Human herpesvirus 8 (HHV-8) is the etiological agent of Kaposi’s sarcoma, primary effusion lymphoma, and some forms of multicentric Castleman’s disease. Although latent HHV-8 DNA can be detected in B cells from persons with these cancers, there is little information on the replication of HHV-8 in B cells. Indeed, B cells are relatively resistant to HHV-8 infection in vitro. We have recently shown that DC-SIGN, a C-type lectin first identified on dendritic cells (DC), is an entry receptor for HHV-8 on DC and macrophages. We have also demonstrated previously that B lymphocytes from peripheral blood and tonsils express DC-SIGN and that this expression increases after B-cell activation. Here we show that activated blood and tonsillar B cells can be productively infected with HHV-8, as measured by an increase in viral DNA, the expression of viral lytic and latency proteins, and the production of infectious virus. The infection of B cells with HHV-8 was blocked by the pretreatment of the cells with antibody specific for DC-SIGN or with mannan but not antibody specific for a cystine/glutamate exchange transporter that has been implicated in HHV-8 fusion to cells. The infection of B cells with HHV-8 resulted in increased expression of DC-SIGN and a decrease in the expression of CD20 and a cystine/glutamate exchange transporter that has been implicated in HHV-8 fusion to cells. The infection of B cells with HHV-8 was blocked by the pretreatment of the cells with antibody specific for DC-SIGN or with mannan but not antibody specific for xCT, a cystine/glutamate exchange transporter that has been implicated in HHV-8 fusion to cells. The infection of B cells with HHV-8 resulted in increased expression of DC-SIGN and a decrease in the expression of CD20 and major histocompatibility complex class I. HHV-8 could also infect and replicate in B-cell lines transduced to express full-length DC-SIGN but not in B-cell lines transduced to express DC-SIGN lacking the transmembrane domain, demonstrating that the entry of HHV-8 into B cells is related to DC-SIGN-mediated endocytosis. The role of endocytosis in viral entry into activated B cells was confirmed by blocking HHV-8 infection with endocytic pathway inhibitors. Thus, the expression of DC-SIGN is essential for productive HHV-8 infection of B cells.

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma-associated herpesvirus, is the etiological agent of KS, primary effusion lymphoma (PEL), and some forms of multicentric Castleman’s disease (MCD). The virus is found in endothelial cells of KS lesions but is also detected in B cells of PEL and MCD lesions and the peripheral blood of KS patients (5). However, B cells from normal individuals are relatively resistant to in vitro infection with HHV-8 (8). Attempts to establish productive infections by using B-lymphoblastoid-cell lines have also met with limited success (8). On the other hand, B-cell lines established from B cells from PEL patients, which harbor HHV-8, can be induced to replicate virus by treatment with phorbol esters (37). These PEL B-cell lines have greatly helped studies of lytic and latent HHV-8 infections but are of limited use as models of natural viral infection.

We hypothesized that the lack of permissive infection of B cells and B-cell lines with HHV-8 in vitro is related to the differential expression of the appropriate virus entry receptors. Several proteins have been reported to serve as HHV-8 entry receptors (3, 25, 33). We have shown previously that DC-SIGN, a C-type lectin first identified on dendritic cells (DC) (18), is an entry receptor for HHV-8 on DC and macrophages in vitro (33). DC-SIGN and its isomer DC-SIGNR were initially shown to be restricted in expression in vivo to dermal and lymphatic DC, activated macrophages, and vascular endothelial cells (38, 40, 44). Recent studies from our laboratory and others have demonstrated that B lymphocytes from peripheral blood and tonsils express DC-SIGN and that this expression significantly increases after B-cell activation mediated by CD40 ligand (CD40L) and interleukin 4 (IL-4) (22, 34). These data suggest that DC-SIGN may also serve as an entry receptor on activated B (aB) cells and that its lack of expression on resting B (rB) cells may explain why previous attempts to infect B cells with HHV-8 have been had limited success.

In the present study, we show that activated blood and tonsillar B cells expressing DC-SIGN can be productively infected with HHV-8, as determined by an increase in the level of viral DNA, the expression of lytic and latency-associated viral proteins, and the production of infectious virus. HHV-8 infection could be blocked by the pretreatment of the B cells with anti-DC-SIGN monoclonal antibody (Mab). These results are the first evidence of a fully productive infection of B cells and confirm the role of DC-SIGN as an entry receptor for this virus. This model can provide insight into the life cycle of HHV-8 and a better understanding of its pathogenesis.

MATERIALS AND METHODS

Blood and tonsil donors. Blood from HHV-8-seronegative, healthy donors was used in this study. The HHV-8 antibody status was determined by an immunofluorescence assay (IFA) (33). Freshly excised tonsils were obtained from per-
sons undergoing tonsillectomy. Informed consent was obtained according to University of Pittsburgh Institutional Review Board guidelines.

