Pathobiology of Papillomavirus-Related Cervical Diseases: Prospects for Immunodiagnosis

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INTRODUCTION

The finding of an association of human papillomaviruses (HPV) with not only genital warts but also lower genital tract neoplasia has resulted in the emergence of technologies that center on the detection and prevention of these diseases. As the association between the presence of HPV nucleic acids and neoplasia has been confirmed, the issue of whether the detection of HPV nucleic acids in the genital tract would identify women at risk for neoplasia has emerged. The discovery of "occult" HPV, however, has complicated the picture and brought with it the realization that a sizable portion of the population has been exposed and may carry the virus without consequence. While this finding has refocused attention to other cofactors involved in the genesis of genital neoplasia, it has left unclear the significance of occult virus, as well as the utility of HPV detection methods in clinical medicine and cancer prevention. Recently, the application of molecular immunology has brought a new dimension to the field, resulting in reagents for detecting HPV-derived protein products in neoplastic epithelium, discoveries of serological immunity to some of these proteins,

and speculation that some of these reagents might identify patients more likely to harbor carcinomas and their precursor lesions. Moreover, recent efforts to produce neutralizing antibodies to HPV suggest indirectly that immunization with HPV proteins may produce a protective immune response (13). The purpose of this review is to summarize the field at present concerning the immunodiagnosis of cancer or cancer precursors by using HPV-derived reagents.

PATHOBIOLOGY OF GENITAL HPV INFECTION

Mechanisms of Infection

Papillomaviruses infect squamous epithelium, and the interval from exposure to the development of a lesion ranges from a few weeks to several months and perhaps longer (63, 77). In theory, the virus enters the female genital tract and gains access to the germinal or replicative cells in the basal epithelium via defects in the surface mucosa. This theory is supported by the demonstration of papillomavirus DNA and RNA in basal cells and the observation that experimental infection of squamous mucosa by HPV is enhanced by disturbing the epithelial surface (and hence exposing the basal cells) prior to exposure (6, 63). Of particular interest is the hypothesis that the viral DNA exists either in or in

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FIG. 1. Tissue localization of HPV nucleic acids and capsid proteins in a low-grade cervical precursor lesion (condyloma). The lesion is characterized histologically by koilocytotic atypia (panel A; arrows). DNA-DNA in situ hybridization with a biotin-labeled HPV-6/11 mixed probe (VIRATYPE; Life Technologies, Gaithersburg, Md.) depicts abundant HPV DNA by the dark staining in the superficial epithelium (panel B). Capsid antigen production is illustrated in panel C by immunostaining, using an antibody generated against bovine papillomavirions (Dako Corp., Carpinteria, Calif.) (arrows).

proximity to the epithelium for an extended interval without causing morphological changes (106). This has been termed latent or occult infection; however, the precise definition of latency and the reservoir of latent infection remain unclear.

Whether the viral DNA actually persists within the cells, on the surface, or in proximity to the cells has not been confirmed, but presumably the reservoir of virus is perpetuated in the basal cells until viral DNA replication is initiated (6). As the cells containing the viral DNA approach the upper layers of the epithelium, the virus replicates and assembles into virions, which can be detected by electron microscopy or immunohistochemistry (97, 110, 121). A portion of the superficial cells in the infected epithelium characteristically display enlarged, hyperchromatic nuclei, with or without cytoplasmic halos (koilocytotic atypia), and the mature virus usually concentrates in this cell population (60, 121) (Fig. 1). Whether koilocytosis per se is due exclusively to viral replication is controversial, principally because this cytological phenomenon may exist in the absence of abundant capsid proteins or virions (19, 97, 110, 116). The implication is therefore that koilocytotic cells may be host to multiple virus-related events. One is a fundamental change in the epithelium that has been morphologically altered by viral infection and exhibits viral cytopathic effect (koilocytotic atypia) in the terminal differentiated cells. Another event may relate more directly to the dependence of actual viral propagation on epithelial maturation, in which case the

FIG. 2. Low (A)- and high (B)-grade cervical precursors associated with HPV types 6 and 16, respectively. Lesions associated with HPV-16 frequently demonstrate greater degrees of nuclear atypia (panel B) in all layers of the epithelium and are associated with aneuploid DNA content.

machinery of viral growth is switched on in mature cells, some of which by definition will appear as koilocytes (60, 121). Alternatively, both mechanisms may participate in the genesis of koilocytotic atypia, with greater degrees of nuclear atypia and cytoplasmic halo formation occurring in cells with active viral maturation.

