Genital Viral Infections

Studies on Human Papillomavirus and Epstein-Barr Virus

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Abbreviations

bp	Base pair
CIN	Cervical intraepithelial neoplasia
DNA	Deoxyribonucleic acid
EBER	Epstein-Barr encoded RNAs
EBNA	Epstein-Barr virus associated nuclear antigen
EBV	Epstein-Barr virus
HIV	Human immunodefiency virus
HPV	Human papilloma virus
HSV	Herpes simplex virus
LMP	Latent membrane protein
mRNA	Messenger ribonucleic acid
OC	Oral contraceptives
OHL	Oral hairy leukoplakia
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SIL	Squamous intraepithelial lesions
STD	Sexually transmitted disease

The White Rabbit asked: "Where shall I begin, please your Majesty?"

"Begin at the beginning" the King said gravely, "and go until you come to the end: then stop."

Lewis Carroll

INTRODUCTION

The concept 'sexually transmitted diseases' (STD) was introduced in the nineteen sixties and comprises many diseases of varying importance from scabies to HIV infection. The STD family has grown wider and new members will probably join.

Long before the concept of STD was introduced, syphilis was one of the most serious sexually transmitted diseases, and became known as an epidemic disease in Europe in the last decade of the 15th century. The first known case of syphilis in Sweden occured in the beginning of the 16th century (Thyresson 1991). Even during the 20th century syphilis has been a disease of importance.

During the nineteen seventies and eighties gonorrhea and chlamydia became the most widely spread STD in Sweden. In the last decade the spectrum of STD has changed, with a decreasing prevalence of gonorrhea and chlamydia. This phenomenon might be explained by the effects of the contact tracing which was introduced in the nineteen forties in four Swedish cities with STD clinics and was performed by social workers. This system is now well integrated in the work at STD clinics (Ramstedt 1991). The decrease might also be explained by the increasing use of condoms to avoid HIV infection, which emerged as a new and serious STD in the beginning of the nineteen eighties. Altered sexual behaviour, with fewer sexual partners and a later sexual debut, might also have contributed to the decreasing prevalence of gonorrhea and chlamydia.

Today human papilloma virus (HPV) infection has become the most widespread STD. However, new pathogens are emerging. In studies from England (Sixbey et al, 1986) and from Germany (Näher et al, 1992), and also in this thesis, there is evidence of the presence of Epstein-Barr virus (EBV) in the genital mucosa of both men and women. This has raised the possibility that Epstein-Barr virus might also be a sexually transmitted virus.

Human papilloma virus

Historical background

Even in ancient times condylomata were well-known in literature and art. The descriptions 'fig' and 'condyloma' have survived till our time (Bäfverstedt 1967). Mucosal changes on the penis described in medieval documents might have been condylomata. At the end of the 18th century condylomata were interpreted as a manifestation of syphilis. The first observation that condylomata were not a sign of syphilis was made by Bell in 1793. This was later confirmed independently by Jonadan (1826) and Ricord (1838). In the early 19th century condyloma was instead interpreted as a manifestation of gonorrhea. However, this standpoint was questioned by Aime Martin, who, in 1872, was able to demonstrate that many patients with genital warts showed no signs of gonorrhea (Oriel 1971).

During the 19th century it was also suggested that condylomata were caused by irritation of the epidermis by dirt, smegma and genital discharge. This theory still had support in the beginning of the 20th century.

A relationship between genital warts and skin warts was suggested by Gemy in 1893 because of the resemblance in histological features. It was also noticed that many patients with genital warts also had common warts, although children with skin warts seldom had genital warts. This hypothesis gained further support through experiments in which extracts from excised penile warts were inoculated to other parts of the body. Plane and common warts were in some cases seen at the site of inoculation (Ciuffo 1907, Serra 1908, Wile & Kingery 1919). Papillomaviruses were identified as viral particles by Strauss in 1949 using the electronmicroscope.

The hypothesis that genital warts could be sexually transmitted was proposed in the early nineteen twenties, but no definite proof could be presented. However, in the middle of the nineteen fifties it was observed that many soldiers returning from the Far East had penile warts and that their wives developed vulvar warts. Barrett, Silbar and McGuinley (1954) suggested that the disease could be sexually transmitted, but they were strongly criticised. In the beginning of the nineteen sixties Teokharov presented epidemiological data confirming the hypothesis that genital warts comprised an independent viral venereal disease, different from non-genital warts (Oriel 1971).

Characteristics of the virus

The papillomaviruses belong to the papovavirus group. The virus particle is 52-55 nm in diameter and has an icosahedral capsid, which is built up of 72 capsomers. There is no lipid envelope. The papillomavirus genome is a circular, double-stranded DNA molecule of about 8000 base-pairs (bp) in size. There is a great variety of HPV types but the organisation of their genomes is almost identical. The viral genomes are functionally divided into two long domains, each containing a series of open reading frames (ORF) that code for viral proteins. The early (E1-7) regions code for proteins associated with genome replication and control, while the late (L1-2) regions code for the structural proteins of the virus capsid. Other regions do not possess any coding function but rather contain viral regulatory elements (von Krogh 1991, zur Hausen & de Villiers 1994).

Today, approximately 70 different types of HPV are known and of these more than 20 affect the genital mucosa (Lorincz et al, 1992, de Roda Husman et al, 1994, de Villiers 1994). Significant heterogenecity at

the nucleotide level is found between the different HPV genotype. DNA sequence homology is the basis of HPV genotype classification and a new type of HPV shows less than 90% homology to any known HPV (van Ranst et al,1993). The genital HPV types have been divided into three groups according to their association with cancer development. Type 16, 45 and 56 are called high-risk types, as they are frequently associated with increasing degree of dysplasia and cervical cancer (de Roda Husman et al, 1994). Type 18 is mainly associated with adenocarcinoma and has earlier been thought to be a high-risk type, but later studies have suggested this type to be an intermediate type (Syrjänen et al, 1990(a)). Other intermediate risk types are 31, 33, 35, 51 and 52. The low-risk types are 6, 11, 42, 43 and 44 (Lorincz et al, 1992).

Clinical manifestations

The classical manifestation of genital HPV infection is **condyloma accuminatum**, which in Greek means "a pointed rounded tumour". This manifestation is easily diagnosed by the naked eye. Some warts have been called plane or **papular** and are sometimes difficult to differentiate from lichen ruber planus or verruca seborroica. In these cases a biopsy is often required for differential diagnosis.

One group of manifestations caused by HPV infection is not possible to detect with the naked eye. To visualise them the acetic acid test is useful. They are called macular, flat or microcondylomata and are often said to be **subclinical**. This terminology is somewhat misleading as these manifestations could be symptomatic and in that sense be clinical. The term subclinical was first applied to HPV lesions of the cervix by Reid and co-workers (1982), but this term has later also been used for lesions on the outer genitals.

Lastly, there is latent genital HPV infection, which harbour HPV DNA, although no lesions can be detected either with acetic acid test or by histology.

Diagnostic methods

Different techniques for detection of viral DNA have been developed during the last decades. The **Southern-blot DNA technique** was first described by Southern in 1975. It has been considered the golden standard for HPV DNA detection in cell or tissue preparations.

The cellular DNA is extracted from cell material after the digestion of proteins and RNA. The DNA is cleaved using different restriction endonucleases and separated by agarose gel electrophoresis. The DNA is then denatured and transferred ("blotted") onto a hybridisation membrane. Labelled with either radioactive or non-radioactive compounds the cloned DNA is hybridisised to the cellular DNA on a filter at different temperatures below the melting point, which is by definition the point at which 50% of a given DNA exists as single-stranded molecules. By variation of the temperature, salt and formamide concentrations and size of the DNA probe, different levels of stringency can be applied. After 2-4 days of hybridisation, the membrane is washed, air dried and exposed to autoradiography. Non-radioactive markers are visualised by an appropiate reaction. From 0.1 to 0.01 genome equivalents per cell can be detected.

When using the **dot blot technique** the phenol/ chloroform-extracted total cellular DNA is not digested with restriction nucleases as in Southern blot analysis. It is either spotted on a gel, denatured and blotted onto a membrane or denatured by heat and alkaline treatment and dotted directly onto a membrane. The diameter of these spots can be as small as a few millimetres which

makes it possible to test many specimens on one filter (Schneider 1987).

The **polymerase chain reaction (PCR) technique** is considered to be the most sensitive technique today. Basically, it is a method of amplifying DNA. It was first invented by scientists at the Cetus Corporation and is today one of the most important tools in research and clinical practice. The PCR provides a method of producing millions of copies of a specified piece of DNA. The PCR technique involves the use of an enzyme, DNA polymerase, to make DNA starting from a primer annealed or hybridised to an existing length of DNA. The result is the production of double-stranded DNA molecules. The two sections of DNA produced are identical to the starting section. The whole process may be repeated and at each cycle the number of identical sections of DNA produced is doubled, leading to an exponential amplification of the piece of DNA. Twenty to 30 cycles will produce enough DNA for most purposes. The separation of the double-stranded DNA into single strands at each cycle in order to allow access of the primers requires heating to 96°C. The normal DNA polymerase enzyme will be destroyed by this operation and it is therefore necessary to use a thermophilic organism to avoid adding new polymerase enzyme. Thermus aquaticus is an organism in which DNA polymerase survives 96°C and the enzyme called Taq polymerase. The whole reaction may be set up in one single tube containing the DNA under investigation, Taq polymerase, excess of the primers and the deoxynucleoside triphosphates. The tube is then placed in a programmable thermal cycler. The strand separation occurs at 96°C, the annealing with primers at 37°C and the polymerase reaction at 70°C. Electrophoresis in agarose gel is usually used to determine the size and check the purity of the product (Lo & Feldman 1994).

