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Short Communication

Detection of Epstein-Barr Virus-Infected Mucosal Lymphocytes in Nasal Polyps

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Primary nasal lymphomas of T or NK cell origin are known to be associated with Epstein-Barr virus (EBV). However, it is not known whether EBV is normally present in nasal mucosa as distinct to nasopharyngeal tissue. This study investigates the prevalence of EBV infection in 13 cases of nasal polyps. EBV DNA was detected in 2 of 13 (15%) by Southern blot hybridization and in 9 of 13 (69%) by polymerase chain reaction. In situ hybridization for EBV-encoded small nuclear RNAs (EBER) was positive in 11 of 13 (85%) cases; the virus was present in stromal lymphocytes only and not in the epithelial cells. Immunohistochemistry for EBV proteins in 7 cases revealed EBV nuclear antigen (EBNA)-2, latent membrane protein (LMP)-1, and ZEBRA (the switch protein encoded by gene BZLF1) expression in rare isolated stromal lymphocytes in 3 cases. Double immunostaining in 1 case showed that the LMP-1$^+$ cells were B or T cells. Immunohistochemistry for EBV lytic proteins showed very rare viral capsid antigen (VCA)$^+$ and membrane antigen (MA)$^+$ cells in 1 case and very rare diffuse early antigen (EA-D)$^+$ and VCA$^+$ cells in 1 other case. The expression of ZEBRA, EA-D, VCA, and MA suggested a disruption of latency in very rare stromal lymphocytes leading to a productive cycle. Although the incidence of EBV positivity in nasal polyps in our population is high (85%), very low numbers of EBV$^+$ cells are found in each case. Nevertheless, they indicate that nasal mucosa could be one of the sites of EBV persistence through a low level of infection of the resident lymphocytes and thereby provide a possible setting for the emergence of virally associated tumors in this site. (Am J Pathol 1996, 149:1111–1118)

Epstein-Barr Virus (EBV) is strongly associated with two tumors in the nasal region, nasopharyngeal carcinoma (NPC; as reviewed by Miller[1]) and nasal lymphoma.[2–4] Although nasal lymphoma may also involve the nasopharynx, the site of origin of NPC, the majority of primary nasal lymphomas are found more anteriorly in the nasal cavity.[5] Recently, we have reported that EBV DNA can be detected by polymerase chain reaction (PCR) in 80% of the normal nasopharyngeal tissue from Chinese subjects without apparent EBV-related diseases,[6,7] and the EBV-containing cells were detected by *in situ* hybridization (ISH) within the stroma or rarely intraepithelially.[8] Whether similar EBV infection is present in the nasal mucosa in non-neoplastic conditions is unknown. To examine the prevalence and cellular localization of EBV infection in the nasal as distinct to the nasopharyngeal mucosa, a series of 13 nasal polyps were studied for evidence of EBV.

Although nasal polyps are relatively common, occurring in approximately 4% of the population,[9] their etiology remains obscure. Various theories have been proposed, including allergic and infectious/viral causes and immune disturbance.[10–12] How-

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ever, no virus has been found in nasal polyps by viral culture[11,13] or ISH analysis[11] so far. These polyps provide a readily available source of non-neoplastic nasal mucosa for examining the possibility of chronic EBV infection at that anatomic site. Extranodal lymphomas frequently arise in a background of chronic inflammation (as in lymphomas of gastric mucosa-associated lymphoid tissues) or in a site of chronic immunological reaction such as Sjogren syndrome. Although there is no indication that similar precursor lesions occur in nasal lymphomas, the use of nasal polyps as a source of chronically inflamed mucosa for study of the background tissue is not unreasonable. We were also interested to find out whether the virus was present purely in a latent form or whether a replicative infection is present that may lead to an increased viral load locally and a higher chance of infection of the resident lymphocyte population. In latent infection, a restricted number of regions of viral genome are transcribed.[1] However, cells with latent virus can also be activated into viral replication spontaneously or by some chemical or physical agents resulting in the release of infectious virions and death of the host cell.[1] We have therefore also studied the expression of the viral-coded proteins associated with latently EBV-infected cells (EBV nuclear antigen (EBNA)-2 and latent membrane protein (LMP)-1) and with the lytic infection (ZEBRA (the switch protein encoded by the BZLF1 gene), membrane antigen (MA), diffuse early antigen (EA-D), and viral capsid antigen (VCA))[1] in EBV^+ cells in nasal polyps.

