American Journal of Pathology, Vol. 150, No. 5, May 1997

The Mechanism of Epstein-Barr Virus Infection in Nasopharyngeal Carcinoma Cells

Chin-Tarng Lin,*† Chung-Ren Lin,* Guet-Khim Tan,* Wannhsin Chen,† Aileen Ngo Dee,† and Wing-Yee Chan*

*From the Department of Pathology, * National Taiwan University Hospital, Taipei, and Institute of Biomedical Sciences,†, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China*

To investigate the relationship between Epstein-Barr virus (EBV) and nasopharyngeal carcinoma (NPC) cells, we examined the pathway of EBV infection in NPC cell lines. We used immunolocalization to investigate the EBV receptor (C3d-R) and polymeric immunoglobulin receptor [secretory component (SC) protein]. We incubated IgA anti-EBV and EBV particles with NPC cells and observed the EBV DNA signal by* in situ *polymerase chain reaction hybridization and polymerase chain reaction plus Southern blotting. We also colocalized SC protein and EBV RNA in NPC biopsy specimens. Results showed that: 1) NPC cells did not express the EBV receptor but did express SC protein in each line; 2) SC protein was also expressed in some tumor cells but not in untransformed squamous metaplastic epithelia in NPC biopsy specimens; 3) EBV could infect NPC cells through an EBV-IgA and SC complex and retained an EBV viral genome in their nuclei; SC expression could be down-regulated by EBV proteins; and 4) in biopsy specimens, a fraction of tumor cells showed SC protein expression; only a portion of tumor cells contained EBV, and of these cells only a few expressed SC protein. These findings indicate that EBV cannot infect untransformed nasopharyngeal squamous metaplastic epithelia but can enter NPC cells through IgA-mediated endocytosis. (Am J Pathol 1997, 150:1745–1756)

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in Chinese living in South China, Hong Kong, Singapore, and Taiwan but is uncommon in Western countries.[1,2] The etiological factors of NPC have not yet been totally elucidated, but this carcinoma has a close association with Epstein-Barr virus (EBV).[3–16] To identify the actual relationship between EBV and NPC, we have established nine NPC cell lines in the last several years.[17–19] Our observations on these cell lines and their original biopsy specimens[19] showed that: 1) only five of nine cell lines contained EBV; 2) only a small fraction of tumor cells in each positive cell line contained EBV; 3) the EBV DNA sequence in each positive cell line was an episomal form, not an integrated form; 4) the untransformed squamous metaplastic epithelia of the nasopharynx did not contain EBV; 5) NPC cells that did not contain EBV appeared in every original biopsy specimen [this conclusion was obtained by *in situ* nucleic acid hybridization to localize latent membrane protein mRNA, EBER-1 small nuclear RNA, and *Bam*HI W fragment DNA and by *in situ* polymerase chain reaction (PCR) hybridization[19]]; and 6) EBV was present not only in certain tumor cells but also in some lymphocytes. These results promptly led us to ask how EBV infects NPC tumor cells. We therefore attempted to demonstrate the pathway for EBV entry into these NPC cells.

EBV is a ubiquitous human herpes virus that persists in the human body, usually in lymphocytes, after primary infection.[20] The association of EBV with NPC as mentioned above is supported by findings that patients with NPC generally have high titers of IgA against EBV-specific antigens, such as viral capsid antigen,[21–23] and a large proportion of NPC biopsy specimens contain an EBV genome.[5,8,19] Because most laboratory data so far suggest that EBV is a lymphotropic virus, it is unclear how the virus becomes associated with NPC. One possible expla-

Supported in part by research grants NSC84-2331-B002-302 and NSC85-2331-B002-188-M6 from the National Science Council and a grant from Academia Sinica (C. T. Lin).

Accepted for publication January 9, 1997.

Address reprint requests to Dr. Chin-Tarng Lin, Department of Pathology, National Taiwan University Hospital, Taipei 10000, Taiwan, ROC.

nation is that NPC cells in all patients may also express EBV receptors (C3d-R).[23–26] Another possible pathway might be in association with IgA transport through the mucosal epithelia, because the EBV-specific IgA level seems to be highly related to the clinical course of NPC patients.[27,28] It is well known that at the intestinal mucosa or in salivary gland tissue, plasma cells secrete IgA into the subepithelial space. By means of its J chain, polymeric IgA (pIgA) can bind to the polymeric immunoglobulin receptor [secretory component (SC) protein] on the basolateral membranes of basal epithelial or acinous cells. The ligand-receptor complex is then endocytosed by the epithelial cells and transported to the luminal surface.[29–31] If the ligand-receptor complex carries EBV, the latter may also possibly be transported into the cytoplasm. In fact, a recent report suggests that IgA antibody can enhance infection by EBV via the SC in colon cancer culture cells.[28]

To observe each possible port of entry for EBV infection in NPC cells, we examined whether EBV receptors were present on the NPC cell membrane and then observed the SC in NPC cell lines and original biopsy specimens using immunohistochemistry. We also examined EBV attachment, entry, and retention in NPC cells by PCR and Southern blotting and *in situ* PCR hybridization and compared the relationship between SC and EBV in the original biopsy specimens.

Materials and Methods

Cell Lines and Culture

Nine NPC cell lines,[17,18] two cervical carcinoma cell lines (CaSki and HeLa), a hepatoma cell line (Hep3B), a renal adenocarcinoma line (ACHN), and a human fibroblast line (HFY1) (all from American Type Culture Collection, Rockville, MD, except NPC and HFY1) were cultured in Dulbecco's modified Eagle's medium containing 5 to 10% fetal calf serum in a 10% CO_2 incubator at 37°C. Two B-cell-derived lymphoblastoid cell lines (B95–8, a marmoset lymphoblastoid cell line; and Raji, a latently EBV-infected Burkitt's tumor cell line) were maintained in RPMI 1640 medium containing 10% fetal calf serum in a 5% CO_2 incubator.

Immunohistochemistry

The procedures for immunohistochemistry were performed according to the methods developed in our laboratory as published previously.[18,19] For localization of C3d-R in NPC cell lines, we used a monoclonal antibody against C3d-R, including a monoclonal antibody obtained from a commercial source and the culture medium produced from a hybridoma cell line, which secreted a monoclonal antibody against C3d-R (HMT-145, American Type Culture Collection). We used peroxidase-labeled sheep anti-SC (The Binding Site Ltd., Birmingham, United Kingdom) to stain SC protein in the culture cells and paraffin sections.

