Epithelial Cell Polarization Is a Determinant in the Infectious Outcome of Immunoglobulin A-Mediated Entry by Epstein-Barr Virus

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Diseases of the nasopharyngeal epithelium due to Epstein-Barr virus (EBV) infection typically occur in chronic virus carriers with preexisting virus-specific antibodies. In vitro studies have shown that EBV-specific immunoglobulin A (IgA) promotes infection of human epithelial cells, otherwise refractory to EBV, via the polymeric immunoglobulin receptor (pIgR). To determine if EBV similarly exploits IgA transport mechanisms in vivo, we examined the fate of IgA-EBV complexes in the blood of mice, where pIgR-mediated transcytosis of IgA immune complexes through hepatocytes eliminates exogenous antigens from the circulation. By PCR analysis we showed hepatobiliary transport of IgA-EBV in viremic mice, but without detectable hepatocellular infection by immunostaining. Because efficient transport of EBV immune complexes might avert an infectious outcome, we modulated the transcytotic pathway in polarized Madin-Darby canine kidney (MDCK) cells transfected with pIgR to determine the effect on viral antigen expression. Like hepatocytes in vivo, MDCK cells in polarized monolayers translocated IgA-EBV from the basal cell face into apical medium without evidence for infection. However, when exposed to IgA-EBV as unpolarized single-cell suspensions, MDCK cells expressed EBV immediate-early and early antigens. These results suggest that pIgR-mediated transcytosis of pIgA-EBV through epithelium facilitates endogenous spread of EBV in long-term virus carriers, with infection being confined to cells with altered polarity from prior cytopathology.

Epstein-Barr virus (EBV) is a persistent human gammaherpesvirus causally associated with infectious mononucleosis, Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma. In vitro, the virus exhibits a pronounced tropism for human B lymphocytes based in part on binding of the B-lymphocyte complement receptor CR2 (CD21) by gp350/220 (15, 39, 53), a major viral membrane glycoprotein. Virus entry into human epithelium is less well defined (4, 47, 48). However, demonstration that the epithelial polymeric immunoglobulin receptor (pIgR) can mediate internalization of infectious immunoglobulin A (IgA)-EBV complexes (47) suggests that antibody-enhanced infection plays a role in EBV pathogenesis in chronic virus carriers. A marked, virus-specific IgA antibody response precedes the onset of the EBV-associated epithelial cell disease nasopharyngeal carcinoma by months to years, and detection of antibody in individuals where that cancer is endemic correlates with a 32-fold-increased risk for the disease (59).

The majority of IgA produced enters mucosal secretions by transcytosis through polarized epithelial cells that express pIgR (or secretory component) on their basolateral surfaces (34). The notion that a persistent virus might gain entry into cells by this route is oddly congruent with current views of IgA function in mucosal defense (reviewed in reference 31). Active at both apical and basal faces of the epithelial cell, IgA not only

impairs microbial adherence at the luminal surface but, when bound to foreign protein from organisms invading the mucosal lamina propria underlying the epithelial basement membrane, also promotes excretion by the transepithelial route (24). Epithelial cells expressing pIgR transport the intact immune complex formed with polymeric IgA (pIgA) vectorially from basal to apical cell surface (24), a pathway accessible to endogenous EBV deposited subepithelially by trafficking B lymphocytes. The circumstances that might predispose epithelial cells to infection via this mode of viral entry, rather than simple extrusion of the infectious immune complex from the cell, are unclear. Using two well-characterized models of pIgR-mediated IgA transcytosis in polarized epithelium, specifically the hepatocellular transport of IgA from blood to bile in rodents (16, 22, 42, 56) and Madin-Darby canine kidney (MDCK) cells expressing the pIgR (37), we present evidence to support a role for cell polarization in the infectious consequences of IgA-assisted EBV internalization.

MATERIALS AND METHODS

Mice and virus. Female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine), 6 to 8 weeks old, were used for the production of monoclonal antibody (MAb) as well as studies of hepatobiliary transport of IgA-EBV complexes. Infectious EBV stock was made from an EBV-positive Burkitt's lymphoma cell line, Akata, which can be induced to replicate virus on treatment with anti-human IgG (52). Stock preparations transformed 50% of primary lymphocyte cultures at a dilution of 10^{-2} in a lymphocyte transformation assay (36).

