DETECTION OF EPSTEIN-BARR VIRUS DNA AND HHV-6 DNA IN TISSUE BIOPSIES FROM PATIENTS WITH NASOPHARYNGEAL CARCINOMA BY POLYMERASE CHAIN REACTION

Uraiwan Kositanont[1], Kazyhiro Kondo[2], Cheerasook Chongkolwatana[3], Chokchai Metheetrairut[3], Pilaipan Puthavathana[1], Nivat Chantarakul[4], Chantapong Wasi[1] and Koichi Yamanishi[2]

Departments of [1]Microbiology, [3]Otolaryngology and [4]Pathology, Faculty of Medicine Siriraj Hospital, Bangkok 10700, Thailand; [2]Department of Virology, Research Institute for Microbial Diseases, 3-1 Yamada-oka, Suita, Osaka 565, Japan

Abstract. A total of 34 tissue biopsies were collected from nasopharyngeal carinoma (NPC) patients and 5 controls with non-NPC. Extracted DNA from tissue biopsies were analyzed for presence of specific gene sequences to EBV type A and type B, and HHV-6 by polymerase chain reaction (PCR). The different sequences of EBV type A and B were parts from the highly divergent forms of the EBV nuclear antigen 2 (EBNA 2). The PCR amplified products for EBNA 2A and EBNA 2B were 115 and 119 base pairs respectively whereas that of HHV-6 DNA was 776 base pairs. The results demonstrated that EBV DNA was detected in 32 of 34 cases (94.1%) : 28 (82.3%) with type A, 2 (5.9%) with type B, and 2 (5.9%) with both types. EBV DNA of type A could be detected 1 (20%) of 5 controls. HHV-6 DNA was in 5 of 34 samples (14.7%) whereas HHV-6 DNA was not detectable in biopsy tissues from controls. The results show that in the NPC patient group, A type of EBV is predominant. Detection of HHV-6 DNA in patients group only might be resulted from reactivation of a latent infection or association with EBV-induction of NPC.

INTRODUCTION

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and appears to be responsible for two types of human cancers : nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL). NPC is confined mostly to south China and Southeast Asia while BL is confined to Africa (Klein, 1973). EBV is divided into two types designated type A (B95-8 isolate) and type B (AG876 or Jijoye isolates). These differences stem partly from the highly divergent forms of the EBV nuclear antigen-2 (EBNA-2) coding region within U2 open reading frame (Adldinger *et al,* 1985; Dambaugh *et al,* 1984; Rowe *et al,* 1989). Type A virus has a world-wide distribution and is regarded as the predominant strain in Western countries. By contrast, type B virus has been found mainly in central Africa and New Guinea (Young *et al,* 1987; Zimber *et al,* 1986).

A new human herpesvirus, human herpesvirus-6 (HHV-6), was recently isolated from patients with lymphocytic disorders and acquired immune deficiency syndrome (AIDS) (Downing *et al,* 1987; Salahuddin *et al,* 1986; Tedder *et al,* 1987). Yamanishi *et al* (1988) have presented evidence that HHV-6 is the causative agent of exanthem subitum. In addition, HHV-6 can play a potential role in neurological disorders (Komaroff *et al,* 1988). Several interesting studies have shown the interaction of HHV-6 and EBV. Several B cell lines infected with EBV were refractory to infection by HHV-6 (Ablashi *et al,* 1988a; Ablashi *et al,* 1988b). It was hypothesized that infection by EBV may lead to induction of a receptor for HHV-6 on the B cells. There were some simultaneous rises in serum antibody titers to HHV-6 and EBV (Kirchesch *et al,* 1988). The possible co-tumorigenic role of HHV-6 has been suggested by studies in the NIH-3T3 mouse fibroblast system (Razzaque, 1990). HHV-6 is a possible cofactor in NPC because of its almost universal prevalence, lymphotropism and involvement of salivary glands. HHV-6 could replicate (Harnett *et al,* 1990) and hibernate in a latent form (Krueger *et al,* 1990) in epithelial cells of salivary glands, as does EBV.