Preparation of Biotinylated EBV (b-EBV) Particles and IgA Fraction

EBV was isolated from the supernatant of 12-*O*-tetradecanoylphorbol-13-acetate-stimulated B95–8 cells (a single-cell-subcloned B95–8 line prepared from the original line was used for the present experiment) by dextran T-10 gradient ultracentrifugation.[32] A visible band at the interface between 15 and 30% dextran was collected. One milliliter of virus (OD_{280}, 1.8) was biotinylated by incubation with 0.1 ml of dimethyl sulfoxide containing *N*-hydroxysuccinimido-biotin (2 mg/ml; Boehringer Mannheim GmbH, Mannheim, Germany) for 4 hours at room temperature. Free biotin was removed by dialysis, and the dialyzed sample was stored at −80°C. Purification of IgA from human serum was performed by affinity chromatography on a Jacalin column (Sigma Chemical Co., St. Louis, MO).[33] Sera of EBV-infected NPC patients with an IgA anti-EBV-viral-capsid-antigen titer of 1:640 were obtained from Dr. C. H. Wang (Clinical Pathology Department, National Taiwan University Hospital). Bound IgA protein was eluted with 0.8 mol/L D-galactose. After dialysis of the IgA fraction against phosphate-buffered saline, it was analyzed on a 7% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described.[34,35]

Virus Attachment and Entry Studies

To investigate viral attachment to the plasma membrane, NPC cells (NPC-TW 04, 2×10^6 cells) were grown on a coverslip overnight and incubated with an isolated IgA fraction (40 μl, 0.8 mg/ml) for 1 hour at 4°C and then washed and incubated with b-EBV (100 μl) for 2 hours at 37°C. Cells were then washed with 1X standard saline citrate and 0.1X standard saline citrate[36] and fixed with 3% formaldehyde in 0.1 M phosphate buffer containing 8.5% sucrose and 0.002% $CaCl_2$[18] for 15 minutes. The cells were then incubated with avidin-biotin-peroxidase complex (Vectastain kit; Vector Laboratories, Burlin-

game, CA) for histochemical staining. For comparison, NPC cells were incubated with b-EBV alone or with diluted normal human serum (1:50, containing nonspecific IgA) plus b-EBV under the same conditions. Raji cells incubated with b-EBV acted as the positive control. Similar experiments were performed on the HeLa and CaSki cell lines.

For virus entry studies, both biochemical and morphological observation were used. NPC cells were incubated with IgA at 4°C for 1 hour, washed, and then incubated with EBV at 37°C for various periods (0, 1, 4, and 24 hours) as mentioned above. After treatment with trypsin to remove the surface-attached virus, the total cellular DNA was extracted for biochemical analysis.[36,37] Subsequently, the DNA was analyzed by PCR and Southern blotting according to a published method[19,36] to determine whether the virus had been internalized. The template DNA concentration (0.5 μg) as well as the PCR conditions and the sample volume for gel electrophoresis were the same in each experiment to compare the change in EBV DNA signal. For control studies NPC cells were preincubated with 5 mg of antibody against SC (Sigma) at 4°C for 1 hour. IgA was added after unbound antibody had been removed by washing three times, and the cells were then incubated for 1 additional hour. Subsequently, EBV was added, and cells were incubated at 37°C for 3 hours. The solution was removed, and fresh Dulbecco's modified Eagle's medium was added; then the cells were further incubated in a CO_2 incubator for 21 hours. Cellular DNA was then analyzed by PCR as described above. For comparison, one experiment including preimmune sheep serum (1:50 dilution) was used to replace the antibody against SC.

For morphological investigation, *in situ* PCR hybridization to localize the EBV DNA signal was applied, according to our previously described method[19] with some modification. Cells were grown on coverslips and treated with IgA and EBV at 4°C and then incubated at 37°C for various periods. After fixation by 3% paraformaldehyde for 10 minutes, *in situ* PCR amplification was performed.[19] Before the PCR cycle was performed, each coverslip was transferred to a glass slide containing 20 μl of PCR reaction buffer and sealed with rubber cement to prevent buffer from evaporating during the high temperature for DNA denaturation. After the PCR reaction, coverslips were dehydrated (through 75% and then 95% alcohol for 1 minute each and absolute alcohol for 3 minutes, and then washed twice with phosphate-buffered saline for 5 minutes), acetylated (0.25% acetic acid and 0.1 M triethanolamine, pH 8.0, in phosphate-buffered saline), and prehybridized on the coverslip at 90°C for 5 to 7 minutes on a preheated glass slide containing prehybridization buffer. The coverslip was then transferred to hybridization buffer containing a denatured digoxigenin-labeled probe and hybridized at 42°C overnight (16 hours). The coverslips were then washed, immersed in Tris-buffered saline (100 mM Tris-HCl and 150 mM NaCl, pH 7.5), incubated with alkaline phosphatase-labeled anti-digoxigenin antibody, and the enzyme substrate.[19]

Colocalization of SC Protein and EBV-EBER-1 RNA in NPC Culture Cells and Biopsy Specimens

In situ hybridization of EBER-1 for localization of small nuclear RNA in EBV-infected NPC cells after incubation for 2 to 4 days was done exactly as previously described.[19] For colocalization of EBV-EBER-1 RNA and SC in culture cells and paraffin sections, after hybridization with EBV-EBER-1 antisense riboprobe, cells and sections were further stained overnight with peroxidase-labeled sheep anti-SC, washed, and incubated with peroxidase substrate as described previously.[18] For colocalization of SC protein and EBV-genomic DNA in NPC culture cells, NPC culture cells were infected by an IgA-EBV complex for 24 hours and then fixed for immunohistochemical staining using peroxidase-labeled sheep anti-human SC, followed by *in situ* PCR hybridization to localize EBV-genomic DNA as mentioned above.

Results

Immunolocalization of C3d-R and SC Protein

When we first looked for the EBV receptor (C3d-R) in our cell lines by immunohistochemical and immunodot methods, the results were negative for all NPC cell lines, although the control Raji cells were positive (data not shown). This result was confirmed by direct incubation of EBV with NPC cells (see the following experiment). These findings suggest that the cell membranes of our NPC cell lines may contain either very little or no C3d-R protein. Thus, it seems that EBV cannot infect our NPC cells through C3d-R. Therefore, we started to investigate the other possible pathway, ie, IgA-mediated EBV infection through SC protein. Immunohistochemical staining of our NPC cell lines with the anti-SC antibody revealed that in all NPC cell lines many tumor cells showed strong cytoplasmic and some membranous immunostain-

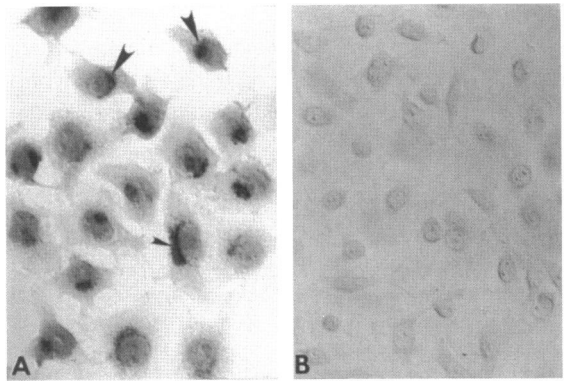


Figure 1. *Localization of SC in NPC culture cells by immunohistochemistry.* A: *The reaction product of anti-SC is seen in the cytoplasm and on some plasma membranes of many tumor cells* (large *and* small arrowheads) *in the NPC-TW 04 line. Some cells reveal immunostaining in the Golgi apparatus region* (small arrowhead). B: *The negative control stained with a nonspecific antibody shows no reaction product. Magnification,* A, B: × *120.*

ing (Figure 1A). The percentage of stained cells in nine NPC cell lines was roughly 45 to 95% in each total cell population (eg, NPC-TW 01 contained 95% positive cells, and NPC-TW 04 contained 83%).

