LARYNGEAL HUMAN PAPILLOMAVIRPS INFECTION

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original studies which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I Aaltonen L.-M., Peltomaa J. and Rihkanen, H., Prognostic value of clinical findings in histologically verified adult-onset laryngeal papillomas. *Eur Arch Otorhinolaryngol*, **254,** 219-222 (1997).

II Aaltonen L.-M., Partanen J., Auvinen E, Rihkanen H. and Vaheri A., HLA-DQ alleles and human papillomavirus DNA in adult-onset laryngeal papillomatosis. *J Infect Dis,* **179,** 682-685 (1999).

III Aaltonen L.-M., Auvinen E, Dillner J., Lehtinen M., Paavonen J., Rihkanen H. and Vaheri, A., Poor antibody response against human papillomavirus in adultonset laryngeal papillomatosis. Submitted.

IV Aaltonen L.-M., Wahlström 1, Rihkanen H. and Vaheri A., A novel method to culture laryngeal human papillomavirus-positive epithelial cells produces papilloma-type cytology on collagen rafts. *Eur J Cancer,* **34,** 1111-1116 (1998).

ABBREVIATIONS

ATCC	American Type Culture Collection
CO_2	carbon dioxide
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
EW	epidermal growth factor
EIA	enzyme immunoassay
FCS	fetal calf serum
HES	human embryonic skin fibroblast
HPV	human papillomavirus
HUCH	Helsinki University Central Hospital
IHC	immunohistochernistry
ISH	*In situ* hybridization
kb	kilobase(s)
mRNA	messenger RNA
PCR	polymerase chain reaction
PPLF	papilloma-patient laryngeal fibroblast
*p*Rb	retinoblastoma gene
pRb	retinoblastoma protein
Th	helper T lymphocyte
VLP	virus-like particle
XR	irradiated

SUMMARY

This work is composed of four studies which focus on different aspects of laryngeal human papillomavirus (HPV) infection, a condition with a variable and unpredictable course. Clinically the most important manifestation of laryngeal HPV infection is laryngeal papillomatosis. Based on case records we evaluated which symptoms or signs at diagnosis could have prognostic value in adult-onset laryngeal papillomatosis. Two risk factors for frequent laryngeal procedures were found: young age at the onset of papilloma and a lesion extending to the anterior third of the vocal folds. As regards the recurrence of disease, the classical division of adult-onset laryngeal papillomatosis into solitary and multiple types was not found to be clinically relevant.

Immunological mechanisms which affect the course of laryngeal papillomatosis are not well understood. To elucidate the role of immunogenetic risk factors we investigated HLA associations of adult-onset laryngeal papilloma patients by polymerase chain reaction (PCR)-based methods. No differences in DQA1 or DQB1 frequencies appeared between the patients as a group and the reference population. A suggestive association was found between the DQB1*0501 allele and the latest HPV-negative laryngeal biopsy. Those patients without detectable HPV DNA had undergone fewer laryngomicroscopies than those whose biopsy contained HPV DNA. The former patient group was also older than the latter. Earlier, the DQB1*0501 allele was reported to be protective against cervical cancer, another HPV-associated disease.

Genital HPV infection is known to give rise to the humoral immune response, but in laryngeal papillornatosis HPV antibody production has not been widely investigated. In our series, HPV 6 and HPV 16 antibodies were equally frequent in patients and controls, with HPV 11 antibodies more frequent in patients. However,

no such difference in HPV 11 antibodies appeared between male patients and controls. In contrast, female patients had HPV 11 antibodies more often than did male patients or female controls. Female patients also had a high prevalence of cytological or histological signs of genital HPV infection, which indicates their need for gynecological examination. Based on our results, serum HPV antibodies are not clinically relevant in adult-onset laryngeal papillomatosis and may be induced by concomitant genital HPV infection.

For further studies concerning mechanisms of laryngeal HPV infection, we created a novel experimental model which is based on the supportive effects of papilloma-patient laryngeal fibroblasts (PPLF) on HPV-infected laryngeal epithelial cells *in vitro*. HPV-positive laryngeal biopsies were first cultured as a monolayer in which irradiated PPLF served as feeder cells. When these fourth- or fifth-passage epithelial cells were transferred, to allow growth on an organotypic growth base (collagen raft) containing unirradiated PPLF, they grew as a multilayer. This layer showed cytological features typical of HPV infection. Based on *in situ* hybridization, the original biopsy sections and epithelial cells from each monolayer passage, as well as the collagen raft sections, contained HPV DNA. The monolayer cell culture and the collagen raft, the latter providing differentiation-promoting effects, appears to facilitate maintenance of HPV-infected cells and of the viral genome.

INTRODUCTION

The most important clinical manifestation of laryngeal HW infection is laryngeal papillomatosis, a disease with often relapsing verrucous epithelial tumors. It causes hoarseness and sometimes even airway obstruction; especially in children, spread to the lower respiratory tract is possible. Clinically the disease is divided into adult-onset and juvenile-onset forms (Lindeberg *et al*., 1986). Laryngeal papillornatosis has a potential for malignant transformation, which occurs in 3 to 7% of the patients (Gaylis and Hayden, 1991; Lie *et al*., 1994). HPV is also associated with laryngeal carcinogenesis without pre-existing clinical papillomatosis but is considered more likely to act as a co-factor than a causative agent (Chang *et al*., 1992). Moreover, HPV DNA is commonly detected by PCR in macroscopically normal laryngeal mucosa, and in benign laryngeal lesions lacking histological features of HPV infection (Nunez *et al.,* 1994a; Rihkarten *et al*., 1994).

Laryngeal HPV infection is not very well characterized. The means of transmission. are uncertain, the course of the disease unpredictable, and despite its viral etiology the treatment is based on surgery. Lack of a good experimental model and the difficulty in producing virions have hindered *in vitro* studies. Before effective prevention and treatment of the disease become available, more knowledge is needed about HPV, the mechanisms by which the virus is acting in the larynx, and the characteristics of the affected individuals. The aims of this study were therefore to discover factors which may affect the susceptibility to or the course of the disease and to develop an experimental model of laryngeal HPV infection.

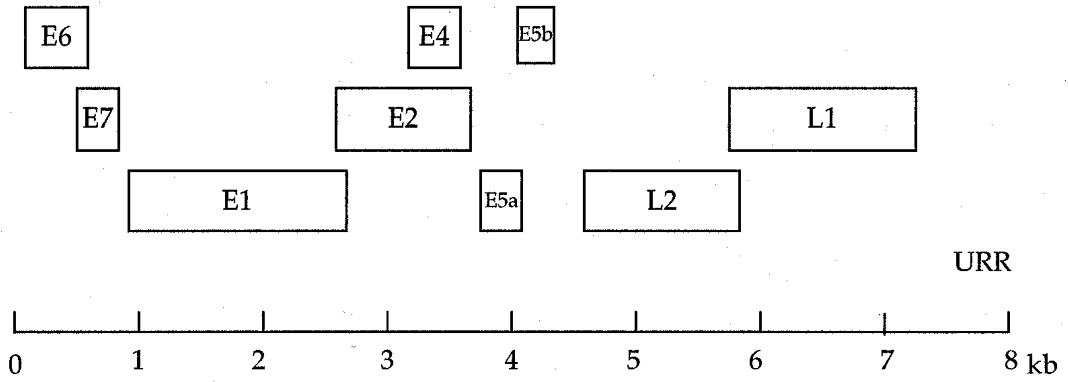
REVIEW OF THE LITERATURE

Human papillomavirus

Human papillomavirus (HPV), which belongs to the *Papovaviridae* family, is a small oncogenic DNA virus which infects epithelial cells. Besides laryngeal papillomas (Lack *et al*., 1980; Mounts *et al*., 1982; Gissmann *et al*., 1983), it also causes anogenital condylomas (Gissmann and zur Hausen, 1980; Gissmann *et al*., 1984), skin warts (Strauss *et al*., 1949; Pfister *et al*., 1979), skin cancer in patients with epidermodysplasia verruciformis (Orth *et al*., 1978), and cervical carcinoma (zur Hausen, 1976; Bosch *et al*., 1995). The structure and composition of the virus was demonstrated by electron microscopy in human warts (Strauss *et al*., 1949), and supercoiled DNA was identified in HPV preparations (Crawford and Crawford, 1963). The existence of different HPV types was demonstrated by the discovery that plantar wart virus complementary RNA did not hybridize with all viral warts and with condyloma DNA (zur Hausen, 1974). This was followed by identification of HPV subtypes (Gissmann and zur Hausen, 1976), and isolation of several new HPV types (Gissmann *et al*., 1977; Orth *et al*., 1977, Orth *et al*., 1978). Nearly 100 genotypes of HPV, many of which are known to be associated with either benign or malignant tumors, have been characterized (Favre *et al*., 1997).