Polymerase chain reaction (PCR) is a technique for the amplification of DNA *in vitro* (Harnett *et al,* 1990; Komaroff *et al,* 1988; Yamanishi *et al,* 1988) of infectious agents that are present in small numbers in clinical samples. The objective of the study was to detect DNA of EBV type A and B,

and HHV-6 by PCR in biopsy tissues from NPC patients and their controls.

MATERIALS AND METHODS

Subjects

Subjects comprised 34 patients with histological proved NPC and 5 controls with non-NPC. NPC patients included 1 case of WHO type 1, 14 cases of WHO type 2, 17 cases of WHO type 3, and 2 cases of unclassified type.

Clinical specimens

Tissue biopsies were collected prior to any clinical treatment from patients and controls attending at Department of Otolaryngology, Siriraj Hospital, Bangkok, Thailand. The samples of approximate 30 mg in weight were kept at -70°C until tested.

Preparation of DNA samples

DNA was extracted from tissue biopsies by modifying the procedure described by Chan *et al* (1988). The tissues were homogenized and then extracted with phenol-chloroform-isoamyl alcohol and followed by ethanol precipitation. The DNA precipitate was digested with RNase A at 37°C for 30 minutes and proteinase K at 37°C, overnight. The mixture was reextracted with phenol-chloroform-isoamyl alcohol and followed by ethanol precipitation. The DNA precipitate was dissolved in 20 µl of tris-EDTA buffer, pH 8.0 and then kept at -20°C until used.

Primer synthesis

Two pairs of primers, which were specific to EBNA 2A and EBNA 2B regions, were synthesized based on the published DNA sequences of EBV genome (Adldinger *et al,* 1985; Dambaugh *et al,* 1984). The sequences of EBNA 2A primers were 5'-AACTTCAACCCACACCATCA-3' and 5'-TTCTGGACTATCTGGATCAT-3'. The sequences of EBNA 2B primers were 5'-TACTCTTCCTCAACCCAGAA-3' and 5'-GGTGGTAGACTTAGTTGATG-3'. The PCR amplified products were 115 and 119 base pairs in length for EBNA 2A and EBNA 2B, respectively.

HHV-6 DNA was amplified using primers derived from the Hashimoto strain with the sequences in which parts of the *SalI* fragment (approximately 6 kilobase pairs) are located in the region encoding a major capsid protein as previous described (Kondo *et al,* 1990; Lawrence *et al,* 1990). The sequences of HHV-6 primers were 5'-GTGTTTCCATTGTACTGAAACCGGT-3' and 5'-TAAACATCAATGCGTTGCATACAGT-3'. The HHV-6 product was 776 base pairs in length. These primers synthesized in a DNA synthesizer (Applied Biosystems, USA).

DNA was amplified in a total volume of 50 µl of a reaction mixture containing 5 µl of sample; 50 pM of the primers; 40 mM of deoxynucleoside triphosphates (dNTP); buffer (50 mM KCl, 10 mM Tris hydrochloride pH 8.3, 0.01% gelatin, 1.5 mM $MgCl_2$) and 2 units of *Taq* polymerase (Perkin-Elmer Cetus, USA). The amplification was carried out in a DNA thermal cycler (Hybaid, Japan). The cycle for EBV DNA amplification consisted of denaturation at 90°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 5 minutes. The cycle for HHV-6 DNA amplification was the same as that described above, except that the annealing temperature was 62°C. The samples were subjected to 30 amplification cycles. A second run of 30 cycles was done for HHV-6 DNA.

Analysis of amplified products

The amplified products were detected by direct gel analysis and southern blot hybridization.

For direct gel analysis, 10 µl of the amplified products was loaded on 1% agarose gels and subjected to electrophoresis. A 3% Nusieve agarose gel was added for detection of EBV DNA. The DNA was visualized by UV fluorescence after staining with ethidium bromide. Molecular weight markers were included in each gel.