Preparation of b-EBV Particles and IgA Fraction

When we first isolated EBV particles from the culture supernatant of B95–8 cells and amplified the DNA by PCR, the PCR-amplified DNA showed a 110-bp band similar to the band from Raji cells (data not shown). The isolated viral particles were then biotinylated, and the activity and physiological function of this b-EBV conjugate was identified by incubating it with Raji cells. Most Raji cell membranes were adhered to by EBV particles (Figure 2A), indicating that b-EBV possesses EBV activity and is able to bind to its receptor. However, this b-EBV alone could not bind to NPC cells (Figure 2B) as mentioned above. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the IgA fraction isolated from human sera (NPC patients), which contained a high titer of IgA against EBV-viral capsid antigen (1:640), identified one 340-kd band, one 160-kd band, and two nonspecific bands under nondenaturing conditions, in contrast to one heavy-chain band (56 kd), and a light-chain band (25 kd) under denaturing conditions (data not shown). This IgA fraction might contain several different antibodies against EBV proteins and membrane glycoproteins. When the pIgA was purified from this affinity-purified IgA fraction by gel filtration, the yield of pIgA was rather low. However, this pIgA fraction and the pIgA band isolated from a nondenatured gel could directly bind EBV on immunodots (data not shown).

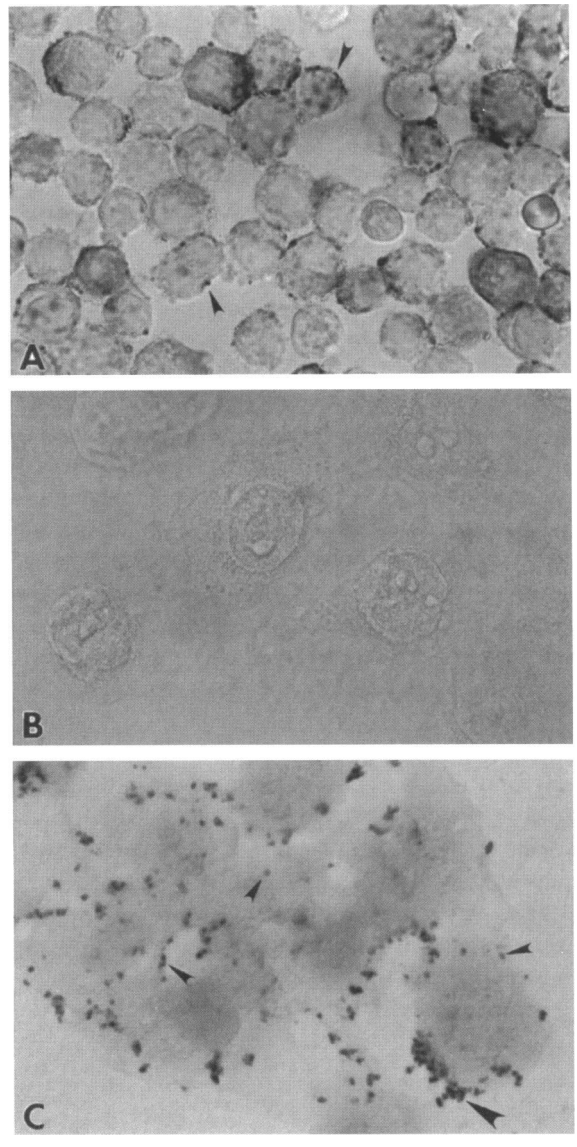


Figure 2. *Identification of b-EBV on Raji and NPC cells.* A: *Raji cells were incubated with b-EBV for 2 hours and fixed for identification of EBV. A spotty pattern of the reaction product* (arrowheads) *of EBV is shown on the surfaces of many Raji cells. Some cells contain more EBV than others. Magnification,* × *220.* B *and* C: *NPC cells. NPC-TW 04 cells were incubated with b-EBV alone* (B) *and IgA and b-EBV* (C) *for 2 hours and fixed for identification of EBV.* B: *No reaction product of b-EBV can be identified on any cells. Magnification,* × *760.* C: *The reaction product of EBV is shown either as spots* (small arrowheads) *or clusters* (large arrowheads) *on the NPC cell membrane. Some cells show more reaction product than others. Magnification,* × *760.*

EBV Attachment Studies

For the EBV attachment study, we used the total IgA fraction. To use the pIgA in the total IgA fraction, we incubated the NPC cells with the IgA fraction at 4°C, washed to remove all non-J-chain-containing mono-

meric IgA, and then incubated with b-EBV at 37°C. After incubation of biotinylated virus and IgA with NPC cells for 2 hours, spots of the reaction product of EBV were seen randomly distributed on NPC cell membranes, with some cells containing more or less reaction product (Figure 2C). The presence of EBV particles in spots or clusters on the surfaces of many NPC tumor cells suggests that EBV-pIgA can bind to SC protein on the plasma membrane. However, if the cells were only incubated with normal human serum (containing nonspecific IgA) plus b-EBV, no reaction product could be seen (data not shown). For comparison, we also used anti-SC to stain other cell types, such as HeLa cells, CaSki cells, lung adenocarcinoma cells (PC-13), hepatoma cells (HepG2 and Hep3B), renal cell adenocarcinoma (ACHN), osteogenic sarcoma (Saos-2), and fibroblasts (HFY1). Only HeLa cells, lung adenocarcinoma cells, and fibroblasts showed no staining. In the CaSki cells, a granular reaction product of anti-SC was present in certain tumor cells. Findings were similar in the other cancer cells (data not shown). Since the HeLa cells did not express SC protein, we also incubated them with IgA plus b-EBV and found no EBV binding. On the contrary, we easily identified the EBV bound on CaSki tumor cells, which contain SC protein (data not shown). Apparently, tumor cells that contain SC protein on their cell surfaces can be bound by the EBV-IgA complex, whereas cells without SC protein expression (such as HeLa cells) cannot be bound by this complex.

Virus Entry Studies by Biochemical Method

To investigate the cell entry of the EBV-IgA-SC complex, NPC cells were incubated with IgA and EBV for various periods and then analyzed by PCR and Southern blotting. When NPC cells were incubated with EBV and IgA, no signal of 110 bp could be initially seen (Figure 3, A and B, lane 4). After 1 hour, a very weak band of 110 bp could be detected by a ^{32}P-labeled EBV probe (Figure 3, A and B, lane 5). A 4-hour incubation showed a stronger 110-bp EBV signal (Figure 3, A and B, lane 6); after 24 hours, the signal was highly amplified (Figure 3, A and B, lane 7). Cells incubated with EBV or IgA alone did not show the 110-bp band even after a 24-hour incubation (Figure 3, A and B, lanes 8 and 9). Our findings indicate that not only can EBV-IgA enter the NPC cytoplasm, but also the EBV genome can exist in the tumor cells. (The increase of EBV signal after a 24-hour incubation may be simply because NPC cells endocytose increasing amounts of EBV over the 24-hour period, resulting in increased viral DNA, but at later times the number of viral genomes is stabilized.) This experiment has been done in five NPC cell lines, including NPC-TW 03–07, with similar results. To confirm the specificity of IgA and SC binding, NPC cells were first treated with excess antibody against SC and then incubated with an EBV and IgA preparation as mentioned above. No EBV DNA could be identified by PCR (Figure 4). This finding supports that SC is important for EBV-IgA entry into NPC cells. We have also treated NPC cells with the anti-SC antibody first and then with EBV and diluted antiserum against EBV. Again, no EBV signal was detectable in NPC cells (data not shown). This finding suggests that EBV-IgG cannot enter NPC cells.