Although genital squamous epithelium appears to be the principal site for HPV infection, there is evidence that infection may occur in germinal or undifferentiated epithelial cells that give rise to both the squamous and glandular components of the cervix mucosa. HPV nucleic acids have been isolated from neoplasms not clearly derived from squamous-committed epithelial cells, most notably, adenocarcinomas and undifferentiated carcinomas (small-cell carcinoma) (113, 117).

Site Specificity

Although all squamous epithelium may be susceptible to HPV infection, squamo-columnar junctions, particularly those in which the glandular portion is being replaced or "transformed" by squamous epithelium (transformation zones), are most vulnerable to the genital papillomaviruses (98). Interestingly, papillomavirus infection has been demonstrated in other mucosal sites in which this process of epithelial transformation takes place, including the larynx (1), oral-pharyngeal mucosa (22), anus (3), esophagus (128), subungual mucosa (nail bed) (82), and conjunctiva (75). Kreider et al. demonstrated that some of the above sites were particularly vulnerable to experimental infection with

HPV-11 virions (62). This observation, combined with the rarity of other genital HPVs (such as type 16) in extragenital cutaneous sites (other than subungual region), indicates that genital HPV types require specific characteristics of the target epithelium for infection. One possibility is that regulation of replication and transcription is cell specific, permitting infection at specific sites. Favoring this hypothesis is the unusual predisposition of HPV type 16 for the cervix over other genital HPVs such as type 6 (88, 96). Such phenomena may be due in part to the transformation zone epithelium which characterizes the cervix. Similarly, epithelium undergoing repair appears uniquely susceptible to some viruses, for example, the occurrence of warts at tracheostomy sites (91) and the recurrence of genital warts in laser excision margins following attempted ablation (34).

Pathobiology of HPV-Related Neoplasia

The shift in focus to HPV as a cause of genital neoplasia came with the observation that a common feature of abnormal Papanicolaou smears, koilocytotic atypia, was a cellular marker for the presence of genital HPV infection (60, 76, 94). This ushered in an accumulation of circumstantial evidence in favor of HPV as a neoplastic agent, not the least of which was the high prevalence of the infection and its clinical and morphological relationship to cervical precancerous lesions (cervical intraepithelial neoplasia or cervical dysplasia). In essence, the initial data supporting HPV as an oncogenic virus in the genital tract were derived almost entirely from

morphological evidence of the strong association between genital condyloma and cervical neoplasia (77).

The cloning of the first genital HPV in 1980 was a critical step which eventually made it possible to link directly the presence of HPV nucleic acids and most condylomata, squamous precancers, and carcinomas of the female genital tract (4, 21, 31, 42). Moreover, it initiated the studies determining that there was a relationship between specific HPV types and certain components of the precancerous cell spectrum (17, 18). Currently, over 60 distinct types of HPV have been identified, many of which are associated with specific clinical and pathological syndromes (129).

These molecular data have prompted a reevaluation of previous concepts about the biological classification of genital precancers. DNA ploidy studies redefined the spectrum of genital squamous cell cancer precursors according to DNA content, including subsets composed of diploid-polyploid and aneuploid lesions (38–40, 112, 127). Although the precise biological relevance of these measurements is unclear, and despite the fact that they do not predict precisely the natural history of a given precancerous lesion, they have made it possible to identify subsets of cervical lesions that statistically will follow different developmental pathways (40). Moreover, this dichotomy may, within limits, be appreciated at the molecular level. Lesion groups that can be distinguished by morphological or morphometric parameters associate (generally) with different HPV types (17, 18, 39). For example, genital warts and condylomata (most of which contain diploid or polyploid DNA contents) are associated with certain viral types (e.g., types 6 and 11), whereas precancerous lesions and invasive cancer are frequently aneuploid and associated primarily with types 16, 18, 31, 33, and 35 (Fig. 2) (6). The most common genital HPV types and associated diseases are summarized in Table 1.

