Human Epithelial Cell Expression of an Epstein–Barr Virus Receptor

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(Accepted 3 December 1986)

SUMMARY

Cultured human epithelium bound and internalized radiolabelled Epstein–Barr virus (EBV) within 1 h of exposure. A similar percentage of cultured cells also were reactive with monoclonal antibodies to the EBV/C3d receptor of B lymphocytes. In cross-sections of fresh frozen, stratified epithelium, receptor expression seemed limited to the less differentiated subpopulation of cells. These findings support the notion of direct infection of epithelial cells by EBV and suggest a viral life cycle in epithelium dependent on the stage of cell differentiation.

INTRODUCTION

The concept of strict B lymphocyte tropism has been central to discussions of the biology of the Epstein–Barr virus (EBV) since the discovery of this herpesvirus in 1964. EBV's restricted host cell range is based on an apparent lack of membrane receptors for the virus on human cells other than B lymphocytes, and virus-associated diseases such as Burkitt's lymphoma, infectious mononucleosis, and diffuse lymphoproliferative disorders of the immunocompromised host all involve infections of the B cell lineage. Yet, important aspects of virus–host interaction remain unclear when viewed in terms of an absolute tropism by EBV for B lymphocytes (Miller, 1984; Rickinson, 1984). First, EBV is linked to an epithelial cell malignancy, undifferentiated nasopharyngeal carcinoma, with viral DNA present in 100% of the tumours worldwide, an association more striking than in Burkitt's lymphoma. Second, despite clear evidence that EBV replicates and is shed at sites in the oropharynx, lymphocytes permissive for viral replication can not be demonstrated in vivo, and certainly experimental infection of B cells in vitro results not in viral replication, but in viral latency and cell immortalization. Recent reports suggest that viral replication in epithelial cells may be a regular feature both of acute EBV infection in man and of the chronic viral carrier state (Lemon et al., 1977; Sixbey et al., 1984; Wolf et al., 1984; Greenspan et al., 1985), but the means whereby the virus gains entry into such cells has been the subject of considerable debate (Bayliss & Wolf 1980, 1981; Sixbey et al., 1983; Takimoto et al., 1983). We present here evidence for viral recognition of a cell surface receptor in vitro on an undifferentiated subset of human epithelial cells, suggesting direct infection of these cells by EBV.

METHODS

Lymphocytes and epithelial cells. Primary explant cultures of normal human ectocervix were initiated from hysterectomy material as previously described (Sixbey et al., 1983) and harvested 2 to 3 weeks after initiation. Experiments were performed with epithelia from 10 separate donors. In all experiments adherent epithelial cell sheets were gently treated with 0.125% trypsin/0.015% EDTA until detached, and were studied as cell suspensions. To monitor effects of trypsinization on cell surface components, EBV receptor-positive Raji (lymphoid) cells, both trypsinized and untreated, were used as controls along with a lymphoid cell line LTR228 (Larrich et al., 1983), currently EBV receptor-negative.
Fresh frozen sections of ectocervix were mounted on glass slides, fixed 10 min in cold acetone and stored at -70 °C for indirect immunofluorescence assays.

**Preparation of tritiated virus.** Virus stocks were obtained from the P3J-HR1 Burkitt's lymphoma cellular subclone 13 which when treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) releases large amounts of virus into culture supernatant fluids (Heston et al., 1982). A tritiated EBV stock was prepared from clone 13 maintained 2 days in medium containing 760 µg/ml hypoxanthine and 18 µg/ml aminopterin and induced with 30 ng/ml TPA in the presence of 2 µCi/ml [3H]thymidine (sp. act. 2 Ci/mmol). At day 3 of induction, 2 µCi/ml [3H]thymidine (sp. act. 44 Ci/mmole) was added. Virus was pelleted from culture supernatant fluids on day 7 and resuspended in 1/160th of the original volume of medium. Particle counting by electron microscope (EM) negative staining revealed greater than 2-7 x 10^6 virions/µl (Tereba & Murti, 1977). Trichloracetic acid precipitation of stock virus yielded 16338 c.p.m. for 50 µl of preparation and 17077 c.p.m. for the same volume of unpelleted virus stock.