**Cell lines.** BCL-1 cells (HHV-8-positive, Epstein-Barr virus [EBV]-negative human B cells) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and used as a source of virus. EBV-transformed Raji cells, Raji cells transduced to express DC-SIGN (Raji-DC-SIGN cells) (48), which do not express DC-SIGN, and Raji cells expressing a truncated form of DC-SIGN lacking 20 N-terminal amino acids (Raji-DC-SIGN-A20 cells) were gifts from D. Litman (New York University) and V. KewalRamani (NCI). K562 cells (American Type Culture Collection), an erythroleukemia cell line that does not constitutively express DC-SIGN, were stably transduced with plasmid pcDNA-DC-SIGN (NIH AIDS Research and Reference Reagent Program) by using Lipofectamine 2000 (Invitrogen) and were selected and maintained in medium containing G418 (Invitrogen). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and l-glutamine and were negative for mycoplasma (as determined by a mycoplasma PCR enzyme-linked immunosorbent assay; Roche).

**Preparation of B cells from blood.** Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Hypaque density gradient separation. To obtain purified B cells, cell populations were depleted of monocytes by two rounds of incubation with anti-CD14 MAAb-coated immunomagnetic microbeads according to the instructions of the manufacturer (Miltenyi). B cells (CD19+ cells) were then isolated from the CD14- cell fraction by incubation with anti-CD19 MAAb-coated magnetic microbeads (Miltenyi) (34). The purity of the fractionated B cells, as determined by staining with anti-CD20 MAAb, was >95%, with <1% CD14+ and CD3+ cells. aB cells were generated by the culture of CD19+ cells in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1,000 U of recombinant human IL-4 (R&D Systems)/ml, and 1 μg of soluble trimeric CD40L (Angen)/ml. We have previously shown that this method of positive selection does not alter the B-cell function or phenotype (34).

**Preparation of B cells from tonsils.** After surgical removal, tonsils were immediately transferred to the laboratory in cold phosphate-buffered saline supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (5 μg/ml), amphotericin B (0.5 μg/ml), and 5% FCS and processed into single-cell suspensions as previously described (47). Lymphocytes from the collected cell suspensions were isolated by Ficoll-Hypaque density gradient centrifugation. The cells RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 10% FCS were prepared. B cells were incubated at 37°C for 1 h with and without the inhibitors and then infected with HHV-8 at 37°C for 2 h, washed, and further incubated in complete medium for 24 h. After the incubation, infection was detected by an indirect IFA for K8.1 and VL-6.

**RESULTS**

**aB cells support productive HHV-8 lytic and latent infection.** We have previously demonstrated the expression of HHV-8 lytic- and latency-cycle proteins in infected DC and macrophages in the absence of productive virus infection (33). That is, after the infection of DC or macrophages with HHV-8, we observed an increase in the number of cells expressing viral lytic- and latency-cycle proteins without increases in viral DNA over several days of culture. This pattern of viral gene expression was consistent with that shown for HHV-8 infection of vascular endothelial cells (28, 33). In the present study, we determined whether B cells, a major target of HHV-8 infection in vivo, can support productive HHV-8 infection in vitro. B cells were isolated to >95% purity from peripheral blood mononuclear cells by CD19-specific magnetic bead separation and either left untreated (rB cells) or treated with CD40L and IL-4 for up to 36 h (aB cells). We first assessed HHV-8 infection of these B cells by analyses of viral lytic- and latency-cycle protein expression. The infection of B cells with HHV-8 did not result in detectable virus infection over 3 days of culture, as shown by the lack of expression of the K8.1 and ORF59 lytic proteins (Fig. 1A, panels a and b). In contrast, similar to our results with HHV-8 infection of DC and macrophages, parallel cultures of purified, infected aB cells expressed both of xCT mRNAs were calculated relative to the expression level of the endogenous control mRNA for β-glucuronidase (β-GUS; Applied Biosystems).

**Generation of anti-xCT polyclonal antibodies.** Rabbit antisem to specific for the xCT peptide located in the extracellular domain (amino acids 218 to 232 [peptide P218-232]; TQNFKDAFGRDSSI) (26) was generated. All rabbit immunizations and serum collections were performed commercially by Genosys-Sigma. IFAs. IFAs of HHV-8-infected and mock-infected cells were done using anti-HHV-8 ORF73 protein rat MAb and mouse MAb directed against ORF-K8.1/AB or ORF59 protein (AB) or polyclonal rabbit anti-viral IL-6 (anti-VI-L6) IgG (AB). Cells were counted and spotted onto poly-L-lysine-coated microscope slides, fixed in 4% parafomaldehyde for 20 min, and permeabilized with buffer (0.55% bovine serum albumin, 0.1% saponin, 0.1% NaNO3) for 20 min at room temperature. Cells were incubated with the primary antibody (AB) for 30 min at 4°C, washed extensively in buffer, and then stained with fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse or anti-rat IgG (Sigma). Controls were the omission of the primary Ab and the staining of uninfected cells. In some experiments, cells were also stained by direct immunofluorescence with FITC-conjugated anti-DC-SIGN MB (clone 1280; R&D Systems) or with K8.1/AB or ORF59 MAb directly conjugated with phycoerythrin or Texas Red dyes by using a Zenon labeling kit (Molecular Probes). To avoid nonspecific binding of IgG, a blocking step using SuperBlock blocking buffer (Pierce) was added. In some experiments, cells were also incubated with anti-xCT rabbit antiserum, washed, and stained with anti-rabbit IgG conjugated with Alexa Fluor 350 or FITC.