Generally speaking, as one follows the spectrum of precursor lesions from those that more closely resemble HPV infection alone (condylomata) through higher-grade squamous cancer precursors, one observes a greater association with the HPV types which ultimately associate strongly with invasive cancer, including types 16, 31, 33, and 35. Importantly, HPV "infection" and high-grade precursors may be observed together (78). One conspicuous departure from this concept occurs with HPV type 18, which is associated infrequently with squamous precursors and more frequently with invasive squamous, glandular, and undifferentiated cervical cancers (37, 65, 113, 118, 119). Examples of low- and high-grade squamous precursors are illustrated in Fig. 2.

Molecular Basis for Type-Specific Neoplasia

Current interest in the host immune response has centered on potentially transforming viral proteins, which are illustrated in Fig. 3 and 4 and explained in greater detail later in the text. In brief, studies of mechanisms of cellular transformation have centered on (i) the expression of gene products with transforming ability, such as the E7 oncoprotein of cancer-associated HPVs; (ii) adjacent reading frames such as the E6 open reading frame (ORF), which in cancer-associated HPVs encodes a uniquely spliced message and truncated protein; and (iii) the process of genomic integration, which occurs progressively more frequently in higher-grade precursors and squamous cancers and, particularly, in HPV-18-associated neoplasms. These mechanisms are summarized in Fig. 4 (7, 32, 92, 106, 126).

TABLE 1. Genital HPV types and associated lesions^a

HPV type	Yr discovered	Characteristics
6	1981	Associated almost exclusively with benign external or exophytic genital condylomata; rarely associated with squamous cancers
11	1983	Associated with genital condylomata and laryngeal papillomas
16	1983	Cloned from a genital cancer; predominant HPV type associated with both genital squamous cancers and high-grade cervical or vulvar precancers
18	1984	Cloned from squamous carcinoma; associated with 10–20% of cancers; preferentially detected in adenocarcinomas and small cell carcinomas; uncommon in cervical precursor lesions
31	1986	Isolated from cervical precursor lesion; shares homology with HPV-16, found in similar lesions (ca. 5%)
33	1986	Isolated from cervical carcinoma; frequency of distribution unclear
35	1986	Isolated from cervical carcinoma; frequency and lesion distribution unclear
39	1987	Isolated from penile intraepithelial neoplasia; found in some cervical intraepithelial neoplasia and cervical cancers
42	1987	Isolated from vulvar condyloma; presumed low-risk papillomavirus
51	1988	Isolated from cervical flat condyloma; associated with spectrum of cervical lesions

^a Data from references 5, 81, and 84.

OCCULT HPV INFECTION: DETECTION AND SIGNIFICANCE

Detection

Considerable evidence has accumulated indicating the presence of HPV DNA in tissue or cell preparations that do not exhibit significant morphological abnormalities. The existence of occult HPV infection has been suggested by the frequent recurrence of disease at initially unaffected sites. In the first molecular analysis of this phenomenon, Steinberg et al. reported finding HPV DNA sequences in normal-appearing laryngeal mucosa from patients with a history of laryngeal papillomas, but who were in apparent remission (116). Ferenczy et al. linked occult infection to clinical disease in their study of patients with vulvar warts or precancers undergoing laser therapy. They found that grossly normal squamous epithelium adjacent to the treatment field often contained HPV DNA and that patients with this clinically occult infection had a higher frequency of recurrences vis-à-vis those who did not (34). MacNab et al. found that the majority of samples of normal mucosa or skin adjacent to cervical and vulvar cancers contained HPV DNA sequences and suggested that the presence of the viral DNA may portend a better prognosis (72).

It must be emphasized that the studies described above addressed populations with documented HPV-associated lesions either concurrently or in the past. Despite its normal appearance, the tissue might have contained HPV DNA because of its proximity to tissue clinically infected by the virus or could harbor HPV DNA sequences either from shed cellular material (contamination) in adjacent lesions or due to extension of the virus beyond the morphologically abnormal areas. Whatever the mechanism, the fundamental ques-

FIG. 3. Prototypical HPV genome (type 16) with ORFs and potential functions of each (6).

tions in determining the clinical importance of occult infection are (i) whether it occurs in women with no history of either HPV infection or abnormal Papanicolaou smears and (ii), specifically, whether it has prognostic importance.

Numerous studies have reported the detection of HPV DNA in women with no history of previous HPV-related disease (61, 69). Although some extremely high estimates have been made using either very sensitive detection techniques such as the polymerase chain reaction or relatively nonspecific techniques such as slot blot hybridization, most studies place the range of occult infection in nonpregnant women at 5 to 12% (41a, 61, 95).

