Viral etiology of juvenile- and adult-onset squamous papilloma of the larynx

(human papillomavirus/immunocytochemistry/Southern transfer analysis)

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ABSTRACT  Juvenile- and adult-onset laryngeal papillomas were examined for the presence of a human papillomavirus (HPV) genome and capsid antigens. DNA was isolated from a portion of tissue removed for therapeutic purposes, and the presence of a papillomavirus genome was detected by Southern transfer analysis. The viral DNA found in the 12 juvenile-onset and the 8 adult-onset laryngeal papillomas examined was identified as HPV-6 on the basis of size, restriction endonuclease digestion patterns, and homology detected under stringent conditions. Restriction endonuclease analysis of the viral genomes revealed at least four different subtypes, designated HPV-6c through HPV-6f. The most common subtype, HPV-6c, was detected in over half of the papillomas studied, including both juvenile and adult types. The remaining tissue was fixed and processed for immunocytochemistry. The immunoperoxidase technique was used with an antiserum that reacts with capsid antigen(s) common to all HPV serotypes. HPV antigen was found in two of the juvenile-onset papillomas and two of the adult-onset papillomas. The antigen was localized to the nucleus and was distributed in the superficial layers of the epithelium. HPV capsid antigen had not previously been detected in cases of adult-onset papilloma, and the HPV genome in both juvenile- and adult-onset laryngeal papillomas had not been characterized. Despite the absence of detectable viral antigen in most of the specimens examined, the presence of the HPV genome provides strong evidence for the papillomavirus etiology of these tumors.

Squamous papillomas of the larynx are a serious clinical problem because of their location, their resistance to treatment, their relentless recurrence, and their tendency to spread throughout the respiratory tract (1, 2). The most frequent age of onset is in the first five years of life, but the disease also occurs in adults. Papillomas of the larynx are tumors of lobulated or papillary form in which each projection has a central core of connective tissue covered with squamous epithelium (3). They histologically resemble cutaneous warts, whose papillomavirus etiology has been established (for review, see ref. 4). Although a papillomavirus etiology has been implicated for laryngeal papillomas, there have been conflicting reports on the presence of virus particles in these tumors when examined by electron microscopy (5–8). More recently, papillomavirus capsid antigen has been detected in juvenile-onset laryngeal papillomas by the immunoperoxidase technique of Sternberger (9), using an antiserum that reacts with genus-specific antigens (10–12). In these studies, papillomavirus antigen was detected in less than half of the juvenile-onset laryngeal papillomas. In the positive cases it was not possible to identify the specific viral serotype present in the lesions because the antiserum is crossreactive with capsid antigens of all papillomaviruses. Despite attempts to isolate papillomavirus or detect the papillomavirus genome in juvenile-onset laryngeal papillomas (13–17), the etiological agent has not been characterized.

In this report, we present evidence for the papillomavirus etiology of both juvenile- and adult-onset laryngeal papilloma. We have demonstrated that the 20 laryngeal papillomas, which we have examined using the Southern (18) transfer technology, contain viral genomes closely related to the human papillomavirus type 6 (HPV-6) present in condylomata acuminata (genital warts). Restriction endonuclease analysis has indicated the existence of four different viral subtypes, which are found in both juvenile- and adult-onset laryngeal papillomas. We have also demonstrated the presence of HPV capsid antigen in both juvenile- and adult-onset laryngeal papilloma by using the genus-specific antiserum with a more sensitive avidin–biotin–immunoperoxidase assay, developed by Hsu et al. (19).

MATERIALS AND METHODS

Biopsy Specimens. Twenty patients with biopsy-proven squamous papilloma of the larynx were identified in the Otolaryngology Clinic at The Johns Hopkins Hospital. The age at diagnosis of clinical disease for 12 patients (7 males and 5 females) with juvenile-onset papilloma was between 1 and 14 years, and for 8 patients (4 males and 4 females) with adult-onset papilloma it was between 23 and 55 years. The duration of symptoms ranged between 2 and 45 years, with juvenile-onset papillomas tending to have a longer duration. Tissue for analysis was obtained at the time of surgical excision. A portion of the tissue was fixed with formalin and embedded in paraffin for standard histopathological examination and was utilized in the immunocytochemical examinations. The remainder of the tissue was frozen in liquid nitrogen and stored at −70°C prior to isolation of its DNA.