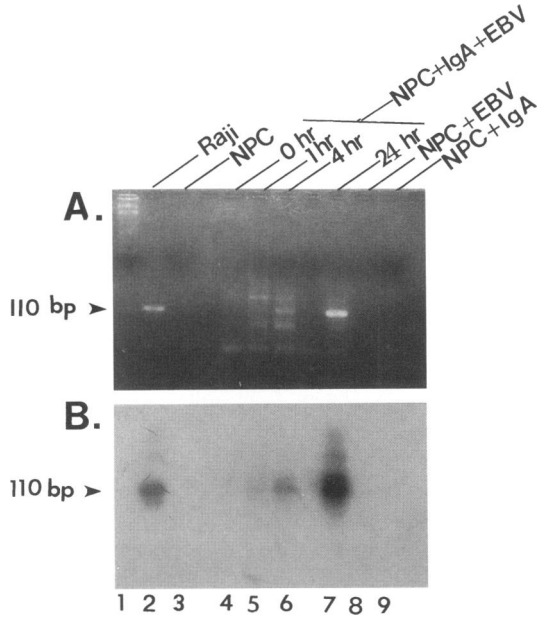


Figure 3. *Southern blot analysis of PCR products for EBV entry studies.* **Lane 1,** *molecular size marker;* **lane 2,** *PCR product of Raji cell DNA;* **lane 3,** *NPC-TW 04 cells alone;* **lane 4,** *NPC-TW 04 cells incubated with IgA and EBV for 0 hour;* **lane 5,** *for 1 hour;* **lane 6,** *for 4 hours;* **lane 7,** *for 24 hours;* **lane 8,** *NPC-TW 04 cells incubated with EBV alone;* **lane 9,** *NPC-TW 04 cells incubated with IgA alone. A single band of 110 bp is seen in* **lanes 2, 5, 6,** *and* **7**. *No band is shown in* **lanes 3, 4, 8,** *and* **9**. *At 0 hour, no band is seen; at 1 hour, a weak band of 110 bp can be seen; at 4 hours a stronger band is shown; at 24 hours a markedly amplified band is seen.* **A:** *Ethidium bromide gel.* **B:** *Southern blot.*

Direct Visualization of EBV DNA Signal in NPC Cells after EBV Infection

When we used *in situ* PCR hybridization as a tool to visualize EBV DNA infection in NPC cells, some granular reaction products were shown on the cell surface, in the cytoplasm, a few in the subplasma membrane region, and some close to the nuclear envelope, but none was in the nuclei after the infected cells were incubated for 2 hours (Figure 5, A

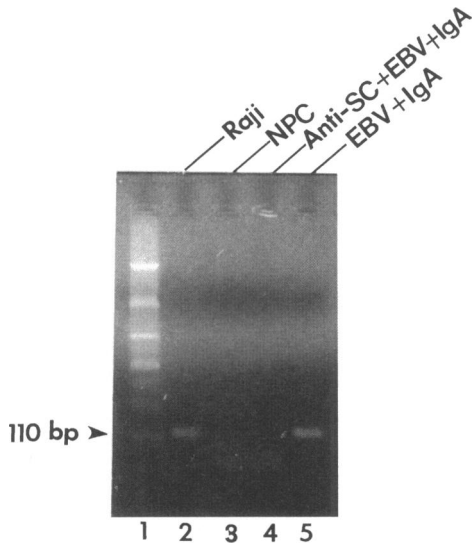


Figure 4. *Ethidium bromide gel of PCR product for inhibition studies.* Lane 1, *molecular size marker;* lane 2, *Raji cell DNA;* lane 3, *NPC alone;* lane 4, *NPC cells pretreated with antibody against SC and incubated with IgA and EBV;* lane 5, *NPC cells incubated with preimmune serum, IgA, and EBV. One single band of 110 bp is shown in* lanes 2 *and* 5. *No band is seen in* lanes 3 *and* 4.

to C, arrows), indicating that EBV particles have attached to the plasma membrane and have been endocytosed into the cytoplasm. After incubation for 24 to 36 hours, some granular reaction products still could be seen in the cytoplasm, and others were aggregated in the nucleus (Figure 5, D and E), suggesting that EBV DNA has been transported into the nucleus. The appearance of aggregated granular reaction products in certain nuclei (Figures 5E and 7D) indicates that EBV genomes have been transported and stabilized in the nuclei in host cells. When EBV-infected NPC cells were passaged six or seven times, the nuclear signal of EBV DNA was slightly decreased. But after incubation for more than 2 months, the EBV DNA signal was markedly diminished (data not shown).

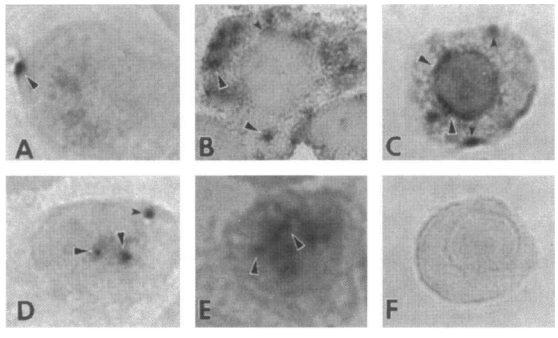


Figure 5. *Montage of EBV infection pathway in NPC cells by in situ PCR hybridization. NPC-TW 01 cells were infected by EBV and IgA anti-EBV and incubated for 2 to 24 hours. The EBV DNA signal was identified by in situ PCR hybridization.* A *to* C: *Incubation for 2 hours.* D *and* E: *Incubation for 24 hours. In the first 2 hours the granular reaction product of the EBV DNA sequence is shown attached to the plasma membrane* (A, arrowhead), *at the subplasma membrane region* (C, small arrowheads), *in the cytoplasm* (B, large arrowheads), *and the nuclear envelope* (B, small arrowhead, *and* C, large arrowheads). *After 24 hours, an occasional granular reaction product is still seen in the cytoplasm* (D, small arrowhead), *but most of the granular reaction product is shown in the nucleus* (D, large arrowheads, *and* E, arrowheads). F: *Negative control NPC cell, blocked by excess antibodies against SC and then incubated with EBV and IgA shows no specific reaction product. Magnification,* × *390.*

For confirmation of the role of SC protein in the pathway, again we used a specific antibody to block SC protein before incubation with EBV and IgA. No reaction product could be seen in any of the NPC cells (Figure 5F). These data suggest that SC rather than Fc receptors plays an important role in the EBV infection pathway in NPC cells.

Localization of SC Protein in NPC Biopsy Specimens

When anti-SC antibodies were used to stain biopsy specimens, only some tumor cells in biopsy specimens contained an anti-SC reaction product (Figure 6, A and B), whereas other tumor cells did not contain a detectable reaction product (Figure 6A), a result similar to previous reports from other groups.[38,39] The percentage of SC positive tumor cells was variable, with a range from 2 to 35% (eg, the NPC-TW 04 biopsy specimen contained 2% positive cells, and NPC-TW 06 contained 35% positive cells). In some areas in NPC-TW 05, certain keratinizing epithelial tumor cells also showed anti-SC staining (Figure 6B). In the normal pseudostratified ciliated columnar epithelia of the nasopharynx, some superficial ciliated columnar cells and a few cells in the middle and basal layers were stained by anti-SC (data not shown), whereas none of the untransformed squamous metaplastic epithelia were stained (Figure 6A). These findings are in accordance with a previous report from another laboratory.[38] Some minor salivary glandular epithelia contained strong staining at the basolateral membrane, in the cytoplasm, and at the apical region. The negative control showed no specific staining when nonspecific antibodies were applied (data not shown).

