Epstein-Barr Virus Enters B Cells and Epithelial Cells by Different Routes

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Epstein-Barr virus (EBV) infects two cell types, B lymphocytes and epithelial cells. Electron microscopic studies have shown that the virus fuses with the lymphoblastoid cell line Raji but is endocytosed into thin-walled non-clathrin-coated vesicles in normal B cells before fusion takes place. To compare early interactions of EBV with epithelial cells and B cells, a fluorescence dequenching assay of fusion was employed, using virus labeled either with the pH-insensitive probe octadecyl rhodamine B chloride (R₁₈) or with 5(*N*-octadecanoyl) aminofluorescein (AF), which loses emission intensity at a pH below 7.4. Fusion of virus labeled with R₁₈ could be monitored with B cells, Raji cells, and epithelial cells. Lowering the extracellular pH or pretreatment of cells with ammonium chloride or methylamine had no effect on these measurements. In contrast, fusion of virus labeled with AF could be measured with Raji cells and epithelial cells, but not with normal B cells unless cells were previously treated with ammonium chloride. Fusion of virus with normal B cells was inhibited with chlorpromazine, chloroquine, and sodium azide, but none of these reagents had any effect on fusion with Raji or epithelial cells. These results suggest that entry of EBV into nonpolarized suspensions of epithelial cells occurs by fusion at the cell surface, that EBV may be incapable of fusing with normal B cells unless it has first been endocytosed, and that pH appears to be irrelevant to either event. A combination of the two probes, R₁₈ and AF, may have general use for determining the sites of entry of enveloped viruses that fuse in a pH-independent manner.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that is implicated in diseases involving infection of two cell types, B lymphocytes and epithelial cells. The virus causes infectious mononucleosis (10), is associated with B-cell neoplasia (4) and nasopharyngeal carcinoma (5), and induces oral hairy leukoplakia in AIDS patients (8). Infection of the B cell is initiated by attachment of virus to the type 2 complement receptor, CR2 or CD21 (6, 20), and a similar, but probably not identical, molecule may be responsible for binding of virus to the epithelial cell (26, 31). Penetration of the virus through the B-cell membrane can then apparently take one of two routes. Analyses of virus entry by electron microscopy indicated that EBV fuses directly with the plasmalemma of the lymphoblastoid cell line Raji, but that it is endocytosed into thin-walled non-clathrin-coated vesicles before it fuses with normal B cells (18, 24). Further studies with immunotoxins bound to the EBV receptor supported these observations by demonstrating that CD21 is internalized with virus entering freshly isolated B cells but not with virus infecting Raji cells (28). Although a complete understanding of the pathogenesis of EBV-induced disease clearly requires knowledge of the infectious process in all cells that the virus infects, nothing is known about how virus penetrates the epithelial cell.

The fact that EBV can fuse directly at the cell surface of Raji cells at neutral pH would suggest that the fusion process is pH independent. However, lysosomotropic agents such as chloroquine and ammonium chloride have been reported to reduce EBV infectivity (18), and calmodulin antagonists that inhibit endocytosis also block EBV infection of normal B cells (19). There is thus some uncertainty about the require-

ments for virus-cell fusion. Does virus fuse more efficiently from a low-pH environment, is the thin-walled endocytic vesicle from which EBV fuses actually acidified, and is the Raji cell unique in its ability to fuse with EBV at the cell surface? Recently developed fluorescence dequenching assays which allow real-time measurement of virus fusion in isolation from the events that precede or follow it provide a new approach with which to examine these questions (11, 12). Amphiphilic fluorophores are incorporated into virus membranes at the high concentrations at which they are self-quenching. When virus and cells fuse, there is a measurable relief of self-quenching and an increase in fluorescence that can be monitored kinetically. We initially used the pH-insensitive probe octadecyl rhodamine B chloride (R₁₈) to measure the rate of EBV fusion with B cells and determine the involvement of one of the EBV proteins, gp85, in the process (9, 15). The availability of a second probe, 5(*N*-octadecanoyl) aminofluorescein (AF), which significantly loses emission intensity at a pH below 7.4, suggested to us that a combination of R₁₈- and AF-labeled virus might be used to determine the pH of the environment in which EBV fuses and examine whether or not pH affects the rate or extent of fusion. We report here that the sites of fusion of EBV with epithelial cells and normal B cells differ and that although the rates and extent of fusion are not influenced by pH, there may be a unique requirement for endocytosis into normal or recently transformed B cells before virus fusion can occur.