**Flow cytometric analysis of cell phenotypes.** The expression of cell surface molecules was assessed by flow cytometry (with a Beckman Couler XLI instrument) using FITC-conjugated MAAb specific for HLA-A, HLA-B, HLA-C, CD20, CD23, CD54, CD40, CD86, and CD83, and DC-SIGN (CD209) for 30 min at 4°C, and then cells were fixed with 1% paraformaldehyde. Cells were stained with isotype antibody or goat anti-mouse IgG–FITC as controls.

**Inhibition of endocytosis.** Chlorpromazine HCl (10 μg/ml), NH4Cl (50 mM), and bafilomycin A1 (BFA-1; 50 μM) were used to inhibit endocytosis, as described previously (1). The stock solutions were prepared in accordance with the manufacturer's recommendations (Sigma), and working dilutions in complete medium were prepared. B cells were incubated at 37°C for 1 h with and without the inhibitors and then infected with HHV-8 at 37°C for 2 h, washed, and further incubated in complete medium for 24 h. After the incubation, infection was detected by an indirect IFA for K8.1 and VL-6.
FIG. 1. HHV-8 infects and replicates in B cells. (A and B) rB cells and aB cells were infected with HHV-8 and stained with MAb against DC-SIGN (green fluorescence) and anti-K8.1 (24 h) or anti-ORF59 protein (48 h) (red fluorescence) or with MAb against ORF73 protein (72 h; red fluorescence) (panels a, b, and c, respectively). Uninfected B cells were negative for each of the three viral proteins (data not shown). (C) Expression of viral lytic and latency proteins in HHV-8-infected aB cells over time (bars represent means ± standard errors [SE]; K8.1, n = 11; ORF59 protein, n = 4; ORF73 protein, n = 5). For each slide, at least four independent fields were counted and the results were averaged. (D) Quantitative HHV-8 DNA results from seven separate experiments with B cells from seven different donors. B cells were assayed by real-time PCR analyses of extracts of rB-cell or aB-cell pellets and the corresponding DNase-resistant, concentrated cell culture supernatants (sups). Both uninfected cell pellets and supernatants were negative for HHV-8 DNA (data not shown). (E) Cell counts (bars) and viability (lines) for uninfected (blue) and HHV-8-infected (gray) aB cells (means ± SE; n = 13).
these proteins after 24 and 48 h of infection (Fig. 1B, panels a and b). The infected aB cell cultures exhibited peak mean expression of the ORF59 protein at 24 h (36% ± 5% of the cells were positive for the protein; n = 4) and K8.1 at 48 h (22% ± 4% of the cells were positive; n = 11) (Fig. 1C). The latency protein encoded by the ORF73 gene (LANA-1) was first evident at 48 h postinfection, with 10% ± 3% (n = 4) of the aB cells being positive (Fig. 1C). By 72 h postinfection, 30% ± 3% of the cells were positive for the ORF73 protein (Fig. 1B, panel c, and C). It should be noted that the data in Fig. 1C reflect the percentages of immunofluorescent-antibody-positive cells among all of the B cells in the cultures, including both DC-SIGN-positive and -negative cells. However, our IFA data indicated that only DC-SIGN-expressing B cells also expressed viral proteins, confirming the hypothesis that DC-SIGN expression is required for the infection of B cells in culture. Finally, we have found that this effect is not unique for the Amgen trimeric CD40L used in this study, as other commercial preparations of CD40L, i.e., those from Alexis and R&D Systems, together with IL-4, also upregulated the expression of DC-SIGN on B cells and made the cells permissive for HHV-8 infection (data not shown).

We next assayed the cells and culture media for levels of HHV-8 DNA. In contrast to the nonproductive HHV-8 infection of DC and macrophages, in which there is no increase in HHV-8 DNA over time and no release of virus into the cell culture supernatant (33), we observed a significant increase in the numbers of copies of HHV-8 DNA in cultures of purified aB cells derived from healthy, HHV-8-seronegative donors (Fig. 1D). Indeed, compared to the amount at time zero, there was an average 10-fold increase in the amount of HHV-8 DNA in aB-cell culture supernatants at 24 h (P < 0.05; paired t test). There was some variation in levels of HHV-8 DNA in the supernatants of cultures of cells from the seven donors at 48 and 72 h. There was a sixfold increase in the HHV-8 DNA levels in the cell pellets at 24 h (P < 0.05), and these levels decreased at 48 and 72 h. There was no increase in HHV-8 DNA in the infected rB-cell cultures. This pattern of viral DNA replication was consistently found in B cells obtained from seven different donors in multiple, replicate experiments (Fig. 1D). The pattern was accompanied by a relatively small but significant decrease in cell viability among the infected aB cells compared to that among the uninfected aB cells, as determined by trypan blue exclusion analysis and cell recovery from the HHV-8-infected cultures (e.g., viability levels were 84.7% ± 6% for uninfected aB cells and 92.8% ± 1.4% for infected aB cells at 48 h; P = 0.05) (Fig. 1E).