Colocalization of SC Protein and EBV-EBER-1 Small Nuclear RNA-1 in Biopsy Specimens

Double localization of EBV and SC in the paraffin sections revealed a reaction product of EBV-EBER-1 small nuclear RNA (deep black) in the nuclei of some individual and certain groups of tumor cells in patches, similar to a geographical

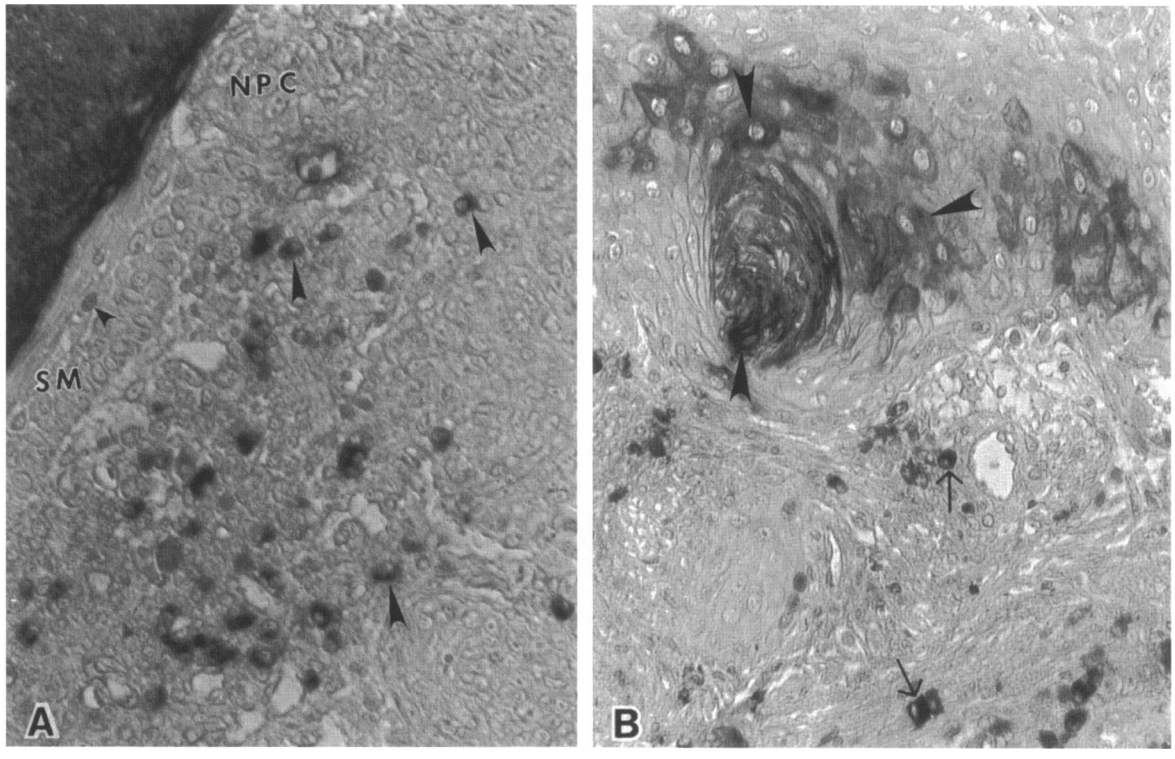


Figure 6. *Localization of SC protein in original NPC biopsy specimens.* A: *NPC-TW 06 cells. The reaction product of anti-SC is shown in the cytoplasm and plasma membrane of certain tumor cells only* (arrowheads). *The majority of tumor cells are unstained. The covering squamous metaplastic (SM) epithelia are not stained, except for nonspecific staining in one nucleus* (small arrowhead). *NPC, nasopharyngeal carcinoma tumor cells. The left upper corner with strong staining is due to the blood clot, which contains endogenous peroxidase activity of hemoglobin.* B: *NPC-TW 05 cells. The keratinizing squamous carcinoma cells* (large arrowheads) *show specific immunostaining. Some nonkeratinizing NPC tumor cells in other areas also show a reaction product* (arrows). *Magnification:* A, × *175;* B, × *170.*

map pattern (Figure 7A) as described previously.[19] The anti-SC reaction product (brown) in the colocalization section was seen in the cytoplasms of some EBV-negative tumor cells and in a few EBV-containing tumor cells (Figure 7B). Most EBV-containing cells did not show or showed a very weak anti-SC reaction product (Figure 7A). Certain tumor cells contained neither EBER-1 nor SC. (Similar findings were also observed in our unpublished data when double localization of SC protein and EBV DNA was performed using anti-SC and *in situ* PCR hybridization of EBV DNA.[19])

Identification of EBER-1 Small Nuclear RNA in EBV-Infected NPC Cells

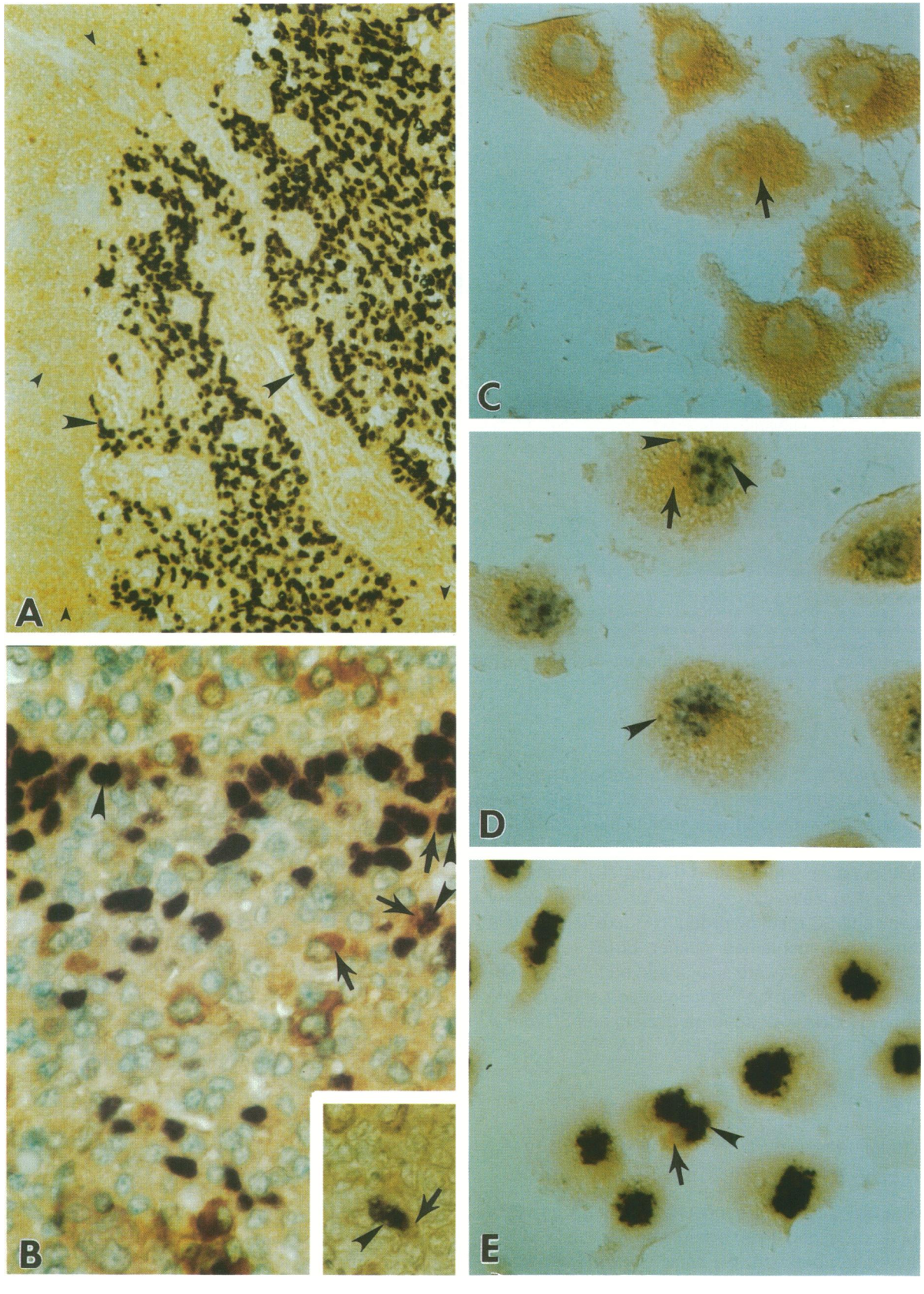
When NPC cells were incubated with IgA and EBV for 2 days and then hybridized with the EBER-1 antisense riboprobe, the small nuclear RNA signal was clearly seen in each EBV-infected cell (Figure 7E).