Materials and Methods

Selection of Cases

Surgically resected nasal polyps with typical morphology and available frozen and paraffin blocks were selected from the files of the Department of Pathology, The University of Hong Kong, Queen Mary Hospital, Hong Kong. All of the patients were Chinese and none of them had clinically identifiable malignancies.

Southern Blot Hybridization

High molecular weight DNA was extracted from cryostat sections of the same frozen blocks of nasal polyps on which immunohistochemistry was performed. SoBH analysis was performed essentially as described.[4] The presence of EBV DNA and the clonality of EBV were detected by using the ^{32}P-labeled 3.1-kb BamHI W and *Eco*RI Dhet fragments of the EBV genome, respectively, supplied by Prof. B. Griffin (London). DNA extracted from the EBV^+ Burkitt's lymphoma cell line Raji and EBV^- T-lymphoma cell line MOLT-4, both obtained from the American Type Culture Collection (Rockville, MD), were used as positive and negative controls, respectively.

Polymerase Chain Reaction

The detection for EBV DNA by PCR was performed essentially as described by Cheung et al.[6] Oligonucleotide primers were used to amplify EBV DNA from base 1399 to 1520, which is within the first internal repeat region (IR1) of the EBV genome.[14] DNA amplification was carried out for 40 cycles using the GeneAmp PCR reagent kit (Perkin Elmer Cetus, Norwalk, CT) using 37°C as the annealing temperature. The specificity of the PCR product (122 bp) was confirmed by SoBH using a ^{32}P-labeled internal probe that spans from base 1424 to 1439. DNA extracted from the Raji cell line was used as a positive control. All of the necessary precautions were taken to prevent possible cross-contamination between samples during the PCR procedure as described by Cheung et al.[6] Using this system, EBV DNA can be detected in as little as 2.5×10^{-7} μg of Raji DNA.[6]

In Situ *Hybridization*

ISH for EBV-encoded small nuclear RNAs (EBER-1 and -2) was carried out mainly using the EBV ISH system from Dako (Kyoto, Japan).[4] In this system, the fluorescein-conjugated EBER oligonucleotide probe was used and detected by alkaline phosphatase-conjugated rabbit anti-fluorescein isothiocyanate antibodies (Fab'_2 fragment). Nitroblue tetrazolium together with 5-bromo-4-chloro-3-indolylphosphate was used as the chromogen. Alternatively, radioactive ISH using ^{35}S-labeled antisense riboprobe specific for EBER was used for some cases.[7] Formalin-fixed, paraffin-embedded blocks of different EBV-infected cell lines, Raji (latent, nonproductive) and B95-8 (productive), and lymph nodes with metastatic NPC were routinely used as positive controls in each run. The BJAB cell line, EBV^- tissues, RNAse-A-treated sections, and hybridization buffer without probe were used as negative controls.

Immunohistochemistry

Monoclonal antibodies to EBV proteins, PE2 against EBNA-2, CS.1-4 against LMP-1, and BZ1 against