Immunocytochemistry. The avidin–biotin–horseradish peroxidase complex modification (19) of the peroxidase–antiperoxidase test (9) was used to localize the papillomavirus antigen in 6-μm sections prepared from paraffin-embedded tissue. The primary rabbit antiserum was prepared against simian dodecyl sulfate-disrupted human papillomavirus virions from pooled plantar warts, and the specificity of the reaction using this antiserum has been described (20). The reagents for the avidin–biotin–immunoperoxidase technique were purchased from Vector Laboratories (Burlingame, CA) and were used according to their specifications. The paraffin was removed from the tissue sections, and they were rehydrated and incubated in 0.3% hydrogen peroxide in methanol. After an incubation with 3% non-immune goat serum, the tissue was incubated in the primary

Abbreviations: HPV, human papillomavirus; SV40, simian virus 40; NaCl/Cit, 0.15 M sodium chloride/0.015 M sodium citrate; kb, kilobase pairs.

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antiserum for benzidine avidin tinylated, methylene blue, the included nonspecific also processed with glass homogenizer. After counter-staining with methylene blue, the sections were mounted for light microscopy. Brown nuclear staining of cells in the superficial layers of the epithelium was scored as positive for viral antigen. A positive control—i.e., sections of cutaneous or genital warts—was included in each test. The sections of laryngeal papilloma were also processed with a rabbit antiserum to sodium dodecyl sulfate-disrupted simian virus 40 (SV40) as a control for identifying nonspecific staining.

DNA Isolation. The frozen tissue was ground in a ground glass homogenizer. The tissue was suspended in 0.15 M NaCl/0.1 M EDTA, pH 8, and the cells were lysed by the addition of sodium dodecyl sulfate to 1%. After an overnight incubation at 37°C with proteinase K (Merck) at 100 μg/ml, the DNA was further deproteinized by extractions with phenol followed by chloroform/isoamyl alcohol, 24:1 (vol/vol). After exhaustive dialysis at 4°C in 10 mM Tris-HCl, pH 7.4/1 mM EDTA/150 mM NaCl, the DNA was incubated with RNase A (20 μg/ml; Calbiochem) at 37°C for 2 hr and extracted with phenol and chloroform. The DNA was dialyzed at 4°C against 10 mM Tris-HCl, pH 7.4/1 mM EDTA (Tris/EDTA).

Hybridization Analysis. Two micrograms of tissue DNA was digested with restriction endonucleases (Bethesda Research Laboratories) under the conditions specified by the vender. After digestion, the DNA was extracted with phenol and chloroform, precipitated with ethanol, and dissolved in Tris/EDTA. The DNA was fractionated by 1% agarose gel electrophoresis, stained with ethidium bromide, and examined by UV fluorescence photography. The DNA fragments were transferred to nitrocellulose filters (Schleicher & Schuell BA85) by the method of Southern (18). Filters were baked in vacuo at 80°C for 2 hr and treated with 10× Denhardt’s solution (21, 22) in 6× NaCl/Cit (1× NaCl/Cit is 0.15 M sodium chloride/0.015 M sodium citrate) at 68°C for 3 hr prior to hybridization. The probe was HPV-6 DNA purified from a condyloma acuminatum (genital wart) of the cervix and inserted into the HindIII site in a derivative of the plasmid pBR322 (pHP6e-1; unpublished procedure). The plasmid was radioactively labeled in vitro by nick-translation (23, 24) and 2 x 10^6 Cerenkov cpm were added to each filter. Hybridization conditions were 68°C for 48 hr in 4× NaCl/Cit/50 mM sodium phosphate, pH 7.2/0.5% sodium dodecyl sulfate/10× Denhardt’s solution in rotating glass cylinders (25). Filters were washed with the salt concentration never lower than 0.66 M in order to maintain unstable hybrids. [Assuming a 41% G+C content, as has been determined for HPV-1 (26), this corresponds to melting temperature t_m – 25°C.] The dried filters were exposed to Kodak XS film at room temperature. To examine the stability of the hybrids, the filters were washed at 68°C for 30 min in 1× NaCl/Cit (t_m – 17°C), exposed, then washed in 0.1× NaCl/Cit (t_m – 0.7°C), and exposed.