Production and characterization of EBV-specific pIgA. Human pIgA was isolated on jacalin affinity columns (Pierce, Rockford, Ill.) from sera of patients with acute infectious mononucleosis as previously described (47). To prepare a murine MAb of the IgA isotype specific to the major membrane glycoprotein of EBV, gp350, naturally glycosylated gp350 isolated by fast protein liquid chromatography (FPLC) from membranes of productively infected B95-8 cells (12) was used in a standard immunization protocol (11, 27, 32). Cells from a mesenteric lymph node fused to the murine myeloma cell line X63-Ag8.653 (25)

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produced a stable, gp350-specific, IgA-secreting hybridoma, designated 1556F2, after multiple subclonings. After injection of 1556F2 intraperitoneally into pristane-primed BALB/c mice, the resultant ascitic fluid was clarified by centrifugation and delipidated with Lipoclean (Calbiochem Behring, La Jolla, Calif.). Immunoglobulin was precipitated by an equal volume of saturated ammonium sulfate and dissolved in phosphate-buffered saline (PBS). Both human and murine IgAs were separated with reference to molecular weight standards (Sigma, St. Louis, Mo.) into polymeric (pIgA) and monomeric (mIgA) fractions by gel filtration (FPLC system; Pharmacia, Piscataway, N.J.) through Superose 6 and 12 columns in tandem (33). Respective pIgA and mIgA fractions were pooled and concentrated by ultrafiltration. The purity of the pooled fractions was determined on 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad) stained with Coomassie blue. The specificity of IgA to gp350 was determined by immunofluorescent staining of T-lymphocyte line CEM, transfected with cDNA encoding gp350 (gift of J. Tanner, University of Montreal). Antibody was quantitated by enzyme-linked immunosorbent assay using whole virus as the substrate.

Preparation of EBV-IgA complexes. Formation of complexes was assayed in a lymphocyte transformation inhibition assay as described previously (47). Briefly, 10-fold dilutions of stock EBV were incubated for 30 min at 4°C with equal volumes of pIgA 1556F2 (titer, 1:400). Stock virus or IgA-bound EBV was added to 3×10^6 peripheral blood lymphocytes collected from an EBV-seronegative donor and previously separated on Ficoll gradients (LSM; Organon Teknika Corp., Durham, N.C.). Cells were distributed to microtest plates, 5×10^5 cells per well with six replicate wells for each virus dilution. Transformation was judged by the establishment of a permanent lymphoblastoid cell line after 8 weeks in culture.

Hepatobiliary transport of EBV. Stock virus (100 µl), EBV-IgA immune complexes prepared as described above, or a PBS control was injected as a total volume of 0.5 ml into the tail veins of BALB/c mice not fed for 3 h to permit accumulation of bile. Eight mice were used in each experiment. At 30 min, 60 min, or 24 h after injection, animals were bled from the retro-orbital sinus and then sacrificed. Bile was aspirated from the gall bladder with a microliter syringe (Hamilton Co. Inc., Whittier, Calif.); the liver was resected at the 24-h time point for frozen sections. To remove PCR inhibitors, bile was processed by using Gene Releaser (BioVentures, Inc., Murfreesboro, Tenn.) according to the manufacturer's protocol. PCR amplification was performed with primers specific to sequences in the reiterated *Bam*HI W EBV DNA fragment (47). Forty cycles of amplification were performed on a DNA thermal cycler with *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's protocol. PCR products were detected by a 32 P-labeled oligonucleotide probe specific to regions internal to primer sequences (47).

Polarized epithelial cell culture. Strain II, or low-resistance (100 to 300 Ω/cm^2), MDCK cells stably transfected with cDNA encoding the rabbit pIgR (from Keith Mostov, University of California, San Francisco) (37) were cultured on filters (pore size, 0.45 µm) in microwell chambers (Transwells; Costar, Cambridge, Mass.) in minimum essential medium with 10% fetal bovine serum. After 96 h in culture, the integrity of each monolayer was tested by measuring transmembrane electrical resistance (TER) with a Millicell-ERS meter (Millipore Corp., Bedford, Mass.). Readings of greater than 100 Ω/cm^2 were required for cell use. To assess the permeability of the cell monolayers, the rate of diffusion of ^3H -inulin (1 to 5 Ci/mmol; Amersham Life Sciences, Arlington Heights, Ill.) through filters covered by an intact monolayer (as indicated by electrical resistance) was compared with that through bare filters or those with incompletely formed cell sheets (8). Polarity of expression of pIgR was determined by indirect immunofluorescent staining of the top or bottom of porous cell supports with a MAAb to the transmembrane portion of the pIgR (Hybridoma Reagent Laboratory, Baltimore, Md.).

