence (from −400 to +1) of BMRF1, was then inserted upstream of the rat CD2-GFP gene.

**Cell lines**

Cell lines were routinely grown in complete culture medium consisting of RPMI 1640 (Invitrogen Life Technologies) supplemented with 8% FCS, 200 U/ml penicillin, and 200 μg/ml streptomycin. Akata is a human EBV Burkitt’s lymphoma B cell line that displays a nonproductive infection, but which can be induced into lytic cycle by ligation of cell surface IgG (20, 23). The AKBM line was derived by electroporation of the EBV Akata line with the pHEBO-BMRF1p-rCD2-GFP reporter plasmid and by selecting stable transfectants with 500 μg/ml hygromycin B (Boehringer Mannheim). 2A8 is an EBV-1 subtype of Akata (24).

**Synchronous reactivation of EBV and isolation of cells in lytic cycle**

Viral activation in EBV-1+ AKBM cells was induced by cross-linking surface IgG with 10 μg/ml goat anti-human IgG Ab (Cappel) for 2 h. After two-wash steps, cells were cultured in fresh medium until harvesting at the time points indicated. For isolation of productively infected cells, the induced AKBM cells were stained with a mAb specific for rat CD2 (OX34) and were then positively selected by magnetic cell sorting using anti-mouse IgG2a/IgG2b microbeads and MS columns (Miltenyi Biotec) according to the manufacturer’s guidelines. In all experiments, mock-induced AKBM cells were taken along as controls.

**Antibodies**

The following Abs have been used in this study and, unless otherwise indicated, were described elsewhere (19, 25–27). OX34 (American Tissue Culture Collection) is a mouse IgG2a mAb specific for the extracellular domain of rat CD2. The EBV-specific mAb used included: BZ.1 specific for the immediate-early BZLF1 protein; L2 kindly provided by Dr. G. Pearson, Georgetown University (Washington, D.C.), recognizing the BALF4-encoded 125-kDa component of the viral capsid Ag (VCA) complex; OT13N2 kindly provided by Dr. J. Middeldorp, Vrije Universiteit Medical Center (Amsterdam, The Netherlands), detecting lytic cycle early Ags (EA-D); and 72A1 (American Tissue Culture Collection) recognizing the BRLF1-encoded gp350/gp220 late membrane Ag. Murine mAbs used to detect human cellular proteins were: W6/32 (American Tissue Culture Collection), recognizing β2-microglobulin-associated HLA class I complexes (HLA-A, HLA-B, and HLA-C alleles); HCA2 (28), specific for HLA class I H chains; DA6-147 and HB10A kindly provided by Dr. P. Cresswell, Howard Hughes Medical Institute (New Haven CT), specific for HLA-DR α-chain and β-chain; CD71 (BD Pharmingen) and H68.6 (Roche), both reactive with human transferrin receptor; 148.3 and 435.4 kindly provided by Dr. R. Tampe, Wolfgang Goethe-University (Frankfurt, Germany) and by P. M. van Endert, Institut Necker (Paris, France), directed toward TAP1 and TAP2, respectively. To control for sample loading on polyacrylamide gels and Western blots, actin was detected with rabbit Abs purchased from Sigma-Aldrich (catalog no. A2066). For some flow cytometry experiments, PE-conjugated mAbs to human cell surface Ags were purchased from BD Pharmingen (CD20, CD71, CD80, and CD86) or from Serotec (product code MCA81PE for HLA class I; code MCA71PE for HLA-DR; and code MCA1266 as an IgG2a isotype control).

**Flow cytometry**

Cell surface expression of specific molecules was determined either using Abs directly conjugated to PE or by indirect detection of unlabeled primary Abs with a second goat anti-mouse IgG PE (Jackson Immuno Research or Serotec); after cell surface staining, cells were fixed with 1% paraformaldehyde before analysis. For detection of the intracellular markers of the EBV lytic cycle, cells were first fixed with 2% paraformaldehyde.
and permeabilized with 0.1% Triton X-100 before indirect immunofluorescence as described (25). Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