It is important to reemphasize at this juncture that the precise epithelial location of HPV DNA sequences in normal epithelium is not known. Numerous studies with relatively sensitive techniques such as in situ hybridization have (with rare exceptions) failed to localize HPV nucleic acids in normal epithelium, despite the confirmation of their presence by Southern blot hybridization (86). However, this does not necessarily refute the potential importance of these nucleic acid sequences. Nuovo found that a large number of HPV-related lesions contain more than one HPV type when analyzed by polymerase chain reaction, even though only one HPV type could be detected by in situ hybridization (87). This would suggest that, when a lesion develops from infection by a single virus type, other virus types are in some

way inhibited from producing morphological changes. In fact, the frequency of histologically demonstrable double infection is <5% (87). Nevertheless, Nuovo and Pedemonte demonstrated that recurrent lesions following ablation are frequently associated with HPV types other than the original (89). While the role of occult infection in these recurrences is unknown, this and other findings suggest that occult infection may have clinical significance under appropriate circumstances.

Prospective Significance of Occult Infection

The previous discussion does not address the issue of whether HPV DNA testing will provide information about the natural history of occult infection in women without a history of an abnormal Papanicolaou smear or clinical warts. This question remains one of the most problematic, because few prospective data are available and two major methodological problems must be overcome. The first problem is the requirement of identifying a "control" population of women who are truly HPV DNA negative because the control group may vary as a function of the number of HPV DNA tests performed on each patient (95). The second is confirming that a specific population of HPV DNA-positive women is truly disease-free at the start of follow-up. Studies indicate that blind re-review of cytologically negative

FIG. 4. Schematic identifying potential mechanisms by which cancer-associated HPVs (types 16, 18, 31, 33, 35, etc.) produce cellular changes (adapted from references (7, 32, 92, 106, and 126)).

women who are HPV DNA positive will uncover significant cytological abnormalities in 15 to 20% which were missed on the first analysis (69, 125). Thus, if careful quality control is not performed, there is the possibility that lesions missed (or not sampled cytologically) and then subsequently discovered will be misinterpreted as having "progressed." Lorincz et al. found that 15 and 5% of HPV-positive and -negative cases, respectively, developed a significant cytological abnormality over a mean follow-up of 2 years (68). However, they noted that the majority of lesions were discovered in women with a prior history of HPV-related disease and represented recurrence rather than first-incidence cases. In all, only 2 of 28 HPV-positive women with no history of HPV-related disease developed a lesion over 2 years of follow-up (68).

Epidemiological Discrepancies and Practical Limitations

From the above data it appears that a significant proportion of women who are HPV DNA positive do not exhibit clinical disease, thus questioning the epidemiological significance of the prevalence of HPV DNA in a population. Kjaer et al. reaffirmed the powerful relationship between sexual behavior and cervical cancer in their study of two similar populations in Greenland and Denmark (57). However, when a comparison of prevalence rates for HPV nucleic acids was completed, it appeared that the low-risk population exhibited a higher rate of HPV DNA positivity (55). Their preliminary conclusion was that the risk of invasive cancer was not directly related to prevalence of HPV DNA, as detected by DNA detection methods. Corroborating evidence has surfaced in other studies as well. Reeves et al. found that the index of HPV detection in Latin America was equally high in women considered at low and high risk for invasive cancer (95). Kiviat et al. found equal prevalence rates in populations of women with different sexual histories (54). The conclusion from these studies would be that perhaps only a subset of HPV DNA-positive women are at risk for cancer and that the assay used to detect HPV DNA is detecting HPV which is not directly related to risk factors associated with cancer (55). This conclusion therefore contrasts with the clear relationship between factors such as number of sexual partners and herpes simplex virus type 2 infection and clinical cervical or genital HPV-related disease (52, 55, 57, 101). Nevertheless, a third study by Kjaer et al. has unearthed a potential explanation for the lower prevalence of HPV DNA in the high-risk group, which was that factors in the high-risk group (such as repeated early exposures to HPV and development of immunity) prevented a precise documentation of HPV exposure by DNA testing (56). In other words, a high-risk individual with numerous previous exposures might be less likely to be HPV DNA positive than a low-risk individual who has fewer exposures but was exposed in the more recent past. In support of this, Burkett et al. found that the detection of HPV DNA is unrelated to lifetime number of sexual partners, but significantly related to the number of sexual partners during the past year (8). Thus, the parameters of a high number of sexual partners and number of years in which intercourse was not accompanied by barrier contraception may identify a high-risk group while paradoxically reducing the likelihood of recovering HPV DNA. In this context, the absence of a correlation between HPV DNA prevalence and cancer argues less against the importance of HPV infection than for the significance of other primary factors and cofactors in the genesis of genital neoplasia (44, 85).

