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Human Papillomaviruses: Are We Ready to Type?

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INTRODUCTION

Human papillomaviruses (HPVs) are the cause of the most common viral sexually transmitted disease, condyloma acuminata. Exact incidence figures are not available, but data analyzed by the Centers for Disease Control (19) indicate that consultations by private physicians for condyloma acuminata increased by 459% from 1966 to 1981. This survey provides an estimate of 900,000 visits to private physicians for condyloma acuminata in 1981. By comparison, the same survey provided an estimate of about 450,000 visits for genital herpes in 1984 (20). In addition, HPVs are associated with the continuum of disease from dysplasias to invasive squamous cell carcinomas, especially in the genital tract.

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Squamous cell carcinoma of the cervix accounts for about 7% of cancer cases among women in the United States and other developed countries but comprises 24% of cancer cases among women in developing countries and contributes significantly to cancer deaths in those countries (139, 144).

Currently, 57 different HPV types, based on deoxyribonucleic acid (DNA) sequence rather than serology, have been identified (E.-M. de Villiers, personal communication). About one-quarter of these are commonly found in the genital tract. The question addressed in this review is whether identification and typing of the HPV present in genital lesions are of diagnostic, therapeutic, or prognostic value. The first part of the review will describe and evaluate the available typing techniques. The second part will contain a compilation of the data gathered on the frequency of association of different HPV types with different genital lesions. Included here will be a brief discussion of the data implicating HPVs in the multistep oncogenic process. The discussion section will then address the question of whether the typing techniques are adequate for large-scale use and whether sufficient data exist to suggest that HPV typing should be routinely conducted. Emphasis will be placed on what effect such typing would have on patient management.

METHODS OF DETECTION OF HPV INFECTION

HPV infection was initially recognized by the clinically apparent lesion condyloma acuminatum. The presence of virus in such lesions was first confirmed by electron microscopy, then by detection of viral antigen, and finally by detection of viral nucleic acid. As the techniques for detecting HPV infection changed from clinical observation to laboratory detection, it became apparent that other HPV infections existed which were subclinical, and indeed, in some instances, apparently latent. The subclinical lesions were detected by the appearance of characteristic cells in Papanicolaou smears, by colposcopy, and by histology. The latent infections were detected only by nucleic acid hybridization. In this section, the various techniques for the detection of HPV (clinical observation, cytomorphology/histology, electron microscopy, immunocytochemistry, and nucleic acid hybridization) will be described with reference to their sensitivity and specificity. Since the purpose of the review is to evaluate whether typing of the HPV in lesions would be beneficial, each technique will be discussed from the standpoint of its ability to provide typing information, its ease of performance, and the types of samples required. As will become apparent, nucleic acid hybridization is the only technique that currently provides information on the HPV type present in the lesion. Commonly used hybridization-based techniques will be discussed in detail in the subsequent section. Although immunocytochemistry does not currently provide typing information, data generated by this technique will be discussed in some detail because of the potential development of typing capability in the future.

Clinical Observations

HPV infection of the genital tract is clinically manifested as condylomata acuminata (and bowenoid papulosis). In addition, subclinical infection can be detected, by colposcopic examination, as the whitening of the epithelium following application of acetic acid. However, while acetowhite cervical lesions contain HPV DNA, extracervical lesions may not. Different HPV types cannot be identified based on the appearance of the lesion.

Cytomorphology/Histology

Cells pathognomonic for HPV infection are the koilocytes, large cells with abnormal (hyperchromatic) nuclei surrounded by a transparent cytoplasm (80), found in the outer layers of a lesion. These are present not only in condylomata but also in dysplasias which continue to exhibit koilocytes or in condylomatous regions adjacent to dysplastic regions. In 1984, Crum et al. distinguished two types of flat condylomata, one of which showed only mild cytological atypia and the other of which showed more marked cytological atypia and abnormal mitoses (28). They proposed that the abnormal mitoses were predictive of the presence of HPV-16 (discussion of the different HPV types will be presented later). However, others have found that such lesions do not exclusively contain HPV-16 and, therefore, that the light microscope cannot be used to type a lesion (67, 70).

Electron Microscopy

Viral particles consistent with the size of papillomavirus virions have been detected in condylomata acuminata, flat condylomata, and dysplasias (35, 41, 54, 62, 90, 117, 118, 131, 145, 176, 177, 188). By using clinical, cytological, or histological criteria as evidence for HPV infection, the sensitivity of electron microscopy for detecting such an infection ranged from 10 to 50% (35, 41, 69, 117, 118, 145, 176, 177). The virions are found only in the koilocytotic and dyskeratotic cells. Since the morphology of all papillomavirus virions is identical and since all papillomavirus types can be found in virions (70), electron microscopy cannot provide typing information. As a means for identifying the presence of HPV per se in a lesion, electron microscopy is limited to those lesions containing at least some highly differentiated cells.

Immunocytochemistry

In 1978, Orth et al. reported that serum from a rabbit bearing a tumor derived from a cottontail rabbit papillomavirus-induced papilloma reacted with human plantar warts (132). The reactivity could be removed by preabsorbing the serum with alkali-denatured HPV virions. Shortly thereafter, Jenson et al. showed that antibodies raised to disrupted papillomavirus virions from plantar warts cross-reacted with all papillomaviruses (68) and Kurman et al. reported that antiserum to disrupted bovine papillomavirus (BPV) virions behaved similarly (86). The papillomavirus antigen is detected by one of several immunohistological methods. Briefly, the peroxidase-antiperoxidase method is performed by incubating a tissue section sequentially with (i) antibodies raised in rabbits to disrupted papillomavirus virions, (ii) excess swine anti-rabbit antibodies, and (iii) a soluble peroxidase-antiperoxidase complex from rabbits. The rabbit antibodies react with the papillomavirus structural antigenic determinants; one combining site of each swine antibody binds to the rabbit anti-papillomavirus antibody, and the second combining site binds to the rabbit antiperoxidase present in the peroxidase-antiperoxidase complex. The reaction is visualized by incubation with peroxide and 3,3′-diaminobenzidine (179). Alternatively, a more sensitive assay, the avidin-biotin complex method, uses a biotinylated second antibody in (ii) above followed by incubation with the avidin-biotin-peroxidase complex (biotinylated horseradish peroxidase combined with avidin) (63). The reaction is visualized as described above. When tested side by side with

the peroxidase-antiperoxidase procedure, the avidin-biotin complex technique was twofold more sensitive in detecting papillomavirus antigen (1).

All sequenced papillomavirus genomes have several potential genes based on nucleotide sequence. The genes are designated early (E) or late (L) depending on their putative time of synthesis (13, 33). The rabbit anti-papillomavirus antiserum, which is now commercially available from several different suppliers, reacts with the major capsid protein, encoded by the L1 gene (44, 93, 123, 198, 201). This protein is predominantly expressed in the outer, highly differentiated cells of the lesions (koilocytes) (1, 41, 45, 68, 69, 84, 86, 191, 193). When the sensitivity of antigen detection was compared parallel with that of detection of virions by electron microscopy, using as evidence for HPV infection the presence of koilocytes, the antigen assay was found to be twice as sensitive as the virion assay; both were less sensitive than cytology or histology (41). It should be noted that the number of antigen-positive cells in an antigen-positive lesion may vary from as few as one to many (61, 147, 180, 217). The unstained koilocytes look identical to the stained ones; thus, it is unclear why some contain detectable levels of antigen and others do not. It has been suggested that failure to detect antigen may relate to sampling error, variation in the time of expression of viral antigens, destruction of antigens during tissue processing, or lack of sensitivity of the assay (61, 69, 84). It has also been suggested that HPV-16 may produce little capsid antigen (7) and thus may escape immunohistochemical detection. Firzlaff et al. have recently reported that antiserum raised to the HPV-16 L1 protein detected antigen in an HPV-31-induced lesion while the commercially available antiserum did not (44). This raises the possibility that new antisera will be developed that increase the sensitivity of the assay.

The specificity of the antigen detection assay (and of the antiserum) has been confirmed in a number of laboratories by analyzing antigen-positive cells for the presence of virions by electron microscopy. Virtually a 100% correlation has been found between antigen positivity and the observation of virions (41, 68, 70, 86, 113, 117). The specificity of the antisera can also be verified by inclusion of appropriate controls: (i) omitting the primary antibody (antibody to virions) from the assay, or substituting it with preimmune serum; (ii) performing the reaction on uninfected tissue; and (iii) removing the reactivity of the primary antiserum by first absorbing it with disrupted virions. While staining is generally confined to koilocytes, staining is occasionally seen in seemingly normal tissue areas (66, 210). The interpretation is unclear but may indicate that some capsid antigen (L1 protein) may be synthesized without overt signs of cytopathic effect.

Currently, the commercially available antiserum can be used to establish the presence of papillomavirus antigens with the following limitations: (i) only approximately 50% of condylomata stain with antiserum raised to disrupted virions (41, 45, 69, 86, 117, 124, 199, 217); (ii) antigen expression is directly related to the extent of differentiation present in the specimen since detection of the L1 antigen is predominantly confined to the nuclei of koilocytes (41, 45, 86, 193, 217). Thus, the percentage of antigen-positive specimens decreases as the severity of the disease increases from mild dysplasia to carcinoma in situ to invasive carcinoma (45, 84, 125, 164, 183, 187, 194, 199). In fact, the percentage of antigen-positive cells in adjacent condylomatous tissue decreases as the severity of the lesion increases (57, 84).

The commercially available antiserum reacts with antigens shared by all papillomaviruses (termed type-common or group-specific antigens) and thus cannot be used as a typing tool. However, efforts are under way to develop antisera to type-specific epitopes (44). Such reagents could then be used in a relatively simple, rapid, and inexpensive typing procedure. In such a case, it would be desirable to detect a papillomavirus antigen present in dysplasias, carcinoma in situ, and invasive cancer as well as in condylomatous lesions.

Nucleic Acid Hybridization

Nucleic acid hybridization offers the only method available for typing the HPV present in infected tissue. Hybridization assays can be conducted under conditions in which virtually all HPVs will be detected, but not specifically typed (low stringency), or under conditions in which the type can be documented (high stringency). Before discussion of the variety of hybridization techniques in use, a brief description of hybridization and the definition of typing will be given in the following section.

DETECTION OF HPV TYPES BY HYBRIDIZATION

In 1979, Law et al. reported that papillomavirus DNA isolated from different species (cottontail rabbit, bovine, and human) contained conserved sequences which could be detected when nucleic acid hybridization conditions were relaxed sufficiently to allow detection of 25% base pair mismatch (91). At that time, neither had the papillomavirus genomes been sequenced nor had their genes been mapped, but two discontinuous regions of homology were identified based on hybridization to restriction fragments separated on gels. Based on the cross-reactivity of capsid antigens, the assumption was that one conserved region must encode a structural protein. These observations have now been extended by both comparison of DNA sequences and heteroduplex analysis to identify the areas of greatest conservation as E1 and L1, followed by E2 and L2 (14, 32, 49). The presence of these conserved sequences has made it possible to detect new papillomavirus types. It is precisely this conservation, however, that has made development of type-specific antisera difficult.