RESULTS

Immunocytochemistry. Papillomavirus antigen was detected in 2 of the 12 biopsy specimens of juvenile-onset papillomas and 2 of 8 specimens of adult-onset papillomas by using the avidin–biotin–immunoperoxidase assay. The viral antigen was localized in the nuclei of cells in the superficial layers of abnormal epithelium. Each biopsy specimen was tested at least two times with the papillomavirus antiserum as well as the control, SV40 antiserum. The positive specimens were reproducibly reactive with the papillomavirus antiserum, and nuclear staining was not observed in any lesion with the SV40 rabbit antiserum.

In three of the four positive specimens, only a small number of cells contained detectable antigen, a finding that is in agreement with previous reports (10–13). Only isolated individual groups of less than 10 cells were found to be antigen-positive. For example, in specimen 81-229, from a juvenile-onset laryngeal papilloma in a male, antigen was detected in six karyocytic cells (Fig. 1A). Antigen was not evident in any other of the several tissue fragments present on the slide. Similarly, there was a single cluster of approximately 20 positive cells in both 81-139, a juvenile-onset papilloma, and in 80-191, an adult-onset papilloma. In contrast, a biopsy specimen from an adult-onset papilloma in a male (81-220, Fig. 1B) showed many positive cells in the superficial epithelium. More than 150 cells identified as containing papillomavirus antigen were present in six fragments. Other fragments with the identical histopathology were antigen-negative. The antigen was present in the nuclei of karyocytic cells in the superficial layers (Fig. 1B). In serial sections, no staining was observed with normal rabbit serum or the SV40 antiserum. Despite a similar histological appearance with hyperplasia of squamous epithelium, no papillomavirus antigen

FIG. 1. Papillomavirus antigen in laryngeal papillomas detected by the avidin–biotin immunoperoxidase technique. (A) Tissue fragment from a male with juvenile-onset laryngeal papilloma (81-229). Viral antigen was detected in the nuclei of six cells. One of the positive cells is indicated by an arrow. The membrane staining is nonspecific, occurring with the control serum as well. (×120.) (B) Tissue fragments from a male with adult-onset laryngeal papilloma (81-220). Many cells in the superficial layers of several pieces of tissue contained nuclear antigen. One such cell is indicated by the arrow. (×50.)
could be detected in the other 16 laryngeal papillomas of either juvenile or adult types.

**Hybridization Analysis.** The papillomavirus genome was detected in DNA isolated from both juvenile- and adult-onset laryngeal papillomas by using the Southern transfer technique with a HPV-6 probe (Fig. 2). DNA purified from laryngeal papillomas of two patients with adult-onset disease (80-191, 80-192) and two patients with juvenile-onset disease (80-136, 81-139) was digested with restriction endonucleases Hpa I (lanes b–e), BamHI (lanes f–i), Pst I (lanes j–m), HindIII (lanes o–r), or EcoRI (lanes s–u), size fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with radioactively labeled HPV-6 DNA (pPH6e−1). The positive controls included a Hpa I digest of DNA from the condyloma acuminatum of the cervix (lane a) that was the source of the viral DNA for the molecularly cloned probe, and a HindIII digest of pPH6e−1 DNA (0.1 pg) with 2 μg of human foreskin fibroblast DNA (lane n). Because there is one Hpa I site in this HPV-6 genome, a linear viral genome was generated and a single band of hybridization was observed (lane a). The HPV-6 genome had been inserted into the plasmid at its single HindIII site, and cleavage of the plasmid with this enzyme yielded the linear viral genome of 8 kilobase pairs (kb) together with the linear plasmid vector of 3.4 kb, as indicated by two bands of hybridization (lane n). The negative control was human DNA from human foreskin fibroblasts digested with EcoRI (lane w) with which no hybridization was detectable.