**Peptide transport assay**

TAP-mediated peptide transport assays were performed as described (27). In brief, cells were permeabilized with 2.5 IU/ml streptolysin O (Murex Diagnostics). Aliquots of 3 × 10⁶ permeabilized cells were incubated with 200 nmol of the fluoresceinated peptide CVNKTETAR, kindly provided by W. Benchajjongs and J. W. Drijfhout, Leiden University Medical Center (Leiden, The Netherlands) in the presence or absence of 10 nM ATP set at 37°C for 10 min. Peptide translocation was determined by adding ice-cold 1% Triton X-100 lysis buffer. Postnuclear lysates were incubated with Con A-Sepharose (Amersham Biosciences) to isolate the glycosylated peptides. After elution from the beads with mannopyranoside buffer, the amount of fluoresceinated peptide that was recovered on the basis of acquired glycosylation was determined using a fluorescence plate reader (Cytofluor; PerSeptive Biosystems). Results are depicted as a percentage peptide transport with the amounts for latently infected cells in the presence of ATP set at 100%.

**Western blot analysis**

SDS-PAGE and immunoblotting were performed as reported previously (19). Briefly, total postnuclear Nonidet P-40 cell lysates were denatured in reducing sample buffer (final concentration: 2% SDS, 50 mM Tris-HCl, pH 8.0, 10% glycerol, 5% 2-ME, 0.05% bromphenol blue) and solubilized proteins equivalent to 2 × 10⁶ cells were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). Cellular and viral proteins were detected by incubating the membranes with specific Abs followed by HRP-conjugated secondary Abs to mouse or rabbit Ig (Jackson ImmunoResearch Laboratories or DAKO). Bound HRP was visualized using the ECL-plus detection kit (Amersham Biosciences).

**Results**

**A reporter for EBV⁺ cells in lytic cycle**

So far, it has been difficult to study productively EBV-infected B lymphocytes because an appropriately fully permissive culture system is not available. We therefore developed a novel strategy to analyze the subpopulation of cells that have entered the lytic cycle, based on induced expression of a reporter protein under the control of an early EBV promoter. To this end, EBV⁺ cells were transfected with a reporter plasmid (pHEBO-BMRFlp-rCD2-GFP; Fig. 1A) that contains the EBV lytic cycle promoter region for the early BMRFl gene, inserted upstream of a chimeric reporter gene encoding the extracellular and transmembrane domains of rat CD2 with EGFP replacing the functional cytosolic domain (rat CD2-GFP). Thus, in reporter-positive EBV⁺ cells, transactivation of the BMRFl promoter upon immediate-early gene expression should not only result in synthesis of viral lytic phase proteins, but also in translation of the rat CD2-GFP reporter.

Akata is a human EBV⁺ Burkitt’s lymphoma cell line that normally displays a nonproductive infection but, following ligation of the BCR with Abs to human IgG, 10–40% of the cells can be induced into lytic cycle. To validate the use of the inducible reporter, Akata cells were stably transfected with the HEBO-BMRFlp-rCD2-GFP plasmid. The resulting cell line, designated AKBM, was used to examine by flow cytometry the kinetics of lytic cycle induction and expression of the rat CD2-GFP marker following viral reactivation (Fig. 1, B and C). Expression of rat CD2-GFP occurred with kinetics consistent with early EBV gene expression: within 2 h after expression of the immediate-early BZLF1 nuclear protein, and 5–6 h before the detection of the late VCA p125 (Fig. 1B). Two-color flow cytometry analysis confirmed that rat CD2-GFP was expressed only in cells supporting lytic cycle (Fig. 1C). In conclusion, the induced rat CD2-GFP reporter protein is a specific marker of (early) EBV lytic cycle gene expression, thereby allowing further analysis of the population of productively infected AKBM cells.