'Ihe papillomavirus genome is a double-stranded, circular structure of 7.9 kb DNA (Figure 1) which is covered by an icosahedral capsid. All open reading frames are located on one strand. The function of the HPV genome has been widely investigated, especially in HPV 16 (McCance, 1994), and the following data are based on the properties of this HPV genotype. The genome consists of three regions: an upstream regulatory region (URR), and the two regions named according to the phase of infection in which they are expressed: the early (E) and the late (L) regions.

HPV 6



HPV 16

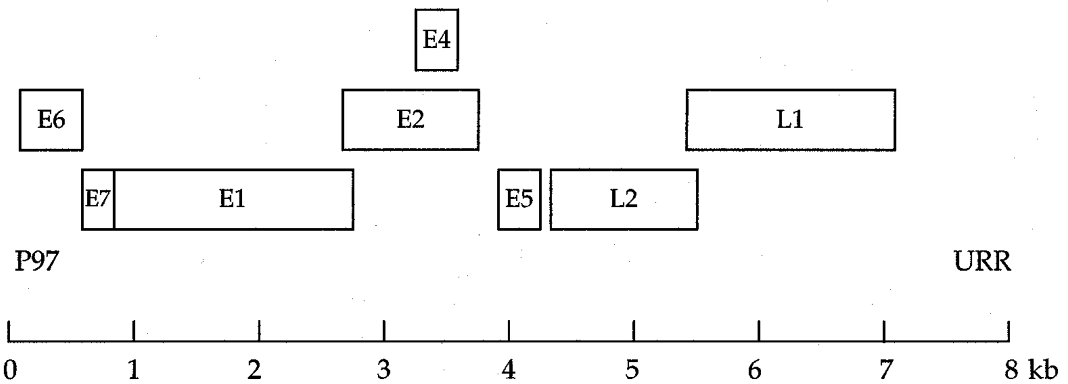


Figure 1. Linear presentation of HPV genomes. Data obtained from Cenbank (HPV 6 X00203, HPV 16 K02718).

Little is known about the interaction of HPVs with their host cells. URR harbors several regulatory sequences such as binding sites for transcriptional activators *(Gloss et al*., 1987). The major transcriptional promoter of HPV 16 is P97 (Smotkin and Wettstein, 1986), which is regulated by a keratinocyte-specific enhancer (Cripe *et al.,* 1987; *Gloss et al*., 1987). The E region consists of eight genes. The E1 and E2 genes are involved in the replication of the HPV genome (Ustav and Stenlund, 1991; Chiang *et al*., 1992). The E2 protein has been found to repress DNA replication by binding to specific E2-binding sites in URR (Romanczuk *et al*., 1990). The functions or gene products of E3 and E8 are unknown. The interaction of E4 protein with host cell intermediate filaments causes their collapse, which may allow the virions to be more easily released from the nucleus (Doorbar *et al*., 1991). Although the mRNA for E4 is detected early in infection, both the mRNA and the protein accumulate during the late phase. The E4 protein is produced in differentiated cells of benign tumors, and this is why it is considered a late gene product (Doorbar *et al*., 1986; Breitburd *et al*., 1987). The E5 is a small membrane-associated hydrophobic protein with transforming activity (McCance, 1994). As reviewed by Auvinen *et al.* (1997) the E5 protein enhances cell proliferation and inhibits the control of cell growth, and it has been shown to be an oncoprotein (Leechanachai *el al.,* 1992; Pim *et al.,* 1992). The most potent oncoproteins are E6 and E7, which are able to transform epithelial cells in *vitro,* which leads to immortalized cultures. They accelerate proliferation of the infected cells by disturbing the function of the tumor suppressor genes *p*53 and *p*Rb (retinoblastoma gene), respectively. The E6 oncoprotein has been shown to bind p53 protein and cause its degradation through the ubiquitin pathway (Scheffner *et al*., 1990). The binding of E7 oncoprotein to hypophosphorylated retinoblastoma protein (pRb) results in the release of a transcription factor E2F, and subsequent transcriptional activation of E2F-responsive genes, which is required for entry into the S phase (Dyson *et al.,* 1989).

The L region has two genes, L1 and L2, which encode the viral structural proteins: major and minor capsid proteins, respectively. The L1 gene in particular is highly conserved between HPV types, which means that antibodies against one HPV type are often cross-reactive with another (McCance, 1994).

HPVs infect basal cells which are the only dividing cells of the epithelium. The viral particles are bound to cell surface receptors, enter the cell, and are transported to the nucleus, where the viral genome is released, and the replication takes place. HPVs can cause either a productive infection in which the late genes are expressed and virions are produced or a non-productive infection in which only some early genes are expressed but no viral production occurs. Productive infection does not lead to host cell lysis, and superficial keratinocytes which contain virus are eliminated by shedding (Breitburd *et al*., 1996).

Classification of papillomavirus types is based on host specificity, and on homology to other papillomaviruses at the genomic level (Delius and Hoftnann, 1994). The percentage of non~identical nucleotides within the L1 open reading frame leads to a new virus type if it is more than 10%, if 2 to 10%, leads to a subtype within the same virus type, and if less than 2%, leads to a variant.

Laryngeal HPV infection

HPV DNA in laryngeal mucosa without clinical papillomatosis

Although the most important clinical manifestation of laryngeal HPV infection is laryngeal papillomatosis, HPV DNA has been found also in macroscopically normal- appearing laryngeal mucosa: the incidence of HPV detected by PCR in macroscopically normal upper respiratory mucosa seems to be around 20%.

Unselected post-mortem biopsies of the laryngeal mucosa were HI'V-positive in 25% (Nunez et al., 1994a) and of the hypopharyngeal mucosa in 18% (Nunez et al., 1994b) of the cases. Vocal cord nodules and polyps harbor HPV 6/11 DNA in 23% of the patients (Rihkanen *et al*., 1994).

The role of HPV in laryngeal carcinogenesis is still unclear. In carcinomas without pre-existing clinical papillomatosis, HPV DNA is detected by PCR in between 8% and 54% of the cases (Perez-Ayala *et al*., 1990; Brandwein *et al*., 1993; Garcia-Milian *et al*., 1998). In verrucous carcinoma, a rare, well-differentiated variant of squamous carcinoma with a low malignant potential, HPV DNA has been detected in 45% of the cases (Fliss *et al*., 1994). Studies of precancerous laryngeal lesions do not support the oncogenic cofactor role of HPV in laryngeal carcinogenesis (Fouret *et al*., 1995; Lindeberg and Krogdahl, 1997); at the present level of knowledge, the issue remains obscure.

Laryngeal papillomatosis

1. Clinical features

The viral etiology of laryngeal papillomatosis was first described by Ullman (1923). Although the disease is rare, the incidence for example in a Danish subpopulation being only 3.8 /million/ year (Lindeberg and Elbrond, 1990), papilloma is the most common benign laryngeal neoplasm, accounting for up to 84% of benign tumors (Strong *et al*., 1979; Jones *et al*., 1984). The main symptom of the disease is hoarseness, but airway obstruction is sometimes present as well (Lindeberg and Elbrond, 1989). Laryngeal papillomatosis is clinically divided into adult-onset and juvenile-onset forms (Lindeberg *et al*., 1986). Especially in children, the disease can be life-threatening; it can disseminate throughout the tracheobronchial tree and end in pulmonary papillomatosis with uncontrollable fatal chest infection

(Padayachee and Prescott, 1993). The means of transmission are in part unknown although adult-onset disease is related to possible orogenital spread, and juvenile-onset to transmission of the infection from mother's HPV-infected genital tract to the child at the time of birth (Kashima *et al.,* 1992).

Depending on the authors, laryngeal papillomatosis is considered to be adult-onset if the patient is older than 16 to 20 years at diagnosis (Lindeberg *et al.,* 1986; Corbitt *et al*., 1988). Unlike the juvenile form with a sex ratio about equal (Lindeberg and Elbrond, 1989; Padayachee and Prescott, 1993), in the adult-onset disease mostly males are affected: the male-to-female ratio has been 2:1 to 4:1 (Capper *et al*., 1983; Lindeberg and Elbrønd, 1989). HPV 6 and HPV 11 virus types are most commonly found in laryngeal papilloma lesions (Bryan *et al*., 1990; Smith *et al.,* 1993; Gale *et al*., 1994; Pou *et al.,* 1995), the former being even more common in adult-onset lesions than is the latter (Corbitt *et al.,* 1988). The most typical site of infection at diagnosis is the vocal folds (Capper *et al.,* 1983). The course of the disease is unpredictable: some patients recover after one laryngeal procedure, while the others run a relapsing course. One reason for this could be that HPV DNA is detected both in biopsies from uninvolved sites and from patients in remission (Steinberg *et al.,* 1983; Abramson *et al*., 1987, Rihkanen *et al*., 1993). Malignant transformation of laryngeal papillomas is a rare event, and occurs only in 3% to 7% of the patients (Gaylis and Hayden, 1991; Lie *et al.,* 1994). Earlier, one of the treatments used for these patients was irradiation therapy, which seemed to increase the risk for malignant transformation, but malignant transformation may also take place with no history of irradiation (Solomon *et al*., 1985), and both low-risk and high-risk HPV types are involved (Lindeberg *et al*., 1989; Doyle *et al*., 1994). Smoking is also a potential cofactor in the conversion of laryngeal papilloma to carcinoma (Kashima *et al*., 1996).