The size of the papillomavirus genome in the laryngeal papillomas was indistinguishable from that of the HPV-6 genome. A single band of hybridization was obtained when DNA from laryngeal papillomas was digested with BamHI (Fig. 2, lanes f–i) or HindIII (lanes o–r) or when DNA from specimen 80-192 was digested with Hpa I (lane b) or EcoRI (lane a). This linear molecule of the viral genome comigrated with the Hpa I linear molecule of HPV-6 DNA (lane a) and the HindIII linear molecule of the cloned DNA (lane n). Some open-circular (form II) viral DNA molecules were detectable in the HindIII digests (lanes o–r) due to incomplete digestion by the restriction enzyme. These could be converted to form III (linear) molecules by the addition of more enzyme. The appearance of supercoiled (form I) viral DNA in the EcoRI digests of 80–191 DNA (lane t), 80-136 DNA (lane u), and 80-139 DNA (lane v) indicates the absence of a recognition sequence for this enzyme in the viral genome. The presence of forms II and III was presumably the result of nicking prior to or during the isolation of the DNA.

Genetic heterogeneity among the HPV genomes in laryngeal papillomas was indicated by the differences in the cleavage patterns obtained with EcoRI (lanes s–u), Hpa I (lanes b–e), and Pst I (lanes j–m). To examine the different subtypes of the HPV present in the laryngeal papillomas, DNA (2 μg) from 8 adult-onset and 12 juvenile-onset laryngeal papillomas, including the four specimens analyzed in Fig. 2, was digested with BamHI, HindIII, EcoRI, and Hpa I. All of the papillomavirus subtypes contained one BamHI site (Fig. 3) and one HindIII site. In one case, 80-13, it was necessary to analyze 20 μg of DNA, instead of the 2 μg shown in Fig. 3 (lane n), to detect the viral genome.

On the basis of the variation observed in the cleavage patterns with EcoRI and Hpa I, four subtypes were identified in the laryngeal papillomas examined (Fig. 4). The most common subtype (HPV-6c) could be identified in four papillomas of adult onset and six papillomas of juvenile onset, and is characterized by the absence of an EcoRI site and the presence of at least six Hpa I sites (Fig. 4, lanes a and b). Subtype d, identified in one adult-onset papilloma, was characterized by no EcoRI site and at least five Hpa I sites (lanes c and d). Subtype e, identified in one papilloma of adult onset and two papillomas of juvenile onset, had no EcoRI site and one Hpa I site (lanes e and f). Subtype f, identified in two adult-onset papillomas, had one EcoRI site and one Hpa I site (lanes g and h). It was not possible to unequivocally determine the subtype in the remaining four lesions due to insufficient quantities of DNA. There was no correlation between subtype and the sex of the patient.

The HPV-6 DNA used as a probe for these hybridization experiments is similar to subtype e on the basis of digestion patterns with EcoRI, Hpa I, and Pst I (data not shown). It is apparent from the intensities of the bands in the Hpa I digests

![Fig. 2. Southern transfer analysis of 2 μg of DNA isolated from laryngeal papillomas and hybridized with radioactively labeled HPV-6 DNA. Lane a, Hpa I digest of DNA from a condyloma acuminatum of the cervix, the source of the molecularly cloned probe. Lanes b and c, Hpa I digests of 80-192 (lane b) and 80-191 (lane c) adult-onset laryngeal papillomas. Lanes d and e, Hpa I digests of 80-136 (lane d) and 81-139 (lane e) juvenile-onset laryngeal papillomas. Lanes f–i, BamHI digests of 80-192 (lane f), 80-191 (lane g), 80-136 (lane h), and 81-139 (lane i). Lanes j–m, Pst I digests of 80-192 (lane j), 80-191 (lane k), 80-136 (lane l), and 81-139 (lane m). Lane n, HindIII digest of pPH6e−1 (0.1 pg) added to 2 μg of human foreskin fibroblast DNA. Lanes o–r, HindIII digests of 80-192 (lane o), 80-191 (lane p), 80-136 (lane q), and 80-139 (lane r). Lanes s–w, EcoRI digests of 80-192 (lane s), 80-191 (lane t), 80-136 (lane u), 81-139 (lane v), and human foreskin fibroblast DNA (lane w).](image-url)
of subtypes c and d (Fig. 4, lanes b and d) that the fragments are not present in stoichiometric amounts and the sum of the molecular weights of, for example, the Pst I fragments (Fig. 2, lanes j–m) do not equal the size of the linear molecule, 8 kb. This is probably related to the distribution and extent of nucleotide sequence homology between the HPV-6 probe and the papillomavirus genome present in the laryngeal papillomas.