We next examined whether there was production of infec-
tious HHV-8 in the aB-cell cultures by determining if we could transfer infectious virus to uninfected B cells by using supernatants from our HHV-8-infected B-cell cultures. For this experiment, supernatants from the 24- and 48-h primary cultures of HHV-8-infected aB cells were centrifuged at 400 g for 10 min and mixed undiluted with a fresh culture of uninfected aB cells. Supernatants and pellets from the secondary cultures of aB cells were examined for HHV-8 DNA and protein at daily intervals, as were the primary aB-cell cultures. The results show that infectious virus was produced in the secondary aB-cell cultures, as evidenced by increases in HHV-8 DNA levels in the supernatants (Fig. 2A) and pellets (data not shown) and the percentages of aB cells expressing lytic and latent proteins (Fig. 2) from 24 to 72 h postinfection.

Taken together, these results indicate that HHV-8 establishes an infection in CD40L- and IL-4-treated aB cells, with the production of lytic and latency proteins, viral DNA, and infectious virus and a moderate decrease in the number of viable cells.

HHV-8 requires DC-SIGN for the infection of B cells. We have recently shown that an average of 8% of peripheral blood B cells constitutively express DC-SIGN (CD209) on their surfaces and that this level increases to an average of 29% after treatment in vitro for 1 day with CD40L and IL-4 (34). We have also reported that DC-SIGN is a receptor for HHV-8 entry into monocyte-derived DC and macrophages (33). To address the question of whether DC-SIGN is a surface receptor for HHV-8 on B cells, we first assessed the binding of radioactively labeled virus to rB and aB cells that were left untreated or pretreated with anti-DC-SIGN MAb or mannan, which is a natural ligand for DC-SIGN.
As shown in Fig. 3A, the level of binding of HHV-8 to aB cells was greater than that of binding to rB cells and this binding was inhibited by pretreatment with anti-DC-SIGN MAb or mannan (P < 0.05). The low level of binding to the rB cells that we measured may be due to the low levels of DC-SIGN or to binding to heparan sulfate, since HHV-8 is known to attach to this surface molecule (4).

We next addressed whether the blocking of DC-SIGN by anti-DC-SIGN MAb or mannan could inhibit the infection of B cells by HHV-8. As shown in Fig. 3B, the pretreatment of aB cells with either anti-DC-SIGN MAb or mannan inhibited HHV-8 infection, as determined by the expression of viral proteins at 24 h. The pretreatment of cells with control, mouse IgG did not inhibit viral infection (data not shown). The pretreatment of aB cells with anti-DC-SIGN MAb also reduced the amounts of viral DNA replication and encapsidated viral DNA in cell culture supernatants by 87% and those in cell pellets by 63% at 24 to 72 h (Fig. 3C). Moreover, the inhibitory effect of the anti-DC-SIGN MAb on HHV-8 infection was dose dependent (Fig. 3D).

We next confirmed our previous results (27) that conventional Raji cells and K562 cells, which do not express DC-SIGN, cannot be infected with HHV-8 but that Raji and K562 cells stably transfected to express DC-SIGN (Raji-DC-SIGN and K562-DC-SIGN cells) are susceptible to HHV-8 infection, as demonstrated by the production of lytic (Fig. 4A and C) and latency (data not shown) proteins. We extended these results by showing that HHV-8 established a productive infection in the DC-SIGN-expressing Raji B-cell and K562 cell lines, as determined by an increase in viral DNA in both cell pellets and cell culture supernatants within 24 h postinfection (Fig. 4B and D).

Taken together, these results support the conclusion that the expression of DC-SIGN is essential for HHV-8 infection of peripheral blood B cells, B-cell lines, and non-B-cell lines.

Role of xCT in HHV-8 infection of B cells. Recently, xCT, the 12-transmembrane light chain of the human cystine/glutamate exchange transporter system xC−, has been reported to be involved in cell fusion and the entry of HHV-8 into several types of continuous cell lines (26). We therefore examined the role of xCT in the infection of B cells by the procedures of Kaleeba and Berger (26) using antisera from rabbits immunized with a peptide derived from the extracellular portion of the xCT protein (peptide P218-232) to inhibit HHV-8-cell fusion and entry into target cells. We first confirmed the observations of Kaleeba and Berger (26) that rB cells do not express xCT protein or mRNA (Fig. 5). However, we found that aB
cells derived from rB cells by treatment with CD40L and IL-4 for 24 h expressed xCT protein (Fig. 5A) and mRNA (Fig. 5B). Moreover, in contrast to Kaleeba and Berger (26), we observed that the K562 erythroleukemia cell line constitutively expressed xCT, as determined by both protein and mRNA analyses (Fig. 5A). The expression of xCT in the K562 cells was confirmed by Western blotting (data not shown). Thus, both K562 and K562-DC-SIGN cells express xCT, while only K562-DC-SIGN cells express DC-SIGN. We have previously reported that K562-DC-SIGN cells but not the parental K562 cells are susceptible to HHV-8 infection (33). In the present study, we confirmed that K562-DC-SIGN cells could be infected with HHV-8 (Fig. 6A), comparably to aB cells (Fig. 6B), and that HHV-8 could not infect either K562 cells or rB cells.