**Viral attachment studies.** Trypsinized epithelial and control cell pellets of 10^6 cells were washed twice, resuspended in 125 µl of tritiated EBV stock in the presence of 100 µg unlabelled thymidine at 37 °C for 1 h, pelleted, and then washed five times. Multiplicity of infection was ≥ 300 viral particles per cell. Infected cells were fixed in 2% glutaraldehyde overnight, washed with 0.1 M-sodium cacodylate buffer, post-fixed for 1 h in 1% osmium tetroxide-dehydrated and embedded in Spurr resin. Sections were either stained with 2% uranyl acetate and Reynolds lead for direct observation or were placed on EM grids and lightly coated with carbon for autoradiographic studies. Ilford L-4 photographic emulsion was applied to grids with a wire loop and air-dried 2 h. Grids were sealed from light in desiccant boxes and kept at 4 °C for 2 to 4 weeks. Radiographic grains were developed with D19 (Kodak) and sections were stained with 3% uranyl acetate for 30 min.

**Immunolabelling of EBV receptor.** Anti-C3d/EBV receptor monoclonal antibodies (MAbs) HB-5 at 5 to 50 µg/ml and anti-B2 (Coulter, Hialeah, Fla., U.S.A.) at 1:10 dilution in phosphate-buffered saline with 1% bovine serum albumin (PBS/BSA) were used in immunolabelling assays. Antibody controls consisted of unrelated IgG2a mouse MAbs M5-F10 against the core region of lipopolysaccharide, T1B (Coulter) an anti-T lymphocyte MAb, B1 (Coulter) which is reactive with all human B cells, and an unrelated IgM mouse MAb, 12A5. Immunolabelling for the C3d receptor was performed on live cell suspensions at 4 °C. After incubation with primary antibody for 1 h, cells were washed three times, then incubated 1 h with either fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin antibodies (Cappel Laboratories) at 1:100 dilution or 5 to 20 nm colloidal gold particles conjugated to goat anti-mouse immunoglobulin antibodies (Janssen Life Sciences, Beerse, Belgium) diluted 1:20 in PBS/BSA. Cells in gold-labelling experiments were then washed, fixed in 1% glutaraldehyde, dehydrated, and embedded in Spurr resin for thin sectioning.

Serial cross-sections of fresh frozen human ectocervical epithelium fixed for 10 min in acetone were stained with anti-C3d/EBV receptor MAbs HB-5 or anti-B2 as above. Rabbit anti-human involucrin antiserum (gift of F. M. Watt) and AE2, a mouse MAb to high molecular weight keratins (gift of T.-T. Sun), were used at dilutions of 1:10 to 1:40 in PBS/BSA on epithelial cross-sections.

**RESULTS**

**Virus binding**

Attachment and penetration of EBV in epithelial cell suspensions were studied by EM to permit ultrastructural characterization of target cells and to exclude chance contamination of primary explant cultures by lymphocytes. By EM 6% of epithelial cell cross-sections bore membrane-bound or intracytoplasmic herpesvirus-like particles (Table 1 and Fig. 1a). Contaminating lymphocytes were not detected in epithelial cell suspensions with MAb B1 and epithelial cells were readily identified on the basis of their distinctive ultrastructural appearance (Shingleton & Lawrence, 1976). Viral particles characteristically attached to cell surface projections or areas of microvilli on epithelial cells as well as on Raji cell controls (Fig. 1), an observation previously reported for lymphocytes (Seigneurin et al., 1977; Nemerow & Cooper, 1984). Intracytoplasmic particles were observed within vesicles and as non-enveloped viral nucleocapsids within the cytoplasmic matrix. No intranuclear particles were visualized.

Labelling of P3J-HR1 virus with [3H]thymidine allowed verification by EM autoradiography that adsorbed particles were in fact input virus (Table 1). Autoradiographic grains indicative of virus were detected on 4% of epithelial cell sections in locations consistent with the short incubation period: on cell surfaces and in the peripheral cytoplasm (Fig. 1c to e). Unlike EBV receptor-positive Raji cell controls which bound multiple particles per cell (Fig. 1f), there was little evidence for viral binding to the receptor-negative lymphoid cell control, LTR228 (Table 1, Fig. 1g).
Fig. 1. EBV attachment and penetration in cultured epithelium. (a) Cultured epithelial cell with viral particle attached to microvillus, favoured site of viral binding. Note fusion of virus to epithelial cytoplasmic matrix. (b) EBV receptor-positive Raji cell control with virus attached to cell surface projection. Autoradiographic grains in (c) and (d) denote attachment of radiolabelled EBV to epithelial cell microvilli. (e) Intracellular radiolabelled viral particle near tonofilaments approaching epithelial cell nucleus at lower left. (f) EM autoradiograph of Raji cell (receptor-positive) control. Note multiple grains, indicative of bound EBV, again on cell surface projections as in (b). The EBV receptor-negative lymphoid line LTR228 did not bind radiolabelled virus, as demonstrated by the autoradiograph in (g). Bar markers represent (a to d) 100 nm and (e to g) 1 μm.
Table 1. Evidence for EBV receptor (EBVR) on cultured human epithelium