For southern blot hybridization, the DNA on gels was transferred to nylon filter membrane (Hybond-N^+, Amersham, UK) with 0.4 M NaOH for 3 hours. The filter was neutralized with 2 × SSPE (0.3 M NaCl, 20 mM NaH_2PO_4 pH 7.4, 2 mM disodium EDTA) for a few minutes. The DNA samples were then hybridized for 12 hours with ^{32}P-labeled cloned probe (1×10^6 cpm/ml) in hybridization fluid (6 × SSPE, 3% skim milk, 0.1% SDS). The filter was then washed twice with 2 × SSPE, 0.1% SDS for 10 minutes each at room

temperature, once for 15 minutes at 65°C, and then twice with 0.2 × SSPE, 0.1% SDS for 20 minutes at 65°C. Bound probes were detected by autoradiography at -70°C for 8 hours with intensifying screens. Probes used to detect amplified products were cloned DNA probes. For the amplified products of EBV DNA, the probes of plasmid pACYC 184, which contains EBNA 2A (1.5-kilobase pairs) and pUC 8, which contains EBNA 2B (0.75-kilobase pairs) were kindly provided by Dr TB Sculley (QIMR, Australia). Cloned DNA probe which contains a part of the *Sal*I fragment (approximate 6 kilobase pairs) as described above was used for HHV-6 DNA detection.

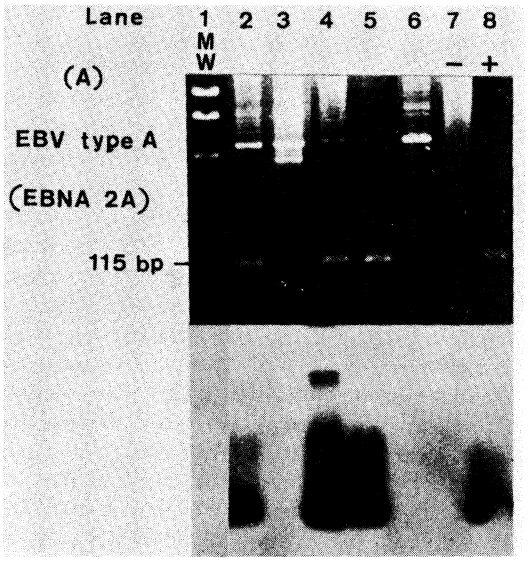
RESULTS

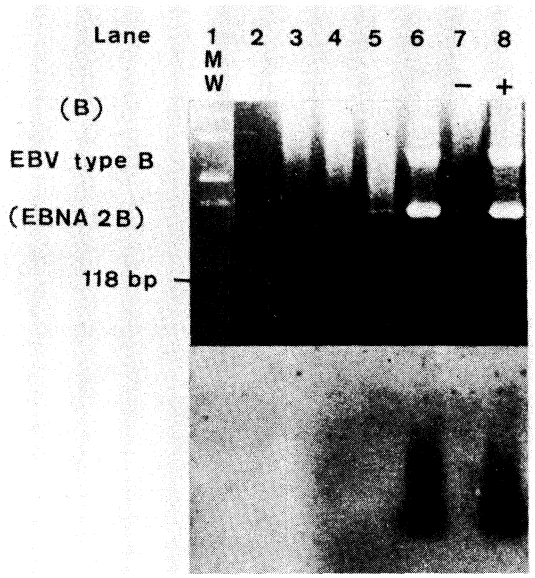
Detection of EBV and HHV-6 DNA by PCR

The oligonucleotides selected as primers for EBNA 2A and EBNA 2B were specific for detection of different genotypes of EBV strains. The PCR amplified products for EBNA 2A and EBNA 2B were 115 and 119 base pairs respectively (Figs 1A, 1B) whereas that for HHV-6 DNA was 776 base pairs (Fig 1C). The results from direct gel analysis and southern blot hybridization for EBV DNA amplified products were comparable. For amplified products of HHV-6, it gave positive signal in southern blot hybridization only.

Detection of EBV DNA in clinical specimens

EBV DNA was detected in 32 (94.1%) of 34 NPC patients : 28 (82.3%) with type A, 2 (5.9%) with type B, and 2 (5.9%) with both types (Table 1).





