(47) Pilepich MV, Perez CA, Walz BJ, et al: Complications of definitive radiotherapy for carcinoma of the prostate. Int J Radiat Oncol Biol Phys 7:1341-1348, 1981

(48) Gibbons RP, Mason JT, Correa RJ Jr, et al: Carcinoma of the prostate: local control with external beam radiation therapy. J Urol 121:310-312, 1979

(49) Neglia WJ, Hussey DH, Johnson DE: Megavoltage radiation therapy for carcinoma of the prostate. Int J Radiat Oncol Biol Phys 2:873-883, 1977

(50) Ray GR, Cassady RJ, Bagshaw MA: Defininoma of the
f experience.

ncer: Manual
adelphia: Lip-

nd staging of
athology: the
adelphia: Lea

tional Library
covering the
ry, veterinary
s.

of the authors
of the Depart-
fense.

1994; revised
r 14, 1994.

Frequent Detection of Human Herpesvirus 6 in Oral Carcinoma

*M. Yadav, A. Chandrashekran, D. M. Vasudevan, D. V. Ablashi**

Human herpesvirus 6 (HHV-6) was initially isolated from patients with lymphoproliferative disorders and acquired immunodeficiency syndrome (AIDS) (*1*). It was later shown to be highly prevalent in various human populations worldwide (*2*). The virus is the etiologic agent of exanthem subitum (*3*), of some types of febrile illness in young children (*4*), and of some cases of heterophil antibody-negative infectious mononucleosis (*2*). HHV-6 has also been reported to be associated with several diseases, including lymphomas (*5-8*), but it has not previously been reported to be associated with epithelial cell tumors of the oral cavity.

Oral carcinoma ranks as the eighth most common tumor in the developed world. In Asia and the Pacific regions, however, it is a major tumor of the head and neck, particularly in populations among whom chewing of betal quid and tobacco is the cultural norm (*9*). Carcinogens have been suspected in the pathogenesis of oral carcinoma (*10*), but the role of viral agents in oral cocarcinogenesis remains unclear.

In a preliminary study (*11*), we found significantly elevated levels of immunoglobulin G (IgG) antibody to HHV-6 in sera from oral carcinoma patients from South India (geometric mean titer [GMT] = 2042) compared with sera from healthy donors from the same population (GMT = 47). In contrast, Yadav and Ablashi (*12*) found normal levels of HHV-6 antibody in sera from patients with nasopharyngeal carcinoma, which is the most common tumor in the Chinese populations of Southeast Asia (GMT = 14) and which has been linked to Epstein-Barr virus (EBV) (*13*).

In the present study, we extend these observations to detection of HHV-6 DNA and viral antigens in tissues of oral tumors.

Fresh tumor tissue samples of histologically diagnosed squamous cell carcinoma of the buccal mucosa (one sample each from nine patients) were obtained from the Medical College of Trichur, India. Fifty nanograms of genomic DNA from each sample was used as the template for polymerase chain reaction (PCR) amplification, using HHV-6–specific nested primers to a conserved region of the virus, and amplified for 30 cycles (*14*). We found that six (67%) of the nine oral carcinoma tissue samples were positive for HHV-6 and the PCR amplicons specifically hybridized to a digoxigenin (Boehringer Mannheim, Mannheim, Federal Republic of Germany)-labeled oligonucleotide probe derived from the plasmid pZVH-14 containing an 8.9-kilobase (kb) insert of HHV-6 DNA (Fig. 1, A and B).