Effect of EBV Infection on the Expression of SC Protein

When NPC cells were infected by EBV through IgA-mediated endocytosis, incubated for 20, 24, and 48 hours, and subjected to colocalization of an EBV genome and SC protein, results showed that at earlier stages of infection, for example, at 20 and 24 hours, the SC protein stainings were almost similar to each other; however, after 48 hours, SC protein expression was reduced (Figure 7, C to E), clearly indicating that SC protein expression in the EBV-infected cells had been down-regulated.

Discussion

In the present experiment we have clearly demonstrated that NPC cells do not contain EBV receptor and, therefore, cannot be infected by EBV directly, but that EBV can infect NPC cells through the EBV-IgA-SC complex, and the EBV DNA can be identified by both PCR and Southern blotting and *in situ* PCR


A
B
C
D
E

hybridization in the whole pathway of EBV infection. Each granular reaction product in the cytoplasm and nucleus may represent one EBV DNA genome or several genomes. Thus, the appearance of several granular reaction products in the EBV-infected nucleus may indicate that a group of EBV genomes has been transported to the nucleus, and the latter is the final destination for EBV genomes.

Some reports have shown that a virus can exploit cellular transepithelial transport mechanisms without actually infecting cells as the virus forms an immune complex and enters the normal host cells.[40–42] This possibility is unlikely in our NPC case, mainly because our cell lines are malignant tumor cells, and they have already lost their polarity. It is conceivable that tumor cells that lose their polarity might disrupt the regulated mechanism of SC transcytosis and excretory function for IgA; thus the immune complex might not be released from the cell membrane, and EBV could still be retained in the tumor cells. This explanation is confirmed by our observation that some nuclei of EBV-infected NPC cells contained several granular reaction products of EBV DNA (Figure 5, D and E), indicating that EBV not only can enter into the NPC cells but also can stay and be stabilized in the cells. Apparently, EBV was not excreted in the early days after infection in our experiment.

An internalized virus might be neutralized by IgA. One report has shown that IgA might intersect the intracellular pathway for synthesis and assembly of a virus to inhibit further assembly and release of the virus.[43] In our case we cannot rule out the possibility that the virus is neutralized by IgA intracellularly, but it seems that this is not the case in this infection model, since EBV was shown to express the EBER-1 small nuclear RNA (Figure 7E) and to suppress SC protein synthesis (Figure 7, C to E) in the infected NPC cells.

EBV entry into epithelial cells, no matter whether via the C3d receptor[44] or via IgA-SC mediation,[28] was not found to be sufficient for long-term viral retention in the nuclei. In our experiments, we have found that most EBV DNA signals are gradually lost after cells are cultured for more than 2 months (data not shown). This phenomenon is also observed in our and other established NPC cell lines, in which some cell lines contained the EBV genome at early passages but lost it at late passages.[19,45] It has not yet been possible to grow a stably infected, EBV-positive NPC cell line *in vitro* in our laboratory; probably, some microenvironmental factors are required to retain EBV DNA in the host cells. The possibility that the early elimination of NPC cells entering the lytic cycle in the culture system might be a reason for loss of the population of EBV-positive cells in the late passage cannot be totally ruled out, although no such lytic cells were observed. On the other hand, the loss of the EBV genome may also be due to other reasons. For example, EBV could not reproduce the genome for other unknown reasons.

EBV can also infect other tumor cell lines by the EBV-IgA complex, such as CaSki cells and the hepatoma (Hep3B) cell line (data not shown), as long as they contain SC protein, similar to a reported colon cancer cell line[28]; if the tumor cells do not express SC protein, such as HeLa cells, they cannot be infected by the EBV-IgA complex (data not shown). However, an unusual case has been reported in which NPC primary culture cells could be infected with EBV by an unidentified mechanism, although this phenomenon was not observed in all other NPC biopsy primary cultures in the same report.[31] Our present experiment provides another mechanism for EBV entry into NPC cells through SC-mediated endocytosis of the EBV-IgA complex. This phenomenon could be used to explain the facts that some NPC biopsy specimens contain a strong EBV signal in certain infected tumor cells,[19,45,46] and that some other tumor cells, such as salivary gland carcinoma,[47] gastric carcinoma,[48] and uterine cervical cancer,[49,50] also contain an EBV signal.

To determine whether the above-mentioned findings obtained from our *in vitro* experiment could also be seen in the *in vivo* biopsy specimens, we have examined paraffin sections from the original biopsy specimens, from which the cell lines had been derived, by immunolocalization of SC protein and dou-

Figure 7. *Colocalization of EBV-EBER-1 RNA and SC protein and colocalization of EBV-genomic DNA and SC protein in NPC-TW 06 original biopsy specimen and culture cells infected with EBV.* **A:** *The reaction product of EBV-EBER-1 small nuclear RNA* (large arrowheads) *(deep black) is shown in many tumor cells in patches. The areas of tumor cells without EBV signal and surrounding the EBV-positive cells show some tumor cells containing an anti-SC reaction product (brown) in the cytoplasm* (small arrowheads). **B:** *Higher magnification of the NPC-TW 06 biopsy specimen showing cells with colocalization of EBV-EBER-1 RNA* (large arrowheads) *and SC protein* (arrows). *Some tumor cells contain either EBV-EBER-1 RNA* (large arrowhead) *or SC protein* (arrow), *but other cells are still not stained.* **Inset:** *Another tumor cell containing EBV-EBER-1 RNA* (arrowhead) *and SC protein* (arrow), *taken from another area of the same section.* **C, D,** *and* **E:** *NPC culture cells infected with EBV and incubated for 20, 24, and 48 hours, respectively.* **C:** *Culture cells stained with anti-SC show the SC reaction product* (arrow) *in the cytoplasm.* **D:** *Colocalization of EBV-genomic DNA and SC protein. The EBV-genomic DNA signal is shown in both the cytoplasm and nucleus* (arrowheads); *the intensity of the SC reaction product* (arrow) *is similar to that in the cells in* (**C**). **E:** *Colocalization of EBER-1 RNA and SC protein. EBV-EBER-1 RNA signal* (arrowhead) *is prominently concentrated in each nuclei. The intensity of the SC reaction product* (arrow) *is weaker than that in the cells in* **D**. *Magnification:* **A,** × *100;* **B** *and* **inset,** × *390;* **C** *and* **D,** × *540;* **E,** × *440.*

ble localization of SC protein and EBER-1 small nuclear RNA. The findings that SC protein was not identified in the untransformed squamous metaplastic epithelia in the nasopharyngeal mucosa but was identified in some tumor cells and minor salivary gland epithelia strongly suggest that EBV cannot infect untransformed squamous metaplastic epithelia but can get into some tumor cells and salivary gland epithelia through this pathway. This notion is supported by the fact that no EBV has been identified in untransformed nasopharyngeal squamous metaplastic epithelia.[19,51,52]