**Surface HLA class I and class II are down-regulated in reporter-positive EBV⁺ cells in lytic cycle**

A common feature of productive infection by herpesviruses is the modulation of surface expression of host cell proteins to escape from immune elimination (1–4). With regards to EBV, we previously reported on two EBV-transformed B lymphoblastoid cell lines (B-LCL) displaying spontaneous entry into lytic cycle in a small subpopulation of cells that cell surface HLA class I and class II were reduced by up to 5-fold in BZLF1⁺ cells (25). The AKBM model now allowed us to extend these findings. AKBM cells were typical of Burkitt’s lymphoma cell lines displaying a latency I type of EBV infection and retaining a biopsy-like phenotype (20); this phenotype included a 2- to 3-fold lower level of HLA class I expression at the cell surface relative to the amount typically expressed on normal EBV-transformed B lymphoblastoid cells (data not shown). However, the Burkitt’s lymphoma lines still express substantial levels of HLA class I and class II (Fig. 2). We anticipated, therefore, that if down-regulation of these surface molecules was a general feature of lytic cycle, we should also observe a significant further reduction with the AKBM model line. We first examined the expression of HLA class I molecules at the surface of latently infected AKBM cells (Fig. 2). Following 20 h of stimulation with anti-IgG, HLA class I (detected by mAb W6/32) was reduced on viable cells within the induced AKBM population that produced green fluorescence due to the expression of rat CD2-GFP (Fig. 2A, top panels). This marked reduction in W6/32 staining was not observed in rat CD2-GFP⁻ AKBM cells in the same culture, nor was it seen when an EBV⁻ subclone of Akata (2A8) was incubated with anti-human IgG Ab (Fig. 2A, bottom panels). The latter observation rendered it unlikely that a reduction in HLA class I is phenotypically associated with plasma cells differentiated upon cross-linking the BCR. Rather, these combined data indicate that the reduction of HLA class I expression at the surface of productively EBV-infected B cells is a consequence of expression of viral lytic phase gene products.

Next, we examined whether surface expression of other cellular proteins was also modulated in the rat CD2-GFP⁺ population of anti-IgG-treated AKBM cells (Fig. 2B). At 20 h postinduction, a reduction was observed for HLA class II and CD20 molecules, in addition to HLA class I. CD71 (transferrin receptor) and CD80 and CD86 (B7.1 and B7.2 costimulatory molecules) remained largely unaffected, pointing toward selective down-regulation of HLA class I and class II molecules and CD20 during productive EBV infection. Expression of CD19, another B cell-specific marker that is expressed on AKBM cells, was not affected by induction of lytic cycle (data not shown). No differences in levels of any of these proteins were observed upon cross-linking of the BCR on the EBV⁻ Akata subclone 2A8 (data not shown).

To exclude the possibility that the down-regulation of HLA class I, HLA-DR, and CD20 are a consequence of imminent cell-death due to induction of the EBV lytic cycle, we measured the cell viability by propidium iodide exclusion over a 7-day period following induction of AKBM cells with anti-human IgG (Fig. 3). The viability of the rat CD2-GFP⁺ population was higher than, or similar to the viability of rat CD2-GFP⁻ population for at least 3 days postinduction. From day 4 onward, a steady decrease was observed in the viability of rat CD2-GFP⁺ cells. Thus, cell death was unlikely to cause the reduction in surface expression of HLA class I and class II and CD20 observed at 20 h after induction of EBV lytic cycle.

Based on these combined results, we conclude that EBV lytic gene products induce selective differences in surface protein expression, which could lead to altered immune detection.
HLA class I down-regulation is an early lytic cycle event

We then examined the kinetics of down-regulation of surface HLA molecules. Following stimulation of AKBM cells with Abs to human IgG, aliquots of the cells were harvested at various times over a 24 h period and the viable cells were stained with PE-conjugated Abs to HLA class I or HLA-DR. The samples were then analyzed.