When PCR was introduced there were some problems with contamination and accompanying higher positive rates of the analysed DNA. With special measures, such as strictly separated working areas for clean reagents, patient material and amplified material as well as the use of special pipettes, the PCR technique has become a most specific and reliable technique (Burmer et al, 1990, Rymark et al, 1993).

The **in situ hybridisation technique** for examining biopsies is another technique often used for virus detection. The principle is based on DNA hydrolysis in histological sections on glass slides. Both radioactive (35 S-labelled) probes and non-radioactive probes, such as biotinylated or digoxigenin-labelled DNA-probes, can be used. The biotinylated probes are more stable than the radioactive probes and do not expose the staff to radioactivity (Schneider et al, 1991). Autoradiography of the tissues and cells will reveal the positive reaction as dark granules in the area of the nucleus (Koss 1987).
The advantage of this technique is that it makes it possible to detect where the DNA is located, but a disadvantage is its lower sensitivity as compared to other detection techniques.

A commonly used clinical detection technique for visualisation of subclinical HPV infection is the application of acetic acid solution to the genital mucosa; i.e. **the acetic acid test.** The phenomenon of acetowhite reactions was first described by Hinselmann (1938). Acetowhite lesions on the vulva, the cervix or the penis, visible after application of 3-5% acetic acid, have widely been considered to be a sign of HPV infection. The acetowhite appearance develops in 2-3 minutes and disappears after some minutes.

It has become obvious, however, that this reaction is not

a specific sign of HPV infection, since it can also be observed in candida or trichomonas infections, in minor abrasions and traumata, and also in psoriatic areas (Wikström et al, 1992). The whitening of the mucosa often looks different when candida, for example, is the cause of the infection. The acetowhiteness is more diffuse, in contrast to the sharp borders usually seen in HPV infection.

Different mechanisms have been suggested to explain this effect of acetic acid. One might be a reversible coagulation of the cytoplasmatic and nuclear proteins of the squamous epithelium, making them opaque and white. Due to the low concentration of the acid solution, this coagulation is visible only if the amount of protein present in the cell is large. Squamous epithelium abnormally loaded with proteins becomes progressively whiter (Cartier 1984). Other explanations for the acetowhiteness phenomenon could be that acanthosis and the lack of a compact layer of stratified cells could produce the positive reaction to acetic acid or that dehydration of the stratum granulosum produces the white colour (Kellokoski et al, 1990). Swelling of infected suprabasal cells, mainly due to an overexpression of cytokeratin, is the latest theory proposed for this phenomenon (Maddox et al, 1994).

A **colposcopic technique** with magnification and illumination was introduced in Germany in the nineteen twenties (Hinselmann 1933). During the last couple of decades colposcopy has become an important complement to cytology for examining patients with suspected HPV infection and dysplasia. As stated by Olsson and Nichols (1960): "A physician who omits such an examination (colposcopy) is much the same as an astronomer who insists that he can see the stars without the use of a telescope".

Later on, the colposcopic technique has also been applied in examination of the penis, referred to as penoscopy.

The typical colposcopic picture of an HPV infection, after application of acetic acid, is a sharply demarcated white area, sometimes with satellites. When the tissue shows signs of cervical dysplasia a mosaic pattern, punctuation and pathological vessels are seen (Stafl & Wilbanks 1991). The colposcopic criteria for HPV infection of the cervix are applied also in penile and vulvar examinations.

With the colposcope the possibility of obtaining a directed biopsy from the affected area increases. The integration of computer imaging and colposcopy may further improve the colposcopic diagnostic accuracy. An inexperienced colposcopist may benefit from computerised support in obtaining the most appropiate histological specimen (Cristoforoni et al, 1995).

The morphological observation implicating HPV as an etiological agent in precancerous states is a virus-induced abnormality of squamous cells known as **koilocytosis.** This term is derived from the Greek words 'koilos' meaning 'hollow' and 'kytos' meaning 'cell'. This phenomenon was first described by Koss and Durfee in 1956 and they characterised the cells by three features: 1/ enlarged, hyperchromatic nuclei, 2/ frequently large cell size and 3/ large, often sharply demarcated, perinuclear halos. Similar cells were observed and repeatedly described by Ayre (1960), who suggested that this abnormality was due to a not-further-defined viral infection.

The tissue counterpart of koilocytosis is a lesion of the squamous epithelium of the uterine cervix described as a "warty" atypia because of its similarity to common

warts. In the nineteen seventies independent observations were presented by two groups, Meisels and Fortin (1976) and Purola and Savia (1977), suggesting that koilocytosis and related tissue lesions may be a manifestation of a cervical equivalent of condylomata accuminata.

The histopathological criteria for the characterisation of a biologically active HPV infection are described by a number of authors (Bergeron et al,1987, Koss 1987, Reid et al, 1987, Syrjänen 1987, Spitzer et al, 1990, Nuovo et al, 1990 (a)). The main features are: epidermal hyperplasia with acanthotic elongation of the rete ridges, hyper and/or parakeratosis, dyskeratosis, binucleation, koilocytosis and the presence of dilated vessels and inflammatory components in the dermis. Koilocytosis is the only criterion, which has been said to be unique for HPV infection, and in view of this widely considered a pathognomonic sign of HPV infection.

Spitzer and co-workers (1990) demonstrated that the histopathological signs of HPV infection on the cervix differed from those on the vulva. One explanation could be that the vulva shows a higher degree of squamous differentiation than the cervix and that the viral effects therefore differ. Even experienced pathologists have difficulty estimating koilocytosis equally. There are also obvious difficulties in differentiating low and high degrees of intraepithelial dysplasia in the anogenital region (Robertson et al, 1989, Carter et al, 1994).

The **Papanicolau smear (PAP)** technique was first described in 1946 and has thereafter been used as a cytological screening method for cervical cancer and cervical intraepithelial neoplasia (CIN). The criteria for abnormal cytopathology of HPV have been discussed over the last decades. Two systems are now used. One is the Bethesda system, which comprises two groups - low and high-grade squamous intraepithelial lesions (SIL) (Lundberg 1989). This classification system has been most widely used in the United States, but is now increasingly used also in Europe. The other system was introduced in 1967 by Richart. The lesions were categorised in three groups: 1) Mild dysplasia, CIN I; 2) Moderate dysplasia, CIN II and 3) Severe dysplasia, CIN III, cancer in situ. This system was earlier the most widely used in Europe. The two classification systems can be summarised as follows:

DYSPLASIA	**CIN**	**SIL**
Mild dysplasia	CIN I	SIL low-grade
Moderate dysplasia	CIN II	SIL high-grade
Severe dysplasia	CIN III	SIL high-grade
Cancer in situ		

Screening programmes with annual cytological examinations aim at decreasing the incidence of cervix cancer. In the Scandinavian countries, except Norway until recently, women from the age of 25- 35 years have regularly been screened in order to detect dysplasia and cancer in early stages (Syrjänen et al, 1990(c), Kataja et al, 1990). The PAP smear is a cheap and simple test but its sensitivity and specificity are rather low. To make the screening programmes more efficient, different combinations of detection techniques have been suggested. One is to add cervicography, which means that the area of the portio cervix is photographed with a cerviscope after acetic acid application. The image is then evaluated by a doctor experienced in this technique (Reid et al, 1991).

Another approach, which may be combined with the

PAP smear, is HPV-testing, in order to detect low-risk or high-risk HPV-types (Reid et al, 1991). The major objections to general HPV-testing are the high spontaneous regression rate of the viral infection, the failure of treatment modalities to eradicate the virus, the negative effect of putting a disease label on a normal person and the financial costs associated with HPV testing and increased referrals to colposcopy clinics (Johnson et al, 1995).

Epidemiology

The incidence and prevalence rates of HPV are difficult to ascertain for several reasons. One is that the clinical materials are usually not possible to standardise. Thus a mix of healthy groups and risk groups, such as STD clinic attenders, makes it difficult to compare the epidemiological data. Another difficulty is that the techniques for HPV DNA detection vary in specificity and sensitivity.

Subclinical and latent infections appear to be the most prevalent HPV manifestations (Hippeläinen et al, 1993). The epidemiological data are complex and difficult to interpret since these clinical entities do not have stringent criteria. Furthermore, HPV infection seems to be a multicentric disease (Sand Petersen et al, 1991, Rymark et al, 1993, DiBonito et al, 1993). Therefore, normal findings on cytological and colposcopic examination do not exclude the presence of HPV DNA (Young et al, 1989, Nuovo et al, 1990 (a)).

Most prevalence studies have been performed in female groups. The peak prevalence rate of HPV in women has in several studies been demonstrated to be in the age group of 20-24 years (Ley et al, 1991, de Villiers et al, 1992) with a decline as age progresses (Melkert et al, 1993) .

The number of sexual partners is the only independent risk factor for HPV infection which has been consistently demonstrated (Rosenfeld et al, 1989, Moscicki et al, 1990, Ley et al, 1991, Fischer et al, 1991, Evander et al, 1995). The number of sexual partners during the last years also seems to be a strong predictor of HPV positivity (Burkett et al 1992, Fairely et al, 1994). Other risk factors of importance could be socio-economic and racial factors (Bauer et al, 1993, Hellberg et al, 1995). It has also been shown in women that an early sexual debut is associated with a significantly increased risk of HPV infection (Shew et al, 1994). It has been suggested that this might be due to an increased biological vulnerability in the early period after the menarche. The increased risk of getting an HPV infection during this period may also play a role in the development of cervical dysplasia (Shew et al, 1994).

The natural course of HPV infection is not fully understood, but several prospective studies have demonstrated, that the majority of HPV-infected women heal spontaneously (Moscicki et al, 1993, Schneider 1993). The persistence of virus might depend on HPV type and immunological status (Nasiell et al, 1986, Kataja et al, 1992).

