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PRINCIPAL INVESTIGATOR: Richard M. Longnecker, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, Illinois 60208-1110

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6. AUTHOR(S)
Richard M. Longnecker, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, Illinois 60208-1110

E-Mail: r-longnecker@northwestern.edu

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13. ABSTRACT *(Maximum 200 Words)*

This proposal aimed to examine the role of a tumorigenic virus, Epstein-Barr virus (EBV), in etiology of breast cancer. EBV is an almost ubiquitous human herpesvirus that establishes lifetime latent infections in humans. It was the first human tumor virus discovered through its association with Burkitt's lymphoma in Africa. EBV infection also associates with various malignancies and proliferative syndromes, mainly affecting lymphoid and epithelial tissues. In lymphoid tissues these include Burkitt's Lymphoma, Hodgkin's disease, some adult T-cell lymphomas, and immunoblastic lymphoma in patients with immune dysfunction. EBV associates with the epithelial pathologies nasopharyngeal carcinoma and oral hairy leukoplakia. Recent studies have also found the EBV genome and viral gene expression in breast cancers using a variety of sensitive detection techniques. In the current proposal for the Department of Defense, we investigated the mechanism of EBV infection of breast epithelial cells.

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Introduction:

EBV infection and humans. EBV is an almost ubiquitous human herpesvirus, with 90% or more of adults carrying the virus (13). Primary infection with EBV is typically self-limiting and may occur asymptomatically during childhood. The tissues infected by EBV almost exclusively are lymphoid or epithelial tissues. During a typical primary infection 0.1 to 1% of circulating B cells show expression of EBV proteins, while during latent infection this decreases to about 1 in a million B cells (13). As with all herpesviruses, EBV infection persists for life (8).

EBV infection is associated with malignancies. EBV is a major viral co-factor in human tumorigenesis. Persistent infection associates with various malignancies and proliferative syndromes (13), mainly affecting lymphoid and epithelial tissues. In lymphoid tissues these include Burkitt's lymphoma, Hodgkin's disease, certain adult T-cell lymphomas and, with immunosuppression, immunoblastic lymphoma. EBV associates with the epithelial disorders nasopharyngeal carcinoma, and in immunosuppression, oral hairy leukoplakia. In immunosuppressed patients EBV associates with leiomyosarcoma, a tumor of smooth muscle origin (12). In vitro EBV efficiently infects, transforms and immortalizes B cells, yielding cell lines called lymphoblastoid cell lines (LCLs).

EBV and breast cancer. Several studies have associated EBV with breast cancer. A recent study detected EBV genomes and gene expression in breast cancer lesions, using PCR analysis, Southern hybridization, and immunohistochemistry employing antibodies against EBV nuclear protein EBNA-1 (2). This confirms the results of Griffin and co-workers who detected EBV by PCR and in situ hybridization in breast cancer lesions (10). There are also reports of EBV-associated lymphomas (1, 9) localizing to the breast and of bilateral breast cancer developing during the rare chronic active EBV infection syndrome (14). The lack of EBV in breast cancer tissue has also been reported (reviewed in (2) however the studies cited above are strengthened by use of multiple detection methodologies to establish the presence of EBV. Historically, EBV association with epithelial tumors has been difficult to reconcile with the observed inability of EBV virions to directly enter epithelial cells. However recent reports point to an alternative mechanism of EBV infection of epithelial cells: by cell-to-cell contact. In experiments in which epithelial cells are co-cultivated with EBV-transformed B cells it has been found that several human epithelial cell lines are infectable by cell-to-cell contact with EBV-bearing B cells (3, 7).

EBV entry into cells. The EBV receptor on B cells is CD21 (5), which interacts with gp220/350 molecules on the virion to bind it to the cell surface. Additional molecules are likely to act in the internalization of EBV. MHC class II molecule HLA-DR (11) acts as a co-receptor in EBV infection of B cells. Recently in this laboratory it has been shown that HLA-DP and HLA-DQ alleles of MHC class II also function in this capacity (6). Infection independent of CD21 has been demonstrated (18) by derivation of drug-resistant cell clones after exposure of gastric carcinoma epithelial cell lines to recombinant EBV bearing a drug resistance gene.

EBV infection by cell-to-cell contact. The role of cell-to-cell spread in EBV pathogenesis remains unclear, however there are recent reports of EBV infection of epithelial cells by the cell-to-cell route. Imai et al (7) reported efficient infection of diverse epithelial lines by this route. Included in the cell lines thus infected were cells derived from gastric adenocarcinoma, biliary carcinoma, laryngeal carcinoma, lung squamous cell carcinoma and adenocarcinoma, human colon adenocarcinoma and renal cell carcinoma. Fingeroth and colleagues (4) used co-cultivation with LCLs to achieve EBV infection of an epithelial cell line (293 cells) and showed that CD21 is weakly expressed on these cells and that infection could be blocked by antibodies to CD21. Chang and colleagues (3) infected nasopharyngeal and keratinocyte cell lines by co-cultivation with EBV-bearing B cells and showed first, that contact between EBV donor and recipient cells is a requirement for infection; second, that while this infection occurs without CD21 expression on recipient cells, it is enhanced by CD21 expression; and third, a small proportion of EBV-infected epithelial cells enter the viral lytic state.