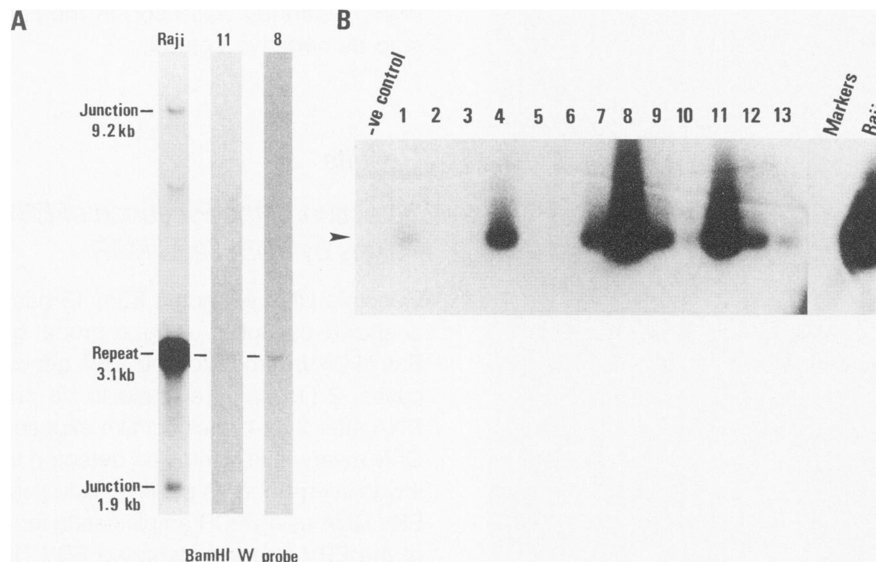


Figure 1. A: *SoBH analysis for the detection of EBV DNA in nasal polyps using a probe specific for the BamHI W fragment of the EBV genome. EBV DNA was detected in the DNA samples of 2 of 13 cases (cases 8 and 11). Only the positive cases are shown here. The band for case 11 was very faint but was interpreted as positive. Raji cell line DNA was used as a positive control for EBV.* B: *PCR amplification for the IR1 region of the EBV genome. DNA from Raji cell line was used as positive control. A distinct EBV-specific DNA band of 122 bp was detected in cases 1, 4, 7, 8, 9, 10, 11, 12, and 13. The band for case 10 was light but was regarded as positive.*

ZEBRA, were kindly provided by Dr. M. Rowe (Birmingham, UK). Monoclonal antibodies for EBV lytic proteins, anti-EA-D and anti-VCA (125 kd), were obtained from DuPont (Boston, MA). Anti-MA was obtained from Chemicon (Temecula, CA). Monoclonal antibodies against different cellular surface antigens, CD3 (leu-4) and CD19 (leu-12), were obtained from Becton Dickinson (Mountain View, CA); CD68 (Kp-1) was obtained from Dako (Carpinteria, CA); monoclonal antibody against chromogranin (used as a control) was obtained from BioGenex (San Ramon, CA); Ki-67 (MIB-1) was obtained from Immunotech (Marseille, France).

The standard alkaline phosphatase anti-alkaline phosphatase (APAAP) method was performed on the cryostat sections (6 μm) of nasal polyps as described.[15] After APAAP staining, sections were lightly counterstained by hematoxylin. Normal rabbit serum or anti-chromogranin was used as a negative control. Cytospin slides of Raji, P3HR1,

Table 1. *Summary of the Results of the Detection for EBV DNA by SoBH and PCR, EBER by ISH, and EBV Proteins by Immunohistochemistry in Nasal Polyps*

Case	Sex age (years)	SoBH for BamHI W	PCR for EBV IR1	EBER ISH	EBNA-2	LMP-1	ZEBRA
1	M 34	−	+	+	ND	ND	ND
2	M 33	−	−	+	ND	ND	ND
3	M 34	−	−	+	−	−	−
4	M 52	−	+	++	−	−	−
5	M 39	−	−	+	ND	ND	ND
6	M 30	−	−	−	−	−	−
7	F 68	−	+	+	−	−	−
8	F 30	++	++	+++	+/−	+	+
9	M 31	−	+	+	+/−	+	+ (EA-D+, VCA+)
10	M 70	−	+	−*	ND	ND	ND
11	F 64	+	++	++	+	++	++ (VCA+, MA+)
12	M 60	−	+	+	ND	ND	ND
13	M 76	−	+	+	ND	ND	ND
n = 13		2/13 (15%)	9/13 (69%)	11/13 (85%)			

For ISH and immunohistochemistry, the positive cells in one section were counted and scored as follows: +++, 20 to 100 positive cells; ++, 10 to 20 positive cells; +, 4 to 10 positive cells; +/−, <4 positive cells; −, no positive cells. M, male; F, female; ND, not done.
*Case with little tissue in the paraffin section.

