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13. Mice were infected intracranially with LCMV as in Fig. 1 and anesthetized with Metofane. The abdominal aortas were clamped, and 1 ml of phosphate-buffered saline (pH 7) was used to perfuse the brains via the left ventricles. Craniums were opened and the brains were removed. Protein immunoblots were performed as described [K. E. LaPan, D. G. Klapper, J. A. Frelinger, *Mol. Immunol.* **28**, 499 (1991)]. Blots were blocked with 1% bovine serum albumin, washed, and incubated with anti–class II MAb KL295 (K. E. LaPan, D. G. Klapper, J. A. Frelinger, *Hybridoma*, in press), for 30 min, then washed, incubated with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G1 (IgG1), and developed with ProtoBlot (Promega).
14. Cell-mediated cytotoxicity assays were performed as in (*9*) with LCMV-infected CHB2 target cells. Spleen cells (10^7) from LCMV-infected mice were incubated in an appropriate dilution of anti-CD4 (MAb 172-4) [R. Ceredig, J. W. Lowenthal, M. Nabholz, H. R. MacDonald, *Nature* **314**, 98 (1985)], anti-CD8 (MAb 31M) [M. Sarmiento, A. L. Glasebrook, F. W. Fitch, *J. Immunol.* **125**, 2665 (1980)], or medium alone and incubated for 15 min at 37°C. Rabbit complement (Low-Tox-M) (Cedarlane) was added at a 1:15 dilution for 45 min at 37°C. Spleen cells were washed and reconstituted to the original volume before CTL assays were done.
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Immunoglobulin A–Induced Shift of Epstein-Barr Virus Tissue Tropism

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Increased immunoglobulin A (IgA) antibodies to the Epstein-Barr virus (EBV) appear months to years before the clinical onset of nasopharyngeal carcinoma and define populations at high risk for this EBV-associated epithelial cancer common in south China. In the human HT-29 epithelial cell line, polymeric IgA (pIgA) specific for EBV promoted infection of the otherwise refractory epithelial cells. When bound to pIgA, EBV entered epithelial cells through secretory component–mediated IgA transport but no longer infected B lymphocytes. Such an immune-induced shift in EBV tissue tropism provides a paradigm for endogenous spread of EBV in the immune host that predicts infectious sequelae of epithelium.

PRIMARY EBV INFECTION IS MARKED by virus replication in oropharyngeal epithelial cells with subsequent spread of the virus to mucosal lymphocytes (*1*). Although there is debate on the tissue reservoir for EBV that sustains the lifelong carrier state (*2*), late manifestations of infection indicate that EBV is a mucosal pathogen: nasopharyngeal carcinoma and endemic Burkitt's lymphoma involve mucosal epithelium and mucosa-associated lymphoid tissue, respectively (*3*). Prominent IgA responses accompany two EBV-related lesions of human mucosal epithelium—nasopharyngeal carcinoma and oral hairy leukoplakia—that arise years after primary infection (*4–7*), but virus-specific secretory antibodies (Abs) are absent from the saliva of most asymptomatic shedders of the virus (*8*). Because IgA appears to herald EBV-associated epithelial disease, we investigated whether the process of IgA transport through epithelium might promote endogenous spread of this mucosal pathogen from B lymphocytes to epithelium in the persistently infected host.

In mucosal secretions more than 90% of IgA is polymeric, whereas in human serum the monomeric IgA (mIgA) molecule predominates (*9*). At the mucosa, plasma cells secrete pIgA into subepithelial interstitial spaces, where it specifically binds, by means of the J (joining) chain, to the secretory component (SC), a transmembrane protein expressed on basolateral surfaces of epithelial cells. This ligand-receptor complex is endocytosed by the epithelial cell and transported to the luminal surface, where the functional Ab, still bound to a portion of SC, is released (*10*). Of the three types of epithelia lining the nasopharynx, two express SC: pseudostratified columnar epithelium and intermediate epithelium (*11*). Both types cover much of the lateral nasopharyngeal wall including the fossa of Rosenmüller, where nasopharyngeal carcinoma arises (*12*). SC is also expressed on thymic and parotid epithelia, known sites of EBV pathology, as well as on tumor cells in some nasopharyngeal carcinomas (*5, 11*).