Thus, while HPV DNA positivity clearly identifies a group of women who are more likely to have a cervical lesion, the value of this technique for predicting the development of a lesion is limited, as is the specificity of the technique, particularly in the general population. Because of the way in which the screening protocols are structured (i.e., around the Papanicolaou smear), the potential value of widespread HPV testing is limited to complementing the Papanicolaou smear and reducing false-negatives (30, 69, 125). Assuming that more sophisticated approaches to Papanicolaou smear interpretation (e.g., with computerized image analysis) may accomplish the above, HPV DNA testing as currently used will have questionable value, particularly given the uncertain predictive value of HPV DNA positivity for disease incidence.

Although HPV DNA testing is an imperfect alternative to the Papanicolaou smear as a cancer preventative measure, a significant proportion of cancer cases do develop despite Papanicolaou screening, and about one-third of them develop in women who have never been screened (30, 83). If more thorough health care delivery including Papanicolaou smear screening were offered to high-risk populations, this latter subset of women might be identified. However, women who do not have access to a gynecological exam cannot benefit from a Papanicolaou smear or viral testing as conventionally applied.

The search for other methods of detecting HPV-related neoplasms has recently centered on immunological tests, the premise being that HPV gene products produced during infection will generate an immune response that will identify patients at risk. At the heart of this issue are several questions that will be addressed, including (i) whether there is general evidence for a protective host immune response to HPV-related disease, (ii) what the nature of viral protein production in tissues is, (iii) whether there are specific viral proteins which may produce type-specific immune responses, (iv) whether there is evidence of a host response to these proteins, (v) whether these host responses are directly related to the diseases observed in the genital tract, and (6) whether there is any correlation between type-specific infection and the immune response observed serologically.

IMMUNOLOGY OF HPV

General Studies of Host Response

Based primarily on historical data documenting spontaneous regression of warts, there is ample evidence to assume that a host response exists to HPV-related genital disease (11, 102). Both humoral and cell-mediated mechanisms have been implicated in this process. Workers using papillomavirions from cutaneous warts have described positive reactions to intradermal tests more often (53 to 79%) in patients with plantar warts than in controls (6%) (11). In vitro tests of leukocyte migration inhibition have corroborated these observations. Leukocyte migration inhibition has been reported positive in up to 16 and 79%, respectively, of patients before and after cure of cutaneous warts (11, 79, 80).

Studies of the immunological response at the morphological level have focused on T-cell subsets and Langerhans cells in and around lesions. Langerhans cells have been identified as participants in antigen processing, presumably activated by infiltrating lymphocytes. They have been observed to vary considerably in number and distribution in genital warts, prompting speculation that this variability may reflect differences in host response to HPV-related lesions

FIG. 5. Schematic of pATH fusion protein vector (122). (Original protocols from which this diagram was constructed were provided by T. J. Kornberg.)

(81). Cellular infiltrates in the vicinity of cervical HPV-related precursor lesions contain both B and T cells, although there is no clear explanation as to the significance of their presence (11, 120).

General studies of humoral immunity to papillomaviruses point to three humoral responses, including antibodies to HPV-derived proteins, the infected cells themselves, and antigens not directly associated with warts. Early studies of HPV-specific immune responses used purified virions obtained by density gradient purification of lesional tissue (53, 90). Problems with these studies include the purity of antigen preparations used to determine host serological response and the heterogeneity of populations tested, both in age and immune status and in type of warts. These factors present obstacles to identifying type-specific immune responses and should be kept in mind when reviewing early studies of host response. For example, Kienzler reported that patients with spontaneously regressing warts had a higher index of humoral antibodies to the above reagents (53). Increasing titers occurred either with resolution or following treatment (33, 41, 53). The latter observation suggested that physical trauma to the affected area influenced production of antibodies. Sequential serological studies further indicated that the antibody response developed only after infection had been ongoing for a few months, was strongest in the first year, and diminished thereafter, and the level became inversely proportional to the length of the disease. Persistence of antibody was observed in 20 to 35% of patients at 7 to 9 years following infection (53).