Further studies were performed on archival formalin-fixed and paraffin-embedded sections of biopsy tissue from seven oral mucosal tumors. The sections were incubated at a dilution of 1:50 with mouse monoclonal antibody (MAb) (GA5G3) to the HHV-6 glycoprotein (gp) 116K/64K/54K component (*15*), which is a late protein in the viral replicative cycle. The tissue-bound GA5G3 MAb was visualized by the immunoperoxidase reaction (*16*), and [illegible] seven (100%) of the biopsy specimens were seen to express HHV-6–associated antigen in the transformed cells (Fig. 2, A and B). The antigen was localized in the cytoplasm and sometimes also strongly localized in the membrane and nucleus of squamous cells, which had a typical epithelial morphology. The squamous cells also stained specifically to cytokeratin MAb (BioGenex Laboratories, San Ramon, Calif.), confirming their epithelial nature. It is interesting that paraffin-embedded sections from three biopsy specimens of nasopharyngeal carcinomas were negative with the GA5G3 MAb, showing that the HHV-6 antigen was not expressed in the nasopharyngeal carcinoma tissues. Control tissue sections from normal oral mucosa were also negative for the GA5G3 MAb. Moreover, the GA5G3 MAb failed to react with oral carcinoma tissue sections following adsorption of the antibody with heat-inactivated HHV-6 particles, thus confirming its specificity.

PCR for HHV-6 was also conducted on DNA extracted from the paraffin-embedded sections that were positive for GA5G3 MAb; of seven samples analyzed, five (71%) were positive. Thus, the immunohistochemical staining was more sensitive in the overall detection

**Affiliations of authors:* M. Yadav, A. Chandrashekran, Department of Genetics and Cellular Biology, University of Malaya, Kuala Lumpur, Malaysia.

D. M. Vasudevan, Department of Biochemistry, Government Medical College, Trichur, Kerala, India.

D. V. Ablashi, Department of Microbiology and Immunology, Georgetown University School of Medicine, Washington, D.C.; and Advanced Biotechnologies Inc., Columbia, Md.

Correspondence to: Professor M. Yadav, Ph.D., Department of Genetics and Cellular Biology, University of Malaya, 59100 Kuala Lumpur, Malaysia.

See "Notes" section following "References."

of HHV-6–associated antigens in tissues than the PCR technique for virus DNA sequence. Additional PCR analysis of the oral carcinoma DNA with EBV nuclear antigen primers (*14*) showed no amplified product; this finding is in agreement with the report that EBV DNA and EBV antigens are absent in oral carcinoma tissue (*16*).

Sera from five patients with advanced oral carcinoma (IgG GMT = 1613) were tested by immunofluorescence assay for immunoglobulin A (IgA) antibody to HHV-6, using HSB_2 cells infected with the prototype GS strain (*17,18*). Patients with the malignancy had elevated levels of IgA antibody to HHV-6 (IgA GMT = 2031; range = 640-5120), and five age-matched, normal, healthy donors lacked the IgA antibody (GMT = <10). These data suggest that the IgA antibody to HHV-6 could be a diagnostic and prognostic marker in oral carcinoma, but further studies using larger numbers of serum specimens from oral carcinoma patients are required.

The frequent detection of HHV-6 DNA in oral carcinoma is similar to the previous demonstration of EBV DNA in biopsy specimens of nasopharyngeal carcinomas (*13*). Infective HHV-6 particles, which may be the source of infection of the oral epithelial cells, are present in saliva (*19,20*) and are probably derived from the salivary and transbronchial glands (*21*).

Oral carcinogenesis is probably the result of a multistep process with a multifactorial etiology. Epidemiologic, clinical, and laboratory studies confirm an etiologic link between tobacco chewing and oral cancer (22). Tobacco-specific nitrosamines, which are genotoxic carcinogens, are suspected to cause molecular aberrations in the proto-oncogenes and tumor suppressor genes and, through accumulated genetic changes, to lead to malignancy (*23*). Some oncogenic viruses, like HHV-6, perhaps acting synergistically with these chemical carcinogens, could facilitate the transformation process. Human herpes simplex virus and human papillomavirus have been thought to play a role in the pathogenesis of oral carcinoma because both viruses are often noted in precancerous lesions. These viruses probably act by a "hit-and-run" mechanism (*10*). HHV-6 has not as yet been suspected as a possible cofactor in the development of oral tumors, however. Recent observations of HHV-6 transactivation of human papillomavirus in cervical carcinoma cell lines (*24*) and HHV-6 transformation of simian virus 40- and adenovirus 7-immortalized cells (*25,26*) lend support to the hypothesis that HHV-6 in combination with other cofactors, e.g., carcinogens, could play a role in the oncogenesis of oral carcinoma.