In addition, the finding that only some tumor cells contained both SC and EBV, whereas most EBV-containing cells were SC negative or showed very weak SC staining, suggests that the former finding may be a result of tumor cells recently infected with EBV through the IgA-SC complex, whereas the latter observation could be due to the fact that the tumor cells have been infected by EBV earlier, and the infected cells have proliferated, resulting in down-regulation of SC protein expression. This explanation is further supported by our other experiment, in which the SC protein expression was gradually decreased when the EBV-infected NPC cells were cultured longer and longer (our unpublished data using reverse transcriptase-PCR amplification of SC mRNA from different incubation time courses also revealed down-regulation of SC mRNA expression by EBV proteins). The findings that some cells are positive for SC but negative for EBV may be explained by the possibility that those SC-positive cells still have not been infected by EBV yet. In other words, not all tumor cells are infected by EBV at a certain point of cell proliferation, as we described in a previous article.[19]

Although this pathway of EBV infection may not be the sole one, we propose that it may be a major route for EBV infection in NPC cells. The expression of SC protein in certain keratinized squamous cancer cells in NPC suggests that EBV may also possibly infect keratinized epithelia whenever their SC protein has been expressed. Similarly, the expression of SC in salivary glandular epithelia may explain the source of the appearance of EBV in the saliva of NPC patients.[40]

The data obtained from the present experiment enable us to propose a possible pathway for EBV infection in NPC cells. Under normal conditions, EBV may not be able to infect the untransformed nasopharyngeal squamous metaplastic epithelia but may infect the transformed squamous epithelia if they express SC protein, through EBV-IgA-SC-mediated endocytosis. The EBV-infected, transformed cells (or NPC cells) could proliferate along with the replication of the EBV genome. During EBV-carrying cell proliferation, SC protein expression may be down-regulated.

Acknowledgments

We thank Dr. C. H. Wang (Clinical Pathology Department, National Taiwan University Hospital) for the gift of human serum against EBV-viral capsid antigen and Dr. M. T. Hsu (Institute of Biomedical Sciences, Academia Sinica) for critical review of the manuscript.

References

1. International Agency for Research on Cancer, International Association of Cancer Registries: Cancer Incidence in Five Continents. Geneva, World Health Organization, 1976
2. Waterhouse L, Muir C, Shanmugaratnam K, Powell J, Peachham D, Whelan S: Cancer Incidence in Five Continents, International Agency for Research on Cancer Scientific Publication 42, vol IV. Lyon, International Agency for Research on Cancer, 1982
3. Nonoyama M, Huang CH, Pagano JS, Klein G, Singh S: DNA of Epstein-Barr virus detected in tissue of Burkitt's lymphoma and nasopharyngeal carcinoma. Proc Natl Acad Sci USA 1973, 70:3265–3268
4. Klein G, Giovanella BC, Lindahl T, Fialkow RJ, Singh S, Stehlin JS: Direct evidence for the presence of Epstein-Barr virus DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of the nasopharynx. Proc Natl Acad Sci USA 1974, 71:4737–4741
5. Andersson-Anvret M, Forsby N, Klein, G, Henle W: Relationship between the Epstein-Barr virus and undifferentiated nasopharyngeal carcinoma: correlated nucleic acid hybridization and histopathological examination. Int J Cancer 1977, 20:486–494
6. Raab-Traub N, Hood R, Yang CS, Henry B II, Pagano JS: Epstein-Barr virus transcription in nasopharyngeal carcinoma. J Virol 1983, 48:580–590
7. Raab-Traub N, Flynn K, Pearson G, Huang A, Levine P, Lanier A, Pagano J. The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. Int J Cancer 1987, 39:25–29
8. Fahraeus R, Fu HL, Emberg I, Finke J, Rowe M, Klein G, Falk K, Nilsson E, Yadav M, Busson P, Tursz T, Kallin B. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int J Cancer 1988, 42: 329–338
9. Hitt MM, Allday MJ, Hara T, Karran L, Jones MD, Busson P, Tursz T, Emberg I, Griffin BE: EBV gene expression in a NPC-related tumor. EMBO J 1989, 8:2639–2651