Summary of DOD Proposal. This proposal aimed to examine the role of a tumorigenic virus, Epstein-Barr virus (EBV), in etiology of breast cancer. EBV is an almost ubiquitous human herpesvirus that establishes lifetime latent infections in humans. It was the first human tumor virus discovered through its association with Burkitt's lymphoma in Africa. EBV infection also associates with various malignancies and proliferative syndromes, mainly affecting lymphoid and epithelial tissues. In lymphoid tissues these include Burkitt's Lymphoma, Hodgkin's disease, some adult T-cell lymphomas, and immunoblastic lymphoma in patients with immune dysfunction. EBV associates with the epithelial pathologies nasopharyngeal carcinoma and oral hairy leukoplakia. Recent studies have also found the EBV genome and viral gene expression in breast cancers using a variety of sensitive detection techniques. In the proposal to the Department of Defense, we investigated the mechanism of EBV infection of breast epithelial cells.

Body:

The proposal consisted of three specific aims which are briefly described below. Results of experiments in each aim will be briefly summarized.

Aim 1: A. Determine if EBV can infect breast epithelial cells efficiently by cell to cell contact.

B. Confirm EBV infection by cell to cell infection of additional breast epithelial cell lines and primary cells.

C. Establish breast epithelial cell lines harboring EBV.

Much of aim 1 was completed. We performed a series of experiments which demonstrated that EBV could infect breast epithelial cells efficiently by cell-to-cell contact, whereas infection with cell-free virus was very inefficient. These results were reported in a manuscript which was published in the Journal of the National Cancer Institute (92:1849-1851). Infection of the epithelial cells was monitored using our

recombinant EBV expressing green fluorescent protein (GFP). Infection of epithelial cells was confirmed using an antibody directed against epithelial-specific antigen (ESA). In addition, related to the experiments proposed in aim 1, we demonstated that antibodies directed against gp350, a major glycoprotein found in the virion envelope, did not block infectivity. Since these antibodies efficiently block infection with free virus, these studies suggested that entry by cell-to-cell contact may require different factors than infection with cell-free virus. In regard to the second series of experiments proposed in aim 1, we were able to confirm that EBV can efficiently infect primary breast epithelial cells immortalized with hTERT and purchased from Clontech. Unfortunately, we were entirely unable to generate breast epithelial cell lines harboring EBV. This was the third portion of aim 1 and success of these experiments were vital to the experiments proposed in aim 2. Although we observed good infection of the T47D, MCF7, and hTERT immortalized breast epithelial cells, we were unable to generate cell lines harboring the EBV genome. In these experiments, we monitored infection by expression of GFP and attempted to select stable clones infected with EBV by use of G418 selection. A G418 resistance gene is adajacent to the GFP gene in our GFP recombinant EBV. It is unclear at this time, despite considerable effort and time during the granting period, why we were unable to generate such lines.

Aim 2: A. Determine the outcome of viral entry into breast epithelial cells using antibodies and/or nucleic acid probes specific for EBV gene products.

In this aim, we intended on determining which viral genes are expressed in breast epithelial cell lines infected with EBV. The major portion of the studies focused during the granting period attempted unsuccessfully in establishing cells lines infected with our GFP EBV. We actively pursued these studies since we believe that they may provide important information in regard to the type of latency EBV establishes in infected breast tissues if EBV is commonly found in breast tissues. This could provide important details in regard to any putative EBV gene expression in human breast cancer and would be important in the analysis of breast biopsies. In these experiments, we infected T47D, MCF7, and the hTERT immortalized breast epithelial cells with our recombinant EBV containing GFP and the G418 selectable marker. Drug selection was applied on monolayers of the breast epithelial cells after co-cultivation with lethally irradiated EBV-GFP cells to select clones of EBV-positive epithelial cells. The optimum drug concentration for each cell line was determined by plating the cell lines in various drug concentrations. Irradiation of the EBV-GFP cells prevents these cells from growing in the culture conditions. Despite good levels of infection of the epithelial cells monitored as we have previously reported (17), we were unable to derive EBV infected breast epithelial cell lines despite culturing the infected cells for several months. Numerous green cells were visible immediately following infection. The infected cells would occasionally double as observed by fluorescent microscopy but they did not form macroscopic colonies. We repeated this experiment several times using the same approach, but were unsuccessful in each trial. In addition, over the period of the funding period, we performed several variations of the general approach outlined above. This included (i) varying the proportion of fetal calf serum contained in the medium, (ii) varying the relative proportions of EBV "donor" and "recipient" cells, (iii) passage of the

infected breast epithelial cells at several timepoints after infection, (iv) varying the state of confluence of the breast epithelial cells prior to infection by cell-to-cell contact, and (v) obtaining T47D and MCF7 cells from several alternative sources in case our difficulties were specific to particular clones of these cells.

Aim 3: A. Examine mechanism of entry.

In this aim, in colloboration with Geoff Kansas (a member of the Microbiology – Immunology Department at Northwestern), we investigated whether B cell surface LFA molecules, which are major mediators of interaction with cell adhesion molecules (ICAMs, present on epithelial tissues), participate in EBV infection of breast epithelial cells by cell-to-cell spread. The approach was to examine whether antibodies to LFA were capable of blocking infection. The lymphocyte function associated antigen 1 (LFA-1) is present on all leukocytes and is an adhesion molecule playing a key role in B cell interactions with epithelial cells. LFA-1 is a heterodimer glycoprotein comprised of CD11a, the 180kDa integrin α chain, and the non-covalently associated CD18, (β2 integrin), a 90-95 kDa transmembrane protein. In our investigations we used a number of different antibodies against CD11a as well as antibodies against CD18. Antibodies were used over a range of concentrations. Protocols for use of the putative blocking antibodies involved their inclusion during cell-to-cell infection and/or in pre-treatment of EBV donor cells. Although this appeared to be a promising avenue of investigation, none of the antibodies we obtained from Dr. Kansas had any effect on the infection of breast epithelial cell lines by to cell-to-cell contact.