pIgA-EBV endocytosis in MDCK cells. Filters containing polarized MDCK cells were rinsed with ice-cold PBS. Approximately 30 µl of stock EBV, pIgA, or pIgA-EBV complexes was adsorbed to the basolateral surface of culture membranes and overlaid with cold medium at 4°C for 3 h to maximize binding. Filter surfaces were then thoroughly rinsed with cold PBS to remove excess virus. Warm medium was added to both sides of the filter, and cells were incubated at 37°C overnight. Samples of apical medium were tested for EBV by PCR, as described above. Cell monolayers were disrupted with trypsin, and cell pellets were washed in PBS prior to immunofluorescent staining for viral antigens. In select experiments, MDCK cells were first removed from membranes by trypsinization, washed twice in PBS, and resuspended in medium as a cell suspension culture for 8 h. Cells were recovered by centrifugation, exposed to EBV or pIgA-EBV complexes for 30 min, and then allowed to attach to culture dishes overnight before fixation and immunostaining.

Detection of EBV antigen expression. Frozen liver sections or MDCK cells were fixed on glass slides in methanol-acetone (1:1) for 3 min at -20°C, then incubated with MAAb BZ.1 (from L. S. Young, University of Birmingham) or EA(D) (Biotech Research Laboratories, Rockville, Md.), and then stained with fluorescein isothiocyanate-labeled anti-mouse IgG (Southern Biotechnology, Birmingham, Ala.).

RESULTS

Specificity and purity of anti-EBV gp350 pIgA. Characterization of human IgA used in these experiments has been previously described (17, 47). Because the humoral pIgA response during acute viral infection is transient and human serum is an unreliable source for virus-specific pIgA (17, 19), we made the IgA-secreting hybridoma 1556F2. Antibody precipitated from mouse ascitic fluid could be separated into both monomeric and polymeric components, with FPLC fractions between markers ferritin (440 kDa) and catalase (232 kDa) containing pIgA (Fig. 1A). Polyacrylamide gel electrophoresis confirmed the composition of both antibody pools, with pIgA represented by the band of approximately 325 kDa. Fractions collected after the catalase marker contained a lower-molecular-weight species consistent with mIgA (Fig. 1A). When tested for antigen specificity, antibody reacted with the T-cell line CEM transfected with the gene encoding EBV gp350, whereas cells containing the vector alone did not immunostain (Fig. 1B and C). Because gp350 is the ligand for the EBV receptor CD21 on lymphocytes, some anti-gp350 antibodies have been shown to impair B-cell infection (21, 47, 54). Viral binding by IgA MAb was confirmed not only by enzyme-linked immunosorbent assay but also by inhibition of primary B-lymphocyte immortalization in the transformation assay. At the highest concentration of EBV used, only two of six replicate lymphocyte cultures transformed in the presence of pIgA, versus six of six with EBV alone. Transformation was completely blocked at subsequent dilutions of virus.

Hepatobiliary transport of pIgA-EBV complexes. In many species of rodent, pIgA is selectively transported from serum to bile by the liver (16, 22, 42, 56). Translocation involves binding of pIgA to the pIgR expressed on the surface of rodent hepatocytes (40, 49). The receptor-ligand complex is endocytosed and transported through the hepatocyte in vesicles for release into bile (38, 43). Because the liver can also remove antigen complexed to IgA from the circulation into bile (6, 41, 46, 50, 51), this mechanism is viewed as a noninflammatory means for disposal of foreign protein. Three separate experiments with FPLC-purified preparations of both human and mouse (1556F2) pIgA are shown in Fig. 2. By PCR analysis, mice injected with pIgA-EBV had viral DNA detected in bile within 30 to 60 min after tail vein injection. Mice injected with virus alone had detectable EBV DNA in serum but not bile. PCR products were not obtained from blood or bile of control mice injected with PBS (data not shown). Although pIgA clearance into bile is maximal at 30 to 60 min after injection (22), immune complexes have been reported to be eliminated at a lower rate (41), which may explain the variable time course for EBV transport seen in the three experiments (Fig. 2).