Although there are fewer prevalence studies on male groups, it seems reasonable to assume that genital HPV infection is as widespread in men as in women (van Doornum et al, 1994). Most studies are performed in groups of males attending STD clinics, and usually these men must be regarded as a risk group. In a study from a Swedish STD clinic, one third of the examined males showed HPV-associated lesions (Strand et al, 1993). In a study of Finnish conscripts, classical genital warts were found in 5.6% and subclinical lesions in 35% (Hippeläinen et al, 1993). In a study of Swedish

conscripts, 12% of the men without acetowhite reactions were HPV-positive using PCR (Kataoka et al, 1991).

Concerning the natural course of HPV infection in males, the same pattern as in female groups was shown - most infections were transient (van Doornum et al, 1994).

Genital HPV infection is mainly a sexually transmitted disease although nonsexual transmission has been discussed (Schneider 1993, Pao et al, 1993). In studies of virgins HPV is seldom found (Fairley et al, 1992, Rylander et al, 1994). An HPV infection in a child under the age of 3 years is generally thought to have been transmitted from the mother during delivery and these warts regress spontaneously (Brandt Traulsen 1995, Puranen et al, 1996). Children over the age of 3 years might get warts by autoinoculation from non genital warts but in these cases the possibility of sexual abuse should be carefully considered (Cohen et al, 1990). Antibodies to HPV 16 have been demonstrated in a high degree in the age group between 11 and 20 years (Jochmus-Kudielka et al, 1989).

Neoplasia

The risk of developing cancer of the cervix uteri is still very high world wide. There are an estimated 500.000 deaths every year from cervical cancer (Jenson & Lancaster 1990). It represents the greatest risk of dying from cancer among women in the underdeveloped countries. In Sweden the total annual incidence of cervical cancer is about 500 (The Swedish Cancer Registry 1992). Women in Scandinavia are offered regular controls with PAP-smears. Syrjänen and coworkers (1990(b)) have demonstrated that the annual incidence of invasive cancer has been reduced, but a relatively constant level was seen in the age groups over 55 years.

The association between HPV infection and the development of cancer of the cervix was first hypothesised by zur Hausen in 1977 and is today well-documented (Syrjänen et al, 1990 (b), zur Hausen & de Villiers 1994). Prospective studies on the development of malignancy in patients with HPV infection, both cervical and vulvar, have been performed in Rochester, Minnesota by Chuang and co-workers (1984 (b)) during 1950-1978, and in Sweden by Sigurgeirsson and co-workers (1991) during 1969-1984. Both studies showed similar ratios of cervical carcinoma in situ, 2.6% and 2.4% respectively.

There are several other factors considered to correlate to the risk of developing cervical cancer. One of these is immunosuppression, for example in patients with renal allografts (Alloub et al, 1989) and in HIV-infected persons (Kiviat et al, 1993, Williams et al, 1994, Chirgwin et al, 1995).

Smoking is considered a substantial risk factor for all anogenital cancers except cancer of the vagina. The risks tend to increase in proportion to the number of cigarettes smoked. In former smokers the risk of getting cancer decreases with increasing time since cessation of smoking. One hypothesis is that local immunosuppression might explain the relationship between smoking and HPV-related anogenital cancer (Daling et al, 1992). Another model, suggesting a synergism between smoking and HPV- infection, has been proposed by zur Hausen (1986). According to this hypothesis, HPV infection might lead to cell proliferation, whereas carcinogens, possibly chemicals found in cigarette smoke, transform the tissue.

Persistence of HPV DNA has been hypothesised to be a determinant of the risk of cervical neoplasia. In a study by Brisson and co-workers (1996), a higher persistence of the HPV DNA was seen in women who had used oral contraceptives (OC) for more than 2 years. On the other hand, Strand and co-workers (1996) found HPV DNA less frequently in subjects using OC. One mechanism behind the relationship between OC and HPV infection might be hormonal factors influencing transcription and/or translation of the HPV genome. The expression of progesterone receptors is significantly associated with high-grade CIN and HPV 16 and HPV 18-positive cervical lesions (Schneider 1993). Pregnancy as a co-factor is controversial, but early age at first birth has come out as an independent risk factor for cervical cancer (Bosch et al, 1992), as has multiparity (Eluf-Neto et al, 1994). Nutritional factors, such as defiencies of vitamin A, vitamin C, beta-carotene and folic acid, have also been discussed as possible determinants of the risk of cervical neoplasia (Schneider et al, 1989).

The state of the viral genome in the cell may represent a parameter of importance to the progression of the virally induced lesion. The HPV genome is normally maintained as an episome in genital warts, but many studies have shown that in tumours at least some of the viral DNA is integrated into the host genome. This integration seems to occur early in the neoplastic development. There is no common point of integration with respect to the chromosome, but there is a remarkable consistency with respect to the viral genome. Integration interrupts the E1 and E2 open reading frames (ORFs). E6 and E7 are consistently retained and expressed in both tumours and tumour cell lines.

It has been suggested that expression of E6 and E7 proteins is important in the development and possibly the maintenance of the malignant phenotype (Vousden 1989).

The natural history of cervical intraepithelial neoplasia has been extensively reviewed by Andrew Östör (1993). In studies from Europe, the United States and Asia in the period from 1950 to 1989 he found a consistent pattern, with regression of CIN I in 60%, persistence in 30% and progression to CIN II in 10% and to invasive cancer in 1%. Concerning CIN II, the corresponding figures were 40%, 40%, 20% and 5%. The probability for CIN III to regress was 33% and to progress to invasive cancer 12%. The morphology of the changes does not predict if the changes will progress or regress.

Vulvar cancer has been reported to be correlated to HPV in the wide range of 0-80% (Ansink et al, 1994). The most common clinical forms are keratinising squamous cell carcinoma, mostly seen in elderly women and rarely associated with dysplasia, and warty and basaloid carcinoma, mostly seen in younger women where dysplasia is frequently seen and HPV is found in 75% of the cases (Hörding et al, 1994).

Bowenoid papulosis is an anaplastic change of epithelial cells and characterised by multiple, pigmented papules on the penis in young men (Lloyd, 1970, Wade et al, 1978). A macular clinical appearance with the histological picture of cancer in situ has also been described (Barrasso et al, 1987, Wikström et al, 1994). This disease is considered to be benign and in most cases self-healing (Schwartz et al, 1991).

Cancer of the penis is a rare tumour. During the last decade interest has been focused on the relationship between HPV and penile cancer in parallelity to cervical cancer. A higher incidence of penile cancer has been

demonstrated in men, who have not been neonatally circumcised (Brinton et al, 1991). Hygienic factors have been pointed out as a possible explanation (Maden et al, 1993). Smoking might be a risk factor, as well as the number of life-time sex partners (Hellberg et al, 1987, Maden et al, 1993).

As in cancer of the cervix, there is evidence that high-risk HPV-infection has a role in the development of cancer of the penis (Maden et al, 1993, Zabbo et al, 1993). Penile intraepithelial neoplasia has been demonstrated in 33% of male partners of women with CIN (Barasso et al, 1987). Strand and co-workers (1995 (a)) have shown that a high proportion of the partners of women with high-risk HPV or CIN were harbouring HPV of a high-risk type. Similar results were obtained in a study by Campion and co-workers (1989), where 46% of male partners of women with CIN were shown to harbour HPV on the penile skin. Another study demonstrated that 41% of patients with penile cancer had HPV DNA, mainly types 16 and 18 (Hellberg et al, 1987). Phimosis and balanitis have also been suggested as risk factors (Hellberg et al, 1987), as have scars of the penis (Maden et al, 1993).

Anal cancer has increased in incidence during the past 30 years (Frisch et al, 1993). The tumour is more common among women and has a peak incidence between 60 and 70 years of age (Goldman et al, 1989). In a recent study, almost half of the women with histologically invasive vulvar cancer showed signs of HPV infection and anal squamous neoplasia (Ogunbiyi et al, 1994). It is also known that HIV-positive, homosexual men with low CD4/CD8 ratio and a history of anal warts have an increased risk of developing anal cancer (Wells et al, 1990, Palefsky 1994).

Finally, HPV DNA has also been found in squamous cell **cancer in the oral mucosa**. What role HPV plays in the progression to oral cancer is still not well elucidated (Shroyer & Greer 1991).

Epstein-Barr virus

Historical background

When Denis Burkitt, a British surgeon working in Uganda, saw children with tumours of the lymphoid tissues the pathogenesis was unknown (Burkitt 1958). The disease was later named Burkitt's lymphoma after him. The virus in the tumour was identified by Epstein and Barr in 1964. They succeded in establishing a procedure for culturing Burkitt lymphoma cells in vitro (Epstein & Barr 1964, Epstein et al, 1964). Virus particles could be observed in the tumour cells after some time in culture. The virus was classified on the basis of its particle structure as belonging to the herpes group of viruses, but was shown to be a new and distinct virus strain. It has been designated as the Epstein-Barr virus (EBV).

Characteristics of the virus

EBV belongs to the herpes viridae group and stores its genetic information in the form of linear double-strained DNA molecules. The infectious particle is composed of a nucleotid that contains the viral DNA in a condensed form. It is surrounded by an icosahedral capsid and a membrane envelope.

The EBV genome has been cloned as a complete library of overlapping restriction endonuclease fragments of the restriction enzymes EcoRI and BamHI. The nucleotide sequence of the genome of the prototype strain has been established.