MATERIALS AND METHODS

Lymphoblastoid cell lines. Cell lines were grown at 37°C and diluted at least biweekly in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% heat-inactivated fetal calf serum, 100 IU of penicillin per ml, and 100 µg of streptomycin per ml. These lines were Raji (22), an EBV

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genome-positive, nonproducing human B-cell line that expresses CR2; BAT, a human B-cell line expressing CR2 which was recently transformed in this laboratory with EBV; Molt-4 (17), an EBV genome-negative human T-cell line that expresses CR2 but cannot internalize the virus (16); and MCV5, an EBV-producing marmoset cell line (a gift of George Miller, Yale University, New Haven, Conn.).

Isolation of T-depleted human leukocytes (normal B cells). Leukocytes were obtained from heparinized blood by flotation on Ficoll-Hypaque and depleted of T cells by a double cycle of rosetting with 2-aminoethylisothiuronium bromide-treated sheep erythrocytes and centrifugation over 60% Percoll (Pharmacia, Piscataway, N.J.). Cells isolated in this manner contained fewer than 2% T cells and as many as 50% monocytes. For convenience, they are referred to as normal B cells since, although it is recognized that a significant proportion of the cells are monocytes, virus does not bind to monocytes and depletion of these cells has no effect on the magnitude of dequenching of labeled and bound virus (15).

Isolation of human epithelial cells. Epithelial cells were obtained from newborn human foreskin tissue. Aseptically collected tissue was rinsed with Dulbecco's phosphate-buffered saline (PBS), and subcutaneous fat was removed. The tissue was cut into 3- to 5-mm² pieces and incubated for 9 to 12 h at 4°C in F-12 medium (Sigma) containing 0.75 U of dispase (Sigma) per ml. The epidermis was peeled away from the dermis and incubated for 35 to 40 min at 37°C with stirring in PBS containing 0.1% trypsin with EDTA (Sigma). Epidermal cells were collected in the supernatant after inactivation of trypsin with serum, washed, resuspended in Dulbecco's modified Eagle's medium (Sigma), and layered onto a discontinuous 30 (3 ml), 40 (3 ml), 45 (3 ml), and 100% (1 ml) isotonic Percoll gradient. The gradient was centrifuged at 450 × *g* for 30 min at 4°C. Cells from the 45/100% interface, which were predominantly basal cells and bound more virus than did other cell fractions (15a), were washed and used in fusion assays.

Virus production. Virus was harvested from spent culture medium of MCV5 cells 7 days after they had reached confluency. The medium was centrifuged at 4,000 × *g* for 10 min to remove cells, 100 µg of bacitracin per ml was added to the clarified supernatant, and the virus was pelleted by centrifugation at 16,000 × *g* for 90 min. Pellets were suspended in 1/250 of the original volume of medium containing 100 µg of bacitracin per ml, reclarified by centrifugation three to four times at 400 × *g*, and filtered through a 0.45-µm-pore filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.).

Virus labeling. A stock solution of 13 nmol of R₁₈ (Molecular Probes, Inc., Junction City, Ore.) per µl was prepared in chloroform-methanol (1:1, vol/vol) and stored at -20°C. The probe was incorporated into virus membranes by a modification of the method of Hoekstra and colleagues (11). Briefly, 3 µl of the stock probe was dried under nitrogen and solubilized in ethanol, and 15 µl of this solution containing 15 nmol of R₁₈ was added to 250 µl of virus with vortexing. The probe and virus were incubated at room temperature in the dark for 1 h, after which the virus and unincorporated R₁₈ were separated by chromatography on Sephadex G-75 (Sigma). Labeled virus was stored at -70°C. Labeled virus was still infectious and retained binding specificity of unlabeled virus (15). A stock solution of AF (50 mg/ml; Molecular Probes) was prepared in dimethylformamide and stored at -20°C. Two microliters of the stock solution was used to label 250 µl of virus. Virus was then treated and stored in the same manner as the R₁₈-labeled EBV. Like R₁₈-labeled

virus, virus labeled with AF retained infectivity and specificity of binding (data not shown).

Fluorescence dequenching assay. EBV that had been labeled with R₁₈ or AF at a self-quenching concentration was added to cells and incubated for 1 h on ice in the dark. Cells were washed four times with ice-cold PBS, resuspended in 400 µl, and transferred to the microcuvette of a spectrofluorometer (SLM SPF500C; SLM Aminco-Bowman, Urbana, Ill.) equipped with a magnetic stirrer and circulating water bath at 37°C. Fluorescence was monitored continuously at an excitation wavelength of 560 nm and an emission wavelength of 585 nm for R₁₈-labeled virus and at an excitation wavelength of 496 nm and an emission wavelength of 522 nm for AF-labeled virus. At the end of the assay, Triton X-100 (1%, vol/vol) was added to allow the measurement of fluorescence that would be obtained upon infinite dilution of the fluorophore; dequenching was expressed as a percentage of this value. If a single virus pool were used, the standard deviation of the mean of dequenching in repeat experiments was 6%. Representative experiments are shown.

Lysosomotropic agents and cellular inhibitors. Stock solutions of ammonium chloride (100 mM), chloroquine (100 mM), methylamine (50 mM), and sodium azide (100 mM) were prepared in PBS. A 5 mM stock of chlorpromazine was prepared in PBS with 1% dimethyl sulfoxide. All working dilutions were made in RPMI 1640. All reagents were purchased from Sigma. Cells were preincubated with 500 µl of each reagent for 35 min at 37°C and pelleted by centrifugation at 400 × *g* for 5 min, and all but 100 µl of supernatant was removed. Fifty microliters of virus was added, and cells were incubated for 1 h on ice. Unbound virus was removed by washing cells three times in PBS, and cells and bound virus were transferred to the fluorometer.

Determination of intracellular pH. The pH in endocytic compartments was measured by modification of published methods (7, 21, 29). Cells were incubated with fluorescein isothiocyanate (FITC)- and tetramethylrhodamine (TRITC)-labeled dextrans (70,000 molecular weight; Molecular Probes) for 35 min at 37°C to allow uptake into cells. Cells were washed free of unassociated dextran and analyzed in the spectrofluorometer by measuring the TRITC fluorescence at an excitation wavelength of 560 nm and an emission wavelength of 580 nm, followed by measuring FITC emission at 522 nm at excitation wavelengths from 450 to 518 nm. Fluorescence was expressed in arbitrary units. Monensin (Sigma; 1 µg/ml) was then added to equilibrate extracellular (known pH) and endosomal pH, and another measurement of FITC fluorescence was made. A change in the intensity of the FITC fluorescence was assumed to indicate that the average pH of the intracellular compartment was either above (increase) or below (decrease) the known extracellular pH; if no change in fluorescence was observed, the intracellular and extracellular pH were assumed to be the same.

RESULTS

Effect of pH on the rate and extent of virus fusion. Since EBV is capable of fusing at the cell surface or after endocytosis, experiments were first done to examine whether lowering the extracellular pH or raising the intracellular pH would have any effect on the rate or extent of virus binding and fusion. Fluorescence dequenching curves obtained with virus labeled with R₁₈ were identical if Raji cells, normal B cells, or BAT cells were incubated in buffer at pH 5.5 or 7.2, and no effect was seen on the ability of virus to bind to cells, as judged by the relative amounts of fluorescence measured

FIG. 1. Effect of pretreatment of cells with ammonium chloride on relief of self-quenching of R_{18} -labeled virus bound to Raji cells (A), normal B cells (B), BAT cells (C), and epithelial cells (D). Cells were preincubated with 20 mM ammonium chloride ($\square$) or Dulbecco's PBS ($\blacklozenge$).

after addition of Triton (data not shown). This lack of effect of pH on binding was confirmed with virus that had been labeled with [3 H]thymidine (15). Twenty-four percent of radioactive counts added associated with cells at pH 7.2, and 23% associated at pH 5.5. Preincubation of Raji cells, B cells, BAT cells, or epithelial cells with ammonium chloride, an agent which raises endosomal pH, also had no influence on dequenching of labeled virus (Fig. 1), and identical results were obtained with concentrations of up to 1 mM methylamine (data not shown). However, a third lysosomotropic agent, chloroquine, had no effect on the dequenching of virus bound to Raji cells (Fig. 2A) or epithelial cells (Fig. 2D) but inhibited dequenching of virus bound to normal B cells and BAT cells in a dose-dependent manner (Fig. 2B and C).

The fact that only one lysosomotropic agent affected entry of EBV raised concern as to whether each of them had actually affected intracellular pH. Endosomal pH was therefore measured in treated and untreated cells, using TRITC-

FIG. 2. Effect of pretreatment of cells with chloroquine on relief of self-quenching of R_{18} -labeled virus bound to Raji cells (A), normal B cells (B), BAT cells (C), and epithelial cells (D). Cells were preincubated with 1 mM ($\blacklozenge$), 0.5 mM ($\circ$), or 0.2 mM ($\bullet$) chloroquine or with Dulbecco's PBS ($\square$).

FIG. 3. Excitation spectra measured before ($\square$) and after ($\blacklozenge$) addition of monensin to BAT cells containing fluoresceinated dextran. Cells were pretreated with buffer (A) or with 20 mM ammonium chloride (B and C) and were maintained at an external pH of 7.4 (A and B) or 7.0 (C). Measurements were made at a fixed emission wavelength of 522 nm. a.u., arbitrary units.

and FITC-dextran. The emission of TRITC-dextran is unaffected by pH and can be used as an internal control for uptake of dextran. In contrast, the emission of FITC-dextran decreases as the pH of the environment decreases. Fluorescence emission at 522 nm was measured for BAT cells that had endocytosed a mixture of FITC- and TRITC-dextran. Emission from untreated cells was low and increased significantly after addition of monensin to collapse the intracellular pH gradient and raise the internal pH to that of the external buffer at pH 7.4 (Fig. 3A). When cells were pretreated with 20 mM ammonium chloride, fluorescence emission at 522 nm was much higher than that of the untreated cells, although addition of monensin still resulted in a slight increase in signal (Fig. 3B). When the pH of the extracellular medium was reduced to 7.0, addition of monensin to pretreated cells had no effect on emission at 522 nm, indicating that ammonium chloride had raised the endosomal pH to this value (Fig. 3C). Similar experiments with methylamine and chloroquine indicated that these reagents also raised endosomal pH to approximately 7.0 (data not shown). It was thus concluded that the inhibition of virus entry by chloroquine could not be attributed to its effects on intracellular pH. However, a comparison of fluorescence emission from TRITC-dextran showed that the cells treated with chloroquine endocytosed 43% less dextran than did control cells or cells treated either with ammonium chloride or with methylamine.

Determination of site at which EBV fuses with different cells. Since alteration of extracellular or intracellular pH had no apparent effect on EBV fusion, the possibility was considered that endocytosed virus fused with cells before acidification of vesicles. To evaluate this possibility further,

FIG. 4. Relief of self-quenching of R_{18} -labeled virus and AF-labeled virus bound to receptor-positive cells. Data are for AF-labeled virus bound to Raji cells at pH 7.2 ($\square$), Molt-4 cells at pH 7.2 ($\blacklozenge$), and Raji cells at pH 5.5 ($\blacksquare$) and for R_{18} -labeled virus bound to Raji cells at pH 7.2 ($\diamond$).

we again took advantage of the differential sensitivity to pH of emission from rhodamine- and fluorescein-conjugated probes. Fluorescence of virus labeled with R_{18} is unaffected by pH, whereas fluorescence of AF-labeled virus is markedly reduced at acidic pH. The kinetics and extent of dequenching of AF- and R_{18} -labeled virus were similar when virus was bound to Raji cells (Fig. 4). No significant dequenching was seen with AF-labeled virus bound to the fusion-incompetent Molt cell line, indicating that there was no measurable diffusion of probe between bound but unfused membranes within the time frame of the assay. If AF-labeled virus was added to Raji cells in media buffered to pH 5.5, fluorescence was quenched throughout the 32 min for which the assay was run and remained quenched when Triton X-100 was added. Addition of 1 M sodium phosphate to the detergent-solubilized material did, however, lead to recovery of fluorescence, confirming that virus was bound (data not shown). Dequenching of R_{18} - and AF-labeled virus was then compared for virus bound, at neutral pH, to BAT cells, normal B cells, and epithelial cells. No difference was seen in the dequenching curves with either virus bound to epithelial cells (Fig. 5C). However, whereas dequenching of virus bound to BAT cells reached 65% of maximum for R_{18} -labeled virus, it reached only 10% of maximum for AF-labeled virus (Fig. 5B). Only if BAT cells were pretreated with ammonium chloride did dequenching curves for R_{18} - and AF-labeled virus become comparable. Similar results were obtained with normal B cells (Fig. 5A). These data suggested that a majority of virus fused with normal and recently transformed B cells from within an acidic compartment, whereas fusion with the epithelial cell, like fusion with Raji, occurred at neutral pH.

Effect of inhibitors of endocytosis on dequenching. All three of the lysosomotropic agents studied had raised intracellular pH, but only one, chloroquine, had had any effect on dequenching of virus bound to normal B cells and BAT cells. Since chloroquine was also the only agent that affected uptake of dextran into cells, we considered the possibility that its inhibitory effects were related to its effects on endocytosis. Two other reagents reported to inhibit endocytosis, sodium azide and chlorpromazine, were examined for their effects on dequenching of virus. Although neither drug had an effect on dequenching of virus bound to Raji cells or epithelial cells, both reduced dequenching of virus bound to normal B cells and BAT cells (Fig. 6). The effects of azide

FIG. 5. Relief of self-quenching of R_{18} - and AF-labeled virus bound to normal B cells (A), BAT cells (B), and epithelial cells (C). Data are for virus labeled with R_{18} ($\square$) or with AF ($\blacklozenge$) and bound to untreated cells and for virus labeled with AF bound to cells pretreated with 20 mM ammonium chloride ($\circ$).

and chlorpromazine on endocytosis were confirmed by examining the uptake of TRITC-dextran into treated cells. Sodium azide at a concentration of 20 mM reduced dextran uptake into BAT cells by 40%; chlorpromazine at a concentration of 50 μ M reduced dextran uptake by 82%. This result further suggested that the inhibition of endocytosis was correlated with failure of virus to fuse with normal B cells and BAT cells.

FIG. 6. Relief of self-quenching of R_{18} -labeled virus bound to Raji cells (A), normal B cells (B), BAT cells (C) or epithelial cells (D) pretreated with 20 mM sodium azide ($\circ$) or 50 μ M chlorpromazine ($\square$) or untreated ($\blacklozenge$).

DISCUSSION

Enveloped viruses enter cells by fusing their envelopes with cell membranes, either at the cell surface or after endocytosis. Several well-studied viruses, such as influenza virus and Semliki Forest virus, have an absolute requirement for exposure to the low pH of the endosome in order for their fusion proteins to become active; they can fuse at the cell surface only if the extracellular pH is artificially lowered. Other viruses, such as Sendai virus, herpes simplex virus, and the human immunodeficiency virus, have no requirement for acidification to trigger fusion (30). Our experiments confirm that EBV falls into this latter category of viruses. First, exposure of virus at the cell surface to an acidic pH had no discernible effect on virus binding. Second, although all three of the lysosomotropic agents that were used raised the endosomal pH, only one, chloroquine, had any influence on the rate or extent of fluorescence dequenching. There seems little doubt, however, that the virus actually fuses with nonmalignant B cells from within an acidic compartment. The loss of fluorescence signal from entering virus labeled with AF, and a reversal of this loss by pretreatment of cells with ammonium chloride to raise endosomal pH, provide reasonable evidence for the conclusion. It is further supported by the experiments with Raji cells with which EBV fuses directly at the plasma membrane. Dequenching of virus labeled with AF and bound to Raji cells could be measured irrespective of whether the cells had been pretreated with drug. The fluorescent probe AF has not been extensively used for analysis of virus fusion. It is considerably more polar than R₁₈ and thus both more difficult to incorporate into virus and more likely to diffuse out. The absence of dequenching of AF-labeled virus bound to Molt-4 cells did, however, give confidence that diffusion from labeled to unlabeled membranes was not a problem, at least within the time frame of these experiments. Comparative analysis of virus fusion with R₁₈ and AF would thus appear to have general potential as an alternative method for determining the site of fusion of enveloped viruses that are not dependent on acidic pH for activity.

Nemerow and Cooper (18) reported that methylamine, ammonium chloride, and chloroquine all affected EBV infectivity, but the reduction in infectivity that they observed with chloroquine far exceeded that for the other two agents (96% versus 16 and 20% inhibition). Furthermore, the point after attachment at which the reagents influenced infectivity was determined for chloroquine alone, and the possibility that the other two reagents affected cell metabolism rather than virus entry cannot be ruled out. The electron microscopic studies of Nemerow and Cooper (18) indicated that chloroquine inhibited penetration of virus through the endocytic vesicle membrane. They observed no effect of drug on the formation of endocytic vesicles. In contrast, however, our fluorescence studies suggest that chloroquine affected the extent of endocytosis and are in agreement with reports that at concentrations exceeding 0.1 mM, it can influence uptake of molecules into cells (1). The fact that two other drugs, chlorpromazine and sodium azide, coincidentally influenced endocytosis and fluorescence dequenching in normal B cells raises the possibility that in this cell type, endocytosis is actually required for fusion to take place. Sodium azide is an inhibitor of oxidative phosphorylation that has been shown to affect uptake of Semliki Forest virus (13) and vesicular stomatitis virus (2). Chlorpromazine is a phenothiazine that binds to calmodulin and has been reported to inhibit formation of large thin-walled vesicles (23)

morphologically similar to those into which EBV is endocytosed (18). Indeed, several phenothiazines and other classes of calmodulin antagonists have been reported to influence endocytosis and infection by EBV (19). Whether the impairment of fusion of virus with the surface of normal B cells is due to the lack of an essential component in the plasmalemma or to the presence of an inhibitory factor is unclear.

The contrasting failure of chlorpromazine, chloroquine, and sodium azide to affect dequenching of virus bound to Raji cells and epithelial cells correlated with the observation that little if any enveloped virus entered an acidified cell compartment in either of these cells and further suggests that virus penetrates not only Raji cells but also epithelial cells by direct fusion with the plasmalemma. This conclusion should be tempered by two caveats. First, dequenching assays of fusion provide information about the behavior of a population of virions, both infectious and noninfectious, not about individual particles. Thus, it is fair only to say that a majority of EBV virions behave in a given fashion. Second, the epithelial tissue used was in an unnaturally nonpolarized and dispersed state. This concern is, however, somewhat mitigated by the fact that such dispersed cells are infectable with EBV (25).

It has been suggested that abnormalities in the cytoskeleton of malignant cells, such as Raji, are responsible for the discrepancy between the route that EBV takes into the B-lymphoma line and its normal counterparts (18, 27). It seems plausible that the superficially similar route that EBV takes into epithelial cells may also reflect the nature of the integration of membrane and cytoskeleton. CR2 is the ligand-binding subunit of a signal transduction complex in the membrane of the B cell which is composed of a number of poorly characterized molecules and the B-cell-specific protein, CD19 (14). Binding of the EBV attachment protein gp350/220 to CR2 has been reported to induce capping in association with surface immunoglobulin and endocytosis (27); the cytoplasmic tail of CR2 is essential to this process (3). The identity of the EBV receptor on epithelial cells is uncertain, but irrespective of whether or not it is a CR2-related molecule, it clearly does not associate with a complex incorporating CD19 or co-cap with immunoglobulin on this cell type, and its interface with the cytoskeleton may be quite different from that of CR2. The apparently disparate routes that EBV takes into epithelial cells and B cells may have considerable significance for the subsequent fate of nucleocapsids in each cell type and for the route(s) and mechanisms responsible for intracellular transport to the nucleus, and they reinforce the important point that the biological aspects of infection of these two cell types are quite different.

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