We next used MAb directed against DC-SIGN and antisera specific for xCT P218-232 to try to block the infection of aB cells and K562-DC-SIGN cells. The pretreatment of K562-DC-SIGN cells with anti-DC-SIGN MAb inhibited HHV-8 infection, as shown by the blocking of the expression of K8.1, while blocking with anti-xCT antisera had a minimal inhibitory effect (Fig. 6C). These data were confirmed using aB cells, for which the blocking of DC-SIGN inhibited HHV-8 infection by over 80% at 24 h but the blocking of xCT resulted in a decrease of only 15% in the level of aB cells expressing K8.1 (Fig. 6D). The pretreatment of the aB cells with anti-DC-SIGN MAb also decreased levels of HHV-8 DNA in cells and supernatants by 68 and 99%, respectively (Fig. 6E). Pretreatment with xCT antisera, on the other hand, reduced HHV-8 DNA levels in cells and supernatants by 8 and 20%, respectively (Fig. 6E).

Taken together, these data indicate that the infection of peripheral blood B cells and B-cell lines with HHV-8 requires the expression of DC-SIGN but is not dependent on xCT expression.

**HHV-8 infects tonsillar B cells via DC-SIGN.** We have previously shown that B cells derived from tonsils constitutively express DC-SIGN at levels comparable to those on in vitro-activated peripheral blood B cells, i.e., an average of 26% of the cells are positive for DC-SIGN, and they coexpress B-cell activation markers (34) (a representative example is presented in Fig. 7A). Therefore, we examined whether this level of expression of DC-SIGN was sufficient to allow the infection of tonsillar B cells by HHV-8, without pretreatment with CD40L and IL-4. We found that the tonsillar B cells were susceptible to HHV-8 infection, with approximately 30% of the total B-cell population expressing viral K8.1 protein by 24 h postinfection (data not shown). Double staining of the infected cells with anti-DC-SIGN and K8.1 MAb demonstrated that only cells expressing DC-SIGN were infected with HHV-8, as evidenced by the coexpression of K8.1 (Fig. 7B). The infection of tonsillar B cells also resulted in a fivefold increase in viral DNA by 24 h and a peak level of virus in the supernatant by 48 h postinfection (Fig. 7D). Furthermore, over 95% of HHV-8 infection of the tonsillar B cells was blocked by the pretreatment of the cells with anti-DC-SIGN MAb (Fig. 7B).

We next examined tonsillar B cells for the presence of xCT. We could not distinguish xCT protein by IFA of tonsillar B cells derived from five individuals due to high levels of background staining with the control rabbit preimmune serum (data not shown). However, we detected xCT mRNA in only one of the five tonsillar B-cell preparations (Fig. 7C). We attempted to infect these tonsillar B cells in the presence of anti-xCT antiserum and stained them for K8.1 expression 24 h

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**FIG. 5.** Expression of xCT on B cells and K562 cells. (A) Freshly isolated rB or aB cells or K562 or K562-DC-SIGN cells were incubated with rabbit antiserum against xCT peptide and goat anti-rabbit IgG–FITC and counterstained with DAPI. (B) DC-SIGN- and xCT-specific mRNA levels in K562, K562-DC-SIGN, rB, and aB cells. The levels are expressed relative to mRNA levels for the β-GUS gene (an internal housekeeping gene) in these cells.
postinfection. As shown in Fig. 7B, bottom right panel, treatment with anti-xCT antisera did not interfere with the infection of these cells with HHV-8. These results indicate that DC-SIGN but not xCT expression is required for the infection of tonsillar tissue B cells.

HHV-8 infection of B cells increases the expression of DC-SIGN. We have previously reported that HHV-8 infection of DC and macrophages induces the downregulation of DC-SIGN expression within 24 h, with a progressive loss through 72 h postinfection (33). Therefore, we studied the expression of DC-SIGN and other costimulatory molecules in infected B cells compared to that in uninfected B cells. We observed a significant increase in the percentage of aB cells expressing DC-SIGN (CD209) by 24 h after HHV-8 infection (Fig. 8) \((P = 0.05\) compared to uninfected aB cells at 24 h or infected aB cells at 0 h). In contrast to the enhancing effect of HHV-8 infection on DC-SIGN expression in aB cells, there was downregulation of the expression of CD20, as demonstrated both by the percentage of positive cells and by the mean fluorescence intensity (MFI) by 24 h postinfection \((P < 0.05\) compared to uninfected aB cells at 24 h or infected aB cells at 0 h). We also noted the downregulation of the MFI for HLA-A, HLA-B, and HLA-C in HHV-8-infected aB cells by 24 h, i.e., from 1,617 MFI in the uninfected aB cells to 989 MFI in the virus-infected aB cells by 24 h postinfection \((P < 0.05\). Finally, there was some downregulation of the MFI for CD86 in the infected aB cells by 48 h postinfection \((P < 0.05\) compared to uninfected aB cells at 48 h or infected aB cells at 0 h). No differences in the expression of CD23, CD54, CD80, CD83, or CD86 between uninfected and HHV-8-infected aB cells were observed. Taken together, these results indicate that HHV-8 infection of aB cells results in the upregulation of DC-SIGN expression and the downregulation of CD20, major histocompatibility complex (MHC) class I, and CD86 expression. This result may be related to the downmodulation of HLA and CD86 expression by HHV-8 gene products MIR1 and MIR2 (12, 13).