The EBV genome contains about 180 kilo-bp. The ends of the linear DNA molecule have terminal repeats that mediate the circularisation of the molecule inside the cell. A unique feature of the molecule is an internal region that contains a number of identical large repeated sequences. The internal repeat is also referred to as the BamHI W repeat. Several other shorter repetitive sequences are distributed throughout the EBV genome. Analysis of possible coding regions in the EBV DNA sequence has shown that the genome contains around 100 different genes. Nine of these encode proteins that are expressed during latent infection. The remaining genes encode proteins that are expressed during the viral replication or lytic cycle (Ricksten 1988).

EBV has the ability to establish a latent infection, which is characteristic of all human herpes viridae. The latency means a silent state of viral infection characterised by low expression of viral genes and minimal cytopathic effects or production of infectious virus.

The viral lytic cycle starts in a primary infection or with the activation of a latent infection and proceeds with the expression of viral early genes as well as the replication of viral DNA and the expression of late genes. Finally, the infected cells release the virus (Dillner 1991). There are two lytic genes, classified as early and late. This classification is based on whether the gene is expressed before or after the onset of viral DNA synthesis. BZLF1 is an early gene protein which plays a role in activating the cascade of lytic gene expression. BCRF1 is a late gene product. It encodes a homologue of the human interleukin-10 molecule. This product has immuno-modulator properties (Dillner 1991).

The latency-associated genes are the EBV-associated nuclear antigens (EBNAs) and latent membrane proteins (LMP). In addition, there are two small nuclear RNAs actively transcribed from the viral genome during latency, EBV encoded RNAs (EBERs). The functions of EBNA are viral DNA replication and immortalisation of B-lymphocytes. The LMPs act as oncogenic proteins. EBERs are the most abundant RNAs in latently infected B-cells. Their function might be the processing of EBV mRNAs (messenger ribonucleic acid) (Dillner 1991).

Until recently EBV was thought to infect only B lymphocytes (Yefenof et al, 1976, Fingeroth et al, 1984, Frode et al, 1985). This restricted tropism has been explained by the distribution of the EBV receptor, an epitope on the complement receptor 2 found on the lymphocytes. However, the virus also infects oropharyngeal and cervical epithelial cells, at least under certain circumstances (Sixbey et al, 1983, Sixbey et al, 1987, Näher & Petzoldt 1992).

When EBV infects B-lymphocytes, its linear genome circularises to form an episome, or an extra chromosomal element within the cell nucleus. This results in the transformation of B-lymphocytes, which are immortalised and acquire the ability to proliferate indefinitely (Kieff & Liebowitz 1990).

The source of circulating EBV-infected lymphocytes has been discussed. It has been suggested that virus in the oropharynx serves as a reservoir to repeatedly infect B-cells, which then migrate to the periphery (Moss et al, 1981). Another model suggests that the EBV-infected cells are longlived and perhaps are the very cells that were originally infected or their progeny (Klein 1989). Another recent theory proposes that EBV- specific secretory IgA-mediated mechanisms can play a role in viral entry into mucosal epithelial cells (Sixbey & Yao 1992).

There are at least two EBV types recognised in human populations. They are designated EBV1 and EBV2, in line with the nomenclature for herpes simplex virus (HSV). Earlier studies suggested that EBV1 was the most prevalent strain in North America and Europe and EBV2 most prevalent in Africa. It is now clear that EBV2 strains are also commonly present in the United States (Pathmanathan 1993).

Clinical manifestations

EBV-infection is common worldwide. Primary infection in children is mostly asymptomatic. Infection in the adolescence is manifestated as **mononucleosis**. The virus is spread by saliva, mainly through kissing, and mononucleosis is therefore often called the "kissing disease". Other routes of transmission are transplantation and blood transfusions, and lately sexual intercourse has been suggested as a possible path of transmission.

Mononucleosis was first described by Pfeiffer in 1899. A more detailed clinical description was given by Sprunt and Evans in 1920. Later Hoagland (1960) established distinct diagnostic criteria for infectious mononucleosis. Blood tests show lymphocytosis and antibodies which can agglutinate red blood cells from sheep, ox or horse. As long ago as in 1942, Per Wising, a Swedish physician, was able to demonstrate that the virus was transmitted by blood from infected persons, but he did not know it was EBV.

Genital ulcers have been described during acute mononucleosis (Brown & Stenchever 1977, Portnoy et al, 1984, McKenna et al, 1994). A vasculitis caused by immuno-complex deposition has been offered as an explanation (McKenna et al, 1994). The virus might be produced and released by epithelial cells, within the ulcers. An orogenital distribution of the virus has also been considered as a possible explanation (Portnoy et al, 1984).

Oral hairy leukoplakia (OHL) was first described in HIV-positive patients as warty infiltration on the side of the tongue. OHL was first thought to be HPV associated, but EBV was later found to cause this clinical appearance (Greenspan et al, 1984, 1985). OHL has been considered to be a bad prognostic sign of AIDS development (Katz et al, 1992). However, in a long-term follow-up Lau and co-workers (1991) could demonstrate that OHL should not necessarily be regarded as an ominous sign. OHL has also been demonstrated in HIV-negative, immunosuppressed patients (Itin et al, 1988, Syrjänen et al, 1989). Furthermore, there are also reports of OHL in HIV-negative and immuno-competent persons (Eisenberg et al, 1992, Felix et al, 1992). OHL is probably main-tained by repeated direct infection of the upper epithelial cells with virus from saliva or other infected cells of the oral mucosa and not by reactivation of latent EBV. The absence of demonstrable EBV latent proteins in the basal and suprabasal cells of OHL supports this hypothesis (Niedobitek et al, 1991). The clinical significance of OHL and the pathogenetic aspects have been extensively discussed in several recent studies (Itin et al, 1993, Brehmer-Andersson et al, 1994).

Histologically, OHL has been shown to contain swollen, partially vacuolated epithelial cells similar to koilocytotic cells found in HPV-related lesions (Greenspan et al, 1984, Eversole et al, 1986). It was later demonstrated that the swollen cells of OHL lacked the extensively transparent cytoplasm and nuclear atypia seen in HPV-related infections (Kanas et al, 1988). Other signs of OHL have been shown to be pale eosinophilic cytoplasm, small

perinuclear clear rings and shrunken pyknotic nuclei. Syrjänen and co-workers (1989) advised that 'koilocytosis' should be restricted only to HPV lesions and not be used to describe cellular changes in OHL caused by EBV.

Neoplasia

Nasopharyngeal carcinoma is endemic in specific populations, e.g. Chinese of Cantonese origin, East Africans from highland regions, Tunisians and Eskimos from Alaska, Canada and Greenland. The tumour presumably develops from the epithelial cells of the space behind the nose. The presence of EBV DNA in nasopharyngeal carcinoma biopsies was first demonstrated by zur Hausen and co-workers in 1970. In anaplastic nasopharyngeal carcinoma 100% of the tumour cells carry multiple copies of EBV. Other EBV-associated carcinomas have recently been identified in the salivary glands and in the stomach (Niedobitek et al, 1993).

T-cell lymphomas are more frequently associated with EBV than **B-cell lymphomas,** which may suggest that T-cells are less well adapted to EBV infection (Niedobitek & Young 1994). However, some B-lymphocyte proliferative diseases and B-cell malignancies have been demonstrated to be EBV-related. Several diseases with relation to acquired or inherited immunodeficiencies are EBV-related, for example cerebral lymphomas in HIV-infected patients. In patients who have undergone organ transplantation there is a hundred fold increase of EBV-related lymphomas (Ernberg et al, 1989).

Increasing evidence indicates that EBV also plays an important etiological role in the development of **Hodgkin's disease** (Herbst et al, 1990, Coates et al, 1991).

Furthermore, the identification of EBV in **epidermotropic cutaneous T-cell lymphoma**, mycosis fungoides and Sézary syndrome suggests that EBV could play a role also in the development of these lymphomas, either as an etiological agent or more probably as a chronic activating agent (Lee et al, 1990, Dreno et al, 1994).

Burkitt's lymphoma is a non-Hodgkin B-cell lymphoma of low-grade differentiation. This disease accounts for nearly half of all cancers in children in the tropical regions of Africa. It also occurs frequently in Papua New Guinea in its endemic form. Outside these areas it occurs rarely. The endemic form has manifestations in yaws and the peak incidence is at 6-10 years of age. In these patients 98% carry the EBV genome. The sporadic form is manifested in long bones, kidneys, adrenals, thyroids, ovaries and testes. The peak incidence occurs at 15 years of age. In these patients 20% carry the EBV genome. An African child with a moderately elevated virus capsid antigen (VCA) titre has a 30 times increased risk of developing Burkitt´s lymphoma compared to the normal population (Dillner 1991).

Some general theories about the association between EBV and human malignancies were proposed by Henle & Henle in 1985:

Firstly, EBV could induce uncontrolled diffuse polyclonal proliferation of EBV-genome-carrying B-lymphocytes in immunodeficient persons, due to the loss of the cytotoxic T- cell response. This is the pattern in immunoblastic lymphadenopathy, immunoblastic

lymphosarcoma or diffuse malignant lymphoma.

Secondly, in Burkitt´s lymphoma EBV serves as an initiator, which, together with environmental factors, most likely malaria, lead to an impairment of the ability of cytotoxic T-cells to control the proliferation of the EBV-infected B-cells, thereby further increasing the pool of EBV-infected B-cells. A translocation of the distal part of chromosome 8 to chromosome 14 leads to activation of the c-myc oncogen and subsequent B-lymphocyte proliferation.

Thirdly, concerning nasopharyngeal carcinoma, the etiological factors identified include, besides EBV, genetic susceptibility, including an HLA-associated risk, potential tumour suppressor gene(s) located on chromosome 3 and environmental factors.

Finally, in a recent review, Niedobitek & Young (1994) concluded that EBV obviously is associated with a far broader spectrum of human malignancies than anticipated.

