Superinfection of epithelial nasopharyngeal carcinoma cells with Epstein–Barr virus

(induction/epithelial cells/virus receptors)

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ABSTRACT Attempts were made to superinfect epithelial explant cell cultures prepared from nasopharyngeal carcinomas with Epstein–Barr virus. Virus-specific markers were observed in such cultures 3 days after superinfection. In addition, expression of Epstein–Barr virus early antigens was observed in epithelial cell explant cultures treated with idodeoxyuridine. The results suggest that epithelial cells of at least certain nasopharyngeal carcinomas possess the receptor for the Epstein–Barr virus and that the latent virus genome can be induced from epithelial cell explants prepared from such tumors.

Data obtained from a variety of studies support an association between the Epstein–Barr virus (EBV) and nasopharyngeal carcinoma (NPC). Results of immunofluorescence (IF) tests have shown that patients with NPC usually possess high titers of antibody directed against EBV-specific virus capsid antigens (VCA) (1), membrane antigen (2), early antigen(s) (3), soluble antigen(s) (4), and nuclear antigen (5). In addition, EBV-specific antibody levels have been shown to be related to the clinical stage of the tumor (1, 5).

When NPC tumor biopsy specimens were assayed for EBV DNA by nucleic acid hybridization, virus-specific DNA was found in the specimens (6). Moreover, it has now been shown that both EBV genomes (7) and the EBV-associated nuclear antigen (EBNA) (8) are associated with the epithelial elements of NPC (9, 10) rather than with the infiltrating lymphoid cells (11).

We have studied the expression and regulation of EBV in mammalian cells. To circumvent the inability of EBV to lytically infect (or transform) cells other than B (bone-marrow-derived) lymphocytes (12), we made somatic cell hybrids of Burkitt tumor cells (13) in order to get the EBV genome into other cell types. The EBV genome could be maintained in a repressed state in epithelial hybrid cells for long periods of time and, under certain conditions, could be expressed in these cells (14, 15). Recent data suggest that EBV expression in epithelial hybrid cells following induction with iododeoxyuridine (IdUrd) is enhanced over that found in producer lymphoblastoid cell lines (HR-1) (16), and that this observation may resemble a host range phenomenon typical of other viruses.

Since data suggest that EBV is a B lymphocyte tropic virus, the way in which EBV enters and transforms epithelial cells of the nasopharynx to induce NPC is a paradox. Preliminary data obtained from a study with one NPC tumor specimen suggest that epithelial cell explants prepared from that tumor could be superinfected with EBV and that the resident EBV genome could be induced with IdUrd (17). The study reported here was performed as an extension of these preliminary results and previous work with Burkitt/epithelial hybrid cells to determine how EBV infects epithelial cells of the nasopharynx and to clarify the role of EBV in NPC.

MATERIALS AND METHODS

Preparation of NPC Epithelial Cell Explants. Tumor specimens classified as typical NPC from patients in Hong Kong (patient numbers 75-1525, 75-1531-1, 75-1531-2, and 75-1576) were cut into approximately 1 mm pieces. The tumor specimens were placed on glass coverslips in 13 mm plastic tissue culture plates (Falcon) which had been pretreated with calf serum to aid in attachment of explants. A small volume of RPMI 1640 medium supplemented with 20% fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml was added to the tissue culture plates. Epithelial cells from the explants were allowed to grow out on glass coverslips prior to treatment or superinfection.

Superinfection of NPC Epithelial Cells with EBV. Seven days after preparation of the NPC explants, the medium was changed and cells were grown in fresh RPMI 1640 medium, as previously described, for 24 hr. Coverslip explant cell cultures were infected with 0.2 ml aliquots containing 10⁵ fluorescence-forming units (18) of EBV derived from HR-1 cells. Virus was allowed to adsorb to the cells for 1 hr at 37°, and 2 ml of complete RPMI 1640 medium was added. The cells were incubated for 3 days at 37° and fixed in acetone for 10 min in preparation for immunofluorescence.

Treatment of NPC Cells with IdUrd. To determine whether the repressed EBV genome in epithelial NPC cells could be induced to express EBV early antigen (EA), studies similar to those previously performed with Burkitt hybrid cells (14, 15) were conducted with IdUrd. NPC epithelial explants were prepared as described in the superinfection study. Twenty-four hours after initial change of medium, complete Eagle’s medium containing 60 μg/ml of IdUrd was added to the cultures. The cells were incubated for 3 days at 37° and were fixed in acetone for 10 min; they were later examined for EBV-specific markers by the direct IF test.