Key Research Accomplishments:

- Cell-free Epstein-Barr virus infects breast epithelial cells poorly.
- Cell-associated Epstein-Barr virus infects breast epithelial cells efficiently.
- Antibodies to gp350 did not block infection with cell-associated Epstein-Barr virus whereas it blocked infection with cell-free Epstein-Barr virus.
- Cell-associated Epstein-Barr virus may provide a means for Epstein-Barr virus to infect breast epithelia.
- Infection of breast epithelial cell lines with a GFP reporter recombinant EBV and drug selection did not result in the isolation of cell lines containing EBV.

Reportable Outcomes:

Papers:

Speck P, Haan K, **Longnecker R.** Epstein-Barr Virus Entry into Cells. Virology. 277:1-5, 2000.

Speck P and **Longnecker R**. Epstein-Barr Virus Infects Breast Epithelial Cells by Cell-to-Cell Contact. ***Journal of the National Cancer Institute*** 2000; 92:1849-1851.

Richard Longnecker

Abstracts:

Speck P, **Longnecker R**. Epstein-Barr Virus Infects Breast Epithelial Cells by Cell-to cell Contact. Lynn Sage Cancer Symposium, Chicago, Illinois, 2000.

Conclusions:

We found that cell free Epstein-Barr virus was unable to infect breast epithelial cells efficiently. In contrast, infection with cell associated EBV was able to efficiently infect several breast epithelial cell lines. The difficulty of establishing EBV infected breast epithelial cell lines, as observed in the study, indicates that there are some factors that are not well understood which govern the establishment of EBV in breast cell lines. Clearly more study is warranted. Finally, although the association of Epstein-Barr virus with breast cancer is still controversial, the studies performed over previous funding period provides a mechanism for Epstein-Barr virus to enter breast epithelial cells.

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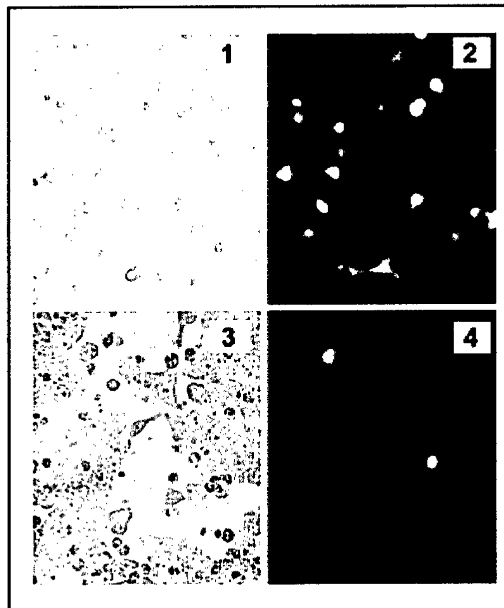

Fig. 1. Enhanced green fluorescent protein (EGFP) expression in breast epithelial cells after contact with EGFP-expressing lymphoblastoid cell lines (LCLs). Subconfluent monolayers of T47D breast epithelial cells in 12-well plates were cocultivated for 24 hours with equal numbers of GFP57 cells, an EGFP-positive LCL derived from primary human B cells, with the use of the EBfaV-GFP virus. After cocultivation, lymphoblastoid cells (which exist in suspension) were removed by washing monolayers three times with medium to remove suspended cells, and monolayers were examined by use of a Leica inverted microscope equipped for fluorescence. **Panels** 1 and 2 show the same field viewed by phase-contrast and fluorescent illumination, respectively. Approximately 1%–3% of cells in the monolayer expressed EGFP. In addition, a small number of rounded EGFP-positive cells, with morphology resembling that of lymphoblastoid cell lines, appeared to be adhering to the top of the monolayer. Cocultivation of T47D cells with Daudi cells induced to transiently express EGFP (**panels 3** and **4**, illuminated as above) by electroporation with plasmid p.EGFP.N1 did not result in EGFP expression in cells within the monolayer. Monolayer cells are negative for EGFP expression (**panel 4**). A small number of rounded EGFP-positive cells, with morphology resembling that of Daudi cells, appeared to be adhering to the top of the monolayer. Original magnification ×400 for all panels.

Infection of Breast Epithelial Cells With Epstein-Barr Virus Via Cell-to-Cell Contact

Peter Speck, Richard Longnecker

Epstein-Barr virus (EBV), a human herpesvirus present in more than 90% of adults, is a major viral cofactor in certain tumors of lymphoid and epithelial tissues *(1)*. Persistent infection is associated with malignancies and proliferative syndromes typically of lymphoid and epithelial tissues *(1)*, including Burkitt's lymphoma, Hodgkin's disease, certain adult T-cell lymphomas, and, in epithelium, nasopharyngeal carcinoma and oral hairy leukoplakia. *In vitro*, EBV efficiently infects, transforms, and immortalizes B cells, yielding lymphoblastoid cell lines (LCLs).