By contrast, six mice inoculated with EBV immune complexes prepared with total (polymeric plus monomeric) IgA specific for EBV gp350 did not translocate EBV into bile (data not shown). Although a physical restriction on hepatic transport of large IgA immune complexes from blood to bile may exist (50), the apparent inconsistency between FPLC-purified and total IgA preparations most likely reflects the excess of mIgA in the latter (Fig. 1). Competition for EBV gp350 by the substantial monomeric component could reduce pIgA-EBV complex formation. EBV-antibody complexes formed with mIgA are not bound by the pIgR (47), and mIgA is not transported into bile in the mouse system (16).

Although EBV transcytosis was observed with pIgA (Fig. 2), examination of up to eight frozen liver sections per animal for expression of BZLF1, a marker of acute epithelial infection (28, 47), did not show significant staining differences between

FIG. 1. Characterization of IgA MAb 1556F2. (A) Protein elution profile (measured at 280 nm) from gel filtration columns of the ammonium sulfate-precipitated antibody in mouse ascitic fluid, superimposed on molecular weight markers. pIgA (325 kDa) elutes between ferritin (440 kDa) and catalase (232 kDa). Protein in pooled fractions from presumed polymeric and monomeric peaks were compared on gradient sodium dodecyl sulfate gels stained with Coomassie blue to determine the purity of each fraction. Lower-molecular-weight bands in the pIgA pool represent contaminating mIgA or degraded pIgA. CEM cells transfected with the gene encoding EBV gp350 (B) or vector alone (C) were stained by indirect immunofluorescence, using the pIgA fraction of 1556F2.

animals receiving virus or mock preparations 24 h postinjection. The absence of infection after IgA-assisted viral entry *in vivo* is at variance with our earlier report of EBV antigen expression in cultured human HT-29 cells (47) and cannot be attributed solely to the restricted host range of EBV. Both mouse lymphocytes (57) and fibroblasts (1) can be infected if manipulated molecularly to express an EBV receptor on cell membranes. While both results confirm EBV utilization of this

endocytotic pathway, we questioned whether the divergent outcomes of IgA-assisted entry (transcytosis versus infection) could reflect the different states of cell polarization present in the two experimental systems. Hepatocytes organized into tissue such as liver are inherently polarized, in contrast to HT-29 cells cultured as reported previously (47, 55).

Outcome of pIgA-assisted entry of EBV in MDCK cells. To evaluate the contribution of epithelial cell polarization to in-

FIG. 2. Hepatobiliary transport of pIgA-EBV complexes. Partial to complete translocation of pIgA-EBV complexes from blood through hepatocytes into bile after tail vein injection, as shown by PCR detection of the reiterated *Bam*HI W fragment of EBV DNA in bile. Time points represent single animals receiving EBV alone or pIgA-EBV that were sacrificed for PCR analysis of blood and bile. For experiment (Exp.) 1, pIgA was derived from a patient with acute infectious mononucleosis (17); EBV DNA was not detected in mice sacrificed at 60 min (not shown). Experiments two and three used the pIgA component of murine MA5 1556F2. BL2, total cellular DNA from EBV-negative Burkitt's lymphoma cell line; Akata, EBV-positive Burkitt's lymphoma cell line.

fection after EBV entry by the IgA-pIgR-mediated route, we used the MDCK cell line transfected with the rabbit pIgR gene (37). These cells spontaneously form well-polarized monolayers with tight junctions and have been widely used in studies of cell polarity and membrane traffic. When introduced into MDCK cells, the pIgR is correctly transported through its complex pathway, and intact IgA immune complexes can be translocated from basolateral to apical cell surfaces (37). Growth on permeable-membrane cell supports provides separate access to basolateral and apical cell surfaces necessary for studies of transcellular transport.