Diagnostic techniques

EBV is difficult to culture. PCR is now available for EBV detection. The in situ hybridisation technique for detecting EBER in the tissue has been a useful test when investigating the association between EBV and malignancies. These techniques have been described under *diagnostic techniques/HPV*.

Serological examinations for detecting antibodies to EBV in mononucelosis are performed with the Paul Bunnell differential test. The mononucleosis patients create antibodies to structural virus antigens, virus capsid antigens (VCA) and early antigen (EA), both restricted (-R) and diffuse (-D). VCA stimulate the patient to create VCA-IgM antibodies, which could be demonstrated early in the acute phase of the disease. Later come the antibodies to EA-D. Antibodies to EBV induced nuclear antigen (EBNA) complex come in the later phase of the disease (Lennette 1991).

Aims

To study the prevalence of HPV and EBV by different detection techniques in an STD population in Sweden.

To relate the clinical appearance of HPV infection to different HPV types and dysplasia.

To examine the presence of HPV DNA and EBV DNA in acetowhite lesions.

To examine the psychological effect of HPV infection.

PATIENTS AND METHODS

The material for the different examinations was sampled from men and women attending the STD Clinic, Department of Dermato-Venereology, Sahlgrenska University Hospital, Göteborg, and from women attending the Department of Gynaecology and Obstetrics, Sahlgrenska University Hospital, Göteborg, during the years 1990-1995.

Paper I

A group of 588 women, consecutively consulting the STD clinic during 14 months (1990-1991) for STD check-up or symptoms, were studied. Samples were collected from the endocervix and the transformation zone using a cytobrush. HPV was detected using the dot-blot/Southern-blot technique. PAP smears were taken for cytological examination. Sampling from the cervix and the urethra was performed for isolation of Chlamydia trachomatis on McCoy's cells. A cotton-tipped swab was rubbed on the wall of the vagina to sample material for culture on Sabouraud agar for Candida albicans. The results of the examinations were correlated to visible condylomata, i.e. filiform or papulous condylomata, to cytological examination and to the presence of Chlamydia trachomatis and/or Candida albicans. Colposcopy was not performed routinely. A follow-up study was performed after three months in 21 of the 48 HPV Southern-blot-positive women.

Sample preparation: Material was collected into tubes containing 1 ml of lytic buffer (Oncor Inc., Gaithensburg, MD, USA) and digested with proteinase K, final concentration 0.5mg/ml, in the same buffer at 60°C. Cells collected by cytobrush were digested for 1 h until the specimen was no longer visible. Thereafter the digested samples were divided into two aliquots, 250 µl for dot-blot hybridisation and 750 µl for Southern blot hybridisation.

Dot-blot hybridisation: The 250 µl aliquots were subjected to two separate dot-blot hybridisation assays performed in parallel. One assay monitored that the samples contained sufficient amounts of DNA by hybridisation with biotinylated probe to human DNA. The parallel assay screened for HPV DNA by hybridisation with a cocktail of biotinylated full genomic HPV DNA probes of the types 6, 11, 16, 18, 31, 33 and 35. All probes were obtained from Oncor Inc., Gaithensburg, MD, and the dot-blot hybridisation was performed according to Kataoka and co-workers (1991).

Southern-blot hybridisation: HPV DNA-positive samples (judged by the dot-blot assay) were further analysed by Southern-blot to determine which HPV genotype or genotypes were present in the samples. The 750 µl aliquots of sample preparations (see above) were then further digested with proteinase K (0.25mg/ml) in the presence of 0.5% SDS at 60°C for 15 minutes to facilitate DNA purification. Proteins were precipitated with salt and vigorous shaking, and removed by centrifugation. The DNA was then precipitated with 99.5% ethanol at room-temperature followed by centrifugation and after two washes with 70% ethanol the DNA-pellet was dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. After restricition enzyme digestion with a mixture of Bam HI and PST I, the sample DNA was separated by 1% agarose gel electrophoresis together with HPV DNA as a control and transferred by vacuum blotting (Hybaid) to positively charged nylon membranes. The membranes were hybridised with biotinylated subgenomic HPV-probes 6, 11, 16, 18, 31, 33 and 35

under the conditions described by Kataoka and co-workers (1991). The probes were obtained from Oncor Inc., Gaithensburg, MD.

Cytological examination: Cervicovaginal smears for cytological examination (PAP smears) were taken with a wooden spatula from the vagina and the ectocervix and from the endocervix with a cytobrush (Medscand AB, Malmö, Sweden). The material was immediately smeared out on a glass-slide and fixed in alcohol.

Paper II

A series of 94 male patients consulting at the STD clinic during a period of 18 months (1989-1991) because of signs of penile HPV infection were examined. The samples for HPV DNA analysis from the different types of condylomata were collected using a cytobrush. HPV was detected using dot blot/Southern blot technique. Probes for HPV types 6, 11, 16, 18, 31, 33 and 35 were available (see Paper I). A punch biopsy was taken for histological examination. The acetic acid test was performed and the clinical appearance was registered as accuminate, papular or macular lesions. The relationships between the clinical appearance, the presence of different HPV types and histopathological diagnosis were evaluated.

Paper III

During a period of 14 months (1990-1992) 23 women with acetowhite vulvar lesions and 19 women without this clinical manifestation were enrolled in the study. The first inclusion criterion for the study group was acetowhite lesions visualised colposcopically as well demarcated plaques, sometimes with satellites in the periphery and sometimes with a pattern of small confluent filiform papules. The patient group was also subjected to biopsy for histopathological diagnosis and an additional inclusion criterion in this group was a histopathological diagnosis of koilocytosis, typical or suspected. The material was sampled using a cytobrush rubbing the inside of the labiae minora and the perineum and a biopsy from the same localisation. HPV was detected using PCR technique. The patients attended the STD clinic mainly because their partners had signs of HPV infection or they themselves had shown signs of an HPV infection.

DNA preparation: The biopsies were thawed, minced thoroughly with scalpels and suspended in 1 ml of L-buffer (10mM Tris-HCl, pH 8.4, 1mM EDTA, 0.08% Triton-X 0.001% SDS). After digestion for 5-12 h at 55°C with 100 µg/ml proteinase K the samples were incubated at 95°C for 10 min to inactivate the proteinase. The DNA was precipi-tated by 95% ethanol at minus 20°C and redissolved in 100 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 0.5 µg DNA was used in the PCR assay.

Cervical cells were centrifuged to pellet the cells. The supernatant was removed. The pellet was washed with phosphate buffer and then resuspended in 100 µl of L-buffer with 100 µg/ml proteinase K. Incubation at 55°C for 1 h was followed by incubation at 95°C for 10 min to inactivate the proteinase. 5-10 µl of the solution was used directly for DNA amplification.

PCR-technique/EBV: The primers used in the nested PCR assay were selected from a repeated sequence (BamHI W fragment) extensively investigated earlier by Ricksten and co-workers (1987,1988) of the prototype strain B 95-8 (Baer et al, 1984). The primers were synthesized commercially (Scandinavian Gene Synthe-sis, Köping, Sweden) and purified as described by the

manufacturer. The outer primer set consisted of 5′CTA GGG GAG AAC GAA GTG AA 3′ (W1; nucleotides 14555-14575 complementary to antisense strand) and nucleotides 5′CTG AAG GTG AAC CGC TTA CCA 3′ (W2; nucleotides 14310-14380 complementary to sense strand). The inner primer set consisted of 5′GGT ATC GGG CCA GAG GTA AGT 3′ (W3; nucleotides 14605-14625 complementary to antisense strand) and 5'GCT GGA CGA GGA CCC TTC TAC 3′ (W4; nucleotides 14777-14797 complementary to the sense strand). The first primer set, W1-W2 amplified a 275 bp long sequence and the nested primer set W3-W4 amplified a 192 bp long sequence. DNA was amplified from 5 µl of samples in a 50 µl reaction solution containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.2 mM MgCl2, 200 µM each dNTP and 20 pmol of the respective primer, 1.25 U of Taq-DNA polymerase (Boehringer-Mannheim, Stockholm) and water to a final volume of 50 µl. The amplification reaction was performed in the Gene Amp PCR system 9600 (Perkin-Elmer Cetus, Göteborg). With the first primer set, amplification was performed by 30 incubation cycles of thermal denaturation at 92°C for 15 s, primer annealing at 66°C for 10 s, and primer extension at 72°C for 15 s. Each extension time was increased by 1 s in each subsequent cycle, 5 µl of the amplified material was used for DNA amplification (40 cycles, as described above) with the second primer set. After the second amplification, a 10 µl aliquot of the reaction mixture was analysed by elctrophoresis in a 2% agarose gel containing 0.5mg/ml ethidium bromide. The results were photographed under ultraviolet illumination. To exclude false positive results due to contamination, every second sample analysed was a negative control containing all PCR reagents except DNA. DNA prepared from the EBV positive cell line P3HR-1 was used as positive control sample. Further confirmation of the amplification products was obtained by Southern-blot analysis. The PCR products were transferred to a nylon membrane and hybridised to an internally biotinylated probe consisting of 14709-14724 (5′CGG ACA GCT CCT AAG AAG GC′3).

PCR -technique/ HPV: Amplification of genital HPV DNA sequences was performed as described by Manos and co-workers (1989). The consensus MY 11 and MY 09 primers promote the amplification of an approximately 450-bp product from at least 25 distinct gential HPVs. Recombinant plasmid DNA containing the entire HPV 6, 11, 16, 18 and 31 genome, respectively, were used as positive controls.