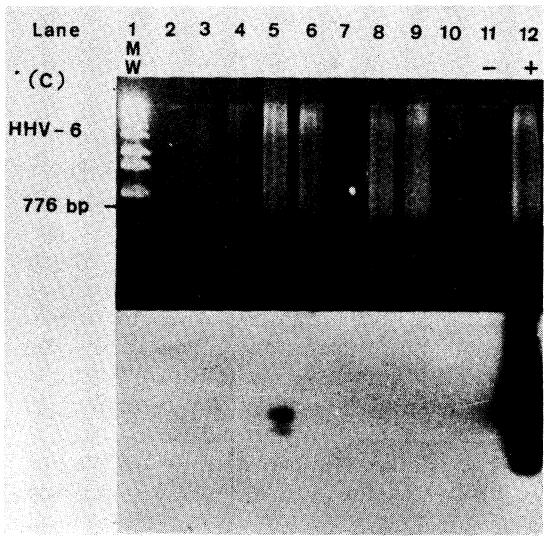


Fig 1—Agarose gel analysis and southern blot hybridization of products amplified by PCR with EBNA 2A primers (A), EBNA 2B primers (B) and HHV-6 primers (C). Molecular weight markers (lane 1) for EBV and HHV-6 products were pUC 19-*Sau3AI* and -*EcoT14I* respectively. Negative controls (-) and positive controls (+) are shown respectively. Lanes 2-10 were applied with DNA amplified products of clinical samples.

Table 1

Detection of EBV DNA and HHV-6 DNA in biopsy tissues from NPC patients.

Histological types	No. of biopsy tissues	No. of biopsy tissues positive for			
		EBNA 2A	EBNA 2B	EBNA 2A and EBNA 2B	HHV-6 DNA
WHO type 1	1	1	0	0	0
WHO type 2	14	11	2	0	2
WHO type 3	17	15	0	1	3
Unclassified	2	1	0	1	0
Total	34	28 (82.3%)	2 (5.9%)	2 (5.9%)	5 (14.7%)
Controls (Non-NPC)	5	1 (20.0%)	0	0	0

Detection of HHV-6 DNA in clinical specimens

HHV-6 DNA was positive in 5 (14.7%) of 34 samples from NPC patients. Detection of HHV-6 DNA was analyzed against classification of histologic sections of NPC according to the World Health Organization (WHO). Five positive samples were found in WHO 2 and 3 (Table 1). HHV-6 DNA was not detectable in biopsy tissues from non-NPC group.

DISCUSSION

In the study, detection of EBV DNA in 94.1% (32/34) of NPC patients was higher than that in 20% of non-NPC controls. The finding has confirmed an association between EBV and NPC performed by serology as reported previously (Hadar *et al,* 1986; Puthavathana *et al,* 1991; Wara *et al,* 1975; Zeng, 1985). The previous reports demonstrated that EBV DNA sequences were detected in 46 (92%) of 50 NPC specimens by PCR (Chang *et al,* 1990) and all the eighteen NPC biopsies tested were positive for EBV DNA detection using *in situ* hybridization (Permeen *et al,* 1990). Detection of EBV DNA found in 20% of 5 controls was similar to 22% in throat washings (Sixbey *et al,* 1989) and 12% in oropharyngeal cells (Gopal *et al,* 1990). Unfortunately, the tissue biopsies from the control group were limited. EBV DNA could be detected in tissues from NPC of all histological types in Thailand. Only one case of WHO type 1 was determined in this study since WHO type 1 NPC was rare in Thailand (Lunchanavanich *et al,* 1988; Puthavathana *et al,* 1991).

The study demonstrated that EBV DNA was detected in 82.3% with type A, 5.9% with type B and 5.9% with co-infection of type A and B of NPC patients whereas 20% of controls were found to be only type A. NPC patients were infected predominantly with type A and some with type B or both types. Type B of EBV was described previously as a poorly transforming type (Dambaugh *et al,* 1984), however, several reports have shown that type B is associated with NPC and BL tumors. The subtypes of EBV found in NPC biopsies were 94.3% type A, 3.8% type B and 1.9% coinfection of type A and B (Shu *et al,* 1991). EBV type B has been found in up to 40% of BL tumors and about 20% in the peripheral blood lymphocytes of healthy adults in BL-endemic areas of Africa (Young *et al,* 1987; Zimber *et al,* 1986). There are some studies that have demonstrated type B EBV infection outside Africa. Young *et al* (1987), for example, detected type B virus in only 3 of 100 spontaneous lymphoblastoid cell lines from Caucasian donors. The findings in Australia by Sculley *et al* (1988) showed a higher portion of anti-EBNA-2B in AIDS patients and HIV-infected persons than that in healthy controls. Additional studies on lymphoblastoid cell lines from HIV-positive subjects and controls showed that 19% were infected