Immunity to wart cells themselves was suggested in studies that used immunofluorescence (73), and other antibodies were detected in patients with warts, including smooth-muscle autoantibodies (74, 104). General defects in immunity have been observed in patients with warts as well, including lower helper/suppressor T-cell ratios and reductions in natural killer cell activity (10). Thus, there was evidence for a complex immune response to warts as well as evidence that certain individuals were susceptible because of defects in immune response to HPV.

In the case of cervical precursor lesions, it is clear that some immune phenomena operate in preventing recurrence following therapy. Richart et al. demonstrated that the risk of developing new cervical HPV-related precursor lesions following ablative therapy was not greater than that for the population at risk, once short-term recurrences were excluded (99). Recent data by Nuovo and Pedemonte suggest further that even short-term recurrences may not signify an immune defect, because these are frequently associated with HPV types other than those recovered from the original

lesion (89). This evidence supports the concept of a host response that occurs locally in the cervix and may be specific for HPV type (89). Narrowing the focus to type-specific immune responses has required a more complete knowledge of the viral genome and its putative proteins, as well as reagents for their detection.

Characterization of the Immune Response to Genital HPVs

Methodology for detection of HPV-derived proteins. Because of the unique problems associated with HPV, in particular, the lack of a system for propagating virus in culture, technology has evolved for expressing specific viral proteins in vitro (35, 59, 93, 101, 122, 124). This technology has made it possible to both explore expression of HPV proteins in tissues and acquire reagents to evaluate the human immune response. Typically used systems involve expression vectors in which an HPV DNA sequence corresponding to a particular transcription unit is cloned in the appropriate orientation directly downstream to a protein that can be induced artificially in bacterial culture (Fig. 5 and 6). By using sequence information from both the vector and the HPV DNA insert, the insert can be cloned in such a way to ensure that, as the vector protein is translated, the insert (HPV) portion is included in the fusion protein. Here, care is taken to insert the HPV DNA in frame with the vector DNA to guarantee that the fusion protein will be a product of the specific HPV reading frame responsible for the protein under study (35, 122). After the proteins are characterized, antisera to individual proteins can be generated in animals (Fig. 7).

Genome organization and potential functions of HPV-derived proteins. The following is a brief summary of the genome organization of genital HPV DNA, the potential proteins produced during the development of HPV-related disease, and the circumstances of production and location of these proteins.

Despite considerable variation in homology among both animal papillomaviruses and HPVs, the genomic organization among these viruses is conserved, and the potential transcription units (ORFs) have been divided into "early" and "late" (capsid) ORFs which are preceded by a non-coding region containing an origin of replication and transcription start sites (6) (Fig. 3). Lowy and colleagues first established that a fragment spanning 69% of bovine papillomavirus type 1 would transform NIH 3T3 and C127 cells in cultures (70). The bovine papillomavirus genome was subsequently sequenced, following which the 69% fragment was designated the early region (12). The remainder of the genome was assigned to the late region, which was found to

FIG. 6. Expression of PATH fusion proteins illustrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Examples include fusion proteins containing HPV-16 E4 and L1 inserts and vector protein alone (pATH). Lanes A to C correspond to uninduced, induced, and salt-precipitated fractions, respectively. Induction of fusion protein expression in bacterial culture produces overexpression of fusion constructs, with large bands depicted by the black and white arrows.

code for structural proteins, and did not have the capability to transform cells *in vitro*. At least seven potential early ORFs (i.e., regions capable of producing proteins) have been identified, with two major late regions coding for the capsid proteins (12). The genomic organization of HPV-16 and the potential function of each of the ORFs is detailed in Fig. 3 (107).