2. Treatment and prevention

The goal of the treatment is to ensure a safe airway and the best possible quality of voice. The many different medical, surgical, and immunological procedures used for the treatment of laryngeal papillornatosis have been reviewed by Clark and MacKenzie (1996). However, even at present the treatment is based on surgical removal of lesions by carbon dioxide (CO_2)-laser, a method introduced as early as the 1970's (Strong *et al*., 1973, 1976) or with a shaver, which is far from optimal because of the viral etiology of the disease. Alpha-interferon treatment is sometimes given, especially to children, because their disease is often more aggressive, with frequent relapses and possible spread to the lower parts of the respiratory tract; but despite the treatment, relapses are common (Haglund *et al*., 1981; Schuurman and Van Den Broek, 1986; Steinberg *et al*., 1988; Leventhal *et al*., 1991). Promising preliminary results have been reported in the treatment of severe laryngeal papillomatosis with intralesional cidofovir injections (Snoeck *et al*., 1998) or flash pump dye laser (Bower *et al.,* 1998).

Prevention of laryngeal HPV infection is difficult because the means of transmission are not exactly known. Vaccines which are based on L1 capsid proteins produced in non-mammalian cells and self-assembled into virus-like particles (VLPs) provide potential means of preventing genital HPV infection and the subsequent development of cervical cancer (Schiller and Lowy, 1996). A polyvalent genital HPV vaccine that includes VLPs of low-risk as well as high-risk types (HPV 6, 16, 18, 31, 45) may be able to prevent recurrent respiratory papillomatosis as well, if the original source of the virus in this disease is the genital HPV infection. Adding only one low-risk type VLP to the vaccine may be sufficient, because of the possible cross-protection against HIV 11 (Roden *et al*., 1996).

3. Cellular and humoral immunity

Typical features of BPV infections are transition from latent infection to clinically recognized disease, the regression of benign or premalignant HIV-induced tumors, and the appearance of malignant HPV-harboring cells (Breitburd *et al*., 1996). Immunological events are possible reasons for these features. Moreover, such events may influence the variable course of laryngeal papillomatosis and the infrequency of the disease, when compared to the frequency of HPV DNA detected in macroscopically normal laryngeal mucosa. Cellular immunity is of major importance for the clearance of infections caused by HPVs, although direct exposure of the virus proteins to immunocompetent cells does not occur (Gissmann, 1996). This is in line with the fact that HIV-related cancers are more common in immunocompromised individuals (Benton *et al*., 1992; Dillner, 1992; Arends *et al*., 1997; Palefsky, 1997). Class I and class II HLA molecules present HI'V-derived peptides to cytotoxic T lymphocytes (CTL) or T helper lymphocytes (Th). Infiltrates of CD4-positive Ths or CD8-positive CTLs and macrophages have been observed in spontaneously regressing HPV lesions (Iwatsuki *et al*., 1986; Tay *et al*., 1987; Coleman *et al*., 1994; Stanley *et al*., 1994). HIV-harboring epithelial cells may modify the immune response by secreting cytokines (Woodworth and Simpson, 1993) and by showing abnormal expression of HLA class I and class II antigens (Stern, 1996)

Down-regulation of HLA class I expression and up-regulation of HLA class II expression are characteristic events in cervical HPV infections. HLA associations have been investigated, especially in cervical intra-epithelial neoplasia and cervical cancer (Apple *et al*., 1994; Odunsi *et al*., 1996), whereas in laryngeal papillomatosis they are poorly verified. Bonagura *et al*. (Bonagura *et al*., 1994), in a series of 16 adult- and juvenile-onset laryngeal papilloma patients, found the HLA DQw3 phenotype to be enriched. They found also HLA class I expression in papilloma

tumor sections to be reduced compared with that in normal laryngeal epithelium, while in HLA class II expression no difference was observed. In natural killer cell activity no specific alteration in laryngeal papilloma patients appeared (Naiman *et al*., 1984).

Humoral immune defects do not seem to predispose to HPV infections (Schiller and Okun, 1996). Several different HPV antigens have been used to measure serological anti-FIPV reactivity: bacterially expressed fusion-proteins containing HPV open reading frames (jenison *et al*., 1988; Jochmus-Kudielka *et al*., 1989), synthetic peptides (Dillner *et al*., 1989a; Müller *et al*., 1990), denatured bovine papillomavirus virions, HPV virions obtained from clinical samples in the case of HPV 1, HPV virions produced in an *in vivo*-mouse model, and assembled VLPs - also called capsids (Cubie and Norval, 1988; Dillner *et al*., 1989b; Christensen *et* al., 1992). A serological assay based on VLPs is the most extensively used and validated method for type-specific serodiagnosis of HPV infection, and most studies on HPV antibody response have focused on genital HPV infection (Kirnbauer *et al*., 1994; Wikström *et al*., 1995a; Eisernann *et al*., 1996; Kjellberg *et al*., 1999). Antibodies to HPV 11 virions have been detected mainly in juvenile-onset laryngeal papillomatosis (Bonnez *et al*., 1992; Christensen *et a*l., 1992). Only one report exists on adult-onset laryngeal papilloma patients, and it included only 15 patients and employed a first-generation HPV serology assay with limited HPV type-specificity (Tachezy *et al*., 1994).

Relation between laryngeal and genital HPV infection

Both low-risk and high-risk HPV types are involved in genital HPV infection: HPV 6 and 11 are typically found in condylomas and HPV 16, 18, 31, and 45 in cervical

cancer. The latter four comprise 80% of the HPV types detected in cervical cancers (Bosch *et al*., 1995).

Genital HPV infection is known to be transmitted in sexual intercourse. In the 1950's the relevance of maternal condyloma to laryngeal papilloma was already being noted (Hajek, 1956). A history of maternal condyloma was found in more than 30% of mothers bearing children with juvenile-onset laryngeal papillomatosis (Quick *et al*., 1980). Juvenile-onset disease occurs most commonly in first-born infants delivered vaginally to young mothers with genital condylomas (Kashima *et al*., 1992), with cases of juvenile-onset laryngeal papillomatosis being rare in caesarean-delivered children (Shah *et al*., 1986). On the other hand, because HPV DNA has been detected even in amniotic fluid (Armbruster-Moraes *et al*., 1994), and because the overall risk of papillomatosis developing in the offspring born to a mother with genital HPV infection is only 1 in 32 (Kashima *et al*., 1996), caesarean delivery for expectant mothers with genital condylomas is thus not routinely recommended.

Transmission of adult-onset laryngeal papillomatosis via orogenital spread is commonly mentioned, but clear evidence for this does not exist. The idea that adult-onset disease is spread by orogenital contact is based on the fact that both laryngeal papillomatosis and genital condylomas harbor the same virus types 6 and 11. Type 6 is, however, even more frequently found both in adult-onset laryngeal papillomatosis and in genital condylomas (Corbitt *et al*., 1988; Greer *et al*., 1990).

In vitro models of HPV infection

The major problem in the *in vitro* studies of HPV infection is that the virus does not replicate in cell culture. Differentiation of the epithelium, a critical point for expression of the complete viral life cycle, cannot yet be fully duplicated *in vitro* (Gissmann, 1994). At present, the basic technique most widely used for *in vitro* studies of HPVs was originally created by Asselineau and Pruni6ras (1984). As described briefly in a review by Meyers and Laiminis (1992), fibroblasts mixed with medium and type I collagen at 0 ºC to 4 ºC are incubated at +37 ºC until the solution solidifies and forms a lattice (collagen raft) which is used as a dermal equivalent. Epithelial cells are seeded on top of the raft and allowed to attach and form a monolayer. The raft is placed on a metal grid, and the epithelial cells stratify and differentiate at the air-medium interface during two or three weeks. This artificial epithelium resembles its *in vivo* counterpart well, with only minor differences found in the morphology of the basal keratinocytes and in the distribution of few keratinocyte-specific antigens. Differences have been detected for example in the expression of cytokeratins K1 and K2 (Kopan *et al.*, 1987), but by varying the growth conditions and the collagen matrix and the fibroblasts used for the experiments, such discrepancies may be diminished.

Efforts to develop cell culture models to study HPV replication have involved immortalizing of primary squamous epithelial cells or culturing them directly from papillomatous lesions. Immortalization of epithelial cells with HPV 16 and 18 DNA (Dürst *et al.*, 1987; Pirisi *et al.*, 1987; Woodworth *et al.*, 1988) results in the integration of the viral genome into the host cell DNA. When epithelial cells are transfected with HPV 11, which does not integrate, episomal viral replication is detected, but only a portion of the virus reaching the nucleus undergoes replication (Auborn *et al.,* 1996).