with B-type EBV, 69% with A-type, and 12% with both types (Sculley *et al,* 1990). These results suggested that both types of EBV may exhibit dual tissue tropism as well as that reported by Sixbey *et al* (1989). They reported that EBV DNA was detected in the throat washings of 34 (22%) of 157 randomly selected donors, 14 (41%) of whom had type B virus, 17 (50%) type A and 3 (9%) both types. Interestingly, preliminary analysis of donors in their study who were shedding both virus types in the oropharynx showed only type A in peripheral blood lymphocytes.

HHV-6 DNA was found in 5 (14.7%) of 34 biopsy tissues from NPC patients while HHV-6 DNA was not detectable in tissues from controls. The results were similar to the 15% in throat washings from NPC and 3% in those from healthy adults (Kido *et al,* 1990). In contrast, 63, 23 and 12 percent of samples of oropharyngeal cells from healthy adults were positive for HHV-6, EBV and both HHV-6 and EBV DNA, respectively (Gopal *et al,* 1990). Detection of HHV-6 DNA in only NPC patients suggested that HHV-6 was reactivated from the latent state or that HHV-6 may be a co-factor in EBV-induction of NPC. These findings revealed that the concept of association between HHV-6 and EBV in NPC is possible. The *in situ* hybridization for determination of HHV-6 and EBV DNA in the same tumor cells may be supportive of a role of HHV-6 in EBV-associated NPC.

ACKNOWLEDGEMENTS

The authors would like to thank Dr TB Sculley for his suggestion of primer sequences of EBNA 2A and EBNA 2B.

REFERENCES

Ablashi DV, Josephs SF, Buchbinder C, *et al.* Human B-lymphotropic virus (human herpesvirus-6). *J Viral Methods* 1988a; 21 : 29-48.

Ablashi DV, Lusso P, Hung C, *et al.* Utilization of human hematopoietic cell lines for the propagation and characterization of HBLV (human herpesvirus 6) *Int J Cancer* 1988b; 42 : 787-91.

Adldinger HK, Delius H, Freese UK, Clarke J, Bornkamm GW. A putative transforming gene of Jijoye virus differs from that of Epstein-Barr virus prototypes. *Virology* 1985; 141 : 121-34.

Chan V, Fleming K, McGee J. Simultaneous extraction from clinical biopsies of high molecular-weight DNA and RNA : comparative characterization by biotinylated and ^{32}P-labelled probes on Southern and Northern blots. *Anal Biochem* 1988; 168 : 16-24.

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 : 2398-402.

Dambaugh T, Hennessy K, Chamnakit L, Kieff E. U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2. *Proc Natl Acad Sci USA* 1984; 81 : 7632-6.

Downing RG, Sewankamba N, Sewadda D, *et al.* Isolation of human lymphotropic herpesviruses from Uganda. *Lancet* 1987; 2 : 390.

Gopal MR, Thomson BJ, Fox J, Tedder RS, Honess RW. Detection by PCR of HHV-6 and EBV DNA in blood and oropharynx of healthy adults and HIV-seropositives. *Lancet* 1990; 335 : 1598-9.

Hadar T, Rahima M, Kahan E, *et al.* Significance of specific Epstein-Barr virus IgA and elevated IgG antibodies to viral capsid antigens in nasopharyngeal carcinoma patients. *J Med Virol* 1986; 20 : 329-39.

Harnett GB, Farr TJ, Pietroboni GR, Bucens MR. Frequent shedding of human herpesvirus-6 in saliva. *J Med Virol* 1990; 30 : 128-30.

Kido S, Kondo K, Kondo T, Morishima T, Takahashi M, Yamanishi K. Detection of human herpesvirus 6 DNA in throat swabs by polymerase chain reaction. *J Med Virol* 1990; 32 : 139-42.

Kirchesch H, Mertens T, Burkhardt U, Kruppenbacher JP, Hoffken A, Eggers HJ. Seroconversion against human herpesviruses and clinical illness. *Lancet* 1988; 2 : 273-4.