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Direct identification of virus</th>
<th>Autoradiography with 3H-labelled EBV*</th>
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<tbody>
<tr>
<td>Epithelial†</td>
<td>16/266 (6%)</td>
<td>12/310 (4%)</td>
</tr>
<tr>
<td>Raji (EBVR⁻)</td>
<td>95/100</td>
<td>54/100</td>
</tr>
<tr>
<td>LTR228 (EBVR⁻)</td>
<td>0/300</td>
<td>2/350</td>
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|                 | Immune labelling of EBVR       | Immunogold staining (EM)             |
|                 | (labelled cells/total counted) |                                      |
|                 | Direct autoradiography         | Indirect immunofluorescence          | Immunogold staining (EM) |
|                 | with 3H-labelled EBV*          | Anti-EBVR MAb HB-5                  | Anti-EBVR MAb HB-5 |
|                 |                                | Unrelated MAb                       | Unrelated MAb         |
|                 | 2/300                          | 114/2549 (6%)                       | 2/300                 |
|                 | 0/100                          | 92/100                               | 0/100                 |
|                 | 2/350                          | 0/100                                | 0/100                 |

* Grain counts were performed independently by two observers in randomly selected fields on separate EM grids.
† Data represent seven experiments performed with cultured epithelia from seven separate donors.
‡ ND, Not determined.

EBV/C3d receptor studies

Recently, Fingeroth and associates reported that the EBV receptor molecule on B lymphocytes is identical to the receptor for the C3d component of complement (C3dR) (Fingeroth et al., 1984; Weiss et al., 1984). To determine whether C3dR was expressed on a subset of epithelial cells, we employed two MAbs which recognize distinct antigenic epitopes on C3dR: HB-5, an IgG2a mouse MAb (Tedder et al., 1984), and anti-B2, an IgM class MAb (Nadler et al., 1981). By membrane immunofluorescence, unfixed cultured epithelial cell suspensions from four tissue donors contained from 1 to 9% C3dR-positive cells when tested with HB-5 (Fig. 2b). No staining was achieved with unrelated MAb or ascitic fluid controls. Bound HB-5 was concentrated on microvilli of 5% of epithelial cell sections when examined by EM with goat anti-mouse IgG-coated gold beads (Table 1, Fig. 2d). Localization of HB-5 to cell surface projections was in a pattern consistent with that seen in Raji cell controls (Fig. 2c) and reflected the binding site of the virus in both cell types (Fig. 1). Although prior trypsinization precluded valid assessment of receptor density, cultured epithelial cells bound virus or gold beads at fewer sites than lymphoid controls, rarely exceeding three sites per epithelial cell cross-section.

C3dR expression on human B lymphocytes is linked to B cell differentiation: the receptor is lost at the plasma cell stage of terminal differentiation (Tedder et al., 1984). Significantly, epithelial cells binding virus in adsorption studies had the ultrastructural appearance of metabolically active, less differentiated cells of the basal and parabasal layers of epithelium. To determine whether C3dR expression in stratified squamous epithelium was restricted to the lower, less differentiated cell layers, we stained by indirect immunofluorescence fresh frozen sections of human ectocervix with HB-5 and anti-B2 (Fig. 3a, b). HB-5 gave brilliant membrane staining at the basal/parabasal levels (Fig. 3b); anti-B2 antibodies produced weak staining in the same region: four unrelated MAbs did not stain the epithelial sections. The pattern of staining with HB-5 and anti-B2 was clearly restricted to the less differentiated cell layers while, in parallel tests, antibodies reactive with two markers of terminal epithelial cell differentiation, involucrin (Rice & Green, 1979) and the 65K to 67K mol. wt. keratins (Tseng et al., 1982), yielded the inverse pattern of fluorescent staining with reactivity only to the uppermost cell layers (Fig. 3c).