Hybridization Theory

A brief discussion of hybridization theory is included in this review to provide an understanding of parameters that are important to hybridization kinetics. The critical points are that (i) two DNA strands anneal due to the complementarity of their nucleotides; (ii) stability of the hybrids is dependent on base composition, salt concentration, temperature, length of hybrid, and degree of mismatch between the two strands [this can be expressed by the formula: $T_m = 81.5 + 16.6 (\log M) + 0.41 (\% G+C) - 0.72 (\% \text{formamide})$, where T_m is the melting temperature, the temperature at which half the duplexes have dissociated; M is the monovalent cation concentration in moles per liter, and % G+C is the percentage of guanine-plus-cytosine residues in the DNA]; (iii) the optimal rate of duplex formation occurs at 25°C below the T_m of the duplex; (iv) raising the temperature to the T_m denatures half of the duplexes. For a duplex of >150 base pairs, each 1% mismatch decreased the T_m by 1°. Hybridization conditions are commonly defined by the number of degrees below the T_m at which they are done (expressed as T_m − number of degrees). When hybridizations

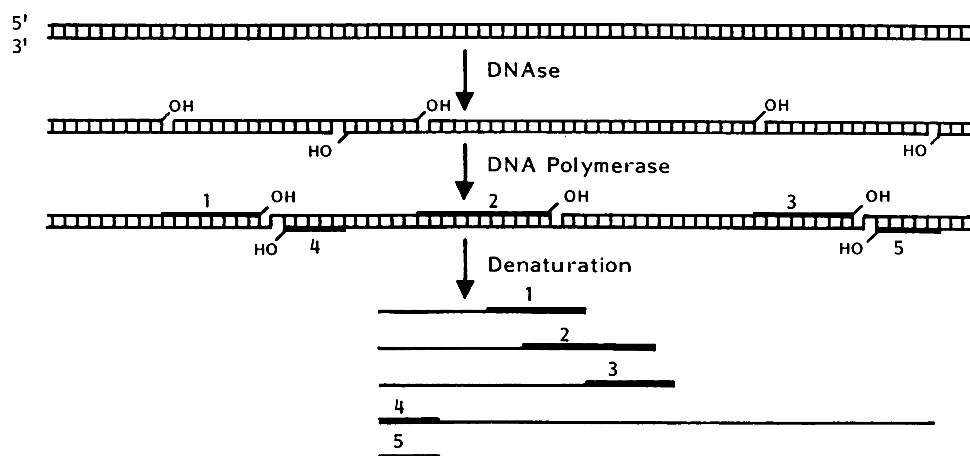


FIG. 1. Generation of a probe by nick translation (157). Single-strand breaks are randomly introduced into the DNA by deoxyribonuclease (DNase) I. *E. coli* DNA polymerase I removes bases in a 5′ to 3′ direction and fills in the gap, resulting in movement of the nick 3′ to its original position. In the presence of radiolabeled or biotinylated deoxynucleotides, the newly synthesized pieces of DNA will become labeled (bold lines). Upon denaturation, a pool of randomly labeled single strands is produced, representative of the entire genome. Vertical lines between the two DNA strands represent multiple base pairs.

are conducted under standard conditions (T_m − 25°), the percent mismatch between the bases on the two strands which is tolerated (i.e., the hybrids will be stable) is approximately 13%. When the hybridizations are conducted under low-stringency conditions (T_m − 43°), the degree of mismatch tolerated is increased to 33% (91). Stringency of hybridization is usually varied by altering the formamide concentration (see formula above). Stringency of the wash conditions is determined by the choice of salt concentration and temperature. The above relationships apply to hybridization between nucleic acids of several hundred nucleotides in length; for shorter oligonucleotides (e.g., those used in the polymerase chain reaction assay; see below), the T_m or dissociation temperature (T_d) is expressed by the formula: $T_d = 4(G+C) + 2(A+T)$, where the letters refer to the number of each base in the oligonucleotide (209). The reader is referred to Lorincz (94) and Wahl et al. (209) for a more complete discussion of hybridization theory.

Generation of Probes

Because the generation of probes plays a central role in evaluating hybridization data, the production of probes used for HPV typing will be briefly described. The HPVs which have been typed have been cloned into bacterial plasmid vectors related to pBR322. Labeling reactions may be conducted on the intact plasmid, in which case both the vector sequences and the HPV sequences will become labeled. Alternatively, the HPV sequence may be released from the vector by digestion with the appropriate restriction enzyme. The HPV sequence is then purified away from the vector by gel electrophoresis. With this method, the labeled DNA is predominantly HPV DNA, although there may be a low level of vector DNA contaminating this reaction. Several reports indicate that biopsy tissue obtained for analysis may be bacterially contaminated. These bacteria may contain DNA which will hybridize with pBR322 DNA (25, 98, 138, 151, 159). Thus, to determine that the hybrids formed between the labeled probe and the extracted DNA are, in fact, HPV DNA duplexes and not bacterial plasmid-related sequences, appropriate controls must be run in parallel to assess the presence of plasmid-related sequences in the biopsy tissue.

In addition to understanding the need for the controls just described, it is also necessary to understand how DNA is labeled to become a probe. This is because the labeling technique affects the interpretation of results generated by the various assays described below. The most common labeling technique is that of nick translation (157) (Fig. 1). In this method, the DNA to be labeled is incubated with deoxyribonuclease I to introduce nicks randomly into the DNA. *Escherichia coli* DNA polymerase I will remove bases processively in the 5′ to 3′ direction (from the nicked site) and fill in that same region with radiolabeled or biotinylated nucleotides which have been added to the labeling reaction. During this synthesis, the original nick is moved 3′ to its original position, giving rise to the name nick translation. The salient feature of this technique is that a pool of labeled DNA fragments of approximately 400 base pairs in length is generated. A hybridization signal (indicative of duplex formation between the probe and DNA isolated from a lesion) may, therefore, indicate that only one fragment from the labeled pool is forming a duplex or that all fragments are involved in duplex formation (that is, that the DNA in the specimen may be identical only to a portion of the HPV type used as probe or may be identical across its entire length to the HPV type used as probe). It is therefore sometimes necessary to refer to the DNA isolated from a biopsy as being "related" to a particular HPV type rather than being identical to it.

There are several alternative labeling procedures, including the random primer method and the synthesis of ribonucleic acid (RNA) probes. The outcome of the random primer technique is similar to that seen by nick translation, but the methodology is different. Primers, consisting of a mixed pool of 6-base-pair oligonucleotides, are annealed to the DNA from which the probe will be generated. These oligonucleotides then serve as primers for DNA synthesis, using the Klenow fragment of *E. coli* DNA polymerase I and radiolabeled nucleotides. The output is a mixture of radiolabeled fragments which span the genome. Alternatively, RNA probes may be synthesized by inserting the DNA of interest into a vector containing an RNA polymerase promoter (128, 181). A radiolabeled RNA probe is then synthesized by using RNA polymerase and radiolabeled nucleotides. The advantage of RNA probes is that RNA-RNA duplexes are more stable than DNA-DNA duplexes, with RNA-DNA duplexes

TABLE 1. HPVs detected in genital tumors

HPV type	Source of cloning	Yr	Reference
6	Condyloma acuminatum	1981	36
11	Laryngeal papilloma	1982	52
16	Cervical carcinoma	1983	39
18	Cervical carcinoma	1984	9
31	Cervical dysplasia	1986	96
33	Cervical carcinoma	1986	5
35	Endocervical adenocarcinoma	1987	97
39	Bowenoid papule	1987	6
42	Vulvar papilloma	1987	6
45	Cervical dysplasia	1987	122
51	Cervical condyloma	1988	129

being intermediate in stability. Thus, the hybridization may be conducted under more stringent conditions (209).

Definition of HPV Types

HPV type designation is based on the degree of DNA sequence homology between different isolates (23). This is unlike other viruses, for which typing is a serological designation. To establish the identity of a new isolate, the following steps are conducted: (i) known HPV types are radiolabeled and used as a panel to which the untyped HPV is hybridized under stringent conditions; (ii) the reactions are treated with S1 nuclease to digest all single-stranded DNA; and (iii) the percentage of radiolabeled DNA which is hybridized to the untyped HPV (and therefore protected from S1 nuclease digestion) is determined. By definition, an untyped HPV DNA which shares >50% homology is called the same type as the HPV DNA with which it forms this duplex. An untyped HPV DNA which shares <50% homology with any of the known HPV types is given a new numerical designation, e.g., HPV-6. Two HPV DNAs which share >50% homology but <100% homology are termed subtypes, designated by a lowercase letter, e.g., HPV-6a and HPV-6b.

Types Isolated from the Genital Tract

The presence of conserved sequences provides a means of detecting new human papillomavirus types through low-stringency hybridization. To assure the orderly designation of new types, the assignment of new HPV types is conducted by E.-M. de Villiers of the University of Heidelberg with the help of an international committee. Currently, 57 HPV types have been accepted by the international committee (de Villiers, personal communication). Those isolated from or frequently found in the genital tract and reported in the literature are shown in Table 1. Several other types have been identified but not yet published.

HPV TYPING TECHNIQUES

Overview

A variety of techniques currently are used to type HPV sequences present in a specimen. These include analyses which require isolation of DNA from the specimen (Southern blot, dot blot, and reverse blot) and those which can be performed directly on tissue sections or scrapes or smears (in situ hybridization, filter in situ hybridization, and, most recently, polymerase chain reaction). As noted above, hybridization conditions can be varied to allow detection of (i) new HPV types (low-stringency hybridization conditions in which case the particular type cannot be designated but the presence of HPV can be established) or (ii) specific HPV types (high-stringency hybridization).

The various procedures for detecting HPV sequences by hybridization will be described below. When data are available, a discussion of sensitivity and specificity of the assay will be given. Pertinent to a discussion of typing is the source of the DNA used in the hybridization, that is, the ease of obtaining sufficient sample for the test and the ability of the test to be applied to previously collected, stored specimens as well as freshly collected specimens. A comparison of the procedures is provided in Table 2.

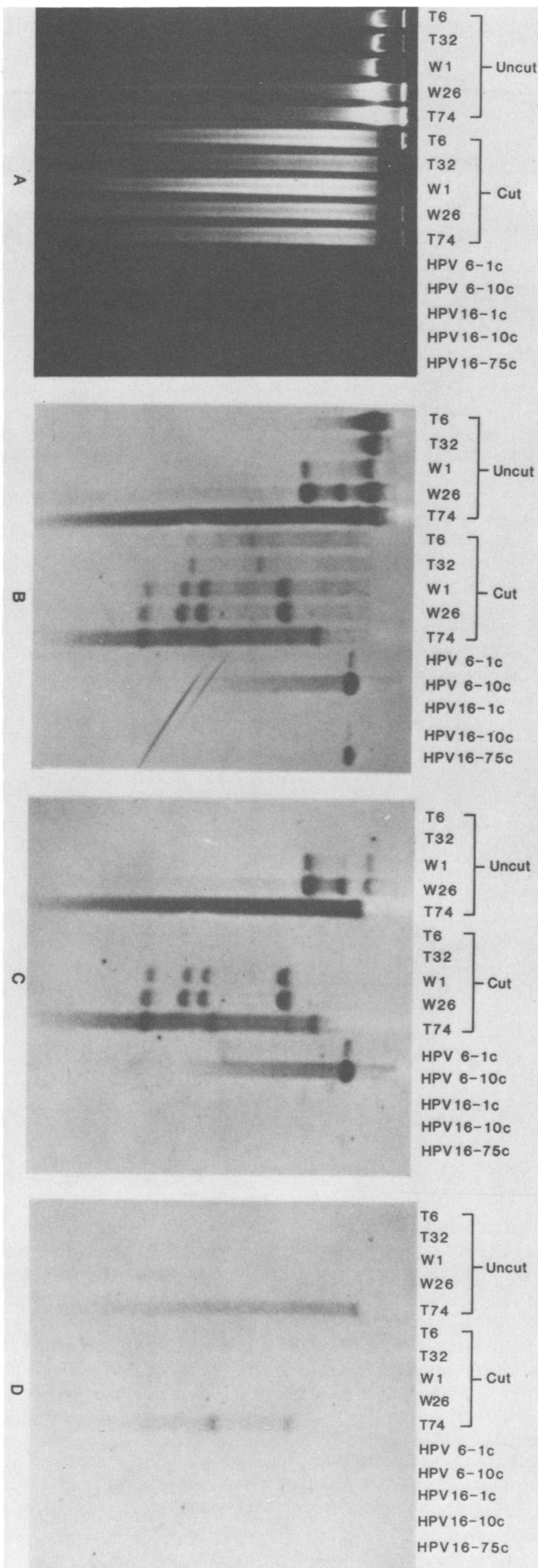
Southern Blot Hybridization

Southern blot hybridization (174) is considered the standard in the field for typing, although, as discussed below, there may be times when genomes will be detected by in situ hybridization and not by Southern blot hybridization. Total cellular DNA is extracted from a specimen and digested with a restriction enzyme, and the fragments are separated by

TABLE 2. Comparison of typing techniques

Technique	Sensitivity (copies/cell)	Advantages	Disadvantages
Southern blot	0.1	Restriction analysis; integrated/episomal; high/low stringency	Requires 10 μg of DNA
Dot blot	1	Requires 500 ng of DNA; rapid	Relatedness only; false-positives; high stringency only
Reverse blot	1	Requires 500 ng of DNA; high/low stringency	Requires individual labeling reactions
Tissue in situ	—[a]	Provides cellular localization in paraffin sections; detects rare + cell	Relatedness only (unless subgenomic probes used)
Filter in situ	—[b]	Allows detection in scrapes/smears; rapid	Similar to dot blot
PCR	0.00001	Highly sensitive; semiautomated	Requires sequence information; contamination; relatedness only

[a] Values vary depending on probe type and detection system used (see text).
[b] Values reported are variable, difficult to express in this format (see text).



electrophoresis on an agarose gel. The restriction endonuclease is sequence specific; thus, the fragments generated are a direct reflection of the presence of this sequence within the genome. Each of the HPV types therefore has a characteristic pattern. This is usually detected by digestion with the restriction enzyme *Pst*I. Following digestion and separation by molecular weight on agarose gels, only a smear of DNA is seen because of the presence of the recognition site throughout the cellular genome. The viral sequences cannot be identified at this stage (Fig. 2A). The DNA is then denatured, neutralized, and transferred to a nitrocellulose (or nylon) filter. The DNA on the filter can first be hybridized with a cocktail containing several labeled HPV types and washed under low-stringency conditions to determine whether any papillomavirus DNA is present (Fig. 2B). The filter can then be washed under high-stringency conditions to determine which of the HPV-containing cellular DNAs contain HPV types present in the probe mixture and which contain other HPVs. HPVs present in the cellular DNA which are identical to the probes will continue to retain the probe on the filter at high stringency, while hybrids formed between the probes and unrelated HPV types will be denatured, releasing the probe from the filter (Fig. 2C).