HHV-8 enters B cells through endocytosis. The entry of HHV-8 into human fibroblasts and endothelial cells occurs by an endocytotic pathway (1). Moreover, human immunodeficiency virus type 1 appears to enter DC by endocytosis after attachment to DC-SIGN (29). To determine if the entry of HHV-8 into B cells is mediated by endocytosis, we first utilized Raji B cells, which do not express DC-SIGN and are resistant to infection with HHV-8 (33). The Raji cells were transfected with DC-SIGN (yielding Raji-DC-SIGN cells) or with a mutant form of DC-SIGN (yielding Raji-DC-SIGN-Δ20 cells), in

![Fig. 6. Effect of anti-xCT and anti-DC-SIGN Ab treatment on HHV-8 infection.](http://jvi.asm.org/)

FIG. 6. Effect of anti-xCT and anti-DC-SIGN Ab treatment on HHV-8 infection. (A and B) K562-DC-SIGN cells (A) or aB cells (B) were infected with HHV-8 and stained for DC-SIGN (green), xCT (blue), or K8.1 (red). Each of the first three panels in each row shows the individual dual combinations, while the fourth panel shows the three-color overlay. (C and D) K562-DC-SIGN cells (C) or aB cells (D) were treated with anti-xCT rabbit antiserum (left panels) or anti-DC-SIGN MAb (right panels) or left untreated (A and B) and were infected with HHV-8 for 24 h and then stained for the expression of DC-SIGN, xCT, and K8.1. Each panel represents a three-color overlay as in panels A and B. (E) Relative levels of HHV-8 DNA in the cell pellets and supernatants of cultures pretreated with either anti-DC-SIGN MAb or anti-xCT rabbit antiserum. The levels of inhibition were measured against those of cultures pretreated with mouse IgG or preimmune rabbit antiserum, respectively.
FIG. 7. HHV-8 infects and replicates in fresh tonsillar B cells. (A) B cells were isolated from tonsils (as described in Materials and Methods) by magnetic bead purification. Cells were then stained for the expression of CD20 and DC-SIGN and analyzed by fluorescence-activated cell sorting. (B) Fresh tonsillar B cells were left untreated or treated with anti-DC-SIGN MAb or anti-xCT antisera prior to infection with HHV-8, cultured for 24 h, and stained with anti-DC-SIGN (green) or anti-K8.1 (red) MAb. Nuclei are stained blue with DAPI. (C) xCT-specific mRNA levels in K562, K562-DC-SIGN, aB, and tonsillar B cells. The levels are expressed relative to mRNA levels for the β-GUS gene (an internal housekeeping gene) in these cells. The results shown are representative of two independent experiments. (D) Quantitative DNA results from real-time PCR assays. HHV-8 DNA was extracted from tonsillar B cells and the corresponding DNase-resistant, concentrated cell culture supernatants. Both uninfected cell pellets and supernatants were negative for HHV-8 DNA (data not shown).
FIG. 8. Expression of cellular markers in activated, HHV-8-infected B cells. aB cells were left untreated (white bars) or infected with HHV-8 (black bars) and cultured for 48 h. At the indicated time points, cells were stained for the expression of B-cell markers and analyzed by flow cytometry. rB and aB cells were also analyzed at the onset of infection (0 h). Bars and lines represent the means (± SE) of results from six independent experiments and refer to percentages of positive cells and MFIs, respectively.
which 20 amino acids from the cytoplasmic, N-terminal domain of DC-SIGN were removed to eliminate the putative dileucine-based internalization motif (29). We confirmed our previous results that HHV-8 can enter and express viral proteins in Raji-DC-SIGN cells (33), as shown by the expression of K8.1 by 24 h postinfection (Fig. 9A). In contrast, the Raji-DC-SIGNΔ20 cells did not express K8.1 protein at 24 h (Fig. 9A). These results support the conclusion that full-length DC-SIGN is required for cell entry by HHV-8 and suggest that viral entry involves endocytosis.

We next examined whether HHV-8 infects DC-SIGN-expressing aB cells through endocytosis. Chlorpromazine HCl is a cationic amphiphilic drug that prevents clathrin-mediated endocytosis, whereas NH₄Cl and BFLA-1 inhibit the acidification of endosomes (1). aB cells were infected with HHV-8 in the presence of the different endocytosis inhibitors and compared to infected untreated aB cells. The endocytosis inhibitors blocked >80% of HHV-8 infection of aB cells compared to that of untreated aB cells, as shown by the number of cells expressing viral proteins K8.1 and vIL-6 (Fig. 9B and C). No effect on cell viability after treatment with the inhibitors was observed (data not shown). These results support the conclusion that HHV-8 enters B cells through an endocytic pathway.

**DISCUSSION**

The infection of B cells by HHV-8 was first suggested by the presence of viral DNA and particles within B cells from pa-
tients with body cavity PEL (6, 10, 39) and the isolation of B-cell lines (termed BCBL or BC lines) harboring persistent HHV-8 infection from such patients (37). HHV-8 DNA has also been found in B cells of patients with KS (21, 23) and MCD (15, 43). To date, however, B cells have been relatively resistant to in vitro infection with HHV-8. Early reports indicated that virus can be transferred to CD19+ B cells (30) and can infect B cells during the immortalization of B-cell lines by infection with EBV (27). However, attempts to infect immortalized B-cell lines have been successful only when viral DNA was introduced directly into the cells (11). In fact, there is little previous evidence of productive HHV-8 infection of cultures of freshly isolated B cells (7, 8).