Immunofluorescence Test. The direct IF test was used to detect EBV-specific EA in order to avoid nonspecific fluorescence due to immunoglobulin G (IgG) in the tumor explants. Previously characterized EBV-positive serum from a Tunisian NPC patient (TU 201) was conjugated directly to

Abbreviations: EBV, Epstein–Barr virus; NPC, nasopharyngeal carcinoma; EA, early antigen; VCA, virus capsid antigen; EBNA, Epstein–Barr-associated nuclear antigen; IF, immunofluorescence; B lymphocyte, bone-marrow-derived lymphocyte.
RESULTS

Superinfection of NPC epithelial cells with EBV

Recent data suggest that epithelial/Burkitt hybrid cells, D98/HR-1, not only possess the receptor for EBV attachment, but are also superinfectable with EBV as demonstrated by the detection of EBV EA in a small percentage of cells (Glaser et al., unpublished data). As a result of this finding, attempts were made to superinfect epithelial cells prepared from NPC tumor specimens. Epithelial explants prepared from four NPC tumor biopsies obtained from three patients were grown on glass coverslips as described; one tumor (75-1531-2) was a second biopsy specimen taken approximately 4 weeks after the initial biopsy. Each coverslip contained two separate explants from which cells had grown out. A preparation of 10^5 fluorescence-forming units of EBV derived from the producer HR-1 cell line was added to cell cultures which were then incubated for 3 days at 37 °C. After fixation with acetone, the cells were examined for EBV EA using the direct IF test. Three of four explants prepared from tumor 75-1531-1 and infected with EBV demonstrated an average of 25% positive cells (Fig. 1 and Table 1). Most EA-positive cells showed nuclear fluorescence, though whole cell fluorescence was also observed. Tumors 75-1576 and 75-1525 were negative. Similar results were obtained on epithelial explant cultures prepared from the second biopsy specimen, 75-1531-2, taken approximately 1 month later (Table 1). Explant cell cultures not superinfected with EBV but tested with the same direct conjugate serum were negative for EBV EA (Fig. 2).

Antigen induction in NPC epithelial explants

Epithelial explants prepared from the same NPC tumor biopsies were grown out on glass coverslips and exposed to 60 μg/ml of IdUrd. After previous failure to recover EBV antigens from other tumor preparations, explants from

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**Fig. 1.** Immunofluorescent photomicrograph of an epithelial cell explant (tumor 75-1531-1) superinfected with EBV derived from HR-1 cells. Note EBV-specific EA in several cells. Approximately x960.

Epitope isothiocyanate (FITC) and adsorbed to explant cell cultures. The cells were washed, mounted in glycerol on glass slides, and examined with a Leitz microscope with an ultraviolet light source.

**Characterization of Serum TU 201.** Since a direct conjugate of serum TU 201 was used in the IF test, and since NPC sera often possess nonspecific anti-nuclear antibodies (19), the serum was tested for such activity. The following cells were used to test serum TU 201: MOLT (an EBV-negative lymphoblastoid line), HeLa, human embryonic lung (HEL) cells, human embryonic nasopharyngeal (HEN) cells, fibroblast cells derived from explants from two NPC tumors (Tunisian TU 66 and TU 54), touch preparations of tumor 75-1531-1 (the tumor in which EBV EA had been observed after superinfection and induction), and D98 cells.

**Fig. 2.** Immunofluorescence photomicrograph of a mock-infected epithelial cell explant (tumor 75-1531-2). Note absence of EBV-positive cells. Approximately x2200.
which only a small number of cells had grown out were used. It was thought that these cells would be the most actively dividing and that this might be important for incorporation of IdUrd into cellular DNA. EBV-specific EA was observed primarily in the nucleus of 5–10% of the epithelial NPC cells in tumor specimen 75-1531-1, and in 10–15% of the positive cells in tumor specimen 75-1531-2, after a 3-day exposure to IdUrd (Fig. 3 and Table 1). Cells showing cytoplasmic (whole cell) fluorescence were also observed in approximately 1% of the total positive cells. The pattern of fluorescence observed in the NPC epithelial cells was similar to that observed in D98/HR-1 epithelial/Burkitt hybrid cells in which EBV EA was expressed after treatment with IdUrd (15). As in the superinfection study, explant cell cultures of tumor 75-1531-2 not treated with IdUrd but tested with the same direct conjugate serum were negative.

**Characterization of serum TU 201**

The direct conjugate serum (EA-, VCA-positive) was tested for the presence of nonspecific anti-nuclear and anti-cytoplasmic antibodies. Results are shown in Table 2. No nonspecific fluorescence was observed when serum TU 201 was tested on the aforementioned cells by the indirect IF test, the anti-complement immunofluorescence test, or the direct test. Touch preparations assayed from tumor 75-1531-1 were negative, as were untreated explant cultures prepared from tumor 75-1531-2 using serum TU 201 (Fig. 2). Therefore, this serum did not contain nonspecific anti-nuclear or anti-cytoplasmic antibodies against a variety of human cell types or against untreated NPC explant cultures detectable by the IF test.