Suggestive information on HPV type can be obtained by the *Pst*I pattern, if the pattern is as expected. However, there are subtypes which may have slightly altered patterns. Thus, the type can be firmly established only by hybridization with a labeled probe containing a single HPV type. Southern blot analysis allows one to determine whether all fragments of the probe HPV type are present in the biopsy. In addition, this assay is highly sensitive. By using ^{32}P-labeled DNA, picogram quantities of HPV DNA homologous to the probe (0.1 copy of HPV DNA per cell, using 10 μg of cellular DNA as starting material) can be detected. Detection of a heterologous type of HPV DNA is 20- to 100-fold less sensitive (Fig. 2B) (138). When the results of hybridization under low and high stringency are combined, HPV DNA is detected in >90% of squamous cell carcinomas of the cervix (9, 89).

The specificity of the technique is dictated by (i) controlling the hybridization conditions sufficiently to rule out nonspecific hybridization, and including controls on the gel to verify this, and (ii) verifying by the restriction pattern that a genome of the appropriate size (8 kilobases) is identified.

FIG. 2. Detection of HPV DNA present in biopsy specimens by Southern blot hybridization. DNA was extracted from the tissue, using the method of Krieg et al. (82). A 10-μg amount of DNA was cleaved with restriction endonuclease *Pst*I. Uncleaved and cleaved DNAs were loaded onto a 1% agarose gel, and fragments were separated by electrophoresis. Also applied to the gel were gel-purified HPV-6 and HPV-16 copy number controls. The DNA was transferred to a nitrocellulose filter (102) and probed with a cocktail containing gel-purified, ^{32}P-labeled HPV-6b, HPV-18, and BPV type 1 DNA. Following hybridization, the filter was washed under conditions of low stringency (T_m − 42°). After exposure to film, the filter was washed under conditions of high stringency (T_m − 10°). The probe was then removed from the filter, and the filter was hybridized to pBR322. (A) Ethidium bromide-stained agarose gel: light areas represent the presence of DNA. It is not possible to distinguish viral from cellular DNA at this stage. (B) Low-stringency wash condition. The dark bands indicate the presence of DNA hybridized to the probe. (C) High-stringency wash conditions. (D) Hybridization to pBR322. T6 and T32, Two squamous cell carcinomas of the cervix; T74, an adenocarcinoma of the cervix; W1 and W26, two vulvar condylomas. Note the difference in the pattern of uncut DNA seen in the carcinomas versus the condylomata.

To determine whether any hybridization could be due to the presence of pBR322-related sequences, the HPV probe can be stripped from the filter and the filter can be rehybridized with labeled pBR322 (Fig. 2D).

This technique also has the advantage of providing suggestive information on the conformation of the HPV DNA within the cell, that is, whether it is integrated into the cellular DNA or extrachromosomal. However, additional experiments are required to determine accurately the physical state of the HPV DNA.

Although the Southern blot technique is widely respected for its sensitivity and specificity, it has some disadvantages. Like other techniques in which DNA is extracted from the specimen prior to hybridization, the procedure is time-consuming and does not provide any information on the localization of the viral DNA within the specimen. In addition, the DNA must be cleaved with a restriction endonuclease and subjected to electrophoresis prior to the hybridization analysis. Finally, relatively large quantities of DNA (10 μg) are required for this assay to achieve the desired sensitivity. While such quantities may be recovered from scrapes or swabs, they are more reliably recovered from biopsies. A very recent comparative study also raises questions about the reproducibility of typing by this technique in different laboratories (J. L. Brandsma, R. D. Burk, W. D. Lancaster, H. Pfister, and M. H. Schiffman, Int. J. Cancer, in press). Replicates of isolated DNA were sent to four separate research laboratories with extensive experience with the Southern blot technique. The typing results from the four laboratories were not always in agreement. In 17 of 38 samples, at least one laboratory disagreed either on the presence or the type of HPV detected.

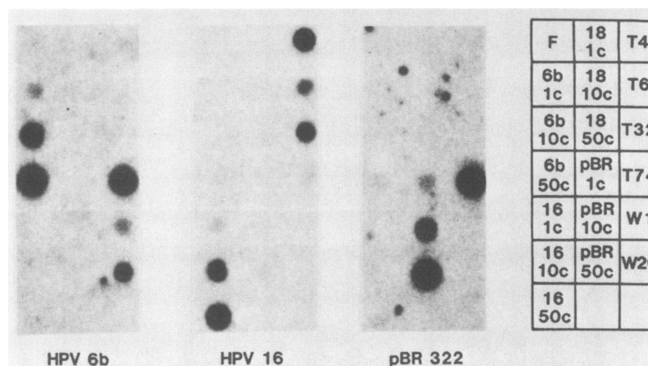


FIG. 3. Detection of HPV DNA present in biopsy specimens by dot blot hybridization. DNA was extracted as in the legend to Fig. 2. A 500-ng portion of DNA was denatured, neutralized, and applied to a nitrocellulose filter. The order of the samples is shown at the right. In addition, 1, 10, and 50 copies of gel-purified HPV-6, -16, and -18 DNA and 1, 10, and 50 copies of pBR322 were included on the filter. The filters were hybridized either with gel-purified, ^{32}P-labeled HPV-6b or HPV-16 or with pBR322 DNA; wash conditions were calculated to yield a T_m − 0.3° (73). These conditions were chosen to improve the specificity of the technique. The darkened areas represent the autoradiographic signal formed by the exposure of film to the radiolabeled, hybridized DNA. F, A foreskin which does not contain HPV DNA (the negative control); 6b, 16, 18, and pBR: HPV-6b, HPV-16, HPV-18, and pBR322, respectively; c, number of copies; T4, a squamous carcinoma of the cervix; other labels as in the legend to Fig. 2. Note that the dot blot results and Southern blot results are identical, although more information is gained from Fig. 2. By comparison of size and intensity of spots in the samples with spots in the controls, it is possible to arrive at an approximate copy number (number of HPV genomes per cell).

Dot Blot Hybridization

As with the Southern blot technique, total cellular DNA is extracted from a specimen in the dot blot technique. However, no further manipulations of the DNA are necessary and only 300 to 500 ng of DNA per membrane is required. The DNA is denatured, neutralized, and applied to a nitrocellulose (or nylon) filter by using a dot blot manifold. Such a manifold contains 96 wells to which DNA can be applied. Replicate nitrocellulose filters can then be probed with the available HPV probes. An example of such a blot is shown in Fig. 3. Appropriate controls on each filter include (i) copy number controls, in which known amounts of each of the HPV DNAs are included to allow confirmation of the specificity of the different probes and an approximation of the number of HPV genomes present per cell; (ii) pBR322, to monitor its presence in the probe; and (iii) 500 ng of total cellular DNA which does not contain HPV DNA as a control for nonspecific hybridization. In addition, a parallel blot should be probed with pBR322 to assess the presence of sequences related to the plasmid. It should be noted that dot blot hybridization can be conducted only under high-stringency conditions. Although there are reports in the literature of low-stringency hybridizations with dot blots, it is more common to find that all samples, negative or positive, will hybridize with the probe under these conditions. This is presumably because all of the DNA is focused on a single spot. In addition, care should be taken in interpreting dot blot hybridization results which indicate that more than one HPV type is present in the total cellular DNA. When a high copy number of one DNA is detected, it has been noted that other HPV probes hybridize as well, but in fact, the latter will be false-positive signals. Following the logic outlined in the previous section, the dot blot technique allows one to say only that the DNA to which the probe hybridizes is related to that probe type, but not necessarily identical across its entire length. This technique allows analysis of DNA when only a small quantity is recovered from a biopsy or scrape or smear and is a relatively quick assay to perform. However, because of the potential pitfalls mentioned above, it is essential to include all of the appropriate controls. It is also desirable, when possible, to confirm mixed infections and unexpected results by Southern blot analysis. The level of sensitivity of the dot blot assay (1 to 2 pg of HPV DNA) is approximately one copy per genome equivalent when 500 ng of cellular DNA is applied to the filter (Fig. 3).

Reverse Blot Hybridization

Reverse blot hybridization is the reverse of the Southern blot analysis. In this case, rather than the cellular DNA being placed on nitrocellulose and labeled HPV probes being used, the known HPV types are placed on the nitrocellulose and the cellular DNA is radiolabeled. This procedure was first introduced by de Villiers et al. (37). A 1-μg portion of each of the cloned HPV types is cut with the appropriate restriction enzyme to release the HPV sequence from the vector sequence, and the two fragments are separated on an agarose gel, running each clone of DNA in a different lane. The DNA from this gel is then transferred to nitrocellulose or nylon and probed with 500 ng of cellular DNA which has been radiolabeled. Any HPV DNA present in the cellular DNA will hybridize to the HPV DNA type to which it is complementary. In addition, if bacterial contamination is present in the biopsy sample, there will be hybridization to

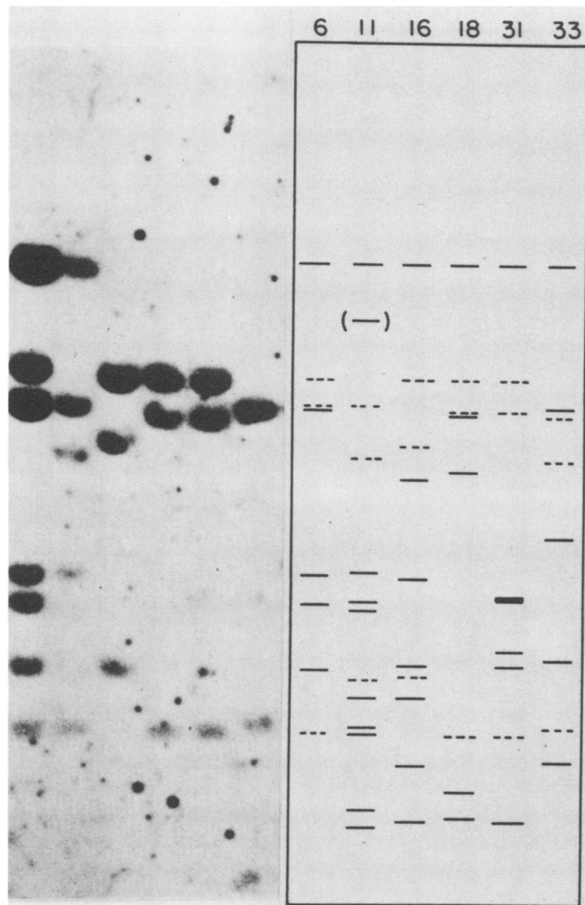


FIG. 4. Detection of HPV DNA in a biopsy specimen by the reverse blot method. HPV-containing plasmids were digested and analyzed as described in the text. T-74 DNA (from an adenosquamous carcinoma of the cervix) was radiolabeled and used as the probe. The drawing at the right indicates the position of HPV fragments (solid lines) and plasmid sequences (dashed lines); the line in parentheses indicates BPV DNA present in the HPV-11-containing plasmid. The top solid line in each lane represents the position of full-length HPV DNA; the other solid bands represent the subgenomic fragments generated by digestion with *Pst*I and the enzyme used to excise the HPV DNA from the plasmid. Note that ^{32}P-labeled T-74 DNA hybridizes to all fragments of HPV-6 (and faintly to HPV-11 due to cross-reactivity) but not to other HPV types. This indicates that the specimen contains HPV-6 DNA but none of the others in this test bank.