**FIGURE 1.** Construction and validation of a reporter for EBV lytic cycle. A, Schematic representation of the pHEBO-based construct containing a chimeric reporter gene, encoding a fusion protein of rat CD2 with its cytosolic domain replaced with GFP, regulated by a promoter corresponding to the 5′ regulatory sequence of the EBV BMRF1 early gene. A hygromycin-resistance gene allows selection of the plasmid following transfection into human cells. B, Kinetics of induction of EBV lytic cycle genes and the rCD2-GFP reporter in AKBM cells following ligation of the BCR with rabbit Abs to human IgG. AKBM cells are hygromycin-selected stable transfectants of pHEBO-BMRF1p-rCD2-GFP in Akata B lymphoma cells. At the indicated times following addition of anti-IgG Abs, replicate aliquots of cells were harvested, fixed with paraformaldehyde, then permeabilized with Triton X-100 detergent before immunostaining with mAbs BZ.1 (anti-BZLF1 immediate-early protein) or L2 (anti-VCA component encoded by BALF4) and PE-conjugated secondary Abs to mouse IgG. Cells were analyzed for red and green fluorescence by flow cytometry, and the percentage of viable cells showing specific fluorescence is indicated. The error bars represent the SEM for three independent samples in one representative experiment of two performed. C, Two-color fluorescence analysis of selected samples from the same time course experiment. The dot plots show the induction of BZLF1 (y-axis) before rat CD2-GFP (x-axis), and the coexpression of lytic cycle proteins in rat CD2-GFP⁺ cells.
for rat CD2-GFP and PE fluorescence to allow determination of the relative intensity of surface HLA staining in the rat CD2-GFP–
(mainly latent infection) and rat CD2-GFP+ (exclusively lytic cycle)
populations within the same cultures. Fig. 4A shows histograms for selected time points (6, 10, 14, and 24 h) after addition of anti-human IgG in a representative experiment. Fig. 4B shows the ratios of the intensity of HLA staining in rat CD2-GFP+ cells to rat CD2-GFP– cells for all time points in this experiment. The intensity of HLA class I staining on the surface of rat CD2-GFP+ cells began to decrease between 6 and 8 h postinduction, and the maximum 3-fold reduction was observed at 14 h. This reduction is comparable to the 3- to 5-fold reduction previously observed with EBV-transformed B-LCL in which a small subpopulation of cells may spontaneously enter lytic cycle (25). Because this reduction of surface HLA class I in AKBM cells started before onset of expression of late lytic-cycle EBV gene products (compare Figs. 1B and 4B), down-regulation of surface HLA class I is most likely induced by early viral proteins. The time course of the reduction in surface HLA-DR expression appeared to be delayed relative to that of HLA class I, but these results are complicated by an apparent increase in HLA-DR expression within the first 6 h (Fig. 4B).

These data show that the reduction in cell surface expression of both HLA class I and class II is an early lytic cycle event.

Isolation of reporter-positive EBV+ B cells in lytic cycle

To elucidate the mechanism responsible for immunomodulation during productive EBV infection, functional and biochemical assays need to be performed with pure populations of cells in EBV lytic cycle. Having shown that the pHEBO-BMRF1p-rCD2-GFP reporter provided a reliable marker of AKBM cells in EBV lytic cycle, we then exploited this marker to isolate the rat CD2-GFP+ cells by immunomagnetic sorting. A representative experiment showing the efficiency of the sorting procedure is depicted in Fig. 5A. Following ligation of the BCR, rat CD2-GFP expression increased from 15% of AKBM cells at 6 h postinduction to 33% at

![FIGURE 2. Rat CD2-GFP reporter to monitor the surface phenotype of cells in lytic cycle. Cells were incubated with or without anti-human IgG Abs, then harvested at 20 h for immunostaining with Abs to cell surface Ags that were detected by flow cytometry. The results are shown as dot plots of GFP fluorescence on the x-axis, indicating lytic cycle activation, and PE-fluorescence on the y-axis indicating surface Ag expression. A, HLA class I expression, detected with W6/32 Ab on the surface of uninduced AKBM cells (top left), anti-IgG-treated AKBM cells (top right), uninduced 2A8 EBV+ subclone of Akata (bottom left), and anti-IgG-treated 2A8 cells (bottom right). The level of HLA class I was selectively reduced only in the GFP+ subpopulation of anti-IgG-induced AKBM cultures. B, Dot plot analysis of uninduced and anti-IgG-treated AKBM cells stained after 20 h with a negative control Ab, or Abs to HLA class II, CD20, CD71, CD80, or CD86.](http://www.jimmunol.org/)