References

(*1*) Salahuddin SZ, Ablashi DV, Markham PD, et al: Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science 234:596-601, 1986

(2) Salahuddin SZ, Kelly A, Krueger GRF, et al: Human herpesvirus-6 (HHV-6) in diseases. Clin Diag Virol 1:81-100, 1993

(*3*) Yamanishi K, Okuno T, Shiraki K, et al: Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet 1:1065-1067, 1988

(*4*) Pruksananonda P, Hall CB, Insel RA, et al: Primary human herpesvirus 6 infection in young children [*see* comment citation in Medline]. N Engl J Med 326:1445-1450, 1992

(*5*) Torelli G, Marasca R, Luppi M, et al: Human herpesvirus 6 in human lymphomas: identification of specific sequences in Hodgkin's lymphoma by polymerase chain reaction. Blood 77:2251-2258, 1991

(*6*) Jarrett RF, Gledhill S, Qureshi F, et al: Identification of human herpesvirus 6-specific DNA sequences in two patients with non-Hodgkin's lymphoma. Leukemia 2:496-502, 1988

(*7*) Josephs SF, Buchbinder A, Streicher HZ, et al: Detection of human B-lymphotropic virus (human herpesvirus 6) sequences in B cell lymphoma tissues of three patients. Leukemia 2:132-135, 1988

(*8*) Yin SY, Ming HA, Jahan N, et al: In situ hybridization detection of human herpesvirus 6 in biopsy specimens from Chinese patients with non-Hodgkin's lymphoma. Arch Pathol Lab Med 17:502-506, 1993

(*9*) Jayant K, Notani P: Epidemiology of oral cancer. *In* Oral Cancer (Rao RS, Desai PB, eds). Bombay, India: Tata Press, 1991, pp 1-7

(*10*) Scully C, Prime SS, Cox MF, et al: Evidence for infection agents in the etiology of oral cancer. *In* Risk Marker for Oral Diseases (Johnson NE, ed). Cambridge, England: Cambridge Univ Press, 1991, pp 96-113

(*11*) Shanavas KR, Kala V, Vasudevan DM, et al: Anti-HHV-6 antibodies in normal population and in cancer patients in India. J Exp Pathol 6:95-105, 1992

(*12*) Yadav M, Ablashi DV: Nasopharyngeal carcinoma (NPC): lack of elevated antibody to human herpesvirus-6 (HHV-6) in these patients. Asean J Clin Sci 10:81-84, 1990

(*13*) Wolf H, zur Hausen H, Becher V: EB viral genomes in epithelial NPC. Nature 244:245-247, 1973

(*14*) Telenti A, Marshall WF, Smith TF: Detection of Epstein-Barr virus by polymerase chain reaction. J Clin Microbiol 28:2187-2190, 1990

(*15*) Balachandran N, Amelse RE, Zhou WW, et al: Identification of proteins specific for human herpesvirus 6-infected human T cells. J Virol 63:2835-2840, 1989

(*16*) Tafacko AA, Teo CG, Griffin BE, et al: Epstein-Barr virus receptor but not viral DNA are present in normal and malignant oral epithelium. J Oral Pathol Med 20:20-25, 1991

(*17*) Ablashi DV, Lusso P, Hung CL, et al: Utilization of human hematopoietic cell lines for the propagation and characterization of HBLV (human herpesvirus 6). Int J Cancer 42:787-791, 1988

(*18*) Henle G, Henle W: Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. Int J Cancer 17:1-7, 1976