We hypothesized that the paradox in which B cells are one of the major cellular targets of HHV-8 infection in vivo while B-cell lines and freshly isolated B cells are refractory to HHV-8 infection in vitro was due to the differential expression of DC-SIGN in these cells. This hypothesis was based on our previous finding that DC-SIGN expressed on DC and activated macrophages serves as a receptor for HHV-8 (33). The infection of these cell types with HHV-8 results in abortive viral replication, with the production of at least some lytic proteins, the establishment of latency, and little evidence of viral DNA replication, similar to the limited replication of HHV-8 in human endothelial cells and fibroblasts (3, 31, 36, 45). Moreover, we (34) and others (22) demonstrated previously that whereas DC-SIGN is constitutively expressed on only a small number of rB cells and at a low level, the expression of this C-type lectin can be enhanced by activating B cells in vitro with a combination of CD40L and IL-4. The present results indicate that activated peripheral blood B cells support fully productive infection with HHV-8, as shown by the detection of the K3,1, ORF59, and vIL-6 lytic proteins in the infected B cells within 24 to 72 h of infection. Furthermore, we were able to detect increased levels of viral DNA in both the cell pellets and supernatants of infected B cells by 24 h postinfection. In contrast, rB cells did not support HHV-8 replication. Importantly, the increase in viral proteins and DNA reflected an increase in the production of infectious virus, as shown by the transfer of productive viral infection to secondary B-cell cultures. The productive infection of aB cells and the lack of infection of rB cells were consistent findings for cells derived from multiple donors. Interestingly, Blackbourn et al. (8) reported that the treatment of cultures of B cells with the lectin mitogen phytohemagglutinin results in limited HHV-8 replication. We have noted that B cells treated with phytohemagglutinin have an increase in the expression of DC-SIGN (our unpublished results), suggesting that this increased expression may be responsible for the replication of HHV-8 in these aB cells.

We confirmed that DC-SIGN was essential for the infection of aB cells with HHV-8 by blocking both viral DNA and protein production by the pretreatment of the B cells with MAb specific for DC-SIGN or with its natural ligand mannan. After 3 days of infection, HHV-8-established latency, as shown by the expression of LANA-1 protein. Our results on productive HHV-8 infection of aB cells were supported by our finding that HHV-8 productively infected the Raji B-cell and K562 cell lines that had been transfected with DC-SIGN but not the parental strain of DC-SIGN-negative Raji B or K562 cells. To our knowledge, this is the first in vitro model for consistent, fully productive infection of cells naturally targeted by HHV-8 in vivo. We are currently investigating if it is possible to reactivate HHV-8 from latency in the infected B cells and return the virus to the full lytic cycle.

Interestingly, tonsillar B cells were able to support a level of productive HHV-8 infection in vitro similar to that of aB cells without requiring in vitro activation with CD40L and IL-4. A greater proportion of these tonsillar B cells than of peripheral blood B cells constitutively express DC-SIGN and B-cell activation markers (22, 34). Moreover, HHV-8 infection of the tonsillar B cells could be blocked by anti-DC-SIGN MAb, similar to the blocking of HHV-8 infection of activated, blood-derived B cells. Thus, regardless of possible differences in activation states between aB and tonsillar B cells, DC-SIGN was required for productive HHV-8 infection of both cell types. Interestingly, DC-SIGN is differentially expressed by B cells in the parafollicular regions of lymphatic tissues (22). Several investigations have found HHV-8-infected B cells in the parafollicular regions of lymph nodes from patients with KS, MCD, and PEL (15, 32, 43). This finding suggests the colocalization of DC-SIGN and HHV-8 in B cells of the lymphatics, which may be a source of virus persistence and development of HHV-8-related lymphomas.

We previously showed that HHV-8 utilizes DC-SIGN to infect DC and activated macrophages (33). The outcome of HHV-8 infection in these cell types was quite different than that of infection in aB cells. First, and most strikingly, the aB cells were permissive for complete virus replication, with the production of infectious virus. In contrast, DC and macrophages, as well as endothelial cells, support the expression of only a limited number of HHV-8 lytic genes early after virus entry and little or no production of infectious virus (28, 33). There also was a limited amount of cell death over 72 h in the infected B-cell cultures compared to that in uninfected cell cultures, suggesting that infectious virus production and subsequent destruction of the cell were restricted to a portion of the aB cells. Second, HHV-8 infection of DC and macrophages resulted in a strong downmodulation of DC-SIGN, whereas DC-SIGN was significantly upregulated in B cells after 24 h of infection with HHV-8. A majority of the DC-SIGN-positive B cells were infected with HHV-8 by 24 to 72 h. This result suggests that the increase in the number of B cells expressing DC-SIGN and the intensity of DC-SIGN expression per cell may be due to direct effects of HHV-8 infection in B cells. The mechanisms for the regulation of DC-SIGN expression are unclear. IL-4 has been shown to upregulate DC-SIGN during the differentiation of monocyte-derived DC through the activation of STAT6, and this effect is oppositely regulated by alpha interferon and gamma interferon (35). Regardless of the basic mechanisms involved, increased expression of DC-SIGN may serve to promote viral spread and replication in the B cells.