Several studies have associated EBV with breast cancer. Bonnet et al. *(2)* detected EBV genomes and gene expression in breast cancer lesions by using polymerase chain reaction (PCR) analysis, Southern hybridization, and immunohistochemistry specific for EBV protein EBNA (i.e., EBV nuclear antigen)-1. Labrecque et al. *(3)* detected EBV in breast cancers by PCR and *in situ* hybridization. There are descriptions of EBV-associated lymphomas *(4,5)* localizing to breast and of bilateral breast cancer developing during the rare chronic active EBV infection syndrome *(6)*.

Recent reports *(7,8)* have described EBV infection of human carcinoma cells on cocultivation with LCLs by a mechanism requiring cell-to-cell contact. These findings and the reported association with breast cancer prompted us to address the question of whether EBV enters breast epithelium by cell-to-cell contact. We have developed an appropriate reagent: EBV bearing the gene encoding and expressing the protein known as enhanced green fluorescent protein (EGFP) *(9,10)*. Cells infected by this virus, designated EBfaV-GFP, are readily detected by their green fluorescence *(9–11)*.

Here, we report that cells of human breast cancer epithelial lines T47D and MCF7, which are not infected on direct exposure to cell-free EBfaV-GFP virus, become infected when cocultivated with LCLs derived with and bearing EBfaV-GFP, as shown by expression of EGFP. This finding is consistent with a possible role for EBV in the etiology of breast cancer.

EBfaV-GFP, with EGFP driven by a strong promoter, within a dispensable region of the viral genome is produced as described previously *(10)*. MCF7 and T47D cells (derived from human breast tumors) and Daudi cells (an LCL immortalized by and bearing wild-type

Affiliation of authors: Microbiology–Immunology Department, Northwestern University Medical School, Chicago, IL.

Correspondence to: Richard Longnecker, Ph.D., Microbiology–Immunology Department, Northwestern University Medical School, Ward 6-231, 303 East Chicago Ave., Chicago, IL 60611 (e-mail: r-longnecker@nwu.edu).

See "Notes" following "References."

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EBV) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, and were cultured according to the recommendations of the ATCC. Binding of monoclonal antibody 323/A3 (Lab Vision, Fremont, CA) against epithelial-specific antigen (ESA), abundant on the surface of T47D and MCF7 cells, and Cy5-conjugated goat-anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) were measured by flow cytometry as described previously *(9)*. Antibody 72A1 *(12)* against EBV glycoprotein gp350 was used in antibody-blocking experiments. Plasmid pEGFP.N1 (Clontech Laboratories, Inc., Palo Alto, CA), used in control experiments to achieve transient expression of EGFP, was transfected by electroporation into Daudi cells as described previously *(10)*.

Monolayers of T47D or MCF7 cells in 12-well plates, at 50% confluence, were overlaid with equal numbers of GFP57 cells, which is an EGFP-positive lymphoblastoid cell line derived from primary B cells, by the method described previously *(10)* with the use of EBfaV-GFP virus. In control experiments, monolayers either were overlaid with equal numbers of Daudi cells transiently expressing EGFP (after adjustment for the proportion of cells expressing EGFP) or were exposed to cell-free filtered EBfaV-GFP virus produced as described previously *(9)* and assayed for infectious titer with the use of Daudi cells. After 24 hours of cocultivation, supernatants were removed, and cell monolayers were washed repeatedly with medium and visually examined with the use of an inverted fluorescence microscope (Leica Microscopes, Wetzlar, Germany). In monolayers cocultivated with GFP57 lymphoblastoid cells, approximately 1%–3% of cells within the monolayer expressed EGFP (Fig. 1, panel 2, showing T47D cells), indicating that infection had occurred. In addition, a small number of rounded EGFP-positive cells, with morphology resembling that of LCLs, appeared to be adhering to the top of the monolayer. No EGFP-expressing cells were present within monolayers exposed to cells transiently expressing EGFP (Fig. 1, panel 4), and again a small number of rounded EGFP-positive cells, morphologically resembling LCLs, appeared to be adhering to the top of the monolayer. Similar results were seen with MCF7 cells (data not shown).

The abundant expression of ESA by T47D and MCF7 cells is shown in Fig. 2 (panels 1 and 2). GFP57 and Daudi cells do not express ESA (panels 3 and 4). T47D and MCF7 cells are not infected by direct contact (Fig. 2, panels 6 and 7)