![FIGURE 3. Viability of B cells in lytic cycle. AKBM cells were incubated with anti-IgG for 2 h, then washed and resuspended in fresh medium in replicate wells. At each time indicated, four replicate cultures were harvested and mixed with propidium iodide to identify viable cells by flow cytometry. The percentage of cells excluding propidium iodide was determined for both the GFP+ (mostly latent EBV infection) and the GFP+ (lytic EBV infection) populations within the anti-IgG-stimulated cultures analyzed. The results for one of two representative experiments are shown, and the SEM of quadruplicate samples was <2% for all time points.](http://www.jimmunol.org/)
24 h after receptor ligation. Staining with the OX34 mAb to rat CD2 and subsequent immunomagnetic sorting using anti-mouse IgG2 beads resulted in the positively sorted population containing 95 and 96% rat CD2-GFP cells at 6 and 24 h postinduction, respectively. Intracellular staining revealed that the immediate-early BZLF1 protein was expressed in a similarly high percentage of sorted cells at both time points (81 and 78%), whereas expression of late VCA was detected in 2 and 68% of the sorted cells at 6 and 24 h, respectively (Fig. 5B). Thus, the reporter-positive cells proceed normally through the productive phase of EBV infection. Moreover, these data indicate that the sorting strategy can be used at different time points postinduction, yielding cell populations at various stages of the replicative cycle. The combined results demonstrate the usefulness of our strategy for obtaining almost pure populations of productively EBV-infected cells, and now permit functional and biochemical studies to elucidate mechanisms of immune evasion exploited by EBV.

Total steady-state levels of HLA class I and class II are not reduced in EBV lytic cycle

To investigate whether the down-regulation of surface HLA class I and class II is dependent on reduced total cellular levels of these proteins, we performed Western blot analysis at 20 h postinduction...
Cell lysates were generated from equal numbers of uninduced AKBM cells and rat CD2-GFP/H11001 cells isolated from induced, productively infected AKBM cells. The cell lysates were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes followed by immunostaining with specific Abs. Parallel blots were probed for EBV lytic cycle proteins, actin, HLA class I H chains, HLA-DR α-chain, and HLA-DR β-chain. These immunoblots did not reveal any marked differences in the
The EBV lytic cycle proteins are selectively sorted for rat CD2-GFP expression. In uninduced control AKBM cells, a model peptide was efficiently translocated across the ER membrane in an ATP-dependent manner (Fig. 6B). In contrast, peptide translocation was substantially impaired in AKBM cells that had entered the EBV lytic cycle (around 70% reduction, Fig. 6B). These results suggest that EBV encodes a protein capable of interfering with peptide transport by TAP. The resulting lack of peptides would cause ER retention of the empty HLA class I molecule, which could explain the observed down-regulation of class I at the surface of productively infected EBV+ cells.

In primary B cells incubated with EBV supernatants containing viral IL-10, TAP transport was deduced to be inhibited because of diminished protein expression of TAP1 (29). Additionally, there are examples herpesvirus proteins interfering with TAP, in which protein expression levels of TAP are affected by targeted degradation (27, 30). We used Western blot analysis to evaluate whether the block in TAP-mediated peptide transport observed in EBV lytic cycle (Fig. 6B) was a consequence of reduced TAP protein expression. Total TAP1 and TAP2 protein levels were both found to be comparable irrespective of whether the lytic program of EBV genes was expressed (Fig. 6C). Because equal amounts of cell lysates were loaded (see actin blots), this observation indicates that the block in peptide transport is not a consequence of targeted degradation of TAP, but rather supports a posttranslational block in TAP function imposed by productive EBV infection.

**Discussion**

A prerequisite for successful analysis of immune evasion strategies during productive EBV infection is the availability of an in vitro culture system that allows efficient progression through the complete viral lytic cycle. In the absence of a fully permissive in vitro infection model for EBV, most studies have focused on induction of the productive phase in latently infected, EBV-transformed B cells, e.g., by surface Ig cross-linking (20, 31), by transfection with expression vectors encoding EBV transactivators (32, 33), or by treatment with chemical inducers (20, 34, 35). For our experiments, chemical induction with broad-acting agents such as phorbol esters and sodium butyrate is not an attractive approach because in addition to activating EBV lytic genes, these agents also induce expression of many cellular genes, including those coding for HLA molecules (36). Thus, in the current study we used the EBV+ Akata cell line that is particularly susceptible to viral reactivation via ligation of the BCR.