For the homeostatic equilibrium of epithelial cells, epithelial-mesenchymal interactions are important (Cunha *et al*., 1986; Streuli *et al*., 1991; Smola *et al*., 1993), and to utilize the stimulating effect of mesenchymal tissue, HIV-infected epithelial cells have been cultured in nude mice and on an organotypic growth base (collagen raft). The first successful propagation of HPVs in the laboratory was provided by Kreider and colleagues who incubated fragments of human neonatal foreskin with an extract from an HPV 11-containing vulvar condyloma, and after incubation grafted them under the renal capsule of nude mice. The grafts developed into condylomatous cysts from which virions could be extracted and used to initiate new cycles of cyst formation (Kreider *et al*., 1987). HPV 31 virions have been produced in collagen rafts after addition of a phorbol ester to the medium (Meyers *et al*., 1992), and when pieces of condylomas containing HPV 11 were explanted on collagen rafts, HPV production was observed (Dollard *et al*., 1992). Moreover, when examined in collagen rafts, epithelial cells transfected with HPV 16 displayed an altered pattern of differentiation (McCance *et al*., 1988; Zheng *et al*., 1994). However, it is impossible thus far to produce reasonable amounts of virions by the methods available.

The model for culturing normal human epithelial cells was described by Reinwald and Green (1975). These cultures included a feeder layer of lethally irradiated mouse fibroblasts which supported the growth of epithelial cells and inhibited that of fibroblasts. Moreover, epithelial cells have been successfully cultured imbedded in collagen gels (Yang *et al.,* 1980). A method that allows laryngeal papilloma-derived squamous epithelial cells containing HPV 6 or HPV 11 DNA to be cultured has been introduced by Steinberg and colleagues (1982). Epithelial cells were grown in tissue culture in a hydrated collagen gel including Nutrient Mixture F12 supplemented with 15% fetal calf serum and 10 μg/rid hydrocortisone. The cells remained viable in culture for more than 6 months and could be serially transferred two to four times before senescence. However, the viral DNA is rapidly lost from

the cell cultures with passaging, possibly because the infected cells show a selective disadvantage in plating efficiency compared with that of uninfected cells (DiLorenzo *et al*., 1992).

AIMS OF THE STUDY

The course of laryngeal papillomatosis is variable and unpredictable, and the lack of an experimental model has hindered in vitro studies. The aims of the study were to:

1. find whether some clinical features at diagnosis can predict the course of adult-onset laryngeal papillomatosis.

2. investigate the role of HLA DQ alleles in adult-onset laryngeal papillomatosis.

3. investigate whether adult-onset laryngeal papillomatosis induces a humoral immune response.

    Because adult-onset females had HPV 11 antibodies more often than did male patients or female controls, and they also had often a history of genital condylomas, further studies investigated whether these female patients' HPV antibodies could have been of genital origin.

4. develop an experimental model of laryngeal HPV infection.

MATERIALS AND METHODS

Patients and samples

From all case records of patients who were treated at Helsinki University Central Hospital, Department of Otorhinolaryngology - Head and Neck Surgery during 1975 to 1994 with a diagnosis of benign or undefined laryngeal neoplasm, those with a histologically confirmed diagnosis of laryngeal papilloma (n=160) were selected. A schematic presentation of patient selection is shown in Figure 2. The material of the retrospective work (Study I) included case records of the 74 adultonset patients (age at diagnosis ≥ 17 years) without malignant transformation whose first laryngeal procedure was performed at Helsinki University Central Hospital and whose follow-up exceeded one year. Studies II and III were based on this same adult-onset laryngeal papilloma patient population without malignant transformation. Of the 113 patients, 43 were excluded: 5 had died, 17 were >80 years old, and 21 did not live in the hospital district. Of the 70 invited patients 8 did not attend. Thus, 62 patients filled in a questionnaire and had their larynxes videorecorded. Peripheral blood lymphocytes and serum samples for HPV antibody assay were drawn, and lymphocytes were isolated as a source of DNA. The patients' paraffin-embedded most recent laryngeal biopsies were used for determination of the type of laryngeal HPV infection by PCR. All female patients were invited for a second visit to an otorhinolaryngologist, which included also an examination by a gynecologist. Mouth-wash and mouth-brush samples, Pap smears, and colposcopically directed cervical biopsies were taken, and a second serum sample was drawn. For the experimental part of the work (IV), fresh laryngeal tissue biopsies were obtained from 30 laryngomicroscopies (corresponding to 15 adult-onset and 2 juvenile-onset laryngeal papilloma patients) and of one total laryngectomy (from a patient with an esophageal carcinoma. infiltrating the trachea). These patients too, were treated at Helsinki University Central Hospital. The HIV-positive epithelial cells grown on

collagen rafts (IV) were taken from papilloma tumors of two adult-onset patients and one juvenileonset patient and of normal-appearing laryngeal mucosa (false vocal fold) of an esophageal carcinoma patient.

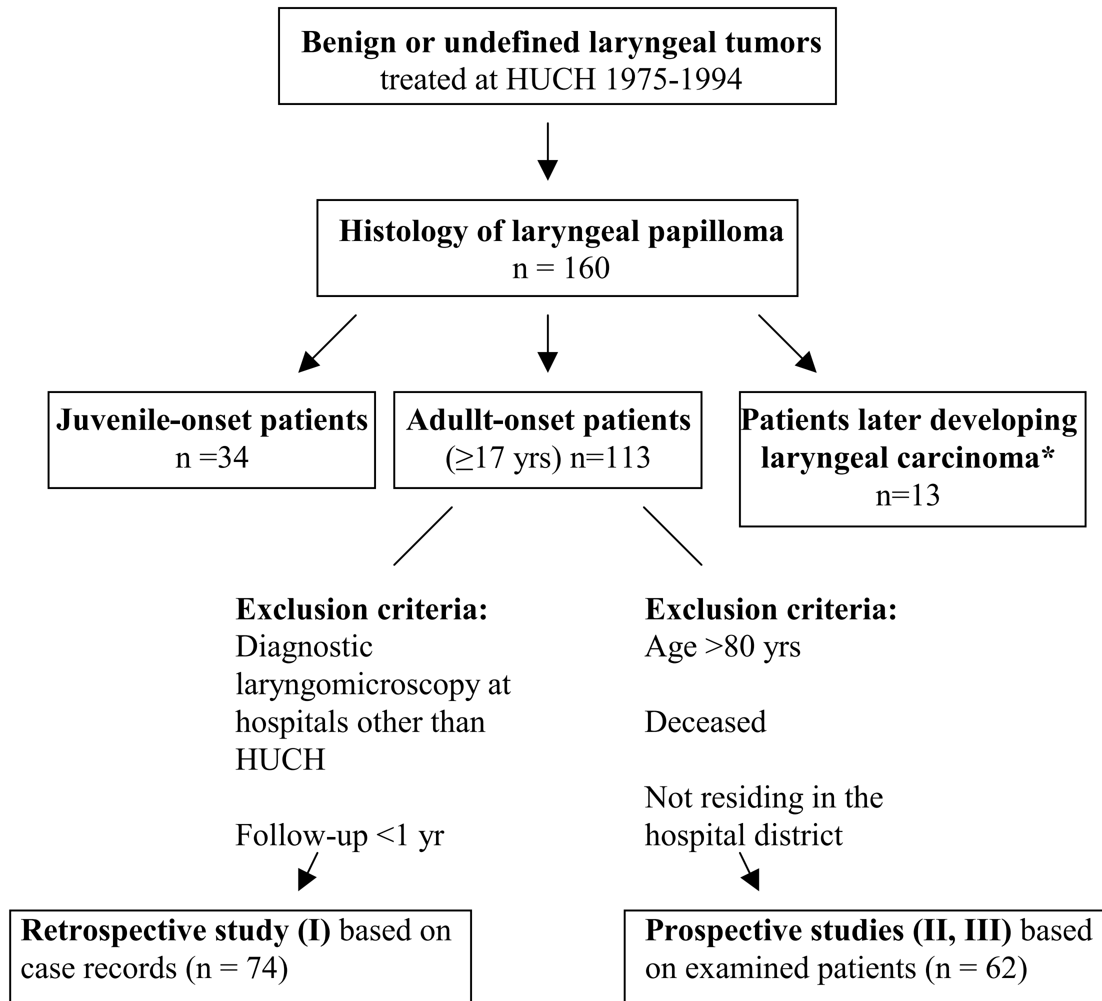


Figure 2. Schematic presentation of patient selection for Studies I, II, and, III.

*3 of these patients had a clinical papillomatosis which ended in malignancy, 10 laryng.eal carcinoma patients' histology of laryngeal papilloma was confirmed. in sporadic biopsies.

Our reference populations were, in the HLA studies, 93 cadaver kidney donors (Partanen and Westman, 1997), and in the HPV antibody assay, 53 age- and sex-matched (13 females, 40 males) anonymous blood donors. For five male and two female patients of advanced age it was impossible to find age-matched blood donor controls. Informed consent was obtained from all patients in this study. The human experimentation guidelines of Helsinki University Central Hospital were followed, and the study was approved by the institutional ethics committees of the Department of Otorhinolaryngology - Head and Neck Surgery and the Department of Obstetrics and Gynecology.