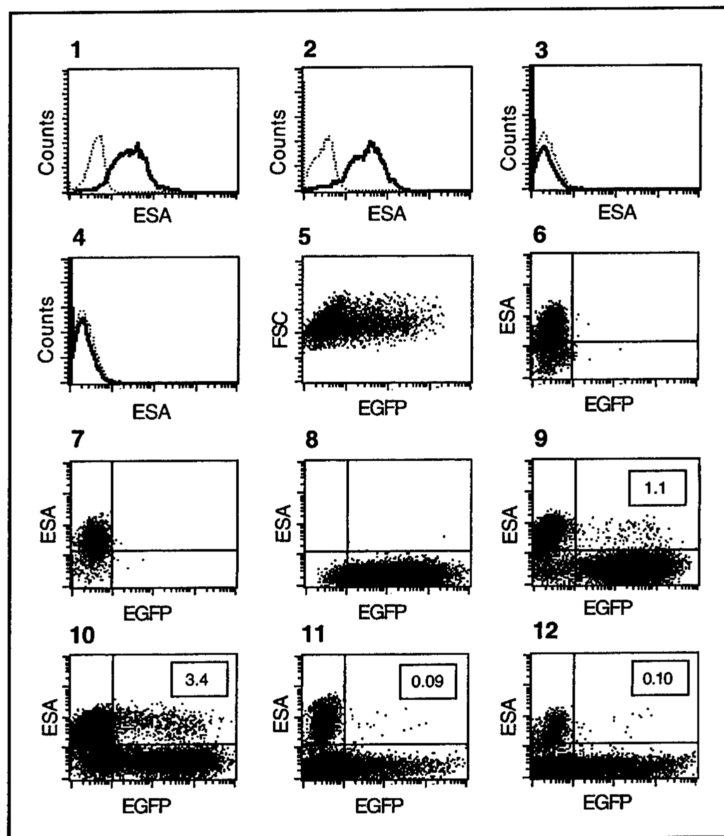

Fig. 2. Two-color flow cytometry confirms Epstein-Barr virus (EBV) infection of MCF7 and T47D breast epithelial cells by cell-to-cell contact. In two-color flow cytometry, expression of epithelial-specific antigen (ESA) serves as a marker to distinguish epithelial cells cocultivated with GFP57, an enhanced green fluorescent protein (EGFP)-expressing lymphoblastoid cell line (LCL) derived by immortalizing primary B cells with EBfaV-GFP virus. Binding of monoclonal antibody 323/A3 against ESA and Cy5-conjugated goat-anti-mouse secondary antibody was measured by flow cytometry as described previously *(9)*. ESA expression is present in abundance on MCF7 cells (**panel 1, heavy line**) and T47D cells (**panel 2, heavy line**). ESA is not present on GFP57 (**panel 3**) or Daudi (**panel 4**) cells. **Dotted lines** are negative control signals produced by omission of primary antibody. MCF7 and T47D cells are not infected by direct contact with EBfaV-GFP virus: On exposure of 2.5×10^5 cells to sufficient free virus to infect 20% of Daudi cells (**panel 5**), neither MCF7 cells (**panel 6**) nor T47D cells (**panel 7**) become infected. Cytometer was calibrated to place uninfected (EGFP-negative) cells (on the *x*-axis) and ESA-negative cells (on the *y*-axis) within the first power of 10 on logarithmic scales spanning 4 powers of 10. Relative fluorescence intensity is depicted on an arbitrary logarithmic scale and does not have units. Abundant expression of EGFP on GFP57 cells is depicted in **panel 8.** Cocultivation of MCF7 or T47D cells with GFP57 cells resulted in the presence of many doubly positive cells—i.e., ESA-positive and EGFP-positive cells. Doubly positive cells are in the **upper right quadrant,** and the **number in the box** is the percentage of total cells falling in this quadrant. In the case of MCF7, after cocultivation, doubly positive cells (1.1%, **panel 9**) were present at a 12-fold greater number than in the control (**panel 11**). In T47D cells, the effect was more marked, with a 34-fold increase in the number of doubly positive cells (3.4% [**panel 10**] versus 0.10% [**panel 12**]). **Panels 11** and **12** show controls, with MCF7 and T47D cells, respectively, cocultivated with Daudi cells transiently expressing EGFP, with percentages of doubly positive cells being ≤0.10% of the total. The same background level of ESA-positive/EGFP-positive cells was evident when GFP57 cells were mixed with T47D cells immediately before cytometry, suggesting that the background level did not arise during cocultivation (data not shown). Differences in cell numbers in the **lower right quadrants of panels 9** compared with **panel 11** and **panel 10** compared with **panel 12** reflect the relatively tight scatter of GFP57 cells in flow cytometry, compared with the very wide scatter displayed by electroporated Daudi cells. This difference in scatter resulted in relatively fewer of the EGFP-positive Daudi cells impinging on the gate employed in flow cytometry to visualize T47D or MCF7 cells. Each cytometry plot shows at least 20 000 events.

with a 100-μL inoculum of cell-free EBfaV-GFP virus that contained sufficient virus to infect 20% of 10^5 Daudi cells, as measured by the proportion of cells expressing EGFP (Fig. 2, panel 5).

To confirm that EGFP expression was occurring in breast epithelial cells in the monolayer and not in GFP57 cells, two-color flow cytometry was applied with the use of a marker present only on the epithelial cell population. Cells were removed, resuspended, and reacted with antibody 323/A3; infected epithelial cells—defined as positive for both ESA and EGFP—were then enumerated. Results (Fig. 2, panels 9 and 10) show that 1.1% and 3.4% of cocultivated MCF7 and T47D cells, respectively, were ESA positive and EGFP positive, confirming infection by cell-to-cell contact. Control experiments, in which cocultivated breast cell monolayers with Daudi cells transiently expressed EGFP (Fig. 2, panels 11 and 12), yielded minimal background (≤0.10%) of dual-positive cells. The same level of background was seen when EGFP-expressing Daudi cells and T47D or MCF7 cells were mixed immediately before flow cytometry, suggesting that the background level did not arise during cocultivation (data not shown).