All the aforementioned induction methods share the drawback that only a minority of cells enters the EBV lytic cycle (12, 20). To overcome this limitation, the subpopulation of EBV-producing cells was isolated by immunomagnetic sorting on the basis of cell surface expression of specific molecules associated with replicative infection. One possible approach was to immunosort for virus-encoded lytic phase membrane Ags, such as the major envelope protein gp350. However, the EBV envelope proteins appear with late kinetics (12), so that only cells that have almost completed lytic cycle could be isolated in this way. In addition, many EBV+ B cell lines express cellular receptors for EBV, including the gp350 counterpart CD21. When virus particles, carrying the late membrane Ags in their lipid envelope, are released from lytic cells, these can bind to neighboring latently infected cells that carry the specific receptor, thereby reducing the purity of productively EBV-infected cells sorted on the basis of late membrane viral Ag expression.

**FIGURE 6.** A, Analysis of steady-state levels of total cell HLA by Western blot. AKBM cells were induced by ligation with anti-IgG for 20 h, then immunosorted as in Fig. 5 to obtain a population of AKBM cells in lytic cycle. Samples of uninduced AKBM and lytic AKBM cells were lysed and separated by SDS-PAGE then analyzed by Western blot with Abs specific for different viral and cell Ags: mAb OT13N2, reactive with EBV early Ags; polyclonal rabbit anti-actin; the mAb, 148.3, recognizing TAP1; and the mAb, 435.4, recognizing TAP2. B, Analysis of steady-state levels of total cell HLA by Western blot. AKBM cells, a model peptide was efficiently translocated across the ER membrane in an ATP-dependent manner (Fig. 6B). C, Analysis of steady-state levels of total cell TAP1 and TAP2 by Western blot. Samples of uninduced and lytic populations of AKBM cells, obtained as described, were analyzed by Western blot using: a polyclonal rabbit anti-actin; the mAb, 148.3, recognizing TAP1; and the mAb, 435.4, recognizing TAP2.
We circumvented the difficulties associated with immunosorting for a virus-encoded membrane protein by developing an alternative, EBV lytic cycle-induced cell membrane marker, rat CD2-GFP. As a proof-of-principle, EBV + Akata cells were stably transfected with a plasmid coding for the rat CD2-GFP reporter, the expression of which was regulated by the upstream insertion of the promoter region for the early lytic cycle gene BHRF1 (Fig. 1). Following ligation of surface IgG on the resulting AKBM cells, a subpopulation representing 10–40% of cells entered the lytic phase of EBV infection. Expression of the rat CD2-GFP reporter was restricted to productively infected cells and occurred with kinetics indistinguishable from those of early EBV lytic cycle gene expression (Fig. 1). This method allowed immunosorting of cells at both early and late stages of lytic cycle, typically with >90% purity (Fig. 5). Thus, we have shown the feasibility of this approach in Akata cells; in addition, this reporter system could be introduced into any EBV + cell line for the study of lytic cycle functions. Furthermore, a similar approach may be applicable to other viruses that do not result in efficient productive infections in vitro, e.g., Kaposi’s sarcoma-associated herpesvirus.

The purpose of immune escape during lytic cycle would be to create a window for EBV production and transmission. In other words, the longer the productively infected cells live and the better they hide from immune elimination, the more viral progeny can be produced. In this context, we examined the life span of our reporter-positive EBV + cells in detail upon synchronous activation of the lytic cycle from latency. During the first 2 days, AKBM cells induced into EBV lytic cycle appear to have a slight survival advantage over latently infected cells (Fig. 3). This could be a consequence of expression of the viral bcl-2 homolog, encoded by the early BHRF1 gene, which has been shown to enhance survival of B lymphocytes (37) and would represent a general evasive strategy by blocking the induction of apoptosis. Viability of productively infected, compared with latently infected EBV + cells, was not reduced until 4 days postinduction, and even after 5 days 50% of lytic cells were viable (Fig. 3). These data substantiate previous circumstantial evidence suggesting that EBV lytic cycle can last for several days (13, 14), and they favor a role for immune evasion by cells in lytic cycle, although ultimately these cells will die.