Cell cultures

The human cells used for this work were grown from fresh tissue biopsies. They were cultured first in monolayers in which irradiated (XR) fibroblasts (dose of irradiation 6000 rad, radiation source Ce137) served as feeder cells. Onto each 8.7 cm2 plate (Greiner, Frickenhausen, Germany) we seeded 2x105 irradiated fibroblasts. The biopsies were cut into small pieces and allowed to grow first on these irradiated mouse fibroblasts (3T3-J2, kindly provided by Dr. Michele De Luca, Genoa, Italy). In these cultures, papilloma-patient laryngeal fibroblasts (PPLF) took over, and because of the poor support provided by mouse 3T3-J2 cells to human HPV-positive epithelial cells, PPLF were in further experiments irradiated (PPL17-XR) and used as feeder cells. The fibroblasts were cultured in Dulbeeco's modified Eagle's medium (DMEM) supplemented with 10% heatinactivated fetal calf serum (FCS), 2mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (henceforth referred to as supplemented DMEM), and the medium was changed three times a week. The patient who supplied these laryngeal fibroblasts was a 24-year-old male with adult-onset disease of a low-risk HPV type. The HPV-infected epithelial cells were then cultured in a monolayer cell culture until

the fourth or fifth passage without loss of HPV DNA, and were then transferred to allow growth on collagen rafts.

1. Monolayer cell cultures

The biopsies were brought to the laboratory in supplemented DMEM, sectioned into small pieces, and allowed to grow on irradiated fibroblasts. Irrespective of the type of irradiated fibroblasts used, their growth medium, with or without epithelial cells, was supplemented DMEM further supplemented with 5 µg/ml insulin (Sigma, St. Louis, MO), 5 µg/ml transferrin (Sigma) 400 ng/ml hydrocortisone (Sigma), 10^{-10} M cholera enterotoxin (ICN, Aurora, OH), and 10 ng/ml epidermal growth factor (EG17) (Austral Biologicals, San Ramon, CA). This medium and the method used to propagate the epithelial cells is modified from that of Rheinwald and Green (1977). The medium was changed three times a week. Before the fibroblasts were seeded, coverslips coated with 1% 3-aminopropyltriethoxysilane (Sigma) were placed on the culture dishes to obtain cells from monolayers for *in situ* hybridization. At each passage, the coverslips were removed, rinsed in phosphate-buffered saline, pH 7.4, and fixed in cold acetone at -20 ºC for 10 min. The cultures were maintained in a 5%-CO_2 incubator at 37 ºC, and if not used for experiments at once, the cells were frozen in growth medium containing 10% dimethyl sulfoxide (Merck, Darmstadt, Germany) at -70 ºC. On average, the cells reached the fourth passage on the 28th day of culture.

2. Organotypic cell cultures (collagen rafts)

The method of preparing collagen rafts has been described in detail earlier (Asselineau and Pruniéras. 1984; Zheng *et al.,* 1994). Twelve-well plates (4 cm^2; Costar, Cambridge, UK) were precoated with 1.6% sterile agarose in 2 x MEM containing glutamine, and antibiotics to prevent the fibroblasts from adhering to

the plates. The fibroblasts were suspended in 2 x MEM containing 20% FCS, glutamine, and antibiotics and mixed with type I collagen (Rat tail; UBI, Lake Placid, NY). For each raft we used 4 x 10^5 PPLF fibroblasts; the final collagen concentration was 0.7 mg/ml. The total volume of the mixture of cells and collagen was 2.5 ml per well. The plates were then cultured in a 5%-CO_2 incubator at 37 ºC for one day. After that, the shrunken collagen gels were transferred to a 24-well plate (1.75 cm^2; Greiner) into which the fourth or fifth passage HPV-positive epithelial cells ($2x10^5$ cells from 90% confluent culture dishes) were seeded. The next day the collagen rafts were transferred onto metal grids and cultured at the air-medium interface for two weeks. The medium (DMEM with 10% FCS, antibiotics, glutamine, insulin, hydrocortisone, transferrin, cholera toxin, and EGF) was changed three times a week. The rafts were then fixed in formalin, embedded in paraffin, and sectioned for detection of HPV DNA. Staining of sections with hematoxylin and eosin was also performed for histological evaluation.

***In situ* hybridization**

Both the tissue biopsies from the larynx and the collagen rafts were embedded in paraffin and sectioned. From these sections, as well as from acetone-fixed cells from the monolayers, HPV-DNA was tested by *in situ* hybridization (HPV *in situ* Screening Test®; Biohit, Helsinki, Finland). The details of this method have been described by Syrjdnen (1990). Five-µm sections were deparaffinized, digested with proteinase, and hybridized with a cocktail of probes (corresponding to 32 HPV types) at 37 ºC overnight. The biotinylated hybrids were detected by alkaline phosphatase conjugated to streptavidin, with 5-bromo-4-chloro-3-indoylphosphate as substrate and nitroblue-tetrazolium as chromogen. The incubation time in the substrate solution was 3 hours for paraffin-embedded sections and 0.5 hour for cell samples. Fixed human uterine cervical carcinoma CaSki cells (ATCC CRL-1550;

American Type Culture Collection, Rockville, MD) which contained HPV 16 DNA were used as positive controls when biotinylated HPV DNA probes Were added, and as negative controls when biotinylated pBR322 control probes were used for hybridization. Moreover, our own additional controls included paraffin-embedded biopsies of ovarian tube (negative controls), uterine cervix with condyloma and dysplasia, and a laryngeal papilloma known to be of low-risk HPV-type (positive controls).

DNA extraction

DNA from fresh samples and from deparaffinized tissue samples was extracted by standard phenol-chloroform methods (Sambrook *et al.*, 1988).

Chemiluminescent molecular hybridization assay

Detection of HPV DNA in PPLF cell cultures and in original tissue biopsies (IV) was performed by the Hybrid Capture^{TM} System (Digene Diagnostics, Beltsville, MD). This sandwich-capture molecular hybridization assay which classifies the samples according to the virus type present (low-risk types 6, 11, 42, 43, 44, or high/intermediate-risk types 16,18, 31, 35,45, 51, 52, 56) utilizes chemiluminescent detection: the principles of the technique have been described elsewhere (Rothrock, 1992). In short, the specific HPV RNA probe cocktail of the test hybridizes with specimens containing the target DNA. A tube surface coated with anti-RNA-DNA, antibodies captures the RNA-DNA hybrid. An anti-hybrid antibody conjugated to alkaline phosphatase is then reacted with the hybrid and detected with a chemiluminescent substrate. The bound alkaline phosphatase cleaves the substrate, and the light which it emits is measured on a luminometer as relative light units,

with amount of target DNA in the specimen proportional to intensity of light emitted. Biopsies of 2-5 mm in diameter or cell suspensions containing 1-2 million cells from a culture dish served as samples. The reported sensitivity of the test is 1 copy/cell, when 1-2 million cells are assayed.

Polymerase chain reaction for detection of β-globin and HPV DNA

For all the paraffin-embedded laryngeal papilloma samples, PCR was performed both with g-globin primers (Saiki *et al*., 1985) and with type-specific primers for HPV 6, 11, and 16 (van den Brule *et al*., 1989). Specimens negative with type-specific primers were subjected to PCR with general HPV primers GP5+/6+ (de Roda Husman *et al*., 1995). Because of the small quantity of DNA, PCR was performed in two phases: first 20 cycles and then 35 cycles in which 5 μl of the former PCR product served as a template. The reaction mixtures contained 1.5 mM $MgCl_2$, 0.4 μM of each primer, dNTP 0.2 mM each, and 1 U AmpliTaq Gold™ DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ). The PCR reaction was initiated with a "hot star" (10' +95 ºC) followed by 1' +95 ºC denaturation, 2' annealing, 1.5' +75 ºC chain elongation, and another 4' +72 ºC chain-elongation step after the last cycle. The annealing temperature was +40 ºC for GP5+/6+, +58 ºC for β-globin, HPV 6, and 11 primers, and +62 ºC for HPV 16 primers. All PCR reactions were performed with a PTC-100™ automated PCR machine (MJ Research, Watertown, MA). For the fresh mouth-washes, mouthbrush, and cervical biopsy samples (III), PCR was performed with β-globin and GP5+/6+ primers in one phase (40 cycles).