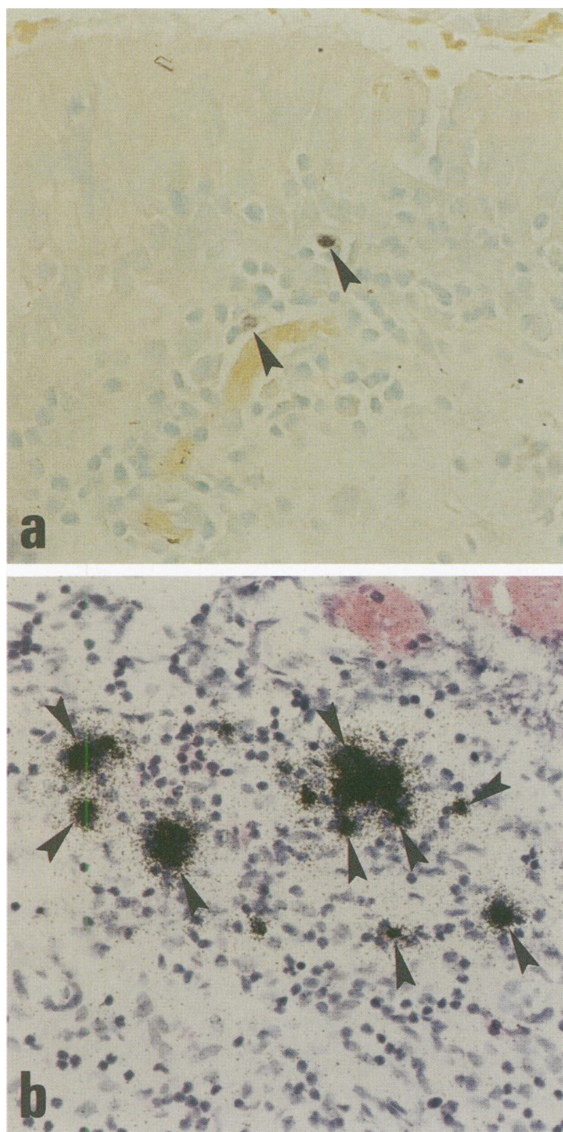


Figure 2. *ISH for EBER in nasal polyps.* a: *Rare EBER⁺ lymphocytes were detected in the stroma beneath the epithelium* (arrow) *but not in the epithelial cells of the nasal mucosa detected with fluorescein-conjugated probe. Some intraepithelial lymphocytes were also seen and were negative for EBER.* b: *Focal cluster of EBER⁺ cells* (arrow) *in the deep stroma of case 8 detected with ³⁵S-labeled probe. Magnification, ×450.*

and B95-8 cell lines were used as positive controls. BJAB and EBV$^-$ tissues were used as negative controls. Indirect immunofluorescence was also used for LMP-1 and ZEBRA staining.[15] Double immunostaining was carried out as described by Tao et al[15] by combining APAAP for CD markers and indirect immunofluorescence for LMP-1. Irrelevant antibody of the same isotype as the LMP-1 antibody was used in the second staining step as negative control.

Results

Detection of the Presence of EBV in Nasal Polyps by SoBH and PCR

Genomic DNA extracted from 13 nasal polyps was analyzed by SoBH using a probe specific for the BamHI W fragment of the EBV genome. Of the 13 cases, 2 (15%) were found to be positive for EBV DNA after 2 to 4 weeks of film exposure (Figure 1a). Only a very faint band was detected in case 11, but it was interpreted as positive. Additional analysis for EBV DNA using PCR amplification for the IR1 region of the EBV genome detected EBV DNA in 9 of 13 cases (69%), including the 2 SoBH$^+$ cases (Table 1; Figure 1b).

Determination of the clonality of EBV in the 2 SoBH$^+$ cases was unsuccessful because the virus load was too low in the samples to be detected by SoBH using the terminal region *Eco*RI Dhet probe.

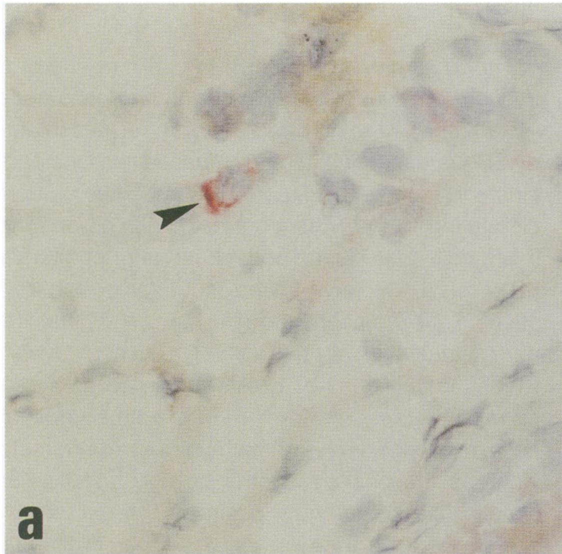
ISH for EBER

ISH showed EBER$^+$ cells in 11 of 13 cases (85%; Table 1). Rare to a few scattered EBER$^+$ cells were found in the stroma of nasal mucosa but not in the epithelium (Figure 2a). Most of these EBER$^+$ cells were recognized as small lymphocytes by morphology. The number of EBER$^+$ cells varied between cases, and the EBER signal intensity over individual cells was heterogeneous. Numerous eosinophils were seen in all cases but were always negative for EBER. In case 8, which was SoBH$^+$ for EBV DNA, focal collections of 3 to 10 EBER$^+$ cells along with individually scattered EBER$^+$ cells were observed (Figure 2b). Case 10, which was PCR$^+$ but EBER$^-$, did not have much tissue left in the paraffin block, which may explain the negative ISH result.