(*19*) Jarrett RF, Clark DA, Josephs SF, et al: Detection of human herpesvirus-6 DNA in peripheral blood and saliva. J Med Virol 32:73-76, 1990

(*20*) Levy JA, Ferro F, Greenspan D, et al: Frequent isolation of HHV-6 from saliva and high seroprevalence of the virus in the population. Lancet 335:1047-1050, 1990

(*21*) Krueger GR, Wassenmann K, De Clerck LS, et al: Latent herpesvirus-6 in salivary and bronchial glands [letter] [*see* comment citation in Medline]. Lancet 336:1255-1256, 1990

(22) Jussawalla DJ, Deshpande VA: Evaluation of cancer risk in tobacco chewers and smokers: an epidemiologic assessment. Cancer 28:244-252, 1971

(*23*) Field JK: Oncogenes and tumor suppressor genes in squamous cell carcinoma of the head and neck. Eur J Cancer B Oral Oncol 28B:67-76, 1992

(*24*) Chen M, Popescu N, Woodworth C, et al: Human herpesvirus 6 infects cervical epithelial cells and transactivates human papilloma virus gene expression. J Virol 68:1173-1178, 1994

(*25*) Razzaque A: Oncogenic potential of human herpesvirus-6 DNA. Oncogene 5:1365-1370, 1990

(*26*) Razzaque A, Williams O, Wang T, et al: Neoplastic transformation of immortalized human epidermal keratinocytes by two HHV-6 DNA clones. Virology 195:113-120, 1993

Notes

Supported by funds from the Ministry of Science, Technology, and the Environment, Malaysia.

We thank Dr. Bala Chandran, University of Kansas, Kansas City, for providing us with the monoclonal antibody to herpesvirus 6. We also acknowledge the editorial assistance of Mrs. Kristine L. Ablashi.

Manuscript received April 8, 1994; revised July 22, 1994; accepted September 15, 1994.