HPV 6 and HPV 11 plasmid DNA (5 ng/reaction) or HPV 6- and HPV 11-positive laryngeal specimens served as positive controls. Cenomic DNA (5 ng/reaction) isolated from SiHa cells (ATCC HTB-35) served as a positive control in HPV 16

PCR, and DNA (5 ng/reaction), isolated from an HPV 16-containing cervical carcinoma biopsy, for GP5+/6+ PCR. Our negative controls were HPV 6-, 11-, and 16-negative laryngeal specimens, respectively, and for GP5+/6+, DNA extracted from laryngeal fibroblasts of a patient with no known history of HIV-related disease. Both for paraffin and for water controls, glycogen was added, to serve as a carrier for possible DNA contamination. Five µl of each PCR product was run in 2% agarose gel which was visualized with ethidium bron-dde, and the products of HPV-type-specific PCR reactions were transferred to nylon membranes for detection. The sensitivity of the test was determined by performing PCR with GP5+/6+ primers on a dilution series of samples containing HPV 11 plasmid DNA. The test detected 100 ag HPV plasmid DNA corresponding to approximately 12 copies of HPV DNA.

Southern blot hybridization

The PCR products were transferred to Hybond N+ nylon membranes (Amersham Life Science, Buckinghamshire, UK). These membranes were prehybridized and hybridized with digoxigenin-labelled oligonucleotide probes. The hybrids were immunologically detected with alkaline phosphatase-conjugated anti-digoxigenin Fab, fragments (Boehringer Mannheim, Mannheim, Germany), followed by a chromogenic reaction (4-nitro blue tetrazolium chloride and x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate, Boehringer Mannheim).

PCR-based HLA class II typing

The DQA1 alleles were determined as described by Ota *et al*. (1991). The assay distinguishes 7 different DQA1 allele-groups, i.e. *0101/2/4, *0103, *02, *03, *04, *05 and *06. The DQB1 alleles were determined by means of the Inno-Lipa DQB1 reverse-dot blot kit (Innogenetics, NV, Zwijndrecht, Belgium), which detects 25 of the 26 currently known DQB1 alleles.

HPV serology

HPV antibodies were detected by a standard enzyme immunoassay based on baculovirus-expressed capsids (Kirnbauer *et al*., 1994). Purified capsids were diluted to 50 ng/ml in ice-cold PBS and coated onto EIA plates overnight at +4 ºC. The plates were blocked with 10% horse serum in PBS for 60' at +37 ºC and incubated with human serum for 90' at +37 ºC. Bound IgG antibodies were detected by incubation with a monoclonal antibody against gamma chain (Eurodiagnostics, Apeldoorn, The Netherlands) for 90' at +37 ºC. Bound monoclonal antibodies were detected with a goat anti-mouse gamma-chain horseradish-peroxidase conjugate (Southern Biotechnology, Birmingham, AL). ABTS peroxidase was applied, and the results were measured as differences in optical density between antigen-coated and negative control plates reacted with the same serum and developed identically. The patient and control sera were run simultaneously in the EIA plates. The cut-off level used to assign seropositivity from absorbance values was pre-assigned and relative to the same internal standards used in previous studies (Wikström *et al*., 1995a).

Statistical analysis

Nonparametric statistical methods were applied to continuous variables (Kruskall-Wallis test and Mann-Whitney U test, I, II). The differences between groups in HLA allele and HPV antibody frequencies were tested with Pearson's Chi-square test or Fisher's exact test (II, III). The latter was used when the number of an expected value in at least one cell was less than 5.

RESULTS

Clinical features predicting the course of adult-onset laryngeal papillomatosis (I)

A total of 74 patient records with adult-onset disease were evaluated: 57 males (mean age 44 years at diagnosis, range 20-80) and 17 females (mean age 39 years at diagnosis, range 21-71 years). The mean follow-up time was 6.2 years (range 1.0-32.4 years).

The most frequent symptom was dysphonia (84%), but dyspnea (3%) and pain in the throat (7%) were also occasionally present. The location of the primary lesion typically extended to the anterior (59%) or middle third (64%) of the vocal folds; lesions extending to the posterior third (31%) were less common. If a lesion was found at diagnosis elsewhere in the larynx, the vocal folds were also affected in 67% of the patients. Lesions covering less than a third of a vocal fold were considered to be small, and those covering more, large. Most of the patients (62%) each had at least one large papilloma lesion. Solitary lesions (68%) were more common than multiple ones.

Laryngeal procedures were performed for each patient on average 5.4 times (range 1-66). Since the year 1985, laser treatment has been available at our clinic, and 50% of the patients in this series were treated with CO_2-laser, solely or combined with mechanical removal. Interferon treatment was given to four patients. Two tracheostomas in this series were performed before laser treatment was available.

To investigate which symptoms and signs at diagnosis might predict the course of the disease, number of laryngeal procedures was chosen as the indication of the severity of the condition. Patients who underwent 1-2 (seldom relapsing disease), 3-6 (often relapsing disease), or over 6 (very often relapsing disease) procedures

were similar in most respects. No statistically significant differences existed between these groups in symptoms, size, or number of primary papilloma lesions. Those 25 patients who had only one small papilloma lesion at diagnosis were equally distributed among the groups. Two clinical findings predicted the course of the disease: those with very often relapsing disease were younger (mean age 33.8± 11.5 years) than the others ($p < 0.05$, Kruskall-Wallis test), and they had a lesion extending to the anterior third of the vocal folds more often ($p< 0.01$, Kruskall-Wallis test) than did the rest of the patients.

HPV type distribution and impact of HLA DQ alleles in adult-onset laryngeal papillomatosis (II)

Patient description

The male-to-female ratio was three to one: 47 male patients, mean age 53 years (range, 26-77), and 15 female patients, mean age 48 years (range, 27-71). The mean follow-up time was 10 years (range, 1-35). No hereditary component was discovered for the disease, and in otorhinolaryngological examination no HPV-associated lesions were detected outside the larynx. A laryngeal tumor relapse was found in 14/62 patients (2 females, 12 males), and in two of these males the relapses proved, according to subsequent histopathology of the biopsies, to be carcinomas.

HPV types detected

Formalin-fixed paraffin-embedded biopsies were available from 61 of the 62 patients. Of all the specimens, 45 (74%) were amplifiable in our PCR. Most of these, 20 (33%) were HPV-6 positive, 8 (13%) were HPV 11-positive and 2 harbored some other HPV type (Figure 3). One specimen was both HPV 6- and

HPV 11-positive. No HPV 16- positive samples were detected. The group of HPV-negative samples and samples not amplifiable in our PCR both comprised 16 samples (26% each). Those with an BPV 6- or HPV 11-positive laryngeal biopsy did not differ from each other with respect to age, sex, number of laryngeal procedures performed, or number of relapses found in the clinical examination.

HLA DQ alleles related to HPV type of laryngeal biopsy, and clinical features

No differences in the DQA1 and DQB1 frequencies were detected between patients. as a group and the reference population. When patients were divided into groups according to number of laryngeal procedures performed (1 or 2, 3-6, >6), no HLA association was noticed. Neither did the presence of HPV 6 or HW 11 DNA in the laryngeal specimen correlate with HLA type. Patients whose latest laryngeal specimen showed a typical histology of HW infection, but was HPV-negative, had an increased frequency of the DQB1*0501 allele compared with those whose laryngeal biopsy was HPV-positive (uncorrected p=0.0053, Pearson's Chi-square test; Bonferroni corrected, p=0.053) or compared with the reference population (uncorrected p<0.001, Pearson's Chi-square test; Bonferroni corrected, p=0.002). These patients had also undergone fewer laryngomicroscopies (mean 2.8; range, 1-17) than those whose biopsy contained HPV DNA (mean 5.7; range, 1-70) (p=0.003, Kruskall-Wallis test). The former patient group was also older than the latter at diagnosis (p<0.001, Kruskall-Wallis test).

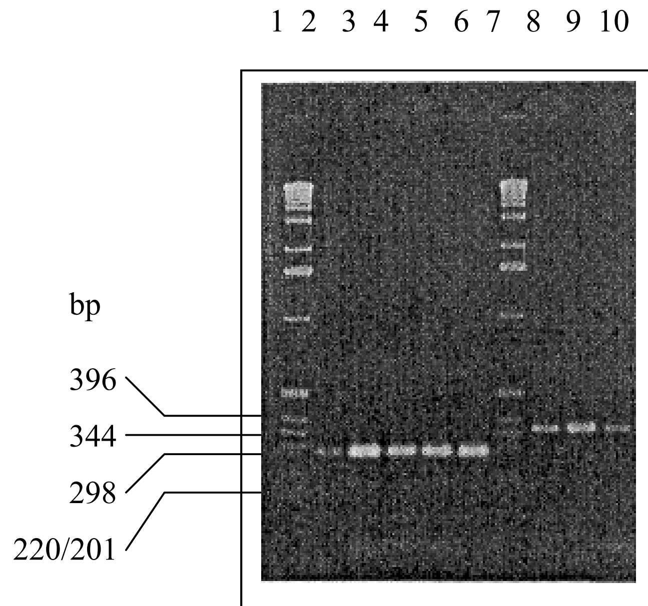


Figure 3a. HPV 6- and HPV 11-positive specimens run in agarose gel and stained with ethidium. bromide. Lanes 1 and 7: DNA molecular-weight marker X (Boehringer Mannheirn). Lanes 2 through 6: HPV 6-positive PCR products (280 bp). Lanes 8 through 10: HPV 11-positive PCR products (360 bp).