the pBR322 (or related vector) fragment. This technique is similar to the dot blot technique in that it allows one to conclude only that the DNAs are related but not whether they are related over their entire length. A modification introduced by Webb et al. in 1987 addresses this limitation (212). For a given HPV type, the agarose gel contains not only HPV DNA released from the vector, but also a *Pst*I digest of the DNA (Fig. 4). Thus, one can determine not only whether the cellular DNA hybridizes to full-length HPV DNA, but also whether it hybridizes to all of the *Pst*I subfragments of the HPV genome. This technique also has the advantage over dot blots in that, by separating the DNA by electrophoresis, the DNA is not all focused on one point of the nitrocellulose, and thus low- as well as high-stringency hybridization can be conducted. The disadvantage of the protocol is that it requires a separate labeling reaction for each of the cellular DNAs to be analyzed. The level of sensitivity of this assay has been estimated to be one copy per cell (212). Of 20 clinical specimens assayed by both Southern blot and reverse blot for comparison of sensitivity, Fife et al. (43) found that 13 of 20 specimens were positive by both Southern blot and reverse blot, 1 of 20 was positive only by reverse blot, and 6 of 20 were positive only by Southern blot.

In Situ Hybridization

The in situ hybridization technique (also referred to as tissue in situ hybridization to distinguish it from filter in situ hybridization) differs from those discussed thus far in that DNA is not isolated from the tissue, but rather tissue sections (or smears) are probed directly (8, 58, 60). It therefore provides information on the localization of HPV genomes within the specimen. The in situ hybridization technique detects many more HPV-positive cells than does the immunological detection of antigens (8, 24, 58, 121, 135, 216). In condyloma, the viral DNA copy number increases as the keratinocytes differentiate (8, 55, 110) (see Fig. 5), with viral DNA and RNA occasionally being detected in the basal and parabasal cells of condylomata (8, 181, 196). In cervical intraepithelial neoplasia (CIN) and invasive carcinomas, clusters of labeled cells are found, generally in the areas of differentiation (30, 181). HPV-containing cells are also occasionally detected in the basal or parabasal cells of CIN lesions (24, 137). In carcinomas it is often difficult to detect HPV DNA in any cells; however, isolated cells or groups of cells occasionally will have a high copy number (18, 135, 137).

That the copy number varies within a specimen (be it a condyloma or a carcinoma) indicates that the copy number determinations made on extracted DNA provide one only with an average copy number per cell. This makes it very difficult to assign a level of sensitivity to the in situ assay. The probes used for this technique have usually been ^{3}H-labeled (135), ^{35}S-labeled (58), or biotinylated (8) DNA. Based on the ability to detect HPV DNA in tissue culture cell lines containing known copy numbers of HPV DNA, the best estimate for the sensitivity of the ^{35}S-labeled DNA probe is 50 to 100 copies per cell (24) and that for the ^{3}H-labeled DNA probe is 5 copies per cell (137). The sensitivity of biotinylated DNA probes may vary from as low as 800 copies per cell (30) to comparable to or better than ^{35}S-labeled probes (195). Each probe has its advantages: (i) the biotinylated probe is more stable than radiolabeled probes, provides a result in a few hours after completion of the hybridization, and avoids exposing the worker to radioactivity; (ii) the tritiated probe gives a very low background, and thus the emulsion can be exposed for long periods of time; and (iii) ^{35}S-labeled probes have a higher background but yield results more rapidly than the tritium probes. For the same reasons given above, these results again reflect only the relatedness of two DNAs to each other, not full genome identity, unless subgenomic probes are used.

With DNA probes it is possible to selectively detect either DNA or RNA: if the tissue sample is treated with ribonuclease and the DNA is denatured, DNA will be detected; if there is no denaturation step (and no ribonuclease is added), RNA will be detected. Detection of RNA increases the sensitivity of the assay since the number of HPV messenger RNA molecules per cell is greater than the number of DNA molecules. Specificity controls for these hybridizations include the following: (i) hybridization with plasmid alone to

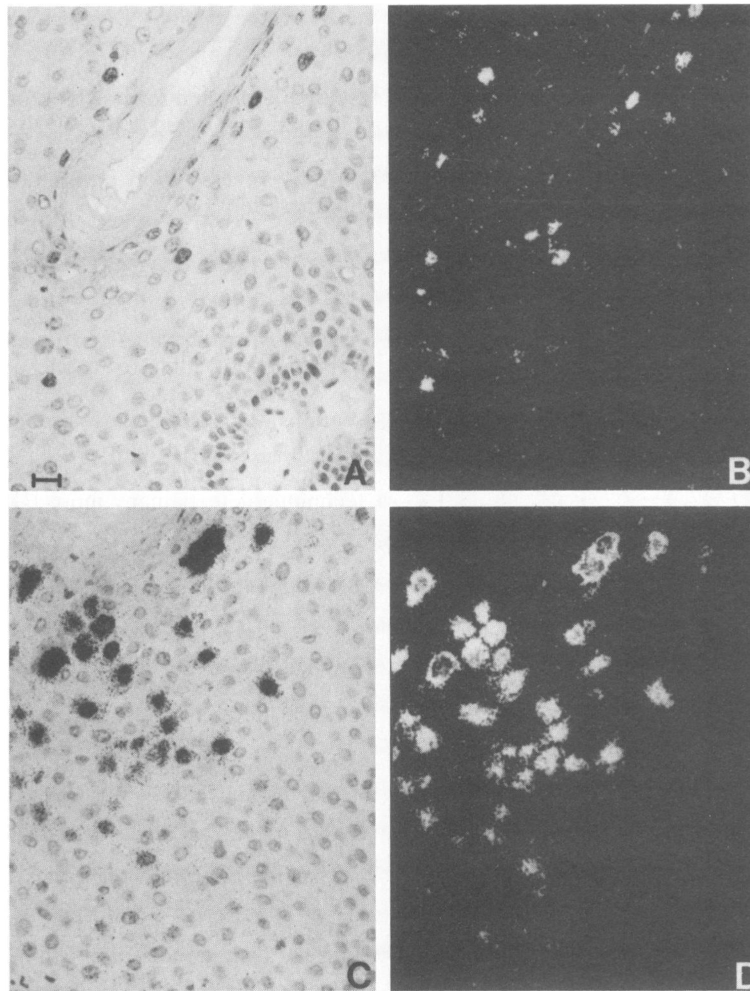


FIG. 5. Detection of HPV DNA and RNA in a vulvar condyloma acuminatum by in situ hybridization. (A, B) To detect DNA, sense ^{3}H-labeled HPV-11 RNA was hybridized to tissue which had been denatured. The signal is confined to the nucleus and is most intense in the superficial cells. (C, D) To detect RNA, antisense ^{3}H-labeled HPV-11 RNA was hybridized to an adjacent tissue section which had not been denatured. The RNA signal is more intense and diffuse and is present in both the nucleus and the cytoplasm. The bright field (A, C) emphasizes morphology; dark field (B, D) emphasizes silver grains representing autoradiographic signal. Note the greater sensitivity achieved with the antisense probe and dark-field illumination. Bar, 25 μm. (Figure courtesy of Mark H. Stoler.)

detect any pBR322-related sequences, (ii) treatment with deoxyribonuclease I prior to hybridization, and (iii) no denaturation of DNA but inclusion of the ribonuclease step.

Recently, radiolabeled and biotinylated RNA probes have also been used (31, 129, 167, 181) (Fig. 5 and 6). Sense RNA probes (RNA which is identical to messenger RNA) will detect DNA copies; antisense RNA probes, hybridized to nondenatured tissue sections, will detect messenger RNA. The RNA probes are more sensitive and specific than the comparable DNA probes (Fig. 5 and 6) because (i) they can be labeled to a higher specific activity, (ii) they have a higher affinity for RNA, and (iii) the T_m for RNA-RNA hybrids is 10°C higher than for the comparable DNA-DNA hybrids (31, 181, 209, 216). The background signal can be reduced by treating the sections with ribonuclease after hybridization. The use of dark-field rather than bright-field illumination further increases the assay sensitivity (181) (Fig. 5 and 6). The degree of relatedness of the cellular DNA to the HPV type present in the probe can be established by using a series of RNA probes representing different regions of the HPV genome.

The in situ hybridization technique has several advantages. It can be performed in retrospective studies with paraffin-embedded tissue and provide information on the location of genomes within the tissue. In fact, in situ hybridization can be used to document the continuity of an HPV type in a histological section which contains dysplastic regions merging into invasive carcinoma (215). When the DNA is present only in a subpopulation of cells, hybridization might be detected by using the in situ method but not the Southern method (30, 60, 135). In addition, in the other procedures, when two different HPV types are detected in isolated DNA it is not clear whether those DNAs reside in the same cell or different cells. This question can be addressed by in situ hybridization when sequential sections can be probed with different HPV types (60). Finally, the in situ technique can provide information on gene expression in different cells within the lesion (12, 22, 121, 128, 181).

Filter In Situ Hybridization

In filter in situ hybridization, which allows the detection of DNA sequences present in smears or scrapes, cells are applied directly to a filter, and then the filter is treated so as

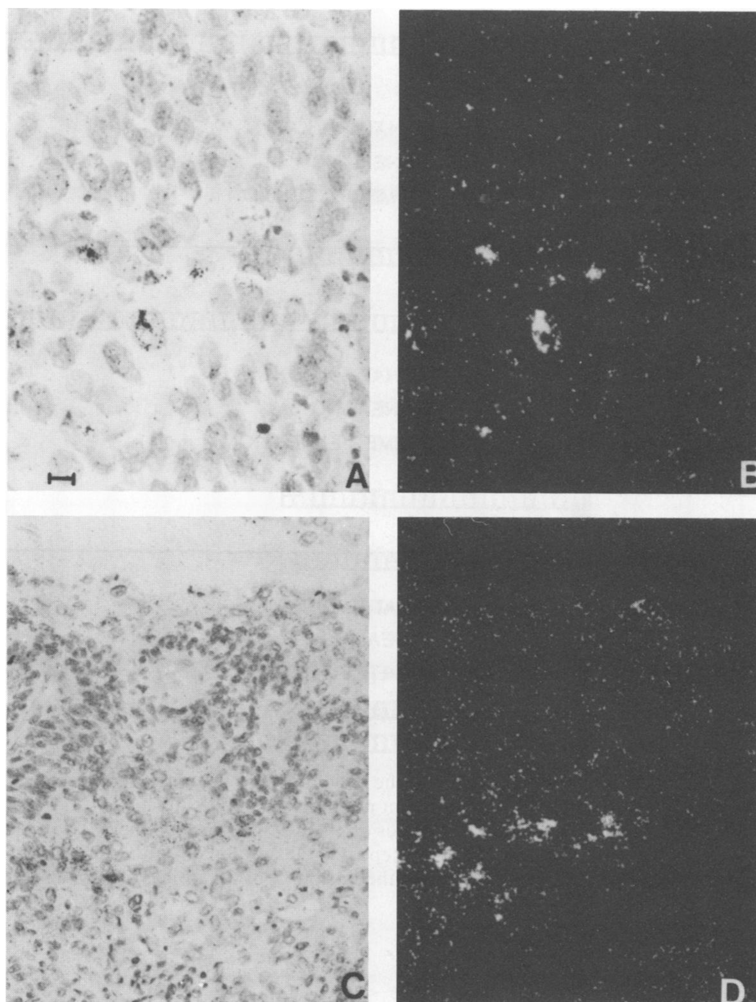


FIG. 6. Detection of HPV DNA and RNA in an invasive cervical squamous cell carcinoma by in situ hybridization. (A, B) DNA was detected by hybridization of a sense ^{3}H-labeled HPV-16 RNA probe to denatured tissue. (C, D) RNA was detected by hybridization of antisense ^{3}H-labeled HPV-16 RNA to an adjacent nondenatured tissue section. (A, C) Bright field; (B, D) dark field. Note that the concentrations of DNA and RNA seen in the carcinoma are significantly lower than those seen in the condyloma (Fig. 5). The bar in panel A represents 12.5 μm in panels A and B and 25 μm in panels C and D. (Figure courtesy of Mark H. Stoler.)

to disrupt the cells and denature and neutralize the DNA (38, 105, 127, 151, 166, 208). This technique avoids DNA extraction procedures but, similar to the dot blot, suffers from a potential to give false-positive results. Also, a high copy number of one type may result in detection of hybridization to other types as well (38).