The papillomavirus genome in all of the laryngeal papillomas was shown to be closely related to HPV-6 on the basis of the stability of the hybrids under stringent conditions. When the filters shown in Fig. 3 were washed in 1× NaCl/Cit at 68°C (tm – 17°C) for 30 min and exposed, no significant decrease in the intensity of the bands was observed. However, when the filters were washed in 0.1× NaCl/Cit (tm – 0.7°C), some of the hybrids were eluted, as indicated by a decreased intensity of approximately 50%. Furthermore, even under nonstringent conditions (1 M Na+, tm – 30°C), there was no detectable homology between the HPV genome in laryngeal papillomas and HPV-1a, HPV-2, or HPV-4 [which were obtained from P. Howley (27)].

**DISCUSSION**

We have presented evidence for the papillomavirus etiology of both juvenile- and adult-onset squamous papillomas of the larynx. Papillomavirus capsid antigen was detected by using an immunoperoxidase assay in 16% of the cases of juvenile-onset and in 25% of the cases of adult-onset laryngeal papillomas. In addition to the remarkable absence of detectable antigen in histologically similar biopsy specimens, there is an apparent lack of antigen in cytologically identical cells adjacent to positively staining cells. A specimen may be negative due to the focal distribution of the antigen, because the staining can be seen to diminish even in a series of six consecutive sections. In addition, the antiseraum, which is raised against sodium dodecyl sulfate-disrupted virions, recognizes a genus-specific common antigen(s) that may not be present or accessible during all stages of virus production. The presence of virions has been demonstrated in positively staining regions that have been reprocessed for an electron microscopic examination (10, 27), but the antigen(s) has not been characterized.

The presence of the papillomavirus genome was demonstrated in all of the laryngeal papillomas examined by using the Southern transfer technique with a HPV-6 probe. This hybridization analysis has the advantage of assaying all of the tissue removed and is sensitive enough to detect less than a single viral genome per cell. For example, the hybridization to 0.1 pg of pHPe-1 added to 2 μg of human DNA was easily detected (Fig. 2, lane n). This represents a reconstruction of one viral genome...
in 10 cell genome equivalents. Although the intensity of hybridization in the laryngeal papillomas is comparable to that in the reconstruction, this technique cannot distinguish between 1 cell in 10,000 infected with 1,000 copies of the viral genome, and 1 cell in 10 infected with a single copy of the viral genome. The distribution of the viral sequence in the lesions can be investigated by hybridization in situ.

The hybridization analysis of DNA extracted from the laryngeal papillomas indicated the presence of a papillomavirus genome having considerable homology with the HPV-6 probe. The classification of the human papillomaviruses is based on DNA sequence homology between the viral genomes as determined by restriction endonuclease analysis and nucleic acid hybridization, as well as serological crossreactivity (for review, see ref. 28). Type-specific antisera are not readily available because of the inability to propagate papillomaviruses in a cell culture system and the paucity of virions in lesions. There are eight types of HPV associated predominantly with specific epithelial lesions: types 1 and 4 are associated with plantar warts, types 2 and 7 with common warts; types 3, 5, and 8 with lesions of epidermodysplasia verruciformis; and type 6 with condyloma acuminata.

Within a given serotype of HPV, genomes with different restriction enzyme cleavage patterns are classified as subtypes (29). We have identified four distinct subtypes of HPV-6, two of which have been found in both juvenile- and adult-onset laryngeal papillomas. Subtypes HPV-6c and HPV-6e have been identified in condyloma of the genital tract, where five different subtypes have been identified (unpublished data). These HPV-6 subtypes are distinct from the subtypes identified by Gissmann and zur Hausen (HPV-6a; ref. 30) and de Villiers et al. (HPV-6b; ref. 31).

Because the incidence of laryngeal papillomas in the United States has been estimated at 7.1 per million (2) and laryngeal papillomas have not been reported as being contagious even among family members, it is not surprising to find another reservoir—i.e., genital tract lesions—for the maintenance of the virus in the population. Maternal transmission has been proposed in reports of laryngeal papillomas occurring in children whose mothers had condyloma at the time of delivery (7, 5, 32, 33).

The possibility remains that the viruses found in laryngeal papillomas are closely related to, but different from HPV-6 and contain distinct sequences that will be detected in find structure mapping. This possibility can be explored by using molecularly cloned genomes from laryngeal papillomas.

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