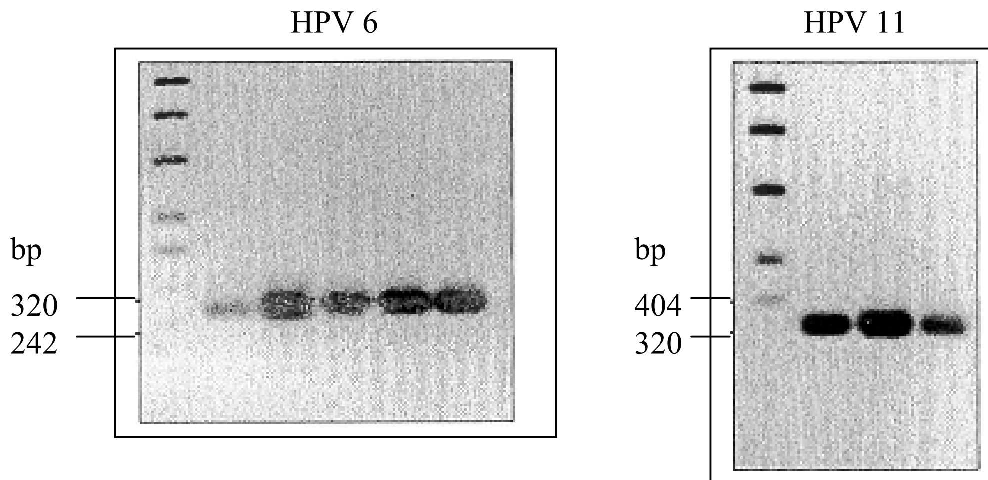


Figure 3b. Corresponding PCR products transferred to membrane and hybridized. Left, HPV 6-positive PCR products equivalent to lanes 2 through 6. Right, HPV 11-positive PCR products equivalent to lanes 8 through 10 in Figure 3a. Markers: Digoxigenin-labeled DNA molecular-weight marker VIII (Boehringer Marmheim).

HPV antibody response in adult-onset laryngeal papillomatosis and over-representation of cytological and histological signs of genital HPV infection in female patients (Ill)

The patient population in Studies H and III was the same. Serum samples of 60 adult-onset laryngeal papilloma patients and 53 age- and sex-matched controls (13 females and 40 males) were available for HPV 6, 11, and 16 antibody assays. For five male and two female patients of advanced age, it was not possible to find age-matched blood-donor controls. HPV 6 and 16 antibodies were equally frequent in patients and controls (23% vs. 21% and 22% vs. 23%, respectively), with HPV 11 antibodies more frequent in patients (33% vs. 15%, p=0.03, Pearson's Chi-square test). However, no such difference in HPV 11 antibodies appeared between male patients and controls. In contrast, female patients had HPV 11 antibodies more often than did male patients (60% vs. 24%, p=0.01 Pearson's Chi-square test) or female controls (60% vs. 15%, p=0.02, Pearson's Chi-square test), but there was no difference in HPV 11 antibodies between female and male controls (15% vs. 15%). The presence of serum HPV antibodies did not correlate with HPV type in laryngeal biopsy, with time-interval (less than 1 year, 1-3 years, more than 3 years) between laryngeal biopsy and serum sampling for HPV antibody assay, with number of laryngeal procedures, or with relapse detected by clinical examination.

We further studied whether our female patients' HPV 11 antibodies could have been caused by oral or genital HPV infection. Of the 15 females, 9 attended the thorough re-examination (see Patients and samples), and 5 of them (56%) had cervical cytology consistent with an HPV infection; and 3 of them also had histological features of HPV infection in cervical biopsy. HPV DNA was detected in one cervical biopsy with general HPV primers. Only three female patients had HPV 11 antibodies in their second blood sample: they all had Pap smear cytology consistent with an HPV infection. Patients with both laryngeal and gynecological HPV

infection had no specific HLA DQ profiles, when these were compared with those of other Finnish Caucasians. The two patients suffering a laryngeal relapse had no HPV 11 antibodies, and no patients had cytological features of HPV infection in oral samples which were also negative for the general HPV primers in PCR.

A novel experimental model of laryngeal HPV infection (IV)

Of the 30 laryngeal papilloma tumor biopsies, 20 biopsies were not co-cultured with PPLF-XR cells, with 19 of these lost during early passages. They did not grow on either 3T3-XR or irradiated human embryonic skin fibroblasts (HES-XR), and a few cultures were lost because of fungal infection. We failed to culture single biopsies without feeders or in soft agarose. Moreover, some specimens were lost when inhibition of the growth of accompanying fibroblasts on calcium-free growth medium was attempted without success. Only once were HPV-positive laryngeal papilloma cells able to grow until the fourth passage on 3T3-XR cells. We then decided to culture all our biopsies on PPLF-XR cells.

Ten biopsies were cultured on PPLF-XR cells and five of them at least until the fourth passage. The biopsy derived from normal-appearing laryngeal mucosa of the esophageal carcinoma. patient with a widely spread disease was also cultured on PPLF-XR cells, and grew successfully until the fourth passage. Of these monolayer cell cultures, *in situ* hybridization was performed on those four which were later transferred to grow on a collagen raft. These cultures showed persistence of HPV-DNA-positive epithelial cells during the passages. When these epithelial cells were transferred to allow growth on an organotypic growth base (collagen raft with un-irradiated PPLF), they produced cytology with typical features (koilocytes, dyskeratotic cells, and parakeratosis) of a laryngeal papilloma tumor. *In situ*

hybridization, which was performed on sections of these collagen rafts, showed that the epithelial cells had maintained the HPV genome.

DISCUSSION

In this work we have studied adult-onset laryngeal papillomatosis from different points of view which could relate to the prediction, course, and severity of the disease, and created a novel experimental model of laryngeal HPV infection.

The manifestations of laryngeal HPV infection can be divided into three clinical entities in which the role of the virus is more or less understood. First, the asymptomatic presence of HPV DNA in macroscopically normal laryngeal mucosa and in benign laryngeal lesions without a histology of papillomas is possible. Second, HW DNA is sometimes detected in premalignant laryngeal lesions and in laryngeal carcinomas without pre-existing laryngeal papillomatosis. Third, and the clinically most important manifestation, is laryngeal papillomatosis which has also the capacity for malignant transformation.

Laryngeal papillomatosis is not well characterized, and many basic problems may emerge in the treatment of these patients. The means of transmission are in part unknown, and the course of the disease is unpredictable and variable. The treatment is based on surgery, although the etiology of the disease is viral. Alphainterferon, which in our series of adult-onset patients was used in only 4/74 (5%), is the drug most often used for treatment although it is expensive and it has side-effects. The treatment takes up to 6 to 12 months and must be given in injections. For these reasons, interferon is the drug of choice, mainly for children with an. aggressive, relapsing disease. Long-term administration of alpha-interferon has led to complete remission in approximately 40% and partial remission in an additional 40% of patients (Leventhal *et al*., 1991). However, because alpha-interferon does not seem to eliminate the latent virus (Steinberg *et al*., 1988), and relapses even decades later are possible (Bergstrbm, 1982; Erisen *et al*., 1996), clinical studies in which the curative effect of interferon could be investigated are difficult to carry out.

Certain clinical and immunogenetic features predict the course of laryngeal papillomatosis

The course of laryngeal papillomatosis varies greatly, and it would be of benefit to recognize some predictive signs which could help us to focus the most attention on patients who are at the greatest risk for frequent relapses. In some earlier reports (Lindeberg and Elbrond, 1989; Quiney *et al*., 1989) solitary papillomas have been signs of a good prognosis, but in our study this finding could not be confirmed. The average number of laryngeal procedures performed for each patient was 5.4 during the mean follow-up time of 6.2 years. Although the number may reflect not only the severity of the disease but also the effectiveness of the surgery and the patient's ability to accept hoarseness, it provides some information about the course of the disease. The distribution of the number of laryngeal procedures required showed that there were roughly two extreme groups of patients: those who needed only 1 to 2 laryngeal procedures for remission and those who had undergone more than 6 laryngeal procedures.