Immunostaining for EBV Latent and Lytic Proteins

Immunohistochemistry for EBV proteins was performed on cryostat sections of seven cases with sufficient remaining frozen tissue. Rare isolated LMP-1$^+$ and EBNA-2$^+$ and very rare ZEBRA$^+$ cells

Figure 3. *Immunohistochemical detection of EBV proteins on cryostat sections of nasal polyps. Expression of LMP-1* (a *and* b), *ZEBRA* (c *and* e), *and MA* (d *and* f) *in rare scattered stromal lymphocytes is shown here* (arrow). a *to* d: *Nasal polyps. Magnification, ×450.* e *and* f: *B95-8 cell line control. Magnification, ×720. APAAP staining* (a *and* c *to* f) *and indirect immunofluorescence* ((b).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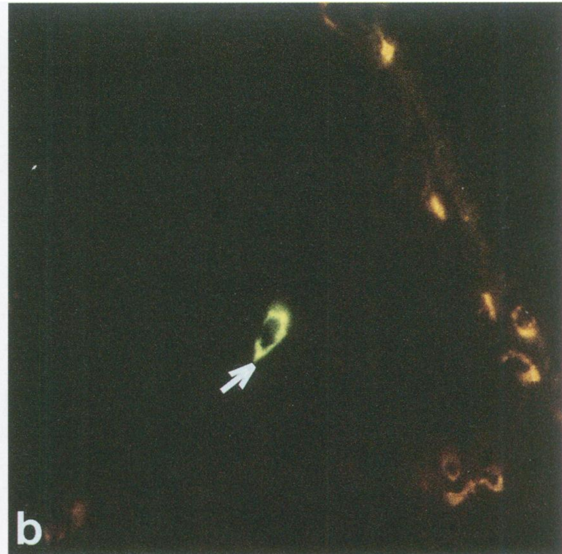
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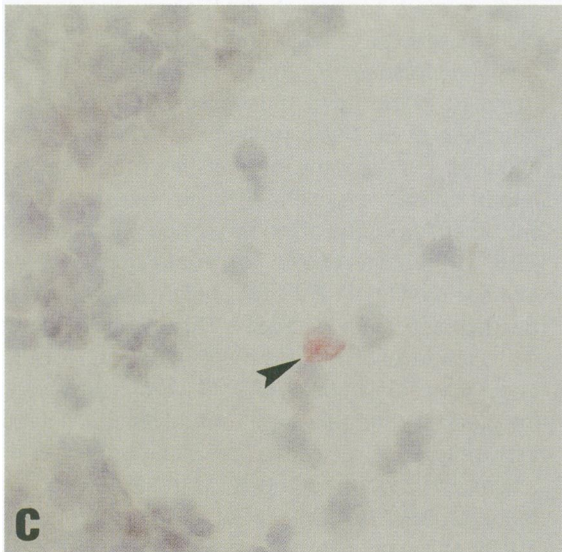