To test whether IgA enhances epithelial infection, we identified a source of pIgA specific to EBV but (unlike the local Ab) not already complexed with SC. Although serum IgA is largely monomeric, acute viral infections often induce transient serum pIgA responses (*13*). Thus, we isolated IgA from the sera of patients with acute infectious mononucleosis, using a lectin (jacalin) that selectively binds human polymeric and monomeric IgA1 (*14*). When the molecular size distribution of IgA from the jacalin eluate was analyzed by sucrose density gradient ultracentrifugation (*15*), two IgA peaks with specificity for the EBV membrane glycoprotein gp340 were detected (Fig. 1). Sucrose gradient fractions containing the faster sedimenting, polymeric form of Ab cosedimented with beef liver catalase (11.4*S*), which was used to mark the approximate position of dimeric IgA (10.2*S*) (Fig. 1). To determine if our IgA preparations could bind SC after separation, we

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incubated pooled fractions from polymeric or monomeric peaks with HT-29.74 cells (*16*), a human colonic carcinoma line in which approximately 20% of the cells express substantial amounts of SC on their surface and which is used extensively for studies of IgA transport. Immunofluorescence with fluorescein isothiocyanate (FITC)–conjugated Ab to human IgA revealed that only cells exposed to polymeric IgA fractions bound the Ab, indicating that the pIgA retained functional integrity (Fig. 1).

We tested whether the purified Ab could neutralize EBV in the B cell transformation assay. EBV-encoded gp340, the most abundant component of the viral envelope, mediates attachment of the virus to the EBV/C3d receptor on B cells (*17*). IgG monoclonal antibodies to gp340 inhibit B cell infection (*18*), and the immunization of cotton-top tamarins with gp340 protects against the development of EBV-induced lymphomas (*19*). IgA pools with specificity for gp340 blocked the B cell immortalization seen with virus alone (Table 1).

Although both the polymeric and monomeric forms of the Ab abrogated infection of B lymphocytes, pIgA to gp340 facilitated the binding of EBV to the HT-29.74 epithelial cell line. By electron microscope (EM) autoradiography (*20*), we determined that 31 (11%) of 279 cells incubated with [^{3}H]EBV bound to pIgA bore autoradiographic grains on their surfaces or within cytoplasmic vesicles (Fig. 2). In contrast, 8 (3%) of 242 cells incubated with virus alone had grains on their surfaces only (P = 0.0007, two-tailed Fisher's exact test), which presumably represents nonspecific adherence. To examine whether the virus had been internalized, we treated a portion of cells from the above experiment with trypsin to remove the surface virus and then analyzed total cellular DNA by polymerase chain reaction (PCR) for a 110-bp sequence from within the large internal repeat region (IR1) of the EBV genome (*21*). Southern (DNA) blots of the amplification product revealed EBV sequences only in cells exposed to pIgA-EBV complexes, suggesting that pIgA had mediated cell binding and subsequent endocytosis of EBV (Fig. 2).

To confirm that pIgA-mediated entry of the virus into epithelium depends on SC, we attempted to infect cells that had first been incubated with rabbit Ab to human SC or with excess amounts of a nonspecific pIgA from an EBV-seronegative donor. When the epithelial receptor for pIgA was blocked by Ab to SC, pIgA-bound virus was found predominantly in cell supernatant washings, not in cell pellets (Fig. 2). In contrast, cells first incubated with an irrelevant rabbit Ab could bind pIgA-EBV; little virus was in the wash fraction. Like with the Ab to SC, pIgA-EBV complexes could be competitively displaced from SC with pIgA that lacked EBV specificity. Successful amplification of the cellular gene N-*ras* confirmed the absence of PCR inhibitory factors.