The sensitivity of this assay was estimated by Wagner et al., who determined that 100 BPV-transformed cells, carrying 100 copies of BPV per cell, had to be present on the filter for a positive result (208). Similarly, Caussy et al. were able to detect 100 CaSki cells (cervical carcinoma cells containing 500 copies of HPV-16 DNA per cell [220]) (18). In contrast, Cornelissen et al. reported the lower limit of detection to be 10^{5} CaSki cells (25). A limited number of studies have been conducted comparing this method with others. Schneider et al. reported that five samples positive by the filter in situ hybridization technique were also positive by Southern analysis (166). de Villiers et al. (38) estimated that the filter in situ hybridization technique is one-half to one-third as sensitive as the Southern technique, in part, because in a number of instances the signal was too close to the noise level and thus was conservatively scored as negative. Comparing the percentage of cervical smears scored as HPV positive by dot blot versus filter in situ hybridization, Cornelissen et al. found the latter to be approximately one-third as sensitive as the former (25). Specificity controls have included (i) having a second filter in the same reaction containing cells that do not carry HPV DNA and (ii) removing the probe and hybridizing with pBR322, or using pBR322 as a probe on a second filter (25, 151, 166). McCance et al. compared the results of sampling the entire cervix and detecting HPV DNA by filter in situ hybridization with Southern analysis of DNA extracted from a colposcopically directed biopsy and found good correlation between the two. However, in some instances, either the smear or the biopsy, but not both, was positive (105). Two swab samples taken 1 week to 3 months apart were analyzed cytologically and by filter in situ hybridization by Schneider et al. (166). In two-thirds of the patients there was repeated cytological evidence of disease and repeated evidence of HPV DNA; specimens from one third of the patients were initially positive by both assays but were negative by both assays the second time.

The specificity and sensitivity of Southern analysis, tissue

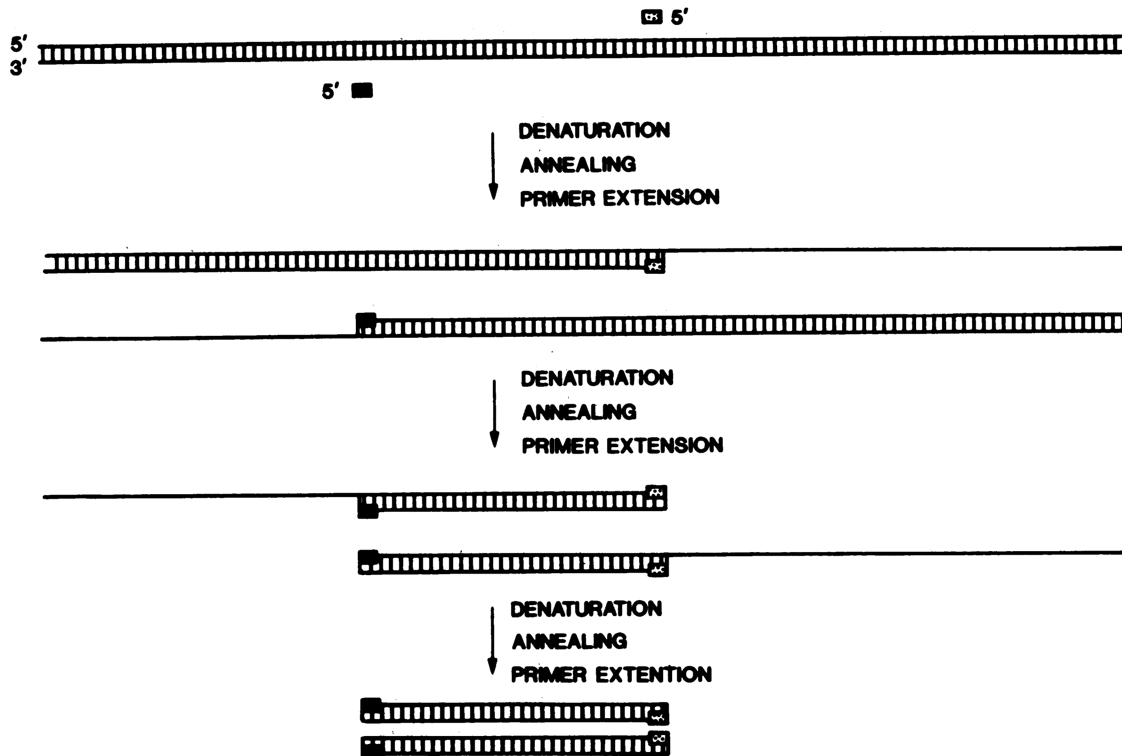


FIG. 7. Amplification of a targeted sequence of DNA by PCR. The solid and stippled block represent two oligonucleotides bordering the sequence of interest (amplifying primers). The vertical bars represent multiple base pairs. When mixed with denatured DNA and allowed to anneal, these primers will hybridize to their complementary sequences on the DNA. DNA polymerase will then use the DNA as template and the oligonucleotide as primer to synthesize a new strand of DNA. Repeat cycles of denaturation, annealing, and primer extension will yield a pool of amplified target sequence; one such sequence is shown at the bottom. For a more detailed illustration of the method, the reader is referred to Oste (134).

in situ hybridization, and filter in situ hybridization have recently been compared on parallel samples by Caussy et al. (18). In this case the standard against which all were compared was the diagnosis of disease. They found that all techniques gave comparable results for condylomata (a sensitivity of 72 to 76%) but for carcinomas, the tissue in situ hybridization was less sensitive (30%). The specificities of all three techniques were roughly comparable (ranging from 87% by Southern analysis to 93% by tissue in situ hybridization). However, given that HPV may be present without signs of disease, it is difficult to be certain that those specimens scored as positive but with no indicators of disease are, in fact, false-positives.

PCR

Polymerase chain reaction (PCR) is the newest technique to be introduced (119, 161, 162). Like the in situ hybridization technique, the PCR can be conducted on paraffin-embedded sections (without extracting DNA first) and thus can be used in retrospective studies (169). The procedure relies upon being able to identify a region of the HPV genome that is conserved even among subtypes of a given type so that all subtypes can be detected. It would be most advantageous, but not an absolute requirement, if this sequence also was type specific. A stretch of nucleotides at the 5′ end of the targeted region and a second stretch complementary to the 3′ end of this region are synthesized (Fig. 7). These oligonucleotides are referred to as the amplifying primers. When these amplifying primers are allowed to anneal to the total cellular DNA (following denaturation of the latter), they will form duplexes with the HPV DNA if it is present. In the presence of DNA polymerase each strand will be elongated. If this cycle is repeated on the order of 30 times, approximately 1 μg of the targeted region of HPV DNA will accumulate. This product is easily visible on an agarose gel, thus suggesting the presence of the HPV sequence in the tissue. This can be verified by transferring the piece of DNA to nitrocellulose and probing with the HPV type presumed to be present. Alternatively, one may take advantage of known restriction sites within the HPV DNA to demonstrate that the amplified DNA contains the expected sequence. Verifying the authenticity of the amplified sequence may be necessary for two reasons: (i) to ensure that sequences complementary to the amplifying primers do not fortuitously happen to be present in cellular DNA which does not contain HPV DNA, and (ii) to verify the HPV type. The need for verification is the following: if the sequences chosen for amplification are conserved among HPV types, then the finding of an amplified product does not implicate a particular HPV type. Second, it should be possible, based on the known sequence, to predict the size of the amplified product. Thus, when that product is found, one should feel reasonably confident that the particular HPV is present. However, two different HPVs may give the same size product, or subtypes of a given HPV may give different size products. Thus, hybridizations should be used to confirm identity until confidence is built that the interpretation of the results is straightforward. It should be pointed out that, as

with some of the other techniques described previously, the PCR technique identifies only type-related sequences, unless multiple targets are amplified, since only a small portion of the genome is analyzed.

The advantages of the PCR technique are that of (i) allowing detection of DNA within a sample without extracting the DNA and (ii) increasing the sensitivity of the assay by amplifying the DNA present in the lesion. It is estimated that as few as one copy per 10^5 cells can be detected by this technique (161). In addition, parts of the procedure can be automated, although the technique is moderately labor intensive as currently performed. A potential limitation of PCR, however, is that it requires some knowledge of the DNA sequence which one wishes to detect. To use PCR as a general typing technique, a series of primers will be needed which can either, of themselves, identify types or sort out types following hybridization (or digestion with restriction enzymes). Then a series of paraffin sections could be incubated with sets of primers (the number of primer pairs that can be added to one reaction has not been established) and the products could be analyzed. Whether currently untyped isolates can be detected by this means depends on whether a universal pair of primers exists which can be used to detect HPV sequences (i.e., a region conserved among all HPV types). A further concern with use of this technique is the possibility that false-positive results may occur due to DNA contamination (or carryover); any contaminants are greatly amplified by this system.

Other Methods

Nucleic acid sandwich hybridization. Nucleic acid sandwich hybridization was designed to avoid the use of autoradiography to detect the presence of hybrids (149). In brief, nonoverlapping fragments of a given HPV type are cloned into bacteriophage M13 and bacterial plasmid pBR322. The M13-HPV DNA is bound to a filter. The pBR322-HPV sequences are radiolabeled. The radiolabeled DNA and disrupted cells from a cervical smear are then added to the filter under hybridization conditions. If HPV DNA is present in the specimen, it will anneal to (be captured by) the M13-HPV DNA on the filter and also anneal to the radiolabeled HPV DNA. Unbound radiolabeled HPV DNA can be washed off and the radioactivity remaining bound to the filter can be counted rapidly in a scintillation counter. The bound radioactivity is indicative of the presence of HPV DNA in the specimen. The detection level is one- to fivefold greater than with the dot blot method (140, 141). Although this method has been used to detect other viral infections, it has not been used extensively to type papillomaviruses.

Commercially available hybridization kits. Kits for determining HPV types are now available, for research purposes only. Enzo Diagnostics, Inc. (New York, N.Y.), provides a kit that uses biotinylated DNA probes for in situ hybridization to frozen or Formalin-fixed tissue sections. Life Technologies, Inc. (Gaithersburg, Md.), has three kits available, one for detection of HPV DNA present in cervical swabs, one allowing specific typing of the HPV DNA from cervical swabs or tissue sections, and one for typing DNA within tissue sections. The first two kits use radiolabeled RNA probes and a modification of the dot blot technique. The last kit uses a biotinylated probe to detect DNA by in situ hybridization. The literature accompanying all kits indicates that they have a high sensitivity and specificity when compared with Southern blot hybridization. None of the kits has been tested widely in many laboratories; thus, their general utility remains to be determined.

SPECTRUM OF HPV INFECTION

Manifestations of Genital Papillomavirus Infection

The association of HPV with warts has long been recognized. The recently developed sensitive assays for HPV nucleic acid sequences described above have demonstrated that a variety of other conditions may be associated with HPV infection as well. This spectrum of clinical manifestations ranges from inapparent to overt lesions and includes both benign and malignant conditions.

Asymptomatic infection. The propensity of HPV-induced lesions to recur has led to the speculation that HPV is able to persist in tissues in an asymptomatic or latent state. This phenomenon was first directly demonstrated in the respiratory tract of children with recurrent respiratory papillomatosis (178), a condition which usually results from contamination of an infant with HPV while passing through an infected birth canal. In this study, HPV DNA was demonstrated in both the respiratory papillomas and surrounding, clinically and histologically normal tissue. Similarly, HPV DNA has been demonstrated in normal skin of the genital area in patients with genital warts (42), in the normal cervix (26, 38, 43, 165, 201), and in histologically normal tissue in the area of HPV-associated genital cancer (101). HPV DNA has also been identified in a small number of normal newborn foreskins (159). Whether the latter finding represents contamination or true infection remains to be determined. The DNA present in these normal tissues is extrachromosomal; the extent to which viral genes are expressed has not been studied.