As EBV-infected cells progress from the latent state to the replicative cycle of infection, surface expression of HLA class I and II is down-regulated (Fig. 2) (25), thereby reducing the chances for T cell-mediated elimination. Our experiments with synchronous induction of lytic cycle in AKBM cells demonstrated that this reduction was initiated after expression of early EBV genes and became more prominent in the course of 24 h of productive infection (Figs. 1 and 4). Therefore, the reduction in cell surface HLA appears to be induced by expression of lytic phase EBV gene products, rather than as a result of preferential viral reactivation in a subpopulation of cells already expressing low levels of HLA molecules. An interesting consequence of the early lytic phase kinetics of class I down-regulation would be that the efficiency of Ag presentation via HLA class I falls with progress of the EBV lytic cycle. This prediction is supported by recent findings that the epitope specificities of EBV-reactive CD8 + T cells generated from the blood of infectious mononucleosis patients are biased toward immediate-early and early viral gene products, and are only rarely directed against late EBV gene products (38).

Immune evasion through down-regulation of HLA class I and class II molecules at the surface of virus-infected cells could result from general evasive strategies, from more specific interference with the Ag processing and presentation machinery, or a combination of both. Regarding specific viral interference with Ag processing for HLA class I presentation in productively EBV-infected cells, we report for the first time that peptide transport by TAP is hampered upon entry into the lytic cycle (Fig. 6). The TAP transporters appear to be attractive targets for immune evasive strategies, because subfamily members α-, β-, and γ-herpesviruses have been shown to block TAP-mediated peptide transport as a means of corrupting the MHC class I processing pathway for Ag presentation to T cells (see reviews in Refs. 1–4). Different mechanisms are used to this end. HSV-encoded ICP47 competes with cytosolic peptides for the peptide binding sites on TAP (39–41), and HCMV US6 associates with ER-luminal parts of TAP, resulting in conformational alterations that prohibit ATP hydrolysis and thereby translocation of peptides across the ER membrane (42–44). Thus, these α- and β-herpesviruses proteins directly interfere with the peptide-transporting function of TAP. In addition, there are examples in which protein levels, rather than the functioning, of TAP are affected as a consequence of herpesvirus gene expression. For example, the murine γ-herpesvirus, MHV-68, encodes an MK3 protein with the ability to target TAP for ubiquitination and proteosomal degradation (30). Interestingly, the recently identified α-herpesvirus BHV-1-encoded inhibitor of TAP uses a combination of the two mechanisms by locking the TAP transporter in a translocation-incompetent state as well as by inducing degradation of its components (27, 45).

For EBV, others have reported that viral IL-10 when provided exogenously leads to a reduction of TAP1 protein expression and down-regulation of HLA class I at the surface of B cells (29). Although this property of viral IL-10 may contribute to immune evasion by EBV, it cannot explain our observations. Firstly, viral IL-10 is a late lytic cycle gene product, whereas our data (Fig. 3) (25) show that the reduction of surface HLA expression is due to early EBV gene expression. Secondly, at 20 h following viral reactivation, steady-state protein levels of TAP1 remain unaffected, but the ability to translocate peptides is diminished by >70% (Fig. 6). In this respect, EBV seems to share the features of HSV and HCMV. However, EBV encodes no obvious homologue of either ICP47 or US6. Future experiments will focus on identification of the EBV early lytic phase gene product responsible for inhibition of TAP-mediated peptide transport.

T cell responses to several peptides of immediate-early, early, and late EBV Ags have been detected (38, 46–50) but, owing to the difficulty of isolating cells in lytic cycle, these effectors have never been demonstrated to specifically eliminate productively infected EBV + target cells. Our reporter system opens the way to test this experimentally by isolating the small population of cells spontaneously entering lytic cycle among EBV transformed B-LCL derived from the same donor as the effector T cells. These studies will also be informative to evaluate the extent of immune evasion during productive EBV infection. To escape from recognition by CD8 + CTL, the combination of impaired TAP function and down-regulated HLA class I surface expression should result in a relatively efficient block in presentation of lytic Ag-derived peptides. In the case of HLA class II Ag presentation, the reduced amount of class II molecules present at the cell surface will increase the chances of EBV gp42 covering all class II complexes to avoid CD4 + Th cell detection.

In conclusion, this study shows that by using a novel strategy to study productive EBV infection, we were able to demonstrate for the first time that early lytic phase EBV gene expression causes a block in TAP-dependent peptide transport. The resulting down-regulated surface expression of HLA class I is likely to contribute to EBV immune evasion to allow for generation and spread of viral progeny.
References