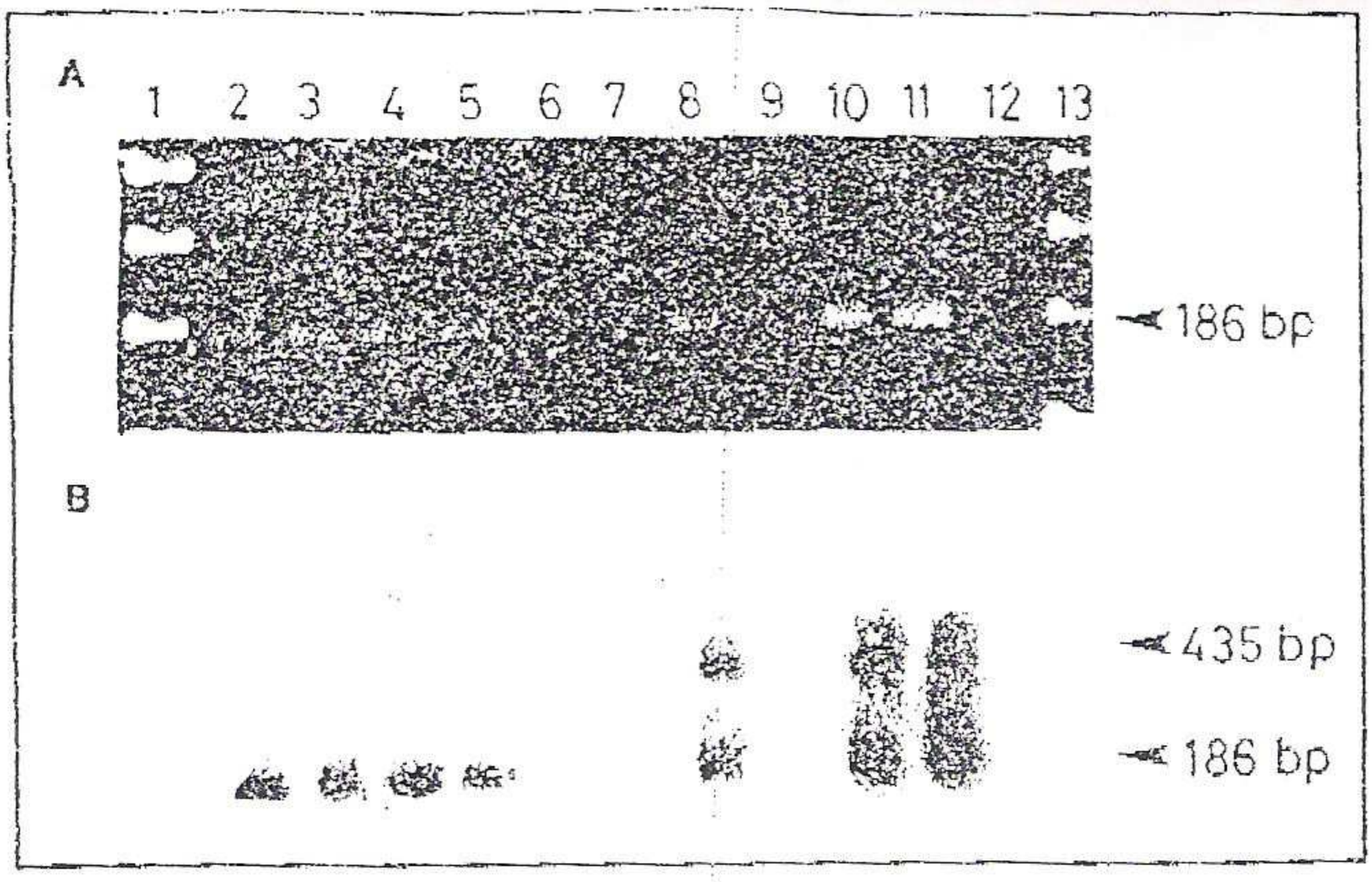

Fig. 1. Detection by nested PCR of HHV-6 sequence in genomic DNA extracted from fresh oral carcinoma tissue. A) The PCR-amplified nested fragment (8 µL) was electrophoresed in 2% agarose gel and stained with ethidium bromide. Lanes 1 and 13: molecular weight markers, 100-base-pair (bp) ladder. Lanes 2-10: oral tumor DNA amplified by PCR. The 186-bp fragment is visible in lanes 2-5, 8, and 10. Lane 11: positive control DNA derived from plasmid pZVH-14 with an 8.9-kb HHV-6 insert. Lane 12: negative control. B) DNA from the gel was transferred to a Hybond (Amersham International, Buckinghamshire, England), positively charged, nylon filter and then hybridized to a digoxigenin-labeled oligonucleotide HHV-6 probe. The 186-bp PCR products amplified by inner primers are noted in lanes 2-5, 8, and 10, and these amplified products and the positive control in lane 11 hybridized to the probe. In lanes 8, 10, and 11, hybridization signals can also be seen to the 435-bp PCR fragment that was amplified by the outer primers.