Classical condylomata. Classical condylomata, a benign, hyperplastic lesion, is probably the most frequently recognized manifestation of HPV infection. The lesions may have various gross and histologic appearance depending on the type and location of the infected epithelium. For example, lesions occurring on cutaneous epithelium (such as the vulva, penis, or perianal area) are keratinized, while those on mucosal epithelium (such as the vagina, cervix, or rectal mucosa) are softer and show little or no keratinization. Most hyperplastic lesions show proliferation of the suprabasal layers of the epithelium, the stratum spinosum, and the stratum granulosum, which may occur by either continued cell division in these layers (as evidenced by suprabasal mitoses) or delayed maturation of the infected cells. Lesions occurring on cutaneous epithelium also show an increase in the outer, keratinizing layer, the stratum corneum. As noted in an earlier section, viral DNA is present in all layers of infected epithelium down to and including the basal layer. Expression of HPV genes in hyperplastic lesions is closely linked to cellular differentiation, with structural proteins expressed predominantly in the upper, more differentiated layers of the epithelium. Although transcription of HPV DNA in lower layers of the epithelium has been difficult to demonstrate, recent studies suggest that expression of HPV genes occurs down to the basal layer (12, 22, 121, 128, 181).

Subclinical infection. The term *subclinical papillomavirus infection* was first applied to the cervix (154) and referred to lesions that were not visible on routine inspection but that showed histologic changes similar to those found in warts. It had been shown previously that such lesions could be visualized by applying 3% acetic acid and examining the cervix under magnification with a colposcope (153). Other investigators have referred to this lesion as a "flat condyloma" (111, 112). The distinction between this lesion and dysplasia (see below) is sometimes unclear, but the term

should be limited to those lesions that show koilocytosis but no basilar cell maturation abnormalities. Difficulties arise because the superficial layers showing koilocytosis may show nuclear atypia which is suggestive of dysplasia (28). More recently, the concept of subclinical HPV infection has been extended to other anatomic sites such as the penis (4). As in the cervix, these lesions can been seen by applying 3% acetic acid and examining the area under magnification. There is evidence of HPV gene expression in these subclinical lesions in that papillomavirus structural protein can often be demonstrated by immunohistology in biopsies of the lesions (152).

Dysplastic changes. Dysplasia implies an abnormality of cellular growth and development. Its use in a pathological description suggests a premalignant abnormality. The association of HPV with dysplastic growth has been best studied in the cervix. CIN is commonly used to describe these cellular abnormalities. CIN is graded depending on the proportion of the thickness of the epithelium that is replaced by abnormal cells. According to this system, lesions with less than one-third of the epithelium replaced with immature (basal-like) cells are graded as CIN I, those with one-third to two-thirds of the epithelium replaced are graded as CIN II, and those with more than two-thirds of the epithelium replaced (including carcinoma in situ) are graded as CIN III (156). By using HPV capsid antigen detection (86, 108, 180) and DNA hybridization techniques (9, 28, 39, 95, 104, 166, 208, 212, 214), numerous studies have shown an association between HPV infection and CIN. CIN can occur in the absence of cytologic features of HPV infection (i.e., koilocytosis), although many of these lesions can be shown to contain HPV DNA (38, 212). It is currently unclear whether lesions showing no koilocytosis and no HPV DNA occur without HPV infection or whether all CIN lesions are HPV associated, with some containing HPV DNA which is not detected by current methods. As noted earlier, the amount of HPV DNA present and the degree to which HPV genes are expressed are inversely related to the degree of dysplasia; that is, CIN I lesions generally have larger amounts of HPV DNA and are more likely to express HPV capsid antigen than CIN III lesions (167, 180). HPV DNA can be demonstrated throughout the epithelium (167), and synthesis of HPV transcripts has also been shown to occur in all layers of the epithelium in CIN lesions (31, 121, 128). Dysplastic changes have also been associated with HPV in the vulvar (17, 27, 59) and penile (4) epithelium.

Malignant changes. Given the association between HPV and dysplasia discussed above and the fact that dysplasia is considered a premalignant lesion, an association between HPV and malignancy is logical. The association is strongest with two types of cancers, skin cancers occurring in patients with epidermodysplasia verruciformis (133, 136) and genital tract cancers. Skin cancers will not be further discussed. Numerous studies have demonstrated the presence of HPV DNA in cervical cancers (5, 9, 39, 46, 47, 50, 56, 83, 97, 104, 137, 172, 221), and several have also demonstrated transcription of HPV genes in these cancers (64, 92, 181, 203). In addition to the demonstration of HPV DNA in primary cervical tumors, HPV DNA has also been demonstrated in metastatic lymph node lesions in patients with HPV-containing cervical cancer (3, 88). Cell lines derived from cervical cancers (such as the long-established HeLa cell [49]) have likewise been shown to contain and express HPV DNA (2, 9, 64, 143, 168, 170, 171, 173, 175, 203, 220). Other genital tract cancers such as vulvar (130, 148, 184, 222) and penile (106, 206) carcinomas have also been shown to contain HPV DNA. In contrast to the benign and dysplastic HPV-associated lesions, the HPV DNA in most (but not all) malignant lesions has been found to be integrated into cellular DNA (21, 40).

HPV Types Associated with Different Lesions

Shortly after the recognition of multiple different HPV types, it became clear that certain HPV types were more closely associated with specific lesions than were others. Conversely, certain clinical or histologic HPV-associated lesions were usually found to contain a limited number of HPV types. In the genital tract, certain generalizations about HPV types associated with specific lesions have resulted from many studies (3, 9, 21, 28, 34, 36, 37, 39, 46, 51–53, 59, 74, 83, 85, 95, 104, 106, 108, 114, 142, 151, 166, 184, 197, 201, 208, 212–214). Several of the larger surveys of HPV type-specific association with certain lesions are summarized in Tables 3, 4, and 5. It should be noted that these studies were conducted at different times, on different populations, and using different techniques and thus cannot be directly compared. In addition, a number of studies found more than one HPV type in a lesion, and such lesions are listed in the tables as positive for each type found. In aggregate, these studies suggest that HPV types 6 and 11 are most commonly associated with overt condylomata, "condylomatous" cervical abnormalities, and mild dysplasias, while HPV types 16 and 18 are most commonly associated with more severe dysplasias and carcinomas. It has been suggested that CIN lesions showing koilocytosis are usually associated with HPV-16 (29). However, because over one-quarter of the specimens showing CIN with koilocytosis contained either multiple HPV types or types other than HPV-16, the presence of CIN with koilocytosis does not constitute compelling evidence for the presence of HPV-16. One study (3) found that HPV-18 was associated with high-grade cervical cancers significantly more often than HPV-16. The authors suggested that HPV-18-associated cervical cancers may be more aggressive than those associated with other HPV types. The observation that HPV-18 is found more commonly in cervical cancer than in dysplasia has been used to support the hypothesis that HPV-18 may cause rapidly progressive lesions (85). The possibility that HPV-18-associated lesions may have a prognosis different from those associated with other HPV types will require further investigation.

In most series examining specific lesions, a small number of lesions contain HPV DNA of a type not included in the screening battery used in the study; i.e., they are detected only under low-stringency hybridization conditions. As these specimens are investigated further, additional HPV types are identified and these new types may also be associated with certain lesions. For example, HPV-31 was cloned from a cervical dysplasia which was identified as positive only under low-stringency conditions when probed with HPV-6, -16, and -18 and was then identified in none of nine normal cervical specimens, none of 6 condylomata, 9 of 44 cervical dysplasias, and 4 of 62 invasive cervical cancers (96). A later study from the same group identified HPV-31 in 24 of 156 cervical dysplasias (almost evenly distributed among the three grades of CIN) and in 3 of 58 invasive carcinomas (85). Studies in two other laboratories have found HPV-31 in 7 of 105 cervical dysplasias and 1 of 26 carcinomas (46) and in 2 of 6 normal cervical samples and 8 of 42 dysplasias (212). HPV-33 was identified in 2 of 29 severe dysplasia lesions and in 1 of 53 invasive carcinoma biopsies (5), and HPV-35 was found in 1 of 6 cervical

TABLE 3. Association of HPV-6 and -11 with specific genital lesions[a]

Country[b]	Method[c]	No. of specimens positive/no. tested								Reference
		Condylomata	Normal	Cervical lesions			Cancer	Vulvar cancer	Penile cancer	
				CIN I	CIN II	CIN III				
Germany	S	62/68	—	—	—	2/6	1/13	0/5	0/2	53
England	S	—	1/3	3/4	4/8	5/7	—	—	—	108
Germany	F	—	4/36		6/13[d]	4/22	—	—	—	208
Germany	F	6/6	1/229		14/45	5/22	2/4	—	—	166
England	S	—	0/17	13/20	10/30	14/28	0/13	—	—	104
Germany	R	61/81	0/2		12/80[d]		1/23	2/7	0/1	37
Scotland	S	0/4[e]	0/3	—	1/16[d]		0/3	—	—	114
Finland	T	5/6[e]	—	8/11	4/6	6/8	—	—	—	197[f]
England	S	—	0/104		2/27[d]		—	—	—	201
USA	R	7/12[e]	2/6	5/12	2/12	0/18	—	—	—	212
Latin America	F	—	7/51	—	—	—	9/46	—	—	151
USA	S	4/6	—	—	1/1	0/1	2/6	—	—	34
USA, Italy	T	20/43	—	—	—	—	2/12	3/26	—	59
USA	D	8/10	—	—	—	1/2	8/23	7/9	—	184
Australia	S	—	0/5		5/11[d]	3/12	12/54	—	—	83
England	D	—	77/202	—	—	—	—	—	—	213
Austria	S	—	0/31	9/33	4/43	10/140	4/44	—	—	46
USA	T	—	—	—	—	—	—	17/46	—	75
USA, Brazil	S	—	—	10/63	9/61	1/32	0/58	—	—	85

[a] Because some studies do not distinguish between HPV-6 and -11, results are pooled for the two types. Some lesions were positive for multiple HPV types and so are listed as positive for each type identified. Patient selection and specimen type are not uniform, so studies cannot be directly compared. —, Not tested.
[b] Germany = Federal Republic of Germany.
[c] Primary typing method used: S, Southern blot; R, reverse blot; D, dot blot; F, filter in situ hybridization; T, tissue in situ hybridization.
[d] Several lesion types pooled in published report.
[e] Condylomatous lesions of the cervix.
[f] Denominator values are total number of lesions positive for any HPV type.

adenocarcinomas, 1 of 50 cervical squamous carcinomas, 2 of 137 CIN lesions, 1 of 13 vulvar carcinomas, and 4 of 436 normal cervical samples (97). HPV types 39 and 42 were found in 6 and 17 of 513 genital lesions, respectively (6). HPV-39 was found only in dysplastic and malignant lesions, while HPV-42 was found only in condylomatous lesions. HPV-45 was cloned from a cervical dysplasia and was also found in 1 of over 400 normal cervical specimens, in 1 of 51 condylomata, in 1 of 52 cervical carcinomas, but in none of 90 additional cervical dysplasias (122). The most recently published genital HPV type, HPV-51, was found in 2 of 50 normal cervical biopsies, 5 of 87 condylomata, 1 of 42 CIN lesions, and 1 of 5 cervical carcinomas (129). In addition, HPV-30, which was initially identified in a laryngeal carcinoma, has also been identified in 2 condylomatous lesions of the genital tract but in none of 23 genital tract dysplasias and carcinomas (71).