Fig. 3. Selective reactivity of MAbs specific for the C3d/EBV receptor in stratified, differentiating epithelium. (a) Cross-section of stratified squamous epithelium from ectocervix stained by haematoxylin and eosin. (b) Serial section of (a) stained by indirect immunofluorescence with HB-5. Arrows delineate basal and superficial cell layers, with fluorescent staining in the less differentiated basal/parabasal layers. (c) Superficial epithelial cell layers stain positive for involucrin, a marker of epithelial cell end-stage differentiation. Bar markers represent 10 µm.
Epithelial cell expression of an EBV receptor

Fig. 2. Reactivity of cultured human epithelium with MAbs specific for the B lymphocyte C3d/EBV receptor. (a) C3d/EBV receptor-positive Raji cell control demonstrating membrane staining with HB-5 by indirect immunofluorescence. Bar marker represents 1 μm. (b) Positive membrane staining of cultured epithelial cell with HB-5. Bar marker represents 1 μm. (c) Localization of the EBV/C3d receptor to cell surface projections (arrows) of human B lymphocytes with HB-5 and immunogold labelling. Bar marker represents 100 nm. (d) Microvillus of epithelial cell labelled by larger 20 nm gold beads indicating site of HB-5 binding. Bar marker represents 100 nm.
Evidence indicating a central role for the epithelial cell in EBV infections has been difficult to interpret in the absence of a satisfactory mechanism for viral entry. Fusion by virus-carrying lymphocytes to cells devoid of receptors suggested by the close proximity of lymphoid and epithelial tissue in the pharynx and by earlier in vitro studies (Bayliss & Wolf, 1980, 1981; Takimoto et al., 1983) lacks the economy of a mechanism likely to be associated with frequent epithelial cell involvement. The data presented here support direct infection of epithelial cells by EBV without lymphocyte intermediaries. The close correlation observed between the percentages of cells binding EBV and HB-5 in epithelial cell suspensions, the similar localization of virus and HB-5 at sites on the epithelial cell surface, and the evidence both from cultured cell suspensions and from tissue sections indicating a selective involvement of less differentiated cells all strongly suggest that the C3d/EBV receptor structure is expressed on epithelia in a differentiation-linked manner much as it is on B lymphocytes. Past difficulty in demonstrating the virus receptor on cultured epithelial cells (Bayliss & Wolf, 1981; Glaser et al., 1980; Shapiro & Volsky, 1983) might indeed be explained by the tendency of such cells to differentiate rapidly in vitro (Banks-Schlegel & Green, 1981), a process reflected in our study by detection of an EBV receptor on just 5% of cells. Enrichment for undifferentiated cells may prove crucial in future studies to characterize further the HB-5/anti-B2-defined molecule on the epithelial cell membrane and to examine its putative role as an EBV receptor.

The present work has been conducted using ectocervical epithelium but its wider relevance is already clear. Cells in the basal and intermediate layers of oronasopharyngeal epithelium show a similar pattern of HB-5 staining (Young et al., 1986). Moreover, the female genital tract has been recently identified as a second site for regular viral shedding (Sixbey et al., 1986). Repeated mucosal trauma, denudation and the rapid cell turnover characteristic of these mucosal sites could make receptor-positive cells available as targets for naturally transmitted virus. Infection of receptor-positive epithelial cells early in the differentiation pathway followed by active EBV replication in desquamating, terminally differentiated cells as suggested by earlier studies (Sixbey et al., 1983, 1984; Thompson et al., 1983) would favour survival of both virus and host and would provide a strategy for viral persistence which does not need to invoke B cell immortalization (Rickinson et al., 1985; Sixbey & Pagano, 1984). Indeed, it is interesting to reflect that the whole involvement of EBV with the B cell system may be a secondary, but nonetheless important, consequence of expression by B lymphocytes of the same surface structure through which the virus gains entry into its natural target, epithelium.

We thank Kuruganti G. Murti and Katherine Troughton for assistance, and Allen Portner and John R. Gilbert for reading the manuscript. This work was supported by grants from the National Cancer Institute, the American Cancer Society, and A.L.S.A.C.

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(Received 17 July 1986)