We used three well-established methods to confirm that cell monolayers were polarized: measurement of TER, permeability of the cell monolayer to the macromolecule inulin, and membrane distribution of the pIgR. Cell monolayers with high TER ($>100 \Omega/\text{cm}^2$), denoting tight junction formation between cells that excludes paracellular diffusion of macromolecules, impeded the movement of radiolabeled inulin across the membrane support from the apical to basolateral medium compartment (Fig. 3A). Once achieved, impermeability was retained (as indicated by a TER of between 100 and 300 Ω/cm^2) for at least 24 h, a time course relevant to our experiments. Freshly seeded cells, bare filters, and older cultures with a low TER were permeable to inulin. When MAb directed against the pIgR was applied to intact cell monolayers either basolaterally through the filter or apically, immunostaining of the basolateral surface only showed a cobblestone pattern of fluorescence indicating localized expression of pIgR characteristic of polarized cells (Fig. 3B and C).

When pIgA-EBV was added to the basolateral surface of MDCK/pIgR cells, viral DNA was detected by PCR in apical medium after overnight incubation (Fig. 4A). Two controls indicated that EBV transcytosis was dependent on the pIgA-

pIgR pathway. Virus alone did not traverse the MDCK/pIgR epithelial cell sheets. Similarly, MDCK cells that contained vector without the gene encoding pIgR did not translocate pIgA-EBV complexes into the apical compartment. As with polarized mouse hepatocytes in vivo, transcytosis occurred without detectable evidence for infection (Fig. 4B). Indirect immunofluorescent staining with MAbs to BZLF1 and BMRF1, two viral proteins expressed during the EBV replicative cycle, was negative. An indication that polarized MDCK cells can indeed be infected by EBV despite their canine origin was provided in control cells, previously transfected with the EBV B-lymphocyte receptor CD21, that stained for BZLF1 after exposure to EBV (Fig. 4C).

In a second series of experiments, the polarity of the epithelium was disrupted prior to exposure to infectious immune complexes. When trypsinized, MDCK cells placed in suspension culture lose their characteristic structural and functional polarity (2, 3, 44, 45, 60). In contrast to polarized monolayers that lacked detectable viral antigen expression after incubation with pIgA-EBV, MDCK/pIgR cells in suspension were susceptible to de novo infection. In three separate experiments using either human or mouse pIgA, infectious immune complexes (but not virus alone) produced expression of the replicative proteins BZLF1 and BMRF1 in the nonpolarized cell population (Fig. 5). These results are in agreement with those reported earlier for human HT29 cells, which were also nonpolarized (47).

DISCUSSION

Recent evidence implicates IgA transcytosis through the epithelium in far more than the mere delivery of antibody to the mucosal surface (31). Novel defense mechanisms to include

FIG. 3. Filter-grown MDCK/pIgR monolayers are polarized and impermeable to macromolecules. (A) Cell monolayer permeability, as indicated by the rate at which the macromolecule ^3H -inulin (1 to 5 $\mu\text{Ci}/\text{ml}$; M_r , 5,000) passed through cell sheets from one medium compartment to another, correlated with TER (ohms per square centimeter). After addition of ^3H -inulin to the apical medium compartment, basolateral medium was collected at the times indicated. Immunolocalization of pIgR on intact monolayers, with anti-pIgR antibodies applied to apical (B) or basolateral (C) surfaces.

both excretion of foreign antigens from the level of the lamina propria and intracellular inactivation of acute viral infection have been proposed (7, 24, 29, 30). Our data indicating IgA-mediated epithelial cell infection as well as transport of EBV across epithelial barriers suggest that this endocytotic pathway is utilized by a persistent pathogen for endogenous spread within the immune host.

Antibody-enhanced infection postimmunization or as described in dengue virus infection (18, 35) differs from that suggested here in two major respects: the responsible immunoglobulin isotype and the clinical setting. Classically, infectious complexes contain IgG and bind cellular Fc receptors (18). By contrast, we have shown enhancement with pIgA through its interaction with the pIgR expressed on basolateral surfaces of the epithelium. Segregation of pIgA to mucosal surfaces (mIgA is the predominant form in the human peripheral circulation) (34) delineates anatomic sites where this immunopathogenic mechanism may occur. Equally distinguishing are the clinical circumstances surrounding the enhancement phenomenon, dengue producing an acute short-lived infection versus EBV's persistent and lifelong interaction with the im-

mune host. The antibody-mediated dengue shock syndrome develops after acute infection either in the first 7 months of life in the presence of maternal antibody or at 3 to 5 years of age in the face of a second infection (18, 35). With EBV, on the other hand, reactivation of latent virus in the chronic carrier state could lead to multiple rounds of enhanced infection at mucosal tissues. Indeed, with the basal face of polarized epithelium as the entry point for pIgA, this mechanism would best offer itself to spread of endogenous virus delivered subepithelially by means of trafficking, EBV-infected B lymphocytes.