10. Chang YS, Tyan YS, Liu ST, Tsai MS, Pao CC: Detection of Epstein-Barr virus DNA sequences in nasopharyngeal carcinoma cells by enzymatic DNA amplification. J Clin Microbiol 1990, 28:2398–2402
11. Niedobitek G, Hansmann ML, Herbst H, Young LS, Dienemann D, Hartmann CA, Finn T, Pitteroff S, Weit A, Anagnostopoulos I, Friedrick R, Lobeck H, Sam CK, Araujo I. Rickinson AB, Stein H: Epstein Barr virus and carcinoma: undifferentiated carcinomas but not squamous cell carcinomas of the nasopharynx are regularly associated with the virus. J Pathol 1991, 165:17–24
12. Gilligan KJ, Rajadurai P, Lin JC, Busson P, Abdel-Hamid M, Prasad U, Tursz T, Raab-Traub N: Expression of the Epstein-Barr virus *Bam*HI A fragment in nasopharyngeal carcinoma: evidence for a viral protein expressed *in vivo*. J Virol 1991, 65:6525–6529
13. Hu LF, Zabarovsky ER, Chen F, Cao SL, Emberg I, Klein G, Winberg G: Isolation and sequencing of the Epstein-Barr virus BNLF-1 gene (LMP1) from a Chinese nasopharyngeal carcinoma. J Gen Virol 1991, 72: 2399–2409
14. Busson P, McCoy R, Sadler R, Gilligan K, Tursz T, Raab-Traub N: Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma. J Virol 1992, 66:2357–2362
15. Brooks L, Yao QY, Rickinson AB, Young LS: Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. J Virol 1992, 66:2689–2697
16. Lung ML, Chang GC: Detection of distinct Epstein-Barr virus genotypes in NPC biopsies from southern Chinese and Caucasians. Int J Cancer 1992, 52:34–37
17. Lin CT, Wong CI, Chan WY, Tzung KW, Ho JKC, Hsu MM, Chuang SM: Establishment and characterization of two nasopharyngeal carcinoma cell lines. Lab Invest 1990, 62:713–724
18. Lin CT, Chan WY, Chen W, Huang HM, Wu HC, Hsu MM, Chuang SM, Wang CC: Characterization of seven newly established nasopharyngeal carcinoma cell lines. Lab Invest 1993, 68:716–727
19. Lin CT, Dee AN, Chen W, Chan WY: Association of Epstein-Barr virus, human papilloma virus and human cytomegalovirus in nine nasopharyngeal carcinoma cell lines. Lab Invest 1994, 71:731–736
20. Yao QY, Organ P, Rowe M: Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carrier: Int J Cancer 1989, 43:67–69
21. Henle W, Ho HC, Henle G: Antibodies to Epstein-Barr virus-related antigens in nasopharyngeal carcinoma. J Natl Cancer Inst 1973, 51:361–369
22. Henle W, Ho JH, Henle G: Nasopharyngeal carcinoma: significant change in Epstein-Barr virus related antibody patterns following therapy. Int J Cancer 1997, 20:663–673
23. Glaser R, de-The G, Lenoir G, Ho JHC: Superinfection of epithelial nasopharyngeal carcinoma cells with Epstein-Barr virus. Proc Natl Acad Sci USA 1976, 73:960–963
24. Young LS, Clark D, Sixbey JW, Rickinson AB: Epstein-Barr virus receptors on human pharyngeal epithelia. Lancet 1986, 1:240–242
25. Sixbey JW, Davis DS, Young LS, Hutt-Fletcher L, Tedder TF, Rickinson AB: Human epithelial cell expression of an Epstein-Barr virus receptor. J Gen Virol 1987, 68:805–811
26. Sixbey JW, Vesterinum EH, Nedrud JG, Raab-Traub N, Welton LA, Pagano JS: Replication of Epstein-Barr virus in human epithelial cells infected *in vitro*. Nature 1983, 306:480–483
27. Henle G, Henle W: Epstein-Barr virus specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. Int J Cancer 1976, 17:1–7
28. Sixbey JW, Yao QY: Immunoglobulin A-Induced shift of Epstein-Barr virus tissue tropism: Science 1992, 255: 1578–1580
29. Brown WR, Osobe Y, Nakane PK: Studies on translocation of immunoglobulins across intestinal epithelium. II. Immunoelectron-microscopic localization of immunoglobulins and secretory component in human intestinal mucosa. Gastroenterology 1976, 71:985–995
30. Solari R, Kraehenbuhl JP: Biosynthesis of the IgA antibody receptor: a model for the transepithelial sorting of a membrane glycoprotein. Cell 1984, 36:61–71
31. Cargo SS, Kulhary R, Prince SJ, Mestecky J: Secretory component on epithelial cells is a surface receptor for polymeric immunoglobulins. J Exp Med 1978, 147: 1832–1837
32. Nemerow GR, Cooper NR: Isolation of Epstein-Barr virus and studies of its neutralization by Human IgG and complement. J Immunol 1981, 127:272–278
33. Roque-Barrerira MC, Campos-Neto A: Jacalin: an IgA-binding lectin. J Immunol 1984, 134:1740–1743
34. Lin CT, Liu JW, Wu JY, Dang H, Li CY, Lam KW: Alteration of acid phosphatase isoenzymes in a human prostate cancer cell line. Lab Invest 1986, 55:666–673
35. Lin CT, Wu HC, Cheng HF, Chang JT, Chang KJ: Identification of β-subunit of GTP-binding regulatory protein in mitotic spindle. Lab Invest 1992, 67:770–778
36. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds): Current Protocols in Molecular Biology. New York, Green Publishing Associates and Wiley-Interscience, 1988
37. Lin CT, Chan WY, Chen W, Shew JY: Nasopharyngeal carcinoma and retinoblastoma gene expression. Lab Invest 1992, 67:56–70
38. Nomori H, Kameya T, Shimosato Y, Saito H, Ebihara S, Ono I: Nasopharyngeal carcinoma: immunohistochemical study for keratin, secretory component and leukocyte common antigen. Jpn J Clin Oncol 1985, 15:95–105
39. Desgranges C, Li JY, De-The G: EBV specific secretory IgA in saliva of NPC patients. Presence of secretory piece in epithelial malignant cells. Int J Cancer 1977, 20:881–886
40. Kaetzel CS, Robinson JK, Chintalacharuvu KR, Vaerman JP, Lamm ME: The polymeric immunoglobulin

receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA. Proc Natl Acad Sci USA 1991, 88:8796–8800

41. Apodaca G, Bomsel M, Arden J, Breitfeld PP, Tang K, Mostov KE: The polymeric immunoglobulin receptor. A model protein to study transcytosis. J Clin Invest 1991, 87:1877–1882
42. Chintalacharuvu KR, Piskwrich JF, Lamm ME, Kaetzel CS: Cell polarity regulates the release of secretory component, the epithelial receptor for polymeric immunoglobulins, from the surface of HT-29 colon carcinoma cells. J Cell Physiol 1991, 148:35–47
43. Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Nedrud JG: Intracellular neutralization of virus by immunoglobulin A antibodies. Proc Natl Acad Sci USA 1992, 89:6901–6905
44. Li QX, Young LS, Niedobitek G, Dawson CW, Birkenbach M, Wang F, Rickinson AB: Epstein-Barr virus infection, and replication in a human epithelial cell system. Nature 1992, 356:347–350
45. Yao K, Zhang HY, Zhu HC, Wang FX, Li GY, Wen DS, Li YP, Tsai CHA, Gleser R: Establishment and characterization of two epithelial tumor cell lines (HNE-1 and HONE-1) latently infected with Epstein-Barr virus and derived form nasopharyngeal carcinomas. Int J Cancer 1990, 45:83–89
46. Zur Hausen H, Schulte-Holthauzen H, Klein G, Henle G, Henle W, Clifford P, Santesson L: EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature 1970, 228:1956–1958
47. Hamilton-Dutoit SJ, Therkildsen MH, Neilsen NH, Jensen H, Hansen JP, Pallesen G: Undifferentiated carcinoma of the salivary gland in Greenlandic Eskimos: demonstration of Epstein-Barr virus DNA by *in situ* nucleic acid hybridization. Hum Pathol 1991, 22:811–815
48. Tokunaga M, Uemura Y, Tokudome T, Ishidate T, Masuda H, Okazaki E, Kaneko K, Naoe S, Ito M, Okamura A: Epstein-Barr virus related gastric cancer in Japan: a molecular patho-epidemiological study. Acta Pathol Jpn 1993, 43:574–581
49. Landers RM, O'Leary JJ, Crowley M, Healy I, Annis P, Burke L, O'Brien D, Hogan J, Kealy WF, Lewis FA, Doyle CT: Epstein-Barr virus in normal, pre-malignant, and malignant lesions of the uterine cervix. J Clin Pathol 1993, 46:931–935
50. Se Thoe SY, Wong KK, Pathmanathan R, Sam CK, Cheng HM, Prasad U: Elevated secretory IgA antibodies to Epstein-Barr virus (EBV) and presence of EBV DNA and EBV receptors in patients with cervical carcinoma. Gynecol Oncol 1993, 50:168–172
51. Yeung WH, Zong YS, Chiu CT, Chan KH, Sham JST, Choy DTK, Ng MH: Epstein-Barr virus carriage by nasopharyngeal carcinoma in situ. Int J Cancer 1993, 53:746–750
52. Pathmanathan R, Prasad U, Sadler R, Flynn K, Raab-Traub N: Clonal proliferation of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. N Engl J Med 1995, 333:693–698