Klein G. The Epstein-Barr virus. In: Kaplan AS, ed. The Herpes Viruses. New York : Academic Press, 1973; 521 p.

Komaroff A, Saxinger C, Buchwald D, Geiger A, Gallo RC. A chronic "Post-viral" fatigue syndrome with neurological features : serologic association with human herpesvirus-6 (HHV-6). *Clin Research* 1988; 36 : 743 A.

Kondo K, Haykawa Y, Mori H, *et al.* Detection by polymerase chain reaction amplification of human herpesvirus 6 DNA in peripheral blood of patients with exanthem subitum *J Clin Microbiol* 1990; 28 : 970-4.

Krueger GRF, Wassermann K, Clerck LS De, *et al.* Latent herpesvirus-6 in salivary and bronchial glands. *Lancet* 1990; 336 : 1255-6.

Lawrence GL, Chee M, Craxton MA, Gomples UA, Honess RW, Barrell BG. Human herpesvirus 6 is closely related to human cytomegalovirus. *J Virol* 1990; 64 : 287-99.

Lunchanavanich P, Sangsa-ad S, Kraitrakul S, Puapairoj A. Nasopharyngeal cancer. *Otolaryngology-Head and Neck Surgery* 1988; 3 : 167-76. (Thai with Eng abstract).

Permeen AM, Sam OK, Pathamanathan R, Prasad U, Wolf H. Detection of Epstein-Barr virus DNA in nasopharyngeal carinoma using a non-radioactive digoxigenin-labelled probe. *J Viral Methods* 1990; 27 : 261-7.

Puthavathana P, Sutthent R, Vitavasiri A, Wasi C, Chantarakul N, Thongcharoen P. Epstein-Barr virus serological markers for nasopharyngeal carinoma in Thailand. *Southeast Asian J Trop Med Public Health* 1991; 22 : 326-31.

Razzaque A. Oncogenic potential of human herpesvirus-6 DNA. *Oncogene* 1990; 5 : 1365-70.

Rowe M, Young LS, Cadwallader K, Petti L, Kieff E, Rickinson AB. Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. *J Virol* 1989; 63 : 1031-9.

Salahuddin SZ, Ablashi DV, Markham PD, *et al.* Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* 1986; 234 : 596-600.

Sculley TB, Cross SM, Borrow P, Cooper DA. Prevalence of antibodies to Epstein-Barr virus nuclear antigen 2B in persons infected with the human immunodeficiency virus. *J Infect Dis* 1988; 158 : 186-92.

Sculley TB, Apolloni A, Hurren L, Moss DJ, Cooper DA. Coinfection with A- and B-type Epstein-Barr virus in human immunodeficiency virus-positive subjects. *J Infect Dis* 1990; 162 : 643-8.

Shu CH, Liu WT, Chang YS, *et al.* Distribution of type A and type B EBV in patients with nasopharyngeal carcinoma. *Chung Hua I Hsuch Isa Chih* 1991; 48 : 427-30.

Sixbey JW, Shirly P, Chesney PJ, Buntin DM, Resnick L. Detection of a second widespread strain of Epstein-Barr virus. *Lancet* 1989; 2 : 761-5.

Tedder RS, Briggs M, Cameron CH, *et al.* A novel lymphotropic herpesvirus. *Lancet* 1987; 2 : 390-92.

Wara WM, Wara DW, Phillips TL, Ammann AJ. Elevated IgA in carcinoma of the nasopharynx. *Cancer* 1975; 35 : 1313-5.

Yamanishi K, Okuno T, Shiraki K, *et al.* Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet* 1988; 1 : 1065-7.

Young LS, Yao QY, Rooney CM, *et al.* New type B isolate of Epstein-Barr virus from Burkitt's lymphoma and normal individuals in endemic areas. *J Gen Virol* 1987; 68 : 2853-62.

Zeng Y. Seroepidemiological studies on nasopharyngeal carcinoma in China. *Adv Cancer Res* 1985; 44 : 121-38.

Zimber U, Adldinger HK, Lenoir GM, *et al.* Geographic prevalence of two types of Epstein-Barr virus. *Virology* 1986; 154 : 56-66.