To address the possibility that LCLs on cultivation yielded free virus that then infected by direct virus-cell contact, cocultivations were repeated with a blocking antibody. Antibody 72A1 against EBV glycoprotein gp350, when included in cocultivations at a range of concentrations (0–40 μg/mL), the highest of which completely abrogates infection of Daudi cells by EBfaV-GFP virus, did not reduce the proportion of T47D or MCF7 cells that became infected (data not shown). We conclude that EBV cell-to-cell infection of these epithelial cells does not require the presence of free virus.

Efficient infection of T47D and MCF7 cells by cell-to-cell contact requires actively growing cells. Repeating cocultivation experiments with the use of completely confluent monolayers yielded cells positive for both ESA and EGFP numbering between 0.2% and 0.3% of total cells, which was two to three times the background level (data not shown).

Previous reports *(7,8)* have shown that EBV infects epithelial cells by cell-to-cell contact. Our observation is consistent with these findings and extends to breast epithelium the range of tissue types potentially infectible by EBV; it also supports the notion that virus-bearing lymphocytes may serve as virus donors for infection of epithelial cells *(8)*. Cell-to-cell spread by fusion of infected cells with uninfected cells has been documented for other viruses, e.g., herpes simplex virus *(13,14)*, pseudorabies virus *(15)*, human immunodeficiency virus *(16)*, and paramyxoviruses such as measles *(17)*. Although the current studies do not elucidate the mechanism of the cell-to-cell spread of EBV, fusion of infected cells with uninfected cells is a possible explanation for this phenomenon. Alternatively, close cell-to-cell contact could augment the accessibility of virus to recipient cells, possibly with viral attachment and entry via a hypothetical low-affinity receptor molecule or molecules. Examination of the mechanism will be a subject of future experiments. EBV association with epithelial tumors has been difficult to reconcile with the apparent inability of EBV virions to efficiently undergo direct entry into epithelial cells, which express little, if any, CD21 (the major receptor for EBV). These observations begin to address the difficulties in understanding a role for EBV in breast cancer etiology by demonstrating EBV entry into breast epithelium by cell-to-cell contact.

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MINIREVIEW

Epstein–Barr Virus Entry into Cells

Peter Speck, Keith M. Haan, and Richard Longnecker[1]

Microbiology–Immunology Department, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611

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Epstein–Barr virus (EBV), a gamma herpesvirus persisting in B cells of most adults, is the prototypic human tumor virus. Persistent infection associates with malignancies and proliferative syndromes mainly affecting lymphoid and epithelial tissues (Rickinson and Kieff, 1996). EBV was discovered after Denis Burkitt, working in Africa in the 1950s, proposed that an infectious agent was involved in the etiology of a childhood tumor, now known as Burkitt's lymphoma (Burkitt, 1962). Herpesvirus-like particles were subsequently observed on electron microscopic examination of cell lines derived from these tumors (Epstein *et al.,* 1964). In lymphoid tissues EBV-associated tumors include Burkitt's lymphoma, Hodgkin's disease, and certain adult T-cell lymphomas. EBV infection associates with the epithelial disorders nasopharyngeal carcinoma, gastric carcinoma, and oral hairy leukoplakia (Osato and Imai, 1996; Rickinson and Kieff, 1996), the latter occurring in immunocompromised patients. In this group the presence of EBV also associates with lymphoproliferative diseases and with leiomyosarcoma, a tumor of smooth muscle origin. EBV genomes and gene expression have been detected in breast cancers (Bonnet *et al.,* 1999; Labrecque *et al.,* 1995). Evidence for this association, e.g., detection of viral genomes in each malignant cell within a tumor, has been reviewed recently by Cohen (2000). As exhibited by the range of pathology described above, EBV clearly gains entry to a variety of cell types, notably B cells and epithelial cells. Studies of EBV biology have been facilitated by construction of viruses containing drug resistance markers, for example that described by Shimizu *et al.* (1996). A recent enhancement of this approach is development of viruses carrying reporter genes, such as that designated EBfaV–GFP (Speck *et al.,* 1999; Speck and Longnecker, 1999), which bears the gene for enhanced green fluorescent protein (EGFP) and produces infectious virus in high titer. Use of this reagent enables the ready visualization and enumeration of infected cells. The strategies EBV has evolved to enter its various target cells are complex and incompletely understood. However, evidence is accumulating that entry of EBV, like many other viruses, involves interactions between several viral glycoproteins and multiple cellular entry mediators.

EBV readily infects human B cells *in vitro,* with initial attachment mediated by binding (Fig. 1) of the EBV major outer envelope glycoprotein, gp350/220, with cellular CD21 (Nemerow *et al.,* 1985; reviewed in Kieff, 1996). Induction of high-level expression of CD21 on normally uninfectable cells such as mouse L cells, human T or erythroleukemia cells, or transformed epithelial cells leads to viral adsorption and inefficient infection (Ahearn *et al.,* 1988; Koizumi *et al.,* 1992; Li *et al.,* 1992; Paterson *et al.,* 1995). From this it has been inferred that the specific role of CD21 in EBV infection is to capture virions at the cell surface. The level of CD21 expression required to bind virions is not known. However, BJAB cells, first shown to be infectable in 1975 (Clements *et al.,* 1975), and which express relatively low levels of CD21 compared to other lymphoblastoid cell lines (Speck and Longnecker, 1999), are readily infectable. As with other herpesviruses, such as herpes simplex, additional molecules are necessary and required for postbinding events such as fusion of virus and cell membranes and virus internalization. Table 1 lists EBV glycoproteins involved in viral entry.