Because viruses can exploit cellular endocytotic activity and transepithelial transport mechanisms without actually infecting cells (*22, 23*), we looked for EBV antigen expression as an indication of successful endocytosis of the virus and initiation of EBV replication. When examined 18 hours after infection for antigens of the replicative cycle (*24*), approximately 4% of cells exposed to pIgA-EBV expressed the EBV immediate-early antigen BZLF1 and early antigen complex {diffuse [EA(D)] and restricted [EA(R)] components} by indirect immunofluorescence staining (Fig. 3). EBV antigens were not detected in cells incubated with the virus alone, virus bound to mIgA, or the virus plus pIgA from an EBV-negative donor. Six weeks after infection, cells infected by the pIgA-SC pathway still retained EBV DNA as determined by PCR analysis after two passages in culture. Up to 8% of cells within individual cellular subclones expressed EBV nuclear antigen (EBNA) when stained by the anticomplement immunofluorescence technique with a polyclonal human serum or with the EBNA 2–specific MAb PE2 (*21, 25*).

Antibody-mediated enhancement of viral infection provides an alternate infectious pathway to entry by virus-specific receptors. Observed with a wide range of viruses in vitro, this phenomenon is clinically significant in human dengue virus infection (*26*).

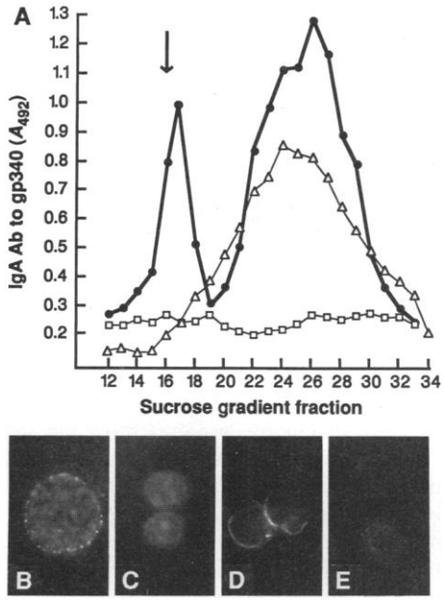

Fig. 1. Sedimentation profiles and SC binding of pIgA and mIgA Ab to EBV gp340. (**A**) Enzyme-linked immunosorbent assay (ELISA) quantitation of gp340 specificity of IgA from sera of patients with acute infectious mononucleosis (●) and nasopharyngeal carcinoma (△) and from supernatant fluids of the IgA secreting human lymphoblastoid cell line Daikiki [American Type Culture Collection (ATCC), TIB 206] (□). IgA separated on jacalin affinity columns (Pierce) was eluted with 0.1 M melibiose and dialyzed against phosphate-buffered saline (PBS) (*14*). IgA (350 μl at 1 mg/ml) was then mixed with 0.05 ml of beef liver catalase (10 mg/ml, Sigma, sedimentation rate 11.4*S*) and 3% glycerol and centrifuged on 5% to 20% (w/v) sucrose gradients at 45,000 rpm for 6 hours at 4°C (*15*). Fractions (125 μl) were collected from the bottom of the tube, and location of the catalase marker (arrow) determined by maximum froth production on addition of 2% hydrogen peroxide and 0.1% SDS (*13*). We determined gp340 specificity of IgA in each fraction by ELISA (*8*), using as substrate naturally glycosylated gp340 isolated by fast protein liquid chromatography from the membranes of B95-8 cells (*28*). Bound Ab was detected with peroxidase-conjugated rabbit Abs to human IgA (1:500 dilution), which was developed with orthophenylene diamine and measured at 492 nm. Fractions containing gp340-specific mIgA or pIgA were individually pooled, dialyzed against PBS, concentrated by Centricon 10 centrifugation (Amicon), then passed through 0.45-μm filters. (**B**) SC on surface of HT-29.74 epithelial cells (*16*), shown with rabbit Ab to human SC (*29*) at 1:1000 dilution. (**C**) Lack of binding of mIgA (0.4 mg/ml) from right peak (●) above. (**D**) SC binding of pIgA (0.1 mg/ml) from left peak (●). (**E**) Blockage of pIgA binding by excess Ab to SC (1:10 dilution). Membrane-bound Ab was detected at 4°C by FITC-labeled Ab to rabbit IgG (B) or to human IgA (C, D, and E).