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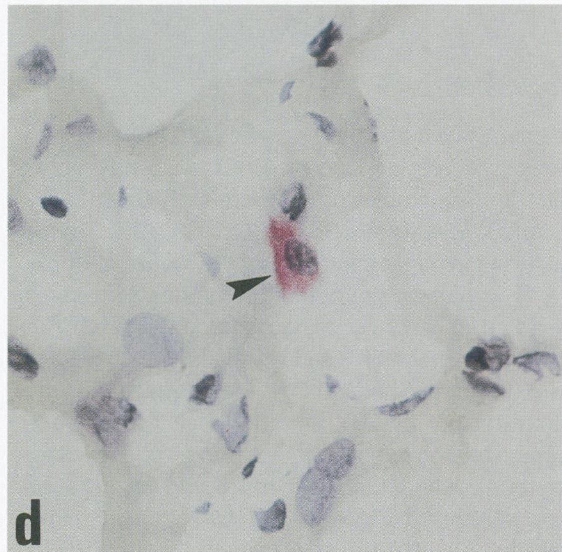
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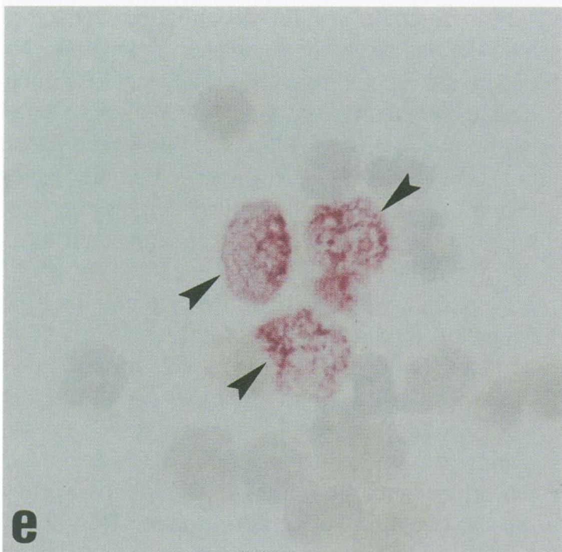
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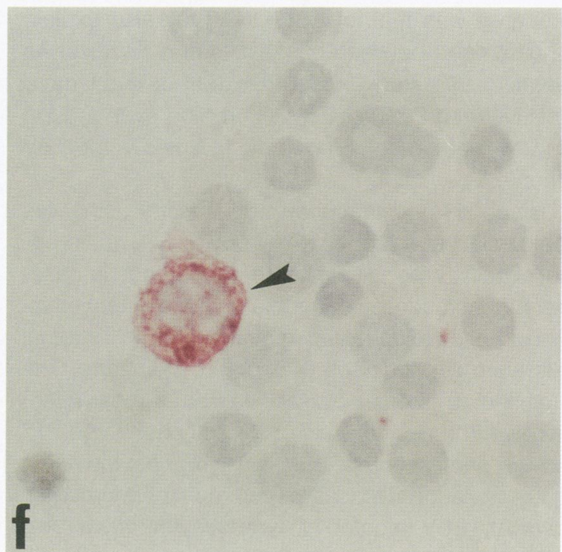
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were detected in three PCR+ cases in the stroma near the epithelium or deeper (Table 1; Figure 3). No positive signal was detected in the clearly identifiable epithelial cells. Immunostaining for EBV lytic proteins revealed very rare positive cells sometimes requiring examination of multiple serial sections before they were detected. Isolated EA-D+ or VCA+ stromal cells were detected in one case, and very rare MA+ or VCA+ stromal cells were detected in another case (Figure 3).