Postbinding events in EBV infection involve a complex of at least three additional EBV glycoproteins: gH, gL, and gp42 (Fig. 1). gH and its homologs are involved in entry of HSV and other herpesviruses (Forrester *et al.,* 1992; Fuller *et al.,* 1989; Gompels and Minson, 1986; Haddad and Hutt-Fletcher, 1989; Miller and Hutt-Fletcher, 1988; Peeters *et al.,* 1992). The EBV gH homolog is designated gp85, product of the BXLF2 gene (Heineman *et al.,* 1988; Oba and Hutt-Fletcher, 1988). As with other herpesviruses, gH associates with an accessory protein,

[1] To whom correspondence and reprint requests should be addressed. E-mail: r-longnecker@nwu.edu.

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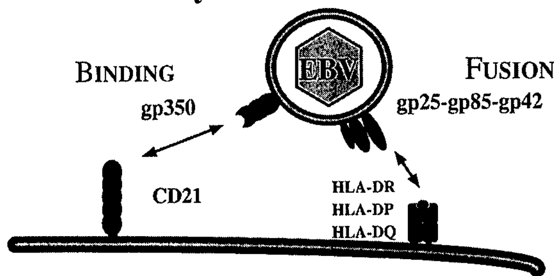

FIG. 1. Epstein–Barr virus infection of B cells. Viral entry into B cells involves the interaction of multiple viral and cellular molecules. The initial attachment of virus to cell is mediated by viral gp350 binding cellular CD21. Postbinding events, mediating internalization of the virus, are known to include interaction between the viral gp25/gp/85/gp42 complex and MHC class II molecules (HLA-DR, -DP, or -DQ) on the cell surface. In contrast, for epithelial cell infection the gH/gL complex does not require gp42 and interacts with a ligand of unknown identity, and gH appears to also have a role in attachment.

a gL homolog designated gp25, product of the BKRF2 ORF (Yaswen *et al.,* 1993). gp85 requires gp25 for its correct folding, transport, and function within the infected cell (Forghani *et al.,* 1994; Hutchinson *et al.,* 1992; Kaye *et al.,* 1992; Liu *et al.,* 1993; Roop *et al.,* 1993; Spaete *et al.,* 1993). Antibodies to gp85 inhibit fusion but not attachment of EBV to B cells (Miller and Hutt-Fletcher, 1988). Virosomes made from EBV proteins bind and fuse with CD21-positive cells; however, without the gH–gL complex they bind but do not fuse (Haddad and Hutt-Fletcher, 1989). In EBV the gp25/gp85 complex associates with the additional glycoprotein gp42, product of the BZLF2 gene (Li *et al.,* 1995). An antibody to the BZLF2 product inhibits infection of B cells but not epithelial cells (Li *et al.,* 1995), implying a role for gp42 in entry of B cells only. The role of gp42 in B cell infection is in penetration of cells rather than attachment, as shown by the finding that EBV lacking gp42 binds B cells normally but cannot penetrate (Wang and Hutt-Fletcher, 1998). Hutt-Fletcher and colleagues showed that MHC molecule HLA-DR is a ligand for gp42 and functions as EBV coreceptor in infection of B cells, confirming an early report of HLA-DR participation in this process (Reisert *et al.,* 1985).

Other molecules are capable of participation in EBV entry (Fig. 1). Haan and colleagues demonstrated that transient expression of HLA-DP or HLA-DQ on CD21-positive, MHC class II-deleted lymphoblastoid cells renders them infectable, showing that these MHC class II isotypes also function as EBV entry mediators (Haan *et al.,* 2000). While it appears that all HLA-DR and -DP molecules function as coreceptors, only -DQ molecules encoding a glutamic acid residue at amino acid 46 of the mature β chain are able to mediate entry (Haan and Longnecker, 2000). This finding is consistent with the groove between the $\beta 1$ and $\beta 2$ domains of the HLA molecule being important for gp42 binding and also raises the possibility that EBV-related sequelae might be related to HLA haplotype. The possibility that B cells possess other EBV entry mediators is suggested by the observation that B cells from bare lymphocyte syndrome patients, which in some cases appear to completely lack expression of HLA-DR, -DP, or -DQ, have been infected with and transformed by EBV. It is not known whether the initial binding of EBV to B cells can be mediated by molecules other than CD21. In our hands transient expression of HLA-DR on mouse B cells does not render them infectable, showing that infection is not mediated by HLA-DR alone (data not shown).

The mechanisms underlying EBV infection of epithelial cells remain unclear, in part because an overwhelming majority of epithelial cell lines are refractory to EBV infection *in vitro.* Several routes for direct infection of EBV virions into epithelial cells have been reported, without a common theme emerging. Nonetheless, mechanisms of viral entry into these cell types show a clear divergence from the mechanisms employed in B cell entry. Yoshiyama and colleagues, using EBV bearing a drug resistance gene, describe EBV infection of CD21-negative gastric epithelial cells as shown by derivation of drug-resistant cell colonies (Yoshiyama *et al.,* 1997). While this result implies a CD21-independent infection route, it is possible that very low level expression of CD21 may suffice to permit EBV entry. Fingeroth and colleagues using an epithelial cell line, 293, which expresses low levels of CD21, have shown CD21-dependent infection of