Table 1. Effect of IgA Ab to gp340 on EBV-induced B cell transformation. Tenfold dilutions of stock EBV (transforming laboratory strain B95-8) were incubated for 30 min at 4°C with Ab to gp340 (titer 1:320) from either polymeric (pIgA) or monomeric (mIgA) sedimentation peaks of the sucrose gradient in Fig. 1. For the EBV lymphocyte transformation assay, stock virus or IgA-bound EBV was added to peripheral blood lymphocytes collected from an adult seronegative donor and separated on Ficoll-Hypaque gradients (Pharmacia). Cells were distributed in a microtest plate, 5×10^5 cells per well with six replicate wells used for each virus dilution. Transformation was judged by establishment of a permanent lymphoblastoid cell line after 6 weeks of culture. Lymphocytes from a second donor gave similar results, with IgA inhibition of transformation occurring at all virus dilutions.

Virus dilution	Transformed wells per total wells infected		
	EBV + B cells	EBV-pIgA + B cells	EBV-mIgA + B cells
10^{-1}	6/6	1/6	2/6
10^{-2}	6/6	0/6	0/6
10^{-3}	6/6	0/6	0/6
10^{-4}	6/6	0/6	0/6
10^{-5}	3/6	0/6	0/6

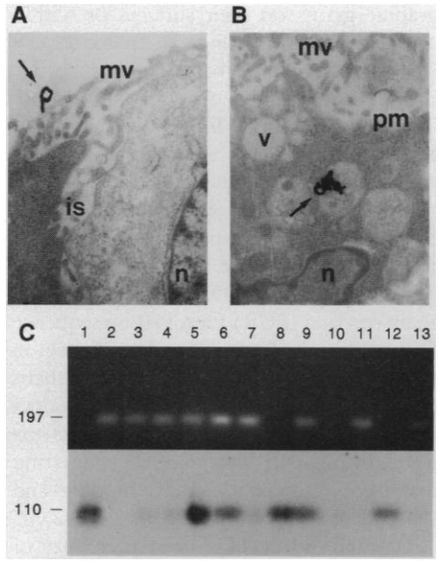

Fig. 2. SC-mediated entry of pIgA-bound EBV into HT-29.74 cells. (**A** and **B**) EM autoradiography, with grains (arrows) denoting bound or internalized [^{3}H]EBV after incubation with pIgA from Fig. 1 (n, nucleus; mv, microvillae; pm, plasma membrane; v, vacuole; is, intercellular space). We made tritiated EBV by inducing EBV replication in the virus-infected Akata cell line in the presence of 2 μCi of [^{3}H]thymidine per milliliter(specific activity 5 Ci/mmol) as described (*20, 30*). [^{3}H]EBV (50 μl) was incubated with 50 μl of pIgA (anti-gp340 titer 1:320) for 30 min, then added to approximately 5×10^6 cells (multiplicity of infection of 0.1 to 0.01 transforming units) with 100 μg of unlabeled thymidine for 30 min at 4°C. Cells were pelleted, washed five times, and processed for EM autoradiography (*20*). Exposure time was 8 weeks. (**C**) Ethidium bromide gel (top) and Southern transfer (bottom) of PCR products from a 197-bp region of the cellular gene N-*ras* and 110-bp region of EBV DNA in HT-29.74 cells infected as in (A) and (B). After infection, cells were treated 5 min at 4°C with trypsin (2.5 g/liter), washed five times, and cell DNA obtained by SDS lysis–proteinase K digestion and phenol extraction. Primers for N-*ras* were 5′-CACCCCCAGGATTCTTACAGAAAAC-3′ and 5′-TCCTAGTACCTGTAGAGGTTAATAT-3′. Primers that recognized the large internal repeat of EBV were 5′-GTTCGCGTTGCTAGGCCACC-3′ and 5′-AGGACCACTTTATACCAGGG-3′; the probe was 5′-AGCGCGTTTACGTAAGCCAGACAGCAGCCAATTGTCAGTT-3′ end-labeled with [gamma-^{32}P] adenosine 5′-triphosphate (ICN Biomedicals) by T4 polynucleotide kinase (US Biochemicals, Cleveland, Ohio) (*21*). PCR