Using nested PCR it was possible to detect 0.5 fg DNA prepared from the EBV positive cell line P3HR-1, corresponding to about 2 genomes of EBV. DNA prepared from cells infected with other herpes viruses were used as negative controls. No amplifications were found after PCR on cells infected with HSV-1, HSV-2, CMV and VZV DNA, respectively. The sensitivity of the HPV/PCR was determined by performing a PCR on diluted HPV plasmid DNA. One fg of cloned HPV genome could be detected which correspond to about 100 copies of the HPV genome.

ß-globin: Samples that were negative for both EBV DNA and HPV DNA were also amplified with ß-globin primers to exclude false negative results (Saiki et al, 1985).

Paper IV

This study consisted of two different parts. The first part included 20 women with acetowhite lesions on the inside of the labiae minora and perineum and with signs of koilocytosis on routine histology, as well as 20

women without acetowhite reactions. The second part included 20 men with acetowhite, koilocytotic lesions on the glans and/or preputium, as well as a group of 20 men without acetowhite reactions. Samples were collected from the oral mucosa in the male study group as well as in the male controls. The material was sampled using a cytobrush. HPV and EBV were detected using PCR technique (see Paper III). A punch biopsy was used for histological examination. The biopsies were reevaluated in both the female and the male group. The study was active during 18 months (1994-1995).

Paper V

A group of 91 women, consecutively consulting at the STD clinic for STD- check up during a period of three months (1991), were screened for the presence of EBV DNA and HPV DNA on the portio cervix. The samples were collected using a cytobrush. The analysis was performed using PCR (see Paper III). The material from these women was also examined using the Southern blot technique in the first study (I).

Paper VI

A study group of 37 women with acetowhite lesions on the portio cervix attending the Department of Gynaecology and Obstetrics for laser treatment, was examined. A control group consisted of 35 woman, who attended the STD clinic without any acetowhite reactions. Patients were included during 6 months (1995). Samples for detection of EBV and HPV were obtained using a cytobrush. The PCR technique was used (see Paper III). A punch biopsy was used for histological evaluation. Five EBV-positive biopsies were examined for the presence of EBER by the in situ hybridisation technique.

In situ hybridisation: The paraffin-embedded biopsies were deparaffinised and the tissue was treated with proteinase K: In situ hybridisation for detection of EBER transcripts was performed using a cocktail of oligonucleotides to EBER-1 and EBER-2 (Dako 4017). The method employed was exactly as specified in the manufacture's instructions.

Paper VII

A group of 41 men who previously had been diagnosed and treated for an HPV infection were reexamined 18 months later for signs of active HPV infection and a follow-up concerning their psychosexual health (1990). A questionnaire concerning sexual contacts, condom use and sexual life after the diagnosis of the HPV infection was given to the patients. The clinical examination of the genitals was performed using inspection with magnification after applying 5% acetic acid for 3-5 minutes.

Statistical methods

Fischer's exact 2×2 table and Stat Graphics Software using multiple, stepwise, linear regression analysis.

RESULTS

Prevalence studies (Papers I,II and V)

Among females attending the STD clinic with worries about HPV infection, discharge, pruritus of the genital area or just for an STD check-up, a group of 588 women were examined using Southern-blot to detect HPV DNA. In the whole group, the HPV prevalence was 8% (48/588). Of the women attending for concern about HPV (n=233), 94 showed clinical signs of HPV infection. The Southern-blot test was positive in 13% (30/233). In the group attending for STD check-up, 1% (4/355) presented clinical signs of HPV infection and 5% (18/355) were Southern-blot positive. Among the 98 women with visible signs of vulvar/vaginal warts, 33 (34%) were HPV positive on the cervix compared to 15 (3%) of 490 woman without clinical signs of HPV infection on the vulva. In 23% (11/48) more than one HPV type was detected; types 6 or 11 were detected in 20% (10/48), types 16 or 18 were detected in 44% (21/48) and type 31 and 33 in 53% (25/48). The prevalence of high/intermediate-risk types in the HPV-positive group was 80% (38/48), compared to 20% (10/48) low-risk types. In patients with cervical HPV infection 26% (11/43) presented a positive Candida albicans culture, compared to 16% (79/504) of the patients without demonstrable HPV DNA. Chlamydia infection was demonstrated in 6% (3/43) of the HPV-positive patients, compared to 12% (61/500) in the HPV-negative population.

In the whole group, 231 women were examined cytologically. Of these, 13% showed an abnormal cytology. Cytological abnormalities were seen in women with all types of HPV, but also where no HPV DNA had been found. Abnormal cytology was demonstrated in 53% (23/43) of the HPV-positive women, compared to 4% (8/188) of the women negative for HPV on the cervix. Dysplasia was seen in 4 women, in 3 with high/intermediate HPV types and in 1 HPV-negative woman.

A follow-up study was performed in the 48 women with a positive Southern-blot test at the screening visit. Only 21 attended the follow-up after three months. In this group, 52 % (11/21) had become HPV-negative (I).

Of 94 men with condyloma-like lesions, 90% (79/88) had HPV DNA detected by dot-blot/ Southern -lot. Of 51 biopsies reevaluated histologically, 88% disclosed an evident koilocytosis. Types 16, 18, 31, 33 and 35 were demonstrated in 8% of the accuminate, in 22% of the papular and in 56% of the macular lesions. Seven men showed clinical manifestations of balanoposthitis. Moderate or severe dysplasia was observed in 29% of 51 lesions examined histologically. There was a significant correlation between high-risk HPV types and dysplasia (II).

A pilot study in 91 women was performed to examine the presence of EBV DNA and HPV DNA on the portio cervix using the PCR technique. EBV DNA was present in 38%, HPV DNA in 33% and both EBV DNA and HPV DNA in 15% (V).

Demonstration of EBV DNA in vulvar, oral and cervical mucosa and penile skin (Papers III, IV and VI):

A study group of 23 women with acetowhite lesions on the vulva was compared to a control group of 19 women without these signs. EBV DNA and HPV DNA were detected using the PCR technique. In the study group EBV DNA was demonstrated in 48% (11/23)

irrespective of the sampling method, i.e. using either cytobrush or biopsy. In the controls, EBV DNA was detected in 11% (2/19) when using a cytobrush. In the study group HPV DNA was demonstrated in 17% (4/23) using biopsy and in 8% (2/23) using a cytobrush. In the controls, the result was positive in 42% (8/19) with the cytobrush (III).

To further evaluate the unexpected results from study III, a study was performed in 20 women with acetowhite vulvar lesions and 20 women without. In the study group HPV DNA was detected in 25% and EBV DNA in 35% and in the control group HPV DNA in 25% and EBV DNA in 10%. In a reevaluation of the histological diagnosis of koilocytosis, this diagnosis was confirmed in only 8 samples and questioned in 4 samples. In 8 samples the final diagnosis was nonspecific inflammation (IV).

In order to investigate the corresponding clinical situation in men, a study was performed in 20 men with acetowhite, koilocytotic lesions on the penis and in 20 controls with non acetowhite, non biopsied lesions. In the study group, HPV positivity was demonstrated in 60% (12/20) and EBV positivity in 20% (4/20). In the controls HPV DNA was detected in 25% (5/20) and EBV DNA in 5% (1/20). Koilocytosis was confirmed in all reevaluated biopsies (IV).

In the oral mucosa of the male study group, HPV DNA was demonstrated in 25% and EBV DNA in 30%. In the control group the figures were 5% for both EBV and HPV (IV).

In the group of 37 women with cervical acetowhite lesions, 30% harboured EBV DNA and 51% HPV DNA and in the control group, where no acetowhite reactions were visible, 57% demonstrated EBV DNA and 23% HPV DNA. Out of the 37 biopsies from the acetowhite lesions of the portio cervix, 2 biopsies showed a normal morphology, 13 a non-specific inflammation (cervicitis), 4 a koilocytosis without dysplasia, 3 a mild dysplasia and 7 a moderate dysplasia. Severe dysplasia was demonstrated in 7 and adeno-carcinoma in 1. Of 22 cervical biopsies disclosing koilocytosis and/or dysplasia, 5 (23%) were EBV positive and 14 (63%) HPV positive. However, in 13 cases disclosing cervicitis EBV was detectable in 6 (50%) and HPV in 3 (25%). Thus, there was no correlation between EBV and koilocytosis and/or dysplasia. EBER was analysed using an in situ hybridisation technique, but was not found in the 5 EBV-positive biopsies examined (VI).

Psychological aspects of HPV-infection (Paper VII):

In this study, 41 men with a previously diagnosed HPV infection were interviewed and examined for signs of current HPV infection. Forty per cent (17/41) reported a negative effect of the disease on their sexual life. Fiftyfour per cent (22/41) were anxious about the association between HPV infection and cancer and 20% (8/41) had reported a decrease in sexual desire because of feeling "dirty". Fifteen of 41 men demonstrated signs of a current HPV infection. Papular or accuminate lesions were present in 12 men and acetowhite lesions in 3 (VII).

DISCUSSION

General considerations

Epidemiological studies, in clinical settings, are usually performed to obtain prevalence and incidence data from a local population. Such studies can produce valuable data to be used for longitudinal follow-up and for geographical, temporal or other comparisons. However, the results must be interpreted carefully, since these studies are often based on materials that are small and selected. This is an obvious limitation in the present studies, as well as in most studies based on patients attending an STD clinic.

It is an important task for the clinician to take an active interest in the evaluation of established as well as new diagnostic techniques. This thesis deals with established diagnostic procedures, like the acetic acid test, as well as modern methods like the PCR. The PCR is so reliable that positive findings must be considered as true positives. However, it is not possible to estimate the number of false negative findings. We have demonstrated a high frequency of HPV and EBV using PCR. One critical question, of course, concerns the clinical significance of these findings. It could be argued that the viruses detected may constitute part of the normal flora with no pathogenic significance. However, both EBV and HPV may, as described above, act as serious pathogens. Therefore, it seems important to discuss our findings in view of this.