**Presence of EBNA in epithelial NPC explants**

The tumors were examined for EBNA activity using touch preparations prepared from each biopsy according to previously published procedure (8–10). Results are given in Table 1. Tumors 75-1525 and 75-1576, which had not re-

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**Table 1. Expression of EBV markers in epithelial cell explant cultures superinfected with EBV or treated with IdUrd**

<table>
<thead>
<tr>
<th>Biopsy (explants)</th>
<th>% EBV-positive cells after treatment*</th>
<th>% EBNA-positive cells in touch preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>75-1531-1</td>
<td>5–10</td>
<td>25</td>
</tr>
<tr>
<td>75-1531-2</td>
<td>10–15</td>
<td>10–15</td>
</tr>
<tr>
<td>75-1576</td>
<td>—</td>
<td>—</td>
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<tr>
<td>75-1525</td>
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</tbody>
</table>

*These numbers represent the average of EBV-positive cells from cell explants examined.
†60 μg/ml in Eagle's medium for 3 days at 37°C.
‡10⁵ fluorescence-forming units of EBV derived from HR-1 cells.

**Table 2. Assay for nonspecific anti-nuclear and anti-cytoplasmic antibodies in serum TU 201**

<table>
<thead>
<tr>
<th>IF test used</th>
<th>Cell type</th>
<th>ACIF*</th>
<th>Indirect†</th>
<th>Direct‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOLT</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>—</td>
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<td></td>
<td>HEL</td>
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<td></td>
<td>HEN</td>
<td>—</td>
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<tr>
<td></td>
<td>TU 66</td>
<td>—</td>
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<td>—</td>
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<tr>
<td></td>
<td>TU 54</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td></td>
<td>75-1531-1§</td>
<td>NT</td>
<td>NT</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>D98</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

ACIF, anti-complement immunofluorescence; NT, not tested.
* Serum diluted 1:8.
† Serum diluted 1:5.
‡ Direct conjugate diluted 1:5.
§ Touch preparations of biopsy.

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**Fig. 3.** Immunofluorescence photomicrograph of an epithelial cell explant (tumor 75-1531-1) treated with IdUrd for 3 days. Note EBV EA-positive cells. Approximately ×2200.
sponded to treatment with IdUrd or superinfection, contained 20–30% EBNA-positive cells. Tumor 75-1531-1 contained >90% EBNA-positive cells and tumor 75-1531-2 contained 70–80% EBNA-positive cells.

DISCUSSION

Since it has not been possible to infect non-lymphoblastoid cell types with EBV and since data concerning the host range for EBV suggest that it is a B lymphocyte tropic virus (9), the way in which EBV enters and transforms epithelial cells of the nasopharynx is unknown. From studies with Burkitt hybrid cells we know that, once EBV gets into an epithelial-like cell, it can be maintained in a repressed state for years and can be induced to replicate after treatment with IdUrd (14–16). Can in vitro cell fusion take place in the nasopharynx, i.e., can the EBV genome in B lymphocytes be transferred into epithelial cells with subsequent transformation (20)? It has been shown by electron microscopy that there may be loss of cell membrane integrity between epithelial and lymphoid cells in normal mucosa of the nasopharynx and in NPC tumors (21, 22). Whether transfer of EBV genetic material occurs under these circumstances remains to be established.

We have data that suggest that a small percentage of epithelial D98/HR-1 Burkitt hybrid cells possess the receptor for EBV (Glaser et al., unpublished data). These data support the concept that if epithelial cells have the proper receptor for EBV, then infection with EBV is possible. Data presented in this report extend these findings and reinforce the notion that epithelial cells of the nasopharynx (progenitors of the carcinoma cells), in at least certain individuals, possess the proper receptor for EBV. We have shown that EBV EA can be synthesized by the resident EBV genome in NPC tumor cells treated with IdUrd. The cells in which EA was observed were epithelial in morphology, confirming by another procedure (induction using IdUrd) the association of the EBV genome and the epithelial elements of NPC tumors. It is not clear whether VCA is synthesized in IdUrd-treated or superinfected NPC cells, since the only direct conjugate serum available was EA- and VCA-positive. Further studies to clarify this point can be performed using a VCA-specific direct conjugate serum. The fact that EBV has been shown to be “shed” in the oropharynx (23) places the virus in the vicinity of nasopharyngeal cells, and infection of these cells may be possible.

The two NPC biopsies taken in which EBV EA was inducible with IdUrd and superinfectable with EBV may have been unusual; more tumor biopsies will have to be studied. The high percentage of EBNA-positive cells may have been a determining factor in the receptivity of these cells to the two treatments; it is also possible that this may have enhanced detectability of virus expression, since the two non-infectable and noninducible tumors contained <30% EBNA-positive cells.

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