We have found two clinical signs at diagnosis which predict the relapsing course of adult-onset laryngeal papillomatosis: patients' young age and a lesion reaching the anterior parts of the vocal folds, the latter perhaps reflecting more the difficulties in surgery in the anterior area than the true biological properties of the tumor. In line. with these findings are the results of our HLA work. Those who had an increased frequency of the protective DQB1*0501 allele and an HPV-negative latest laryngeal papilloma specimen were older at diagnosis and had undergone fewer laryngeal procedures than those whose latest laryngeal biopsies were HPV-positive. The DQB1*0501 allele has been found to be protective in cervical cancer, another HPV-related disease (Gregoire *et al*., 1994; Odunsi *et al*., 1996). This allele may help patients eradicate the HPV genome, and in this way protect adult-onset papillorna patients from frequent relapses. Comparable data are available on the clearance of

hepatitis B virus: the HLA class II allele DRB1*1302 is associated with protection against persistent hepatitis B virus infection (Thursz *et al.*, 1995). However, these HI'V-negative samples may also have contained some other HPV type(s) not detectable by GP5+/6+ primers. It is possible that adult-onset laryngeal papillomatosis is not a single entity but is divided into early-onset more-relapsing and later-onset less-relapsing forms; cellular immunity may, in part, determine this phenomenon. Although the data are somewhat conflicting, the most frequently reported positive association with cervical carcinoma has been that of DQ3 antigen or DQB1*03 alleles (Wank and Thomssen, 1991; Wank *et al.*, 1993; Gregoire *et al.*, 1994), and the DQw3 phenotype has been shown to be enriched in a small series of laryngeal papilloma patients, as well (Bonagura *et al.*, 1994). However, in our series we were unable to confirm the latter finding.

Laryngeal and genital HPV infection

Both laryngeal papillomatosis (Lack *et al.,* 1980; Mounts *et* al., 1982; Gissmann *et al.*, 1983) and genital condylomas (Gissmann and zur Hausen, 1980; Gissmann *et al.*, 1984) are known to be caused by HW types 6 and 11. In the adult-onset form, type 6 is found to be more common (Corbitt *et al.*, 1988) than type 11, which was the result in our study, as well. Moreover, in genital condylomas HPV 6 is more common than HPV 11 (Greer *et al.,* 1990). This similarity in HPV types detected in laryngeal papillomas and in genital condylomas has brought forth the idea that genital HW infection may be the source of the virus, and that the spread of the infection could take place through orogenital contacts. Most of our patients had a self-reported history of orogenital contacts (data not shown) but none of the nine females whose mouth-wash and mouth-brush samples were studied had HPV infection in their mouths as determined by PCR and cytology. Junctional areas of cuboidal and cylindrical epithelium in both the vocal folds and in the uterine cervix may favor the existence of HW infection, and the possible transient HPV load in

saliva could infect the laryngeal epithelium more easily than that of the mouth. The oral mucosa of women with genital HPV infection has been shown to be HPV-positive by PCR in 23% of patients (Kellokoski *et al*., 1992) , which is comparable with the prevalence of HPV infection in the macroscopically normal upper respiratory mucosa in general.

Humoral immunity - a less important mechanism in defence against adult-onset laryngeal papillomatosis?

Cellular immunity is of major importance for the clearance of infections caused by HPVs, but humoral immune defects do not seem to predispose to HW infections (Schiller and Okun, 1996). Genital HPV infection is known to elicit HPV antibody production (Kirnbauer *et al.,* 1994; Wikstrijm *et al.,* 1995a; Eisemann *et al.,* 1996; Kjellberg *et al*., 1999), but HPV antibody production in laryngeal papillomatosis had been examined previously only in small series of patients (Bonnez *et al.,* 1994; Christensen *et al*., 1992; Tachezy *et al*., 1994). What could have been expected is either HPV 6 antibody production among the patients, because laryngeal papilloma tumors are mostly caused by the HPV 6 virus type, or is a weak immune response in general because HPVs most often induce latent infection or chronic disease (Breitburd *et al*., 1996). However, only HPV 11 antibodies were more frequent in patients than in controls, and more careful study showed female patients to have HPV 11 antibodies more often than did male patients or female controls. It is possible that these antibodies were of genital origin, since the presence of HPV antibodies did not correlate with the clinical manifestations of laryngeal papillomatosis, and because female patients had an increased prevalence of cytological and histological signs of genital HPV infection. Unfortunately, because we could not perform HPV PCR on Pap smears, the HPV genotypes of these genital lesions remained unknown. Moreover, the serum antibody response did not correlate with the HPV DNA type found in the larynx, although the type-specificity of the

serological method based on VLPs has been demonstrated in many studies of genital HPV infection (Kirnbauer *et al*., 1994; Wideroff *et al*., 1995; Kjellberg *et al*., 1999). If antibodies against HPV 6, 11, and 16 VLPs had any clinical relevance, this should have been discovered in this kind of large series and especially in male patients because of the 2- to 4-fold male predominance in adult-onset disease. The lack of correlation between HPV antibodies and the number of laryngeal procedures performed or relapse detected in clinical examination both argue against the clinical relevance of HPV antibodies.

Because the serum samples were taken on average 8 years after the laryngeal samples, and no oral HW infection was detected in the females at the second visit, it is possible that HPV antibodies induced by laryngeal infection might have been present earlier but disappeared during follow-up. If so, such antibodies would be less stable over time than antibodies to HPV 16 (af Geijersstam *et al.,* 1998). Stability over time of HPV 6 and 11 antibody levels has not been widely studied, but a decline has been detected in antibody levels after clearance of HPV 6/11 DNA (Wikström *et al*., 1995a). However, the lack of correlation between antibody prevalences and either a current relapse in the larynx or the length of time-interval between laryngeal biopsy and the serum sample-taking suggests an inability to produce a response rather than poor stability over time.

It seems that viral structural proteins which induce the HPV antibodies measured in our assay may not be commonly produced in laryngeal HPV infection. Our preliminary results from immunohistochemistry (IHC) performed with L1- and L2-specific rabbit antibodies (kindly provided by Dr. J. Dillner, Karolinska Institute, Sweden) on sections of laryngeal tumor specimens and collagen rafts containing laryngeal HPV-positive epithelial cells support this possibility. However, previous data on capsid antigen production in laryngeal papillomas are limited and

controversial (Lack *et al*., 1980; Mounts *et al*., 1982). In contrast, in genital condylomas, capsid antigen production seem to be a common event (Sato *et al*., 1986; Wilbur *et al*., 1988), and E6 antigens have also been detected (Tosi *et al*., 1993).

Advantages of the novel experimental model of laryngeal HPV infection

Our method to culture laryngeal HIV-positive epithelial cells is based on the supportive effects of PPLF fibroblasts on laryngeal HIV-infected cells. This is in line with previous findings which emphasize the importance of the origin of fibroblasts in modifying the epithelial phenotype *in vitro,* even when the epithelial cells are malignant (Atula *et al*., 1997). Compared to the method of Steinberg *et al*. (Steinberg *et al*., 1982; DiLorenzo, *et al*., 1992), our procedure offers the advantage of maintaining the viral genome through passages in monolayer cell cultures, and the multilayer on a collagen raft resembles well the cytology of a papilloma tumor. Moreover, it is of benefit to multiply the HIV-infected cells in a monolayer, and if needed, store them frozen until use in experiments. This novel method offers a model of experimental laryngeal papilloma tumor which can be utilized when studying, for example, the effects of interferons, other drugs, and hormones. At present, alpha-interferon is the most widely accepted drug used for laryngeal papillomatosis, but the results of the treatment are often unsatisfactory. If the mechanisms of the action of interferon in HPV infection could be clarified *in vitro,* efforts toward improving the properties of this drug could be guided in the right direction. Moreover, investigating the effects of sex steroid hormones would also be of interest, because of the known male predominance in adult-onset disease, and - on the other hand - because of the direct action of these hormones on HPV-infected epithelial cells (Monsonego *et al*., 1991). In our model, it is possible to investigate the expression of different viral genes, which offers information, for example, about the carcinogenic or virus-producing potential of the infection. Because collagen rafts resemble fresh tissue specimens and can be handled like them, light and electron

microscopy, and molecular biological and immunological tools such as PCR, sequencing, ISH, and IHC can be used in the studies. However, culturing of HPV-positive epithelial cells is not easy, and based on our preliminary results (data not shown) it is possible that in our model, infectious viral particles are not produced, although the viral genome was detected in sections of these collagen rafts by ISH. Perhaps production of viral particles is a rare event even *in* vivo in laryngeal papilloma tumors, because in electron microscopy studies viral particles are rarely found (Svoboda *et al*., 1963; Cook *et al*., 1973), and in antibody assays serum HPV antibodies against capsid proteins are infrequently detected in adult-onset patients.

Concluding remarks

Researchers who study laryngeal HPV infection face some major problems in their work. Statistical significance is not easily established, because the disease is rare, and the number of patients available is severely limited. Moreover, laryngeal tumor biopsies cannot easily be taken without general anesthesia, and as we are dealing with a sensitive voice-producing organ, only small biopsies may be obtained. The impossibility of producing reasonable amounts of viral particles *in* vitro, and the difficulty in culturing HPV-infected epithelial cells - which is related to the former problem - make experimental studies of laryngeal HPV infection laborious. In this work, a novel experimental model of laryngeal HPV infection is presented. Clinical signs at diagnosis which predict a relapsing course and the role of immunogenetic risk factors and humoral immunity in adult-onset disease are elucidated. Moreover, connections between laryngeal and genital HIV infection have been investigated, and it will be of interest to see how studies on genetic variation of the F1PV will expand our knowledge on this issue.

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