was performed on 100 ng of DNA according to manufacturer's protocol with Taq polymerase (Cetus, Norwalk, Connecticut). Lane 1, the PCR product from virus inoculum alone; lane 2, HT-29.74 cells alone; lane 3, HT-29.74 cells and EBV without IgA; lane 4, HT-29.74 with mIgA-EBV; lane 5, HT-29.74 with pIgA-EBV; lane 6, HT-29.74 with pIgA-EBV at 5 days in culture; lane 7, HT-29.74 cells first incubated with rabbit Ab to human SC (1:10 dilution) followed by pIgA-EBV; lane 8, concentrated cell supernatant washings from cells in lane 7; lane 9, HT-29.74 cells incubated with normal rabbit serum followed by pIgA-EBV; lane 10, supernatant washings from cells in lane 9; lane 11, HT-29.74 cells incubated with excess pIgA (0.1 mg/ml) from EBV seronegative donor, then pIgA-EBV complexes; lane 12, washings from cells in lane 11; lane 13, SC-negative epithelial cell line HTB43 (ATCC) with pIgA-EBV. Results are representative of five independent experiments with pIgA from separate donors.

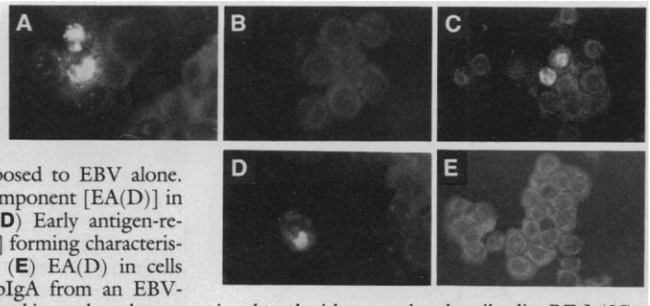

Fig. 3. Indirect immunofluorescence of EBV antigens in HT-29.74 cells after pIgA-dependent EBV entry. (**A**) EBV immediate-early protein BZLF1. (**B**) BZLF1 in control cells exposed to EBV alone. (**C**) Early antigen-diffuse component [EA(D)] in pIgA-EBV exposed cells. (**D**) Early antigen-restricted component [EA(R)] forming characteristic cytoplasmic aggregates. (**E**) EA(D) in cells incubated with virus and pIgA from an EBV-negative donor. Cells were fixed in methanol-acetone, incubated with monoclonal antibodies BZ.1 (*25*), EA(D), and EA(R) (Biotech), then stained with FITC-labeled Ab to mouse IgG.

The potential for Abs of the IgA isotype to promote viral invasion is suggested by studies which show epithelial transcytosis of IgA-immune complexes (*27*). What distinguishes the pIgA-mediated process we describe from the IgG enhancement in prior reports is the capacity of EBV-specific pIgA to promote entry into one cell type while neutralizing EBV infectivity for another. Moreover, the segregation of pIgA to the mucosal compartment, the transience of EBV-specific pIgA in the serum, and the persistence of EBV in mucosal tissues favor a mucosal effect of IgA-dependent infection. With all the human herpesviruses, primary infection in the immunologically naïve host has different clinical manifestations from those seen at recurrence. Our findings suggest one mechanism by which EBV could pass from trafficking B lymphocytes to mucosal epithelium during the chronic carrier state. This route of infection would account for the "reactivation diseases" of epithelium as opposed to the purely lymphoproliferative disorders classically associated with EBV infection.

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