We observed a moderate downregulation of MHC class I molecules in HHV-8-infected B-cell cultures. This finding is in agreement with the observations of Ishido et al. (24) and Coscoy and Ganem (12), which implicated the MIR1 and MIR2 products of the K3 and K5 genes in the downregulation of MHC class I expression on target cells, a common immune evasion strategy employed by herpesviruses. This immunomodulatory effect of the virus in B cells may contribute to the
escape of these cells from targeting by cytotoxic T cells, enhancing the development of HHV-8-related cancers. The immunomodulatory effect of HHV-8 infection on antigen presentation may be further enhanced by HHV-8 infection of DC and macrophages. We (46) and others (20) have shown previously that T-cell responses to HHV-8 antigens in infected individuals are not very robust and that DC infected with HHV-8 in vitro are poor antigen-presenting cells (33). We are currently investigating the effect of infection with fully replicating HHV-8 on antigen presentation by B cells.

The 12-transmembrane light chain of the human cystine/glutamate exchange transporter system xCT, xCT, has been reported previously to be involved in cell fusion and the entry of HHV-8 (26). To address the potential role of xCT in our B-cell system, we determined the pattern of expression of this molecule in B cells and cell lines utilized in our study. For this investigation, we used rabbit antiserum raised against the xCT-derived peptide P218-232, which is the same type of antiseraum used to block virus-cell fusion in the previous study of xCT (26). We could not directly distinguish a role for DC-SIGN compared to xCT in blood B cells, as aB cells that could be infected with HHV-8 expressed both xCT and DC-SIGN. However, Raji B-cell and K562 erythroleukemia cell lines expressed xCT yet were nonpermissive for HHV-8 infection. In contrast, these same cell lines transfected to express DC-SIGN were susceptible to HHV-8 infection, as measured by the expression of viral proteins and the detection of increased levels of viral DNA in the infected cells over time. Thus, xCT was present on DC-SIGN-expressing aB cells and cells of a B-cell line and a non-B-cell line that could be infected with HHV-8 but also on the parental, non-DC-SIGN-expressing cell lines that could not be infected with the virus. Furthermore, blocking experiments showed that the pretreatment of B cells with anti-DC-SIGN MAb resulted in a 99% decrease in the detectable amount of viral DNA in the supernatants of the infected cell cultures by 24 h, as opposed to an 8% reduction observed when cells were pretreated with xCT antisera. It is possible that the lack of inhibitory effect of xCT antisera may be due to lower affinity of the anti-xCT polyclonal antibodies in the rabbit sera than of the MAb specific for DC-SIGN. However, further support for the conclusion that DC-SIGN but not xCT was essential for infection with HHV-8 is that we were not able to detect the expression of xCT mRNA in most of the tonsillar B-cell samples that supported productive HHV-8 replication. Further work is necessary to delineate a possible role for xCT in HHV-8 infection of B cells.

Based on the known endocytic internalization of HHV-8 in human endothelial cells and fibroblasts (1) and the finding that other pathogens that bind DC-SIGN enter cells via endocytosis (16), we reasoned that HHV-8 may be internalized into endocytic vacuoles in B cells after attachment to DC-SIGN. To test this hypothesis, we utilized chemical blockers of endocytosis and also cells transduced to express a form of DC-SIGN lacking the transmembrane domain, essential for its endocytic function (29). Our data indicate that HHV-8 enters B cells through an endocytic pathway, as demonstrated by the lack of infection of cells expressing the truncated form of DC-SIGN versus the full-length DC-SIGN. Previous studies showed that in human foreskin fibroblasts, HHV-8 enters through endocytosis via clathrin-coated pits and that infection can be blocked by interfering with the acidification of the endosomes or the formation of clathrin (1). To confirm that this also was the mechanism used by HHV-8 in aB cells, we employed the same antiendocytotic chemicals used in the study by Akula et al. (1) in our B-cell system. Our data showed that the inhibition of clathrin and endosome pH modifications affected the outcome of infection; i.e., we observed strong inhibition of the expression of viral lytic proteins in the infected B cells. Taken together, these data support the conclusion that HHV-8 enters B cells through endocytosis that involves the DC-SIGN cytoplasmic region.

During the past few years, several lines of evidence have pointed to HHV-8’s using different receptors for attachment, binding, and entry depending on the cell type it is targeting, similar to all other herpesviruses (41). Heparan sulfate serves as an attachment molecule, probably concentrating virus on the cell surface to position it for subsequent binding and entry (2). Integrins are involved in as-yet-undetermined postbinding events when HHV-8 infects adherent fibroblasts and endothelial cells (3, 17), but their role in the infection of other cell types has not been investigated. Our data demonstrate that in the three major, professional antigen-presenting cell types, i.e., myeloid DC, macrophages, and B cells, HHV-8 requires DC-SIGN to result in either abortive or fully productive infection. DC-SIGN is known to internalize its ligands through endocytosis, and our data suggest that this is the mechanism of entry of this virus into B cells. We believe that the use of DC-SIGN-expressing aB lymphocytes and cell lines provides a new and unique model to study in vitro viral entry and replication for a major in vivo cell target of HHV-8. This model may provide valuable insights into the biology of this virus and help in the designing of therapeutic strategies to interfere with virus replication in infected individuals.

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