TABLE 1

EBV Glycoproteins Participating in Attachment and Entry

EBV gene	HSV protein[a]	EBV protein	Known or proposed function
BKRF2	gL	gp25	Complexes with gp42 and gp85[c]
BXLF2	gH	gp85	Complexes with gp42 and gp25[c]
BZLF2	gD[b]	gp42	Complexes with gp25 and gp85,[c] binds HLA class II
BALF4	gB	gp110	Virus maturation
BLLF1	gC[b]	gp350/220	Initial attachment by virion binding to CD21

[a] The herpes simplex virus (HSV) glycoproteins which share sequence homology and/or functional homology with EBV glycoproteins.
[b] Although having no sequence homology with EBV glycoproteins, these HSV glycoproteins may serve as functional homologues.
[c] May serve as fusion complex for EBV B cell entry. Epithelial cell entry does not require gp42 and may require a fusion complex lacking gp42.

these cells by the derivation of drug-resistant colonies, supporting the view that low level surface CD21 expression can enable EBV infection of epithelial cells (Fingeroth *et al.,* 1999). Epithelial cells stably transfected to express CD21 at high levels efficiently bind EBV and undergo a transient infection (Li *et al.,* 1992). Recently Hutt-Fletcher and colleagues reported that gH-deleted EBV binds B cells normally but is impaired in attachment to CD21-negative epithelial cells, implying existence of a gH ligand on epithelial cells and a role for gH in attachment and entry of these cells (Molesworth *et al.,* 2000). gp42 is dispensable for infecting cells of the CD21-expressing epithelial line SVKCR2, yet soluble gp42 inhibits this infection and B cell infection, prompting a model of EBV virions forming two types of gH–gL complex, one including gp42 and the other not, with the two forms having mutually exclusive abilities to mediate infection of B cells or epithelial cells (Wang *et al.,* 1998). A possible role for EBV glycoprotein gp150 in epithelial infection is suggested by the observation that recombinant EBV lacking gp150 infects B cells normally but is enhanced in its ability to infect SVKCR2 cells (Borza and Hutt-Fletcher, 1998).

An alternative entry route into epithelial cells has been demonstrated by Sixbey and colleagues who have shown that certain epithelial cells expressing the IgA receptor can internalize EBV, if bound to polymeric IgA (pIgA) (Sixbey and Yao, 1992). In studies using a polarized epithelial cell system, the EBV–pIgA complex enters the basolateral cell surface and is modified and secreted from the opposite (apical) surface of the cell (Gan *et al.,* 1997). Some individuals who have been exposed to EBV express IgA antibodies to EBV-associated antigens in their serum, so in these individuals infection of epithelial cells could potentially occur by this IgA-mediated mechanism. Recent work with this system shows that in polarized MDCK epithelial cells stably expressing CD21, viral uptake is higher on apical than on basolateral surfaces, despite CD21 expression predominating basolaterally, implying that other cell surface molecules may participate in the virus–cell interaction (Chodosh *et al.,* 2000).

Cell-to-cell contact has been described as an efficient means of EBV entry into epithelial cells. Imai and colleagues working with a diverse range of epithelial cell lines derived from gastric and colon adenocarcinoma and hepatocellular, laryngeal, lung squamous cell, and renal cell carcinomas, found that cocultivation of these cells with lymphoblastoid (EBV-bearing) cells leads to EBV infection of the epithelial cells (Imai *et al.,* 1998). Chang and colleagues confirmed this result using nasopharyngeal carcinoma cells, also finding that efficient infection occurs independently of CD21, that cell-to-cell contact is a requirement for the infection, and that cell-to-cell infection could be further enhanced by inducing expression of CD21 (Chang *et al.,* 1999). Recently in this laboratory we have used lymphoblastoid cell lines established by and bearing EGFP-expressing EBV to show, by transfer of EGFP to epithelial cells cocultivated with EGFP-expressing cell lines, that cell-to-cell spread of EBV occurs to breast epithelial cells (Speck and Longnecker, 2000). This finding extends the range of cell types infected by cell-to-cell spread and reinforces the view that EBV may be involved in breast cancer development, as the viral genome and gene products have been detected in these cancers (Bonnet *et al.,* 1999; Labrecque *et al.,* 1995).

There is a paucity of information regarding the mechanisms underlying cell-to-cell spread of virus, and the identity of virally encoded or cellular molecules participating in the process is not known. Cell-to-cell spread of EBV into the epithelium may be of particular clinical relevance as the cell types infected, which include gastric, nasopharyngeal, and breast epithelium, are among those in which EBV has been implicated in tumor development.

Recent findings reveal that, like other herpesviruses such as herpes simplex virus (Spear *et al.,* 2000), EBV has evolved to utilize multiple entry receptors on the cell surface and to enter cells by multiple mechanisms, involving direct virus–cell entry and cell-to-cell spread of virus. Much of the biology of EBV entry into susceptible cells remains obscure. Areas likely to be focused on in the future could include (i) identification of other EBV entry mediators; (ii) elucidation at the molecular level, such as by X-ray crystallography, of interactions between viral and cellular molecules participating in the attachment, fusion, and entry of EBV; and (iii) study within the infected cells of signaling triggered by events at the cell membrane. In addition, despite the clear implication of EBV in certain T cell tumors, there is very little knowledge regarding EBV infection of T cells. Further research to address these issues will provide new insight into the tropism and pathogenesis of a significant human tumor virus.

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