To identify the lineage of cells expressing EBV proteins, double immunohistochemistry for cellular surface markers (CD19, CD3, and CD68) and LMP-1 was done on the three positive cases. Only in one SoBH+ case were there sufficient LMP-1+ cells available for analysis. The LMP-1+ cells included both B cells (CD19+) and T cells (CD3+) but not CD68+ cells (results not shown).

Discussion

We have shown that the prevalence of EBV infection in nasal polyps in Hong Kong Chinese patients is quite high by employing different techniques (15% by SoBH, 69% by PCR, and 85% by ISH). These results are in direct contrast with the results of Kozak et al,[11] who could not detect EBV in nasal polyps in Canadian patients by using nonradioactive ISH for EBV DNA. The high tissue prevalence of EBV in nasal polyps of Southern Chinese suggests that the natural history of EBV infection may be different, perhaps related to environmental and/or genetic factors, considering that even by SoBH analysis we could still detect EBV DNA in 15% of cases. However, the much higher sensitivity of ISH for EBER[16] compared with that for EBV DNA may also explain the difference between our results and Kozak's. As shown in this study, the EBER ISH is even more sensitive than PCR for EBV IR1 region even though this DNA sequence is repeated 11 times in the EBV genome.

Using ISH and immunohistochemistry, EBV was localized in rare stromal lymphocytes but not in epithelial cells, which is similar to our findings in nasopharyngeal mucosa.[7,8] Double immunohistochemistry in one case revealed that the LMP-1+ stromal cells were lymphocytes of B or T cell lineage. The latter finding is of particular interest as most EBV+ nasal lymphomas have a T or NK cell phenotype,[2,4,5] and the persistence of EBV in T lymphocytes is not generally recognized as a common phenomenon. However, EBV-infected T lymphocytes have also been found recently in normal nasopharynx,[7,8] non-neoplastic lymph nodes,[17,18] or normal spleen and tonsils.[17]

The focal clustering of EBER+ cells in one of our cases can be explained by one of two possibilities: 1) that these were neighboring lymphocytes infected by EBV released in the nasal mucosa itself instead of clustering of latently infected lymphocytes randomly circulating to the nose from other places or 2) that these cells may be the consequence of proliferating EBV+ cells. We have tested the latter possibility by staining the adjacent paraffin section of that case and sections of another SoBH+ case by Ki-67,[19] which identifies the proliferating cells. Results showed that, except for very rare isolated epithelial cells, no positive cells in the mucosa were detected, whereas the positive control of a metastatic nasal lymphoma was strongly positive. These results do not support the idea of active cellular proliferation and are more consistent with the view that the small clusters of EBER+ cells are most probably derived from an episode of lymphocyte infection by EBV released in the nasal mucosa itself. Moreover, the detection of EBV lytic proteins in very rare stromal lymphocytes also implies that the virus may enter into a replicative cycle in this site. The possibility of lytic infection in one of the two cases with positivity of EBV lytic proteins is also correlated with the relative higher quantity of EBV DNA, which was sufficient to be detected even by SoBH. These results indicate that a low level of persistent lytic infection of EBV can occur in nasal mucosa. As other lymphocytes can be infected by virus released from a lytic EBV infection in this site, EBV+ cells can persist through this mechanism and further contribute to the life-long persistence of EBV. However, whether this contributes to the formation of nasal polyps is highly conjectural. As EBV is detected in 88% of normal nasopharyngeal mucosa tissue in our population[7,8] whereas nasal polyps are much rarer, it is unlikely that EBV contributes to causation of nasal polyps. Moreover, the very low number of EBV+ cells in each positive case does not support that EBV is involved in the pathogenesis. Probably, host immunity and other factors are more important in the process.[12] It is significant that we did not detect any EBV in the epithelium of nasal polyps. Although it has been shown that the epithelial cells express EBV receptor CR2 (CD21) on their surface[20] or can be infected by EBV through IgA and the secretory component complex,[21] and EBV can lytically infect epithelial cells *in vitro*,[22,23] no direct evidence for the presence of EBV in the epithelium of the upper respiratory tract in asymptomatic individuals *in vivo* has yet been reported. This negative finding is similar to our findings in normal nasopha-

ryngeal mucosa.[7,8] A recent study by Anagnostopoulos et al[24] has also revealed that the EBV latently or lytically infected cells in infectious mononucleosis patients were exclusively lymphocytes, rather than epithelial cells, which used to be assumed as the primary target cells for EBV. It is interesting to note that, although EBER$^+$ intraepithelial lymphocytes have been detected in normal nasopharynx,[7,8] they were not detected in the nasal polyps. Whether this is due to small sample size or related to the difference between a respiratory-type (pseudostratified columnar) epithelium in nasal polyps and the intermediate or stratified squamous epithelium in nasopharynx where intraepithelial EBV$^+$ lymphocytes were detected needs additional studies.

EBV has been found in normal nasopharyngeal mucosa and three different nasal lesions: nasal lymphoma, NPC, and nasal polyps. The number of EBV-containing cells in nasal polyps or normal nasopharyngeal mucosa[7,8] is significantly lower than that in nasal lymphomas[4] and NPC,[16] despite the productive EBV infection in nasal polyps or normal nasopharyngeal mucosa. This difference of virus load may be due to the healthy host immune surveillance in non-neoplastic nasal or nasopharyngeal mucosa continuously destroying EBV-containing cells, as LMP-1, in addition to other EBV proteins, are antigens recognized by cytotoxic T cells.[25,26] The low number of EBV-containing cells in non-neoplastic nasal and nasopharyngeal mucosa[7,8] probably also reflects this pressure of host immune surveillance. Nevertheless, the frequent finding of a low level of EBV infection in the resident lymphocytes of the nasal mucosa, at least in the context of an inflammatory lesion, supports the view that the nose can be a site of viral persistence and provides a local setting for the emergence of virally associated tumors in this site.

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