The genome can be divided into (i) ORFs with potential transforming activity, (ii) ORFs that encode regulator viral gene products, (iii) ORFs encoding proteins of unknown function, (iv) ORFs encoding late capsid proteins, and (v)

FIG. 7. Western blot analysis of expressed proteins with sera produced against vector protein. Lanes A to C depict identification of vector, E4, and L1 fusion proteins by an antiserum produced for a pATH-E4 fusion protein. Absorption of the antiserum with lysates of fusion protein alone (lanes D to F) abolishes immunoreactivity to other proteins, with specific identification of the E4 protein. Similar treatment of an antibody to pATH-L1 fusion protein (lanes G to I) renders it immunospecific for the L1 protein. Reprinted from *Virology* (15) with permission of the publisher.

the upstream regulatory region (1). Potential transforming proteins include E6, E7, and E5; the principal focus in HPVs is on the E6-E7 region (Fig. 3). This region is virtually always expressed in cancers associated with HPV and preserved in cell lines in which other portions of the HPV genome have been lost or expelled (6). The E6 and E7 proteins can bind to p53 and retinoblastoma anti-oncogene proteins, respectively, both of which are regulators of cell proliferation (7, 32, 92, 106, 126). Hence, E6 and E7 are considered important components of HPV-mediated neoplastic transformation (Fig. 4). The E5 protein has been studied most extensively in bovine papillomavirus systems, in which it is a principal transforming protein (27). Similarly, HPV E5 proteins have been demonstrated to transform established rodent cells in culture (111). Regulatory proteins include principally E2, which produces gene products that modulate the upstream regulatory region of HPV, and E1, which theoretically influences maintenance of the episomal state (71, 115). ORFs with unknown function include E4, which has no demonstrable transforming or regulatory functions but is highly expressed in cutaneous warts (5, 26, 28, 84). The L1 and L2 ORFs encode major and minor capsid proteins, respectively (6).

Conditions under which HPV proteins are expressed. Efforts to characterize the expression of HPV proteins have centered on using antibodies generated to fusion proteins which specifically identified proteins from individual ORFs (Fig. 7). The most extensively studied aspect of HPV expression involves the late proteins (L1 or L2) with most attention focused on L1. These proteins are produced as a function of epithelial maturation and are most prominent in the koilocytotic cells in the superficial epithelium (121). In cutaneous warts containing HPV type 1, the distribution of capsid proteins is high, in contrast to condylomata containing HPV-6, in which the levels of expression vary (6). In precursor lesions, specifically those containing HPV-16, expression is often markedly reduced (13, 64). Whether this is due to the marked morphological alterations in HPV-16-associated lesions, which preclude normal viral maturation, or a fundamental change in the virus *per se* is unclear (Fig. 2). Likely the impact of HPV-16 on the lesion indirectly affects its own expression, in that the fundamental biological changes in the epithelium produced by the virus alter the growth properties to the extent that normal cellular events on which HPV depends for its replication are disturbed (Fig. 2). The invariable result is sporadic viral replication in foci of epithelium that retain the appropriate biological or morphological characteristics required to support viral growth. In this setting capsid protein production is the most profoundly affected, being frequently absent even in areas where viral replication takes place (19, 119).

In short, capsid protein production and virion assembly are events that occur as a function of productive viral infection and are most likely to be found in benign genital warts or precursor lesions in the early stages of neoplastic development (Fig. 1). In general, the morphological features associated with viral production (i.e., koilocytotic atypia) are found in younger patients than those whose lesions lack these features (16).

Expression of the E4 and E2 domains have been likewise characterized by fusion protein technology, with the HPV-1 and HPV-16 E4 proteins identified in the same cell populations that contain capsid proteins (5, 15, 28). As such, they appear to be dependent on the same factors favoring capsid protein production, and their production appears proportional to the amount of capsid protein. In cutaneous warts,

FIG. 8. Cervical precursor lesion stained with antisera generated to fusion patients containing HPV-16 L1 and E4 inserts. (A) Hematoxylin-and-eosin-stained section of a cervical precursor lesion. (B) HPV-16 RNA by RNA-RNA in situ hybridization with an ^{35}S -labeled RNA probe, the silver grains appearing white in a dark-field photomicrograph (arrows). (C) Nuclear staining with the anti-L1 antisera (arrows). (D) Cytoplasmic staining, using an anti-E4 antibody (arrows).

for example, HPV-1 E4 proteins are abundant and are found from the suprabasal layers on up to the surface in a distribution similar to capsid (L1 and L2) proteins (5, 28). In contrast, in HPV-16-related precancerous lesions, E4 expression occurs sporadically, in lower amounts, and usually in the more superficial layers, similar to the late proteins (15) (Fig. 