The general associations of HPV types with lesions has led to a concept that some HPV types are "worse" than others. Thus, HPV types 16, 18, and perhaps 33, 35, and 39 might be viewed as "high risk" types, while HPV types 6, 11, and 42 would be viewed as "low risk" HPV types with respect to carcinogenesis. HPV-31 and -51 might be placed in an intermediate position between high and low risk. While this concept may be useful in some settings, it is important to recognize that the low-risk HPV types are not "no risk" types. For example, HPV-6 and -11 have been identified in some malignant lesions (83, 148, 151, 184, 223). In addition, high-risk types are not always associated with malignancy. HPV-16 has been identified in a number of women without cervical abnormalities whose prognosis is unknown (43, 165, 166, 201). Furthermore, as detailed below, most studies relating a given HPV type with a specific lesion have used retrospective sampling without proper controls, which may skew the results and modify any conclusions. There are few properly controlled studies in the literature evaluating the risk of a given HPV type prospectively.

ASSOCIATION OF HPV WITH MULTISTEP CARCINOGENESIS IN THE GENITAL TRACT

Epidemiologic Data

Carcinoma of the cervix has many features in common with sexually transmitted diseases. In the 19th century, it was noted that uterine (mostly cervical) cancer was uncommon among cloistered nuns and other sexually inexperienced women (158). More recently, the incidence of cervical cancer was linked to age at first intercourse and number of sexual partners (78). This observation coupled with the finding that women with cervical cancer have a higher incidence of antibody to herpes simplex virus type 2 (HSV-2) than comparable controls (150) led to speculation that HSV-2 may have an etiologic role in cervical cancer. However, despite numerous attempts to confirm the association, little direct evidence for a role of HSV-2 in cervical carcinogenesis has been accumulated (207). The possibility that HPV is associated with cervical cancer was also suggested a number of years ago (226), but until relatively recently it was not possible to test the hypothesis.

Although numerous studies have found a strong association between cervical cancer and HPV DNA, there have been few controlled epidemiological studies of this association similar to those that initially implicated HSV-2. This has been due largely to the technical difficulty of testing for HPV infection in large populations. One case-controlled study of 46 women with invasive cervical cancer and 51 matched controls from several Latin American countries has been reported (151). Using filter in situ hybridization on cervical scrape specimens, these investigators detected HPV DNA in

TABLE 4. Association of HPV-16 with specific genital lesions[a]

Country[b]	Method[c]	No. of specimens positive/no. tested								Reference
		Condylomata	Normal	Cervical lesions			Cancer	Vulvar cancer	Penile cancer	
				CIN I	CIN II	CIN III				
Germany	S	2/33	—	2/20[d]		2/9	19/41	2/7	1/4	39
England	S	—	3/17	16/20	24/30	27/28	12/13	—	—	104
Japan	S	—	—	—	—	—	3/9	—	—	203
Germany	R	0/81	0/2	24/80[d]		—	8/23	4/7	1/1	37
Scotland	S	1/4[e]	1/3	—	7/16[d]		1/3	—	—	114
Finland	T	0/6[e]	—	1/10	2/6	2/8	—	—	—	197[f]
Canada	D	—	—	0/13	7/30	27/54	—	—	—	142
Brazil	S	—	—	—	—	—	8/19	—	26/53	106
England	S	—	9/104	8/27[d]		—	—	—	—	201
USA	R	2/12[e]	1/6	1/12	6/12	11/18	—	—	—	212
Taiwan	S	—	—	—	—	—	16/30	—	—	21
USA	S	0/6	—	—	1/1	1/1	3/6	—	—	34
USA, Italy	T	5/43	—	—	—	—	7/12	10/26	—	59
USA	D	6/10	—	—	—	0/2	14/24	3/9	—	184
Australia	S	—	0/5	2/11[d]		5/12	33/54	—	—	83
England	D	—	41/202	—	—	—	—	—	—	213
USA	S	—	—	—	—	—	14/30	—	—	3
Austria	S	—	1/31	3/33	15/43	67/140	25/44	—	—	46
USA	T	—	—	—	—	—	—	8/46	—	75
USA, Brazil	S	—	—	12/63	25/61	21/32	24/58	—	—	85

[a] Studies with mixed probes (e.g., HPV-16 and HPV-18) are excluded from this table. Some lesions were positive for multiple HPV types and so are listed as positive for each type identified. Patient selection and specimen type are not uniform, so studies cannot be directly compared. —, Not tested.
[b] Germany = Federal Republic of Germany.
[c] Primary typing method used: S, Southern blot; R, reverse blot; D, dot blot; F, filter in situ hybridization; T, tissue in situ hybridization.
[d] Several lesion types pooled in published report.
[e] Condylomatous lesions of the cervix.
[f] Denominator values are total number of lesions positive for any HPV type.

91% of cancer patients as compared with 63% of controls ($P = 0.002$). HPV type 16 or 18 was also found more commonly in cancer patients as compared with controls (67 versus 43%; $P = 0.03$). There were some geographic differences in HPV types found, with HPV types 6 and 11 found more commonly in Panama in both cases and controls. The high prevalence of HPV infection among control women was also of interest. This observation highlights the difficulty in accurately assigning specificity values to different detection methods. In this case, further study will be needed to determine whether the high prevalence of HPV infection among controls was an artifact of the detection system or reflects the true prevalence of HPV infection in this population. The prevalence of HPV infection in controls increased with age so that the association between HPV infection and cancer was greatest in the younger age groups.

Because of a 5.7-fold-higher incidence of cervical cancer among women in Greenland compared with women in Denmark, 1,247 women from the two countries were randomly selected from population registers and screened for the presence of HPV DNA in cervical smears by filter in situ hybridization (79). In each country, over 84% of the eligible female population of the targeted region participated in the study and over 95% of the smears were cytologically normal. The frequency with which HPV types 6 and 11 were identified in cervical smears (independent of cytological result) from women from Greenland and Denmark was not significantly different (6.7 and 7.5%, respectively), while the frequency of HPV types 16 and 18 was significantly higher in Denmark (13%) than in Greenland (8.8%). These data suggest that the differences in cervical cancer rates between the two countries cannot be explained by differences in the rate of infection with different HPV types (especially HPV types 16 and 18).

The largest epidemiologic study to date was conducted with 9,295 women who were seen at three hospital clinics in the Federal Republic of Germany (38). These women had cytologic evaluations, and their cervical smears were examined for HPV-6 and HPV-11 DNA and for HPV-16 and HPV-18 DNA by filter in situ hybridization. Cytology revealed that 94.2% had normal smears, 2.1% had koilocytosis only, 1.7% had CIN I or II, 1.3% had CIN III, and 0.7% had invasive carcinoma. Of patients with normal cytology, 9% were positive for HPV, while 35% of those with CIN or invasive cancer were positive for HPV. No figures are cited for the proportion of those with koilocytosis who were positive for HPV DNA, and no breakdown of specific lesions by HPV type was given. The authors did state that HPV-16 and HPV-18 DNA were detected more often than HPV-6 and HPV-11 DNA. It was also noted that a number of samples (exact figures not given) gave indeterminant results that were considered negative but may have been weakly positive.

Morphologic Progression of Cervical Dysplasia

As noted above, dysplastic changes in the cervix are referred to as CIN and are graded according to their progression toward invasive cancer (155, 156). The basis for the grading system is the prospective evaluation of large cohorts of women with cervical dysplasia to determine their risk of developing invasive cancer (81, 156). Cytologic changes suggestive of HPV infection (koilocytosis) have been noted in association with CIN (45, 186). Initially, it was unclear whether HPV infection caused morphologic changes that mimicked CIN, whether HPV infection and CIN were occurring independently, or whether HPV infection caused CIN. As discussed above, CIN lesions of all grades are closely associated with HPV, with most series reporting that

TABLE 5. Association of HPV-18 with specific genital lesions[a]

Country[b]	Method[c]	No. of specimens positive/no. tested								Reference
		Condylomata	Normal	Cervical lesions			Cancer	Vulvar cancer	Penile cancer	
				CIN I	CIN II	CIN III				
Germany	S	0/29	—	0/17[d]		0/8	11/49	0/7	1/10	9
Japan	S	—	—	—	—	—	2/9	—	—	203
Germany	R	0/81	0/2		2/80[d]		1/23	2/7	0/1	37
Scotland	S	1/4[e]	1/3	—	3/16[d]		0/3	—	—	114
Finland	T	1/6[e]	—	2/11	0/6	0/8	—	—	—	197[f]
Canada	D	—	—	3/13	6/30	14/54	—	—	—	142
Brazil	S	—	—	—	—	—	0/19	—	5/53	106
England	S	—	0/104		0/27[d]		—	—	—	201
USA	R	2/12[e]	1/6	3/12	4/12	3/18	—	—	—	212
Taiwan	S	—	—	—	—	—	2/30	—	—	21
USA	S	1/7	—	—	0/3	1/2	2/7	—	—	34
USA, Italy	T	1/43	—	—	—	—	0/12	1/26	—	59
USA	D	3/10	—	—	—	0/2	1/24	2/9	—	184
Australia	S	—	0/5	1/11[d]		2/12	4/54	—	—	83
England	D	—	26/202	—	—	—	—	—	—	213
USA	S	—	—	—	—	—	6/30	—	—	3
Austria	S	—	0/31	2/33	1/43	8/140	4/44	—	—	46
USA	T	—	—	—	—	—	—	11/46	—	75
USA, Brazil	S	—	—	1/63	1/61	2/32	13/58	—	—	85

[a] Studies with mixed probes (e.g., HPV-16 and HPV-18) are excluded from this table. Some lesions were positive for multiple HPV types and so are listed as positive for each type identified. Patient selection and specimen type are not uniform, so studies cannot be directly compared. —, Not tested.
[b] Germany = Federal Republic of Germany.
[c] Primary typing method used: S, Southern blot; R, reverse blot; D, dot blot; F, filter in situ hybridization; T, tissue in situ hybridization.
[d] Several lesion types pooled in published report.
[e] Condylomatous lesions of the cervix.
[f] Denominator values are total number of lesions positive for any HPV type.

70 to 95% of CIN lesions contain HPV DNA. Many of the HPV-containing CIN specimens in these reports showed no koilocytosis. A prospective study in Australia of 846 women with cytologic changes consistent with HPV infection without dysplasia (115) showed that the risk of developing carcinoma in situ was 15.6-fold higher than expected. Overall, 13.4% of the cohort developed cervical dysplasia over a 6-year period. Because this study was based on cervical cytology specimens sent to a central cytologic screening facility and did not include organized follow-up of patients, these numbers probably represent minimum estimates.

Another study of 52 patients with evidence of HPV infection (i.e., koilocytosis) and no other abnormality on cervical biopsy (126) found that 20 patients developed CIN, 18 regressed to normal, and in 7 the cervical abnormality persisted unchanged. These patients were followed with repeat colposcopy and biopsy every 3 to 6 months for up to 35 months. Seven patients were lost to follow-up. These observations are most consistent with the hypothesis that HPV is intimately associated with CIN, perhaps in a causal role. Although the issue still has not been fully clarified, current practice is to proceed with treatment based on the morphologic appearance of the lesion with respect to CIN regardless of the presence or absence of evidence of HPV infection (75).

Prospective Studies of CIN Progression Based on HPV Type

A central issue in determining the value of HPV typing is the question of whether knowledge of HPV type is important in determining prognosis. Several prospective studies have been reported which attempt to address this issue. The largest body of published information on a prospectively followed cohort is from a study of 513 women followed since 1981 by Syrjanen and colleagues in Finland (186, 189, 192, 197). Patients who were found to have HPV-induced cytopathic changes on routine cervical cytologic smears were asked to return at 6-month intervals for colposcopy and repeat cytology. Cervical biopsies with colposcopy were performed on patients who were found to have CIN on cytology, while those without CIN were followed with colposcopy and smears alone until they developed CIN. Patients found to have CIN III on biopsy were treated with cervical conization. The lesions were scored as to whether they regressed, persisted without change, or progressed (from non-CIN to CIN or from CIN to a higher grade of CIN). HPV typing has been performed on lesions from a subset of these patients. At the time of the most recent analysis, the mean duration of follow-up was 28.6 months (192). At that time, 118 of 364 (32.4%) biopsy specimens had been found to contain HPV DNA by several assays, mainly tissue in situ hybridization. The types identified included HPV-6 (24 cases), HPV-11 (22 cases), HPV-16 (31 cases), HPV-18 (18 cases), and HPV-31 (1 case). The remaining 22 cases contained mixtures of two HPV types. There was little difference in distribution of HPV types among patients whose cervical smears were normal, showed HPV changes without CIN, or showed HPV changes plus CIN. Type-specific progression data are not presented in this analysis but an earlier report (197) on 32 typed specimens showed that all 8 cases that subsequently regressed contained HPV type 6 or 11. However, 6 cases containing HPV type 6 or 11 persisted and 10 cases progressed. For HPV types 16 and 18, none regressed, 3 persisted, and 5 progressed. Because this study is still in progress, some information may change with further follow-up.