Prevalence of human papilloma virus infection

Prevalence studies among women in different groups have been performed with the *Southern-blot technique* and the prevalence figures vary between 6 and 38% depending on whether there are visible signs of HPV infection or not (Martinez et al,1988, Kiviat et al,1989, Rosenfeld et al, 1989, Moscicki et al, 1990, Horn et al, 1990, Kataoka et al, 1991, Hjerpe et al, 1992, Lindh et al, 1992). In our study (I) HPV DNA on the cervix was demonstrated in 8% of an STD population. However, in patients with clinical signs of HPV infection of the vulva/vagina this figure increased to 34%. In the group with suspicion of HPV infection the figure was 13%. The present data seem to be in the same interval as those from other studies cited above. Our results point to the fact that HPV infection of the vulva increases the risk of HPV infection on the cervix. Other studies have also demonstrated this association (Evans et al, 1992, Ward et al, 1994, Coker et al, 1994).

When the *PCR-technique* has been used the corresponding figures for HPV prevalence are 18-46%. (Young et al, 1989, Jenison et al, 1990, Rohan et al, 1991, Bauer et al, 1991, Evander et al, 1992, Strand et al, 1993). In our study (V), HPV DNA was detected in 33%, which is in line with earlier findings.

The prospective study was attended by only 21 women after three months (I). Persisting HPV infections (n=9) were seen in the group of patients infected with high-risk types and the doubly infected. Our material is too small to allow any firm conclusions, but the findings of persistence of high-risk types, is in line with other studies (Kataja et al, 1990, 1992, Rosenfeld et al, 1992, Hildesheim et al, 1994).

In our study (I) 53% (23/43) of the HPV-positive women and 4% (8/188) of the HPV- negative women showed abnormal cytological findings. Only 4 patients demonstrated dysplasia and 3 of these patients harboured high risk types and 1 was HPV negative.

Koilocytosis and signs of dysplasia could thus be seen in all groups of women including those who were HPV-negative (I). One explanation could be that these women were false HPV-negatives, because of the limited number of probes in the Southern-blot testing. Of 231 patients examined using cytology, 13% had abnormal cytology including uncharacterisitc atypia and dysplasia. This finding is well in line with other studies, having demonstrated abnormal cytology in 6-41% (Martinez et al, 1988, Hallam et al, 1991, Horn et al, 1991).

The PAP-smear is considered to be a cost-effective screening test. There is, however, discussion concerning at what age to start cervical cytology controls. The fact that HPV positive young women could have CIN I and that these changes mainly heal spontaneously (Moscicki et al, 1992, Schneider 1993, Rosenfeld et al, 1992, Kataja et al, 1992), has raised the question of the consequences of worrying these young women unnecessarily (Bergström et al, 1993).

Another subject of discussion is whether HPV-typing is a useful diagnostic test in the screening context or not. The results from different studies are contradictory (Reid et al, 1991, Meijer et al, 1992, Koutsky et al, 1992). Although it has been shown in one study, that the presence of HPV type 16 seems to be specific and sensitive in indicating CIN II/III, as much as one quarter of the group with CIN II/III were not detected by HPV testing. Thus the evaluation of HPV infection and dysplasia must be based on a combination of different diagnostic tools (Cuzick et al, 1995). In our study group (I) about 50% of the patients were HPV-negative after three months. The persistence of HPV was mainly seen when intermediate or high-risk type of HPV was demonstrated. Screening with HPV-typing might be of importance in woman over 35 years of age, since HPV-types 16 and 18 are seen in 1-2% in this group (Melkert et al, 1993). For research purpose, it is, of course, of great interest to follow these women with colposcopy, cytology, typing and biopsy annually to get a better knowledge of the natural course of the HPV infection. It is important to keep in mind that this is a sensitive group of patients and that we have to be cautious with our diagnostic conclusions.

Human papilloma virus on the penile skin

The morphology of HPV-induced lesions differs. Condylomata accuminata have mainly been associated with low-risk HPV-types. Macular lesions or subclinical HPV infection, on the other hand, have been associated with high-risk HPV-types. These findings were confirmed in study II. Dysplasia was strongly correlated to high-risk type HPV (II). Similar results were demonstrated in another recent study from Sweden (Strand et al, 1996).

One central issue is the acetowhite lesions. In our studies penile acetowhite lesions were strongly correlated to high-risk HPV-types (II,IV). However, today when the PCR- technique is available, alos latent HPV-infection can be detected. HPV was demon-strated in 25% of non acetowhite penile reactions (IV). The same result was demon-strated in non acetowhite vulvar reactions (IV). The clinical relevance of these findings has to be further evaluated.

The application of acetic acid is a non-specific test, since the tissue is capable of turning white, not only in HPV-infection, but also in eczema, psoriasis, candida or after traumata (Wikström et al, 1992). We have shown that acetowhite lesions of the vulva might be EBV associated

(III,IV). Therefore, the acetic acid test usually has to be combined with other diagnostic procedures.

The koilocytosis is generally considered to be pathognomonic for HPV-infection. Our studies on the vulva demonstrated that koilocytosis is not exclusively associated with HPV infection. In penile acetowhite manifestations koilocytosis has also been demonstrated in both HPV-positive and HPV-negative lesions (Strand et al, 1996).

Therefore, koilocytosis can not be the only diagnostic criterion of HPV-infection. However, dysplasia seems to be well correlated to the presence of high-risk HPV-types (Strand et al, 1996) (II). It might be of relevance in this context to point out that the external genital area is covered with relatively stable keratinised stratified squamous epithelium, whereas the cervix consists of mature columnar cells undergoing squamous metaplasia (Law et al, 1991). This might be the basis for the different histological reactions to HPV.

Human papilloma virus in the oral mucosa

Immunocompetent persons harbour HPV in the mouth, but in a low degree and mostly in the latent form. Lichen planus and keratosis in the mouth are strongly associated with HPV (Maitland et al, 1987). There is discussion concerning the association between HPV and cancer of the mouth, but this relationship is still not fully elucidated (Lakshmi et al, 1993). There is a need of further investi-gations to study the interactions with other oncogenes and other oncogenic agents such as chemicals.

Studies on healthy persons without clinical manifestations of HPV infection in the mouth demonstrate HPV in up to 40% using the PCR-technique (Kellokoski et al, 1992, Jalal et al, 1992). In our study (IV) HPV was found in 25% of men with signs of genital HPV infection and in 5% of men without genital HPV infection. These results might indicate an oro-genital transmission. Our finding of different HPV-types in the mouth, as compared to the finding of HPV-types on the genitals, is also in agreement with earlier findings (Kellokoski et al, 1992). The relevance of this finding is not known.

Epstein-Barr virus and genital infection

EBV as a "new" sexually transmitted virus has been discussed (Sixbey et al, 1986, Bevan et al, 1989, Wong et al, 1993). In our present studies (III, IV, V, VI) we have been able to demonstrate EBV in the genital mucosa. However, these findings do not prove sexual transmission.

The virus has been detected from throat washings in patients on immunosuppressive therapy and also in low levels from healthy EBV-seropositive persons (Young & Sixbey 1988). These observations, together with the facts that EBV-transformed lymphoblastoid cell lines in vitro tend to be poor producers of the virus and that B-lymphocytes permissive of viral replication cannot be demonstrated in vivo, suggest that EBV replicates and is shed at epithelial sites in the oropharynx. The possible path of transmission to the genital tract might be by the oro-genital route (Young & Sixbey 1988, Taylor et al, 1994). The fact that EBV DNA can be detected in a non inflammatory tissue with no circulating B-lymphocytes may be an arguement for a genital reservoir of EBV (Näher et al, 1992).

Epstein-Barr virus on the cervical mucosa

Sixbey and coworkers (1986) have demonstrated EBV in cervical samples in a population attending an STD clinic and in a population attending a university health service with signs of infectious mononucleosis. Of five women with signs of infectious mononucleosis two had EBV in cervical cell free washings and three of the women with positive findings of EBV had other STD problems. In a female STD population, without any other sexually transmitted diseases and with no signs of inflammation, EBV DNA was detected on the cervix with the PCR technique in 27.7% (13/47) (Näher et al, 1992). Taylor and co-workers (1994) could demonstrate presence of EBV DNA on the portio cervix with the PCR technique in 40%. Our findings (38%) are in line with these findings (V). As there are still very few studies on this subject, the clinical significance of these findings is not clear. But, since EBV has an oncogenic potential (Henle & Henle 1985) we consider it important to further investigate and correlate the presence of EBV to colposcopic findings and cytology.

The association between EBV infection and cervical cancer development has been studied by several groups. In Greenland and Austria the relationship between EBV and abnormalities in cytological smears has been examined, but no correlations were demonstrated (Schön et al, 1992, Hörding et al, 1992). Wong and co-workers (1993) demonstrated EBV DNA in women with carcinoma of the cervix as often as in controls. Hilton and co-workers (1993) could not demonstrate any association between presence of EBV DNA and carcinoma of the cervix. On the other hand, Ida and coworkers (1991) found that EBV early antigen was more often demonstrated in the semen of husbands of women with cervical cancer compared to controls. Landers and co-workers (1993) found 43% of their carcinoma patients to be EBV-positive as compared to none in the group with normal tissue or CIN I, and 8% EBV-positive each in groups CIN II and CIN III.

Young and co-workers (1988) have suggested that the incorporation of EBV into the genome of the cervical epithelial cells at an early age could be an important early event in cervical carcinogenesis. The same hypothesis has been put forward by Wong and co-workers (1991). The role of co-factors in carcinogenesis, e.g. concerning HPV and carcinoma of the cervix, is important (zur Hausen 1982). A possible synergism between EBV and HPV in this context has also been suggested, perhaps by way of the former expressing the gene called BCRF1 (Moore et al, 1990), which codes for an interleukin-10-like molecule (Hsu et al, 1990), which can modulate the immune reaction to both viruses. As the role of EBV in the carcinogenesis of the cervix is not clear, there is a need for further investigations.