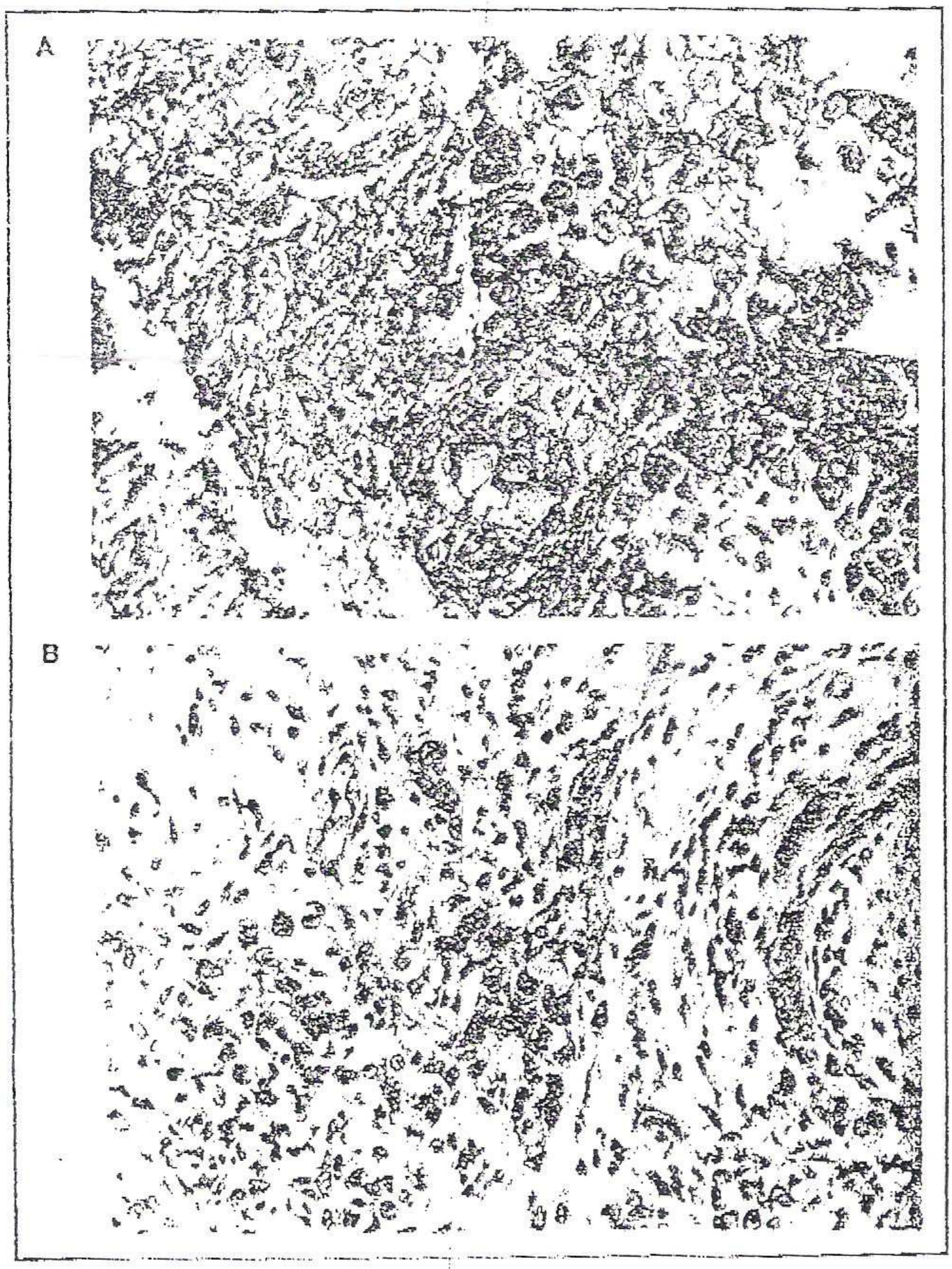

Fig. 2. Immunohistochemical detection of HHV-6–associated protein in formalin-fixed and paraffin-embedded sections of the buccal mucosal tumor. Following deparaffinization, the tissues were successively treated with MAb GA5G3, anti-mouse immunoglobulin labeled with biotin, streptovidin, hydrogen peroxide, and the substrate 3-amino-9-ethylcarbazole (counterstained with Mayer's hematoxylin, original magnification ×1(00). A) Section shows intense reactivity (reddish brown) of the membrane, cytoplasm, and nucleus with GA5G3 MAb to HHV-6 (gp 116K/64K/54K) protein. Nonreactive cells show only blue hematoxylin counterstain. B) Negative controls were achieved by omitting the primary antibody (GA5G3 MAb).