Another study followed 100 women with CIN I documented on three consecutive cervical smears with colposcopy and repeat smears every 4 months (16). Over a follow-

up period of at least 19 months, 67 lesions persisted, 26 progressed to CIN III, and 7 regressed. Cervical cells were assayed for the presence of HPV-6 and -16 DNA by filter in situ hybridization. Specimens from a total of 65 patients were found to contain HPV DNA: 26 contained HPV-6, 19 contained HPV-16, and 20 contained both types. Of the 26 lesions progressing to CIN III, 14 contained HPV-16 DNA, 1 contained HPV-6 DNA, 8 contained both, and 3 contained neither. None of the seven regressing lesions contained HPV-16 DNA, but three did contain HPV-6 DNA. Among the 67 persistent lesions, 22 contained HPV-6 DNA, 5 contained HPV-16 DNA, 12 contained both types, and 28 contained neither. The authors concluded that the presence of HPV-16 DNA was strongly correlated with progression of the lesion to CIN III, although lesions occasionally progressed in its absence. They also noted that their regression rate of 7% was lower than that for most previously published series of CIN I lesions. Because most previous studies had used cervical biopsies, the authors suggested that the natural history of CIN I may be altered by biopsy, i.e., that biopsy may be therapeutic in some cases.

One additional study followed 35 women with CIN II for up to 2 years with repeat evaluations (46). The authors found that 9 of 12 cases associated with HPV type 16, 31, or 33 (based on Southern blot hybridization) progressed or persisted while 11 of 23 associated with other HPV types or with no HPV progressed or persisted. These differences were not statistically significant.

In Vitro Data

Cellular transformation by BPV type 1 was first accomplished in 1980 (99), and since that time morphologic transformation of cultured cells by animal and human papillomaviruses has been the subject of intense investigation. A detailed discussion of these studies is beyond the scope of this review. However, it is pertinent to note that transformation of continuous cell lines has been accomplished with HPV types 6 (73, 76), 16 (72, 87, 116, 203, 219), and 18 (87, 211). In addition, HPV types 16 and 18 have been shown to immortalize primary keratinocytes (77, 146). One recent study has shown that HPV-16 is able to alter epithelial differentiation in a way which resembles intraepithelial neoplasia in an in vitro model system (107). While virus-induced alterations of growth control in vitro do not necessarily correlate with the ability of a virus to induce tumors in the intact organism, the absence of such alterations would make an association with human cancer less tenable.

Role of Cofactors in Cervical Carcinogenesis

In the case of potentially oncogenic animal papillomaviruses such as BPV type 1 and cottontail rabbit papillomavirus, cofactors clearly play a role in the progression of a benign lesion to a malignant one (65, 160). Because HPV (even HPV-16) infection does not invariably lead to the development of cancer, it is quite likely that other factors are also involved in human carcinogenesis associated with HPV (224, 225). As noted above, HSV-2 was previously implicated in cervical carcinogenesis and, although its role as a primary initiator of cervical carcinogenesis is dubious, it is still a viable candidate as a cofactor. The precise role HSV-2 may play is uncertain (109). One of the strongest potential cofactors is a component of cigarette smoke. Several studies have identified cigarette smoking as a risk factor for the development of cervical dysplasia and cancer (10, 11, 100, 202, 204) and components of cigarette smoke have been identified in cervical mucus (165). The case control study of cervical cancer in Latin America discussed above (151) also found a significant difference in smoking among patients (52%) as compared with matched controls (27%). Other possible cofactors that have been proposed in cervical carcinogenesis have included oral contraceptives (185, 204, 205, 218) and vitamin deficiencies (10, 15, 89). In vitro studies have also supported a role for oncogene activation as a cofactor in cervical carcinogenesis (87, 103, 182).

DISCUSSION

The major problem plaguing management of patients with dysplasias is the inability to know whether the lesion will regress, persist, or progress based on morphological criteria. In addition, a certain percentage of patients with dysplasia go undetected by the standard Papanicolaou test and present with carcinoma in situ or invasive carcinoma of the cervix. Therefore, there is an interest in recognizing other parameters that might improve detection or be prognostic indicators. Research of the type summarized in the previous sections has been aimed at determining whether the detection of certain HPV types would provide useful information. Several aspects of these data will be discussed below in this light.

Limitations of Typing Techniques

For large-scale typing, a technique is needed which is rapid, inexpensive, highly sensitive and specific, and applicable to specimens obtained by a relatively noninvasive method. The current standard is generally considered to be Southern blot analysis. This method, however, is neither rapid nor inexpensive, may be subject to interlaboratory variability, and requires quantities of DNA which are most reliably obtained from biopsies. The simplest techniques, dot blot and filter in situ hybridizations, have the advantage that a Papanicolaou smear can be used as starting material. However, they suffer from problems of false-positivity. Advances in tissue in situ hybridization technology have greatly increased the sensitivity of this assay performed on paraffin-embedded sections, but there is little information regarding these parameters in cervical smears (8, 58, 60). Finally, while the PCR is extremely sensitive, more data are needed to assess its specificity.

Assessment of sensitivity and specificity of the current methods is difficult. On the one hand, they could be assessed relative to clinical or histological signs of disease. However, as discussed above, viral DNA has been detected in the absence of any signs of overt or subclinical disease. On the other hand, one could assess sensitivity and specificity of the techniques relative to each other, but when the results of two techniques are at variance it is unclear whether one represents a false-positive or the other represents a false-negative. Again, the Southern analysis seems most "foolproof" in this respect since one can confirm hybridization with predicted fragment sizes.

DNA hybridization procedures in general are more expensive than antigen detection methods. Therefore, typing procedures could be less expensive if based on antibody-mediated detection. Such a procedure requires (i) identification of type-specific epitopes on proteins and (ii) the development of antibodies to these epitopes. These antibodies could then be used as probes on tissue sections, scrapes, smears, or other specimens. An open question is whether

such epitopes, once identified, are in fact expressed at a high enough level to be detected in the full range of lesions.

As mentioned above, the current methods are relatively difficult, time-consuming, and, therefore, expensive. For these reasons, one needs to consider whether the cost to the patient will be worth the benefit. The aim of testing is to single out the minority of patients who need aggressive treatment from the majority of patients in whom the lesions will regress.

Value of Typing Data

Data accumulated on the HPV types present in different lesions have led to the designation of high-risk and low-risk viruses with respect to carcinoma of the cervix. Since HPV types 16 and 18 are the predominant types found in severe dysplasias, carcinoma in situ, and invasive cervical carcinoma, one might argue that it would be useful to screen all Papanicolaou smears for these types and to treat accordingly. Those high-risk patients who are missed by Papanicolaou smear alone until presenting with invasive cancer might then be identified. There are several reasons, however, why such a response would be premature. First, these viruses are found in some patients with no clinical, cytological, or colposcopic evidence of disease but the natural history of such infections is unknown. Second, although a large percentage of the population is infected with HPVs, only a minority develop cervical cancer, indicating that HPV type 16 or 18 alone is not sufficient for tumor formation. Third, it remains unclear whether the lesions progress because of the particular type of viral DNA present or because of the stage of the dysplasia (the latter of which is already ascertained by the Papanicolaou test and used as the determinant of further evaluation and treatment). Data reported recently by Barnes et al. suggest that detection of HPV-18 may allow early detection of a rapid progression through dysplasia to carcinoma (3). If this report is correct, then screening for HPV-18 and careful follow-up of HPV-18-positive patients might well be valuable. Prospective studies and larger sample sizes will be needed to confirm this hypothesis.

Although HPV-6 and -11 are classed as low-risk viruses, they are not without risk; the risk may depend on their location within the genital area (e.g., vulvar, anogenital, or cervical). Not all HPV-6 and -11 lesions regress; some progress and therefore the detection of such viral types should not be ignored.

In addition to these problems of interpreting typing information, another difficulty is the ever-increasing number of HPV types identified. This review has focused on HPV types 6, 11, 16, and 18 because they have been the most studied. However, newer types such as HPV-31, -35, and -39 are clearly found in some dysplastic and malignant lesions but their roles (and the roles of higher-numbered and as yet unidentified types) have yet to be fully defined. Until these newer types are more completely investigated and the rate of discovery of new HPV types slows, typing data on clinical specimens will be incomplete.

Limitations of Epidemiologic Data

The epidemiologic studies reported to date have not determined whether HPV type alone is of sufficient prognostic value to merit modification in therapy even if typing assays were readily available (120). One limitation of all studies attempting to ascertain the type-specific epidemiology and natural history of genital HPV infection has been the lack of a simple, inexpensive test which could be applied to a very large population to determine prevalence. Thus, a large number of infected individuals and uninfected controls has not been identified and followed. Because it has been necessary to use one of the DNA hybridization methods described earlier, nearly all of the patients studied have been preselected for screening and follow-up and very few studies have used matched controls. This selection bias tends to underrepresent individuals in the population with asymptomatic or subclinical infections. These studies may then overestimate the rate or frequency of progression for a given HPV type and may invalidate comparisons between types if a difference in the proportion of subclinical or asymptomatic infections is associated with each type. The epidemiologic study with the least selection bias is the study comparing prevalence of HPV-16 infection in Greenland and Denmark (79), and it does not support the hypothesis that HPV-16 is strongly associated with cervical cancer.

Given that epidemiologic studies are hampered, one is left with the suggestive data cited above for the role of HPV in human carcinogenesis. Certainly these data are consistent with a role for HPV in the multistep process. However, that many more individuals are infected with the virus than ever develop cancer indicates that other cofactors are involved as well. Prospective studies of more representative populations (including HPV-negative matched controls) will need to be completed to assess better the role of each HPV type (or group of types) as an independent factor in carcinogenesis.

Typing to Control Sexually Transmitted Disease

This review has focused on genital tract cancer, especially in females, because that is where the major clinical problem lies and that is the point to which most of the available clinical information is directed. Given that HPVs are responsible for the most prevalent viral sexually transmitted disease (condyloma acuminata), one might suggest that HPV typing (of both sexes) would provide a means of identifying carriers and therefore controlling transmission. In this instance, simply knowing that the virus is present would be sufficient. Infections are currently recognized by clinical inspection or by the presence of koilocytes. The frequency of transmission from an individual not detected by these means (a latently infected individual) versus an individual with subclinical or clinical disease has yet to be determined. Barrasso et al. suggest that some such transmission may occur (4). However, one would want to know whether this is of sufficient concern (when compared with HPV infection detectable by standard procedures) to warrant a more sensitive means of detection. Large-scale typing studies would be necessary to establish the frequency of such transmission. Because of the polymorphisms within each HPV type, typing by antigen detection, even if available, may be insufficient to prove route of transmission. A much more detailed restriction enzyme analysis may be necessary.

CONCLUSION

A full-scale HPV typing program to complement Papanicolaou smear screening does not seem warranted at this time for several reasons. First, an optimal typing procedure for such a program does not yet exist. One might hope this will be accomplished in the future either through further refinement of one of the current nucleic acid-based methods or with the development of antigen detection or serological tests. Second, the extent to which the natural history of the

lesion (or the latent infection) is affected by the type of virus present is not established. It is unclear whether identifying all carriers of high-risk HPVs to follow them more closely will yield a different outcome than following patients identified by Papanicolaou smear. Third, although the viruses can generally be divided into high-risk and low-risk types, these divisions are not absolute, and to some extent the differences may depend on the anatomic location of the infection. The clinician should not be complacent about the presence of a low-risk type (e.g., HPV-6 and -11) even though they are not considered responsible for most malignant cases. Given that there is currently an inexpensive and relatively effective screening program (the Papanicolaou test), at this point it is best to continue to rely on that test for patient management. At this stage in our knowledge, treating a patient according to the HPV type rather than the degree of dysplasia runs the risk of using a more aggressive management regimen than is necessary in some patients and of undertreating patients with lesions that may progress. The available typing procedures, however, are valuable research tools that will help provide answers to many of the remaining questions.

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