Since our results (III, IV) indicated an association between acetowhite, koilocytotic vulvar lesions and EBV, the question was raised if EBV could also be associated with the corresponding cervical acetowhiteness or CIN. Our study (V) demonstrated as much EBV as HPV on the cervix. HPV was correlated to acetowhite lesions on the cervix, but EBV was not (VI). EBV was detected in 30% of the acetowhite lesions, which is in accordance with data from Bevan and co-workers (1989). In normal-looking cervical mucosa EBV was detected in 55%. This finding has to be interpreted with caution, since the study and control groups were selected, coming from a gynaecological and an STD clinic, respectively.

To gain a better understanding of on which tissue level

the EBV-infection is present, different techniques have been applied. Conventional immunohistochemical techniques have not been satisfactory, perhaps due to a highly restricted viral antigen expression and a low viral genome copy number in the affected tissue (Ambinder & Mann 1994). The in situ hybridisation technique, which can detect latent Epstein-Barr virus-encoded RNA (EBER), has been proposed as a possible tool to detect and precisely localise the Epstein-Barr virus. In a first attempt to determine EBV, the in situ hybridisation technique was used on five EBV-positive biopsies to detect EBER. This examination was also performed to exclude the possibility that the EBV DNA was associated with B-lymphocytes. We could not find any EBER neither in epithelial cells nor in lymphocytes. This result, of course, does not exclude the presence of EBV in the tissue.

Epstein-Barr virus on the vulvar mucosa

In our first attempt to detect HPV in acetowhite lesions of the vulva using Southern blot, an unexpectedly low prevalence of HPV was demonstrated (unpublished data). This finding and the clinical resemblance to OHL focused on the possibility of presence of EBV DNA in these lesions.

There are few studies on HPV in the vulvar mucosa. Bodén and co-workers (1988, 1989) have reported good agreement between the clinical, histopathological and virological findings in acetowhite vulvar lesions. There was also a good correlation between the symptoms of vulvo-vaginitis and human papilloma virus infection (Bodén et al, 1988). However, Bergeron and coworkers (1990), could not demonstrate HPV DNA, using the PCR-technique, in a significantly higher degree in biopsies from patients with micropapillomatosis labialis compared to controls. A similar result was obtained in a recent Swedish study (Strand et al, 1995 (b)).

Biopsies from the vulva are more difficult to evaluate concerning the presence of HPV than are samples from the cervix. One possible reason is that the vulva shows a higher degree of squamous differentiation than the cervix. Another explanation may be that several changes indicating a viral effect on the cervix, may represent a normal variation of the vulva (Dennerstein et al, 1994, Strand et al, 1995 (b)). Furthermore, koilocytosis can be demonstrated even when no HPV DNA is detected. Thus, as discussed by Spitzer and co-workers (1990), it is difficult to come to a general agreement on histopathological criteria of HPV infection.

The difficulties in judging what is an evident koilocytosis were illustrated in paper IV. The primary diagnosis of koilocytosis in the vulva biopsies was reevaluated. In only eight cases was the diagnosis of an evident koilocytosis confirmed and in four cases this diagnosis was questioned. The other eight cases were reevaluated as non-specific inflammation without evident koilocytosis. These findings underline the risk of overdiagnosis. Consequently, a false diagnosis of HPV infection might be given to the patient. The combination of acetowhite lesions of the vulva and koilocytosis must be thoroughly considered before the diagnosis of HPV infection is recorded.

EBV was more often detected in acetowhite lesions of the vulva than was HPV, 48% and 17% respectively (III). This finding may appear controversial for several reasons. One is that we have demonstrated a potent oncogenic virus in lesions, which previously have been associated solely to HPV infection. What has also to be questioned and further elucidated is that the expected

virus, HPV, was present in a low degree. One explanation might be that EBV could mimic the clinical picture usually considered to be HPV related.

On the other hand the control group of vulvar patients with no acetowhite reactions was found to be more often HPV positive than the study group. This was an unexpected result as well. One explanation might be use of different collecting techniques by different investigators.

A new series of 20 women with acetowhite lesions on the vulva were examined to evaluate our previous results (IV). Also in this study EBV-positivity was demonstrated more often in the acetowhite lesions, as compared to non acetowhite reactions. Furthermore, HPV was detected as often in the study group as in the control group, which might indicate that HPV only has a minor role in acetowhite vulvar lesions.

Investigating subclinical manifestations of the vulva, Cone and coworkers (1991) demonstrated 14% to be HPV-positive using the in situ hybridization technique. Koilocytosis was demonstrated in only 11% of the biopsied material. Non-specific inflammation was seen in 20%. The low degree of koilocytosis and the relatively high number of nonspecific inflammations are in line with our findings (IV). Altogether, these findings seem to indicate that acetowhite lesions of the vulva may be a nonspecific response to various factors. To elucidate the role of EBV in this context, further studies using the in situ hybridisation technique are mandatory.

Epstein-Barr virus on the penile skin

The findings of HPV and EBV on the vulva prompted us to perform a study on male patients with acetowhite lesions on the penis. HPV DNA was detected in 60%, when using the cytobrush for sampling, and in 35% when examining biopsies. EBV DNA was present in 20% when using the cytobrush for sampling and in none of the biopsies. Näher and co-workers (1992) have demonstrated EBV with PCR in 13.3% (6/45) of a male study group with no clinical signs of inflammation. Our results are in accordance with their findings. In another study Israele and co-workers (1991) could demonstrate EBV DNA in 48% of men with urethral discharge caused by gonorrhea. This result suggests that EBV might be sexually transmitted. The conclusion from our study is that acetowhite lesions on the penis are mainly HPV related, and that these lesions demonstrate koilocytosis histopathologically. However, EBV DNA was present in 20% of these patients, although the clinical relevance of this finding is still uncertain.

The low prevalence of EBV-positive biopsies in males might depend on the preparation of the biopsy material. In the study of the vulva (III) fresh biopsies were examined, whereas in this study of the penis (IV) the biopsies were first fixed in formaldehyde and this is known to decrease the amount of DNA material (Jackson et al, 1990).

Epstein- Barr virus in the oral mucosa

EBV was detected more often in the mouth of men with acetowhite lesions on the penis than in those without these penile reactions. This finding has to be interpreted with caution due to the small number of patients in the study. However, there was one obvious discrepancy between the control group and the study group. Only 5% in the control group had EBV in the mouth and on the penis, whereas in the study group EBV was found in 30% in the mouth compared to 20% on the penis. This finding might indicate sexual transmittance.

Psychological aspects of human papilloma virus infection

A sexually transmitted disease is an undesirable consequence of sexual intercourse and the patients often have feelings of shame. Therefore, it is important that venereologists listen to their patients expressing worries and feelings of guilt. Despite this, there are very few studies on psychological reactions in patients with HPV infection and other STDs.

Filiberti and co-workers (1993) studied female patients with HPV infection in order to better understand the psychological dimension of the disease in relation to different treatments. The investigators demonstrated that about 30% feared their disease might evolve into cancer. Sixteen per cent of the patients reported that the disease gave rise to problems with their partners and the partnership was characterised by aggression, fighting and suspiciousness. Fiftyseven per cent described their sexual life as worse after the illness and its treatment. Persson and co-workers (1993) reported 19% worrying about getting cancer, 10% of the patients stated that the disease had caused separation from their partner and in 17% the disease had influenced the patient's feelings for their partner in a negative way.

Our study of 41 men with earlier HPV infection showed that 40% had been influenced in their sexual behaviour by the knowledge of the presence of the HPV infection. The greatest cause for concern was fear of transmission of a disease that theoretically could give their partners cancer and this feeling was registered in 54%. In 20% the feeling that they were 'dirty' also influenced their sexual life (VII).

Originally 114 men were invited for follow-up. Only 41 men showed up for the reexamination. An analysis of the non-responders was not performed, taking into consideration the delicate situation of these patients.

The present findings underline that physicians dealing with sexually transmitted diseases must be aware of the psychological aspects of their clinical work.

Conclusions

HPV infection is a common STD, found in varying degrees depending on the detection techniques used (I,V). In males, HPV appears clinically as accuminate or papular lesions, associated with low-risk type HPV, as well as subclinical lesions, more often associated with high-risk type HPV and dysplasia (II). Acetowhite lesions of the vulva more often show an association with EBV (III,IV), whereas acetowhite lesions of the penis seem to be associated with HPV (IV). The finding of EBV in acetowhite lesions has not been demonstrated before. The difficulty in judging what is an evident koilocytosis in the vulvar tissue was illustrated. Consequently, a false diagnosis of HPV-infection might be given to the patient. The combination of acetowhite lesions of the vulva and koilocytosis must be thoroughly considered before the diagnosis of HPV-infection is recorded. An association between HPV and acetowhite, koilocytotic lesions was demonstarted on the cervix. EBV was not related to either koilocytosis or dysplasia (VI). This study has also focused on the psychological aspects of HPV infection and on the obvious need for venerologists to keep these aspects in mind (VII).

One critical question concerns the clinical significance of these findings. It could be argued that the viruses detected may constitute part of the normal flora with no pathogenic significance. However, since both EBV and HPV act as serious pathogens, it has been important to discuss our findings in view of this.

"The course of lightning" Alice said very decidedly, for she felt sure about this, "is the thunder - no,no!" she hastily corrected herself. "I mean it the other way". "It's too late to correct it," said the Red Queen. "When you've said a thing, that fixes it, and you must take the consequencies".

Lewis Carroll

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