Our demonstration of transhepatic transport of EBV-pIgA complexes in a well-characterized murine model confirms earlier in vitro findings of antibody-assisted entry of EBV in the human colonic carcinoma cell line HT-29 (47) and indicates that this pathway into cells is relevant in biological systems more complex than tissue culture. The fact that hepatocytes, unlike cultured HT29 cells (47), were not infected may reflect efficient transport of infectious complexes through cells. In-

FIG. 4. Transcytosis of pIgA-EBV through polarized MDCK/pIgR cells. The basolateral surface of cell monolayers was transiently exposed to EBV or pIgA-EBV complexes, once TER was consistent with tight junction formation and cell polarization ($\text{TER} > 100 \Omega/\text{cm}^2$). After overnight incubation, apical medium was tested for EBV DNA by PCR amplification of the *Bam*HI W EBV fragment. (A) PCR products derived from duplicate wells, as detected by Southern blot hybridization. Input virus served as a positive PCR control. (B) Cells translocating virus to apical medium in panel A were trypsinized and stained by indirect immunofluorescence for the EBV immediate-early protein BZLF1. (C) BZLF1 staining of polarized MDCK cells after transfection with the EBV receptor (CD21) and exposure to the Akata strain of EBV.

FIG. 5. Effect of modulation in MDCK cell polarity on EBV infectivity by pIgA-mediated entry. Nonpolarized single-cell suspensions of MDCK/pIgR cells stained for BZLF1 18 h after exposure to EBV alone (A), pIgA alone (B), or pIgA-EBV (C and D). (E and F) Staining for EBV early antigen (BMRF1) after exposure to pIgA-EBV. Findings are representative of three separate experiments.

deed, virus exploitation of transepithelial cell transport mechanisms *in vivo* without infectious consequences has been described in other virus systems (58).

Experiments with MDCK cells suggested that the disparate outcomes of virus internalization by this route do, in fact, hinge on cell polarity. MDCK cells undergo a transition from an unpolarized to a polarized state with every passage in culture (2). Basolateral to apical transcytosis is dependent on intact microtubules to guide vesicles through the cell (5, 23). Cells that are not polarized but express pIgR carry out all transport and processing steps of pIgR except for polarized transcytosis of the bound ligand (14). In passaged MDCK cells, reorganization of the microtubule network to form microtubule bundles occurs only after cell-cell contact has been established in the form of tight junctions (2). Thus, when the basolateral surfaces of MDCK cells were exposed to EBV-pIgA after cell-cell contact was established, virus DNA was detected in apical medium without evidence of antigen expression in the cells themselves. By contrast, single cells in suspension exposed to identical pIgA-EBV preparations expressed viral proteins. What changes occur in postendocytotic sorting of pIgA-EBV or in the intraluminal milieu of the endosome to allow escape of virus into the cytoplasm must now be delineated in this system.

As a model to explain the predictive significance of the IgA response in the development of nasopharyngeal carcinoma, pIgA-mediated entry of EBV into the epithelium raises new

issues regarding the inductive phase in viral carcinogenesis. First, reactivation of latent virus in the immune host, not the long period of viral latency which precedes oncogenesis, may be key in the initiation of tumorigenesis. With virus activation, pIgA-assisted entry might foster spread to cells generally not targeted by virus. Indeed, two EBV replicative proteins, BZLF1 (by inducing transforming growth factor β 1) and BCRF1 (viral homolog of interleukin-10), have the potential of enhancing IgA production (9, 10, 13, 26) and thus the spread of virus by this mechanism. Renewed viral replication as a prerequisite for carcinogenesis is consistent with the marked antibody response to antigens of the EBV replicative cycle that precedes development of nasopharyngeal carcinoma (20, 59). Although primary infection precedes the onset of disease by decades, the time to neoplasm from EBV reactivation may be relatively short. Second, if loss of intrinsic polarization predisposes an epithelial cell to infection as suggested by these data, cytopathology preexisting at the time of viral activation may favor EBV-initiated malignant progression. Under this scenario, the risk for malignancy would be proportional to the likelihood that such a cell will become infected, and it might be substantially altered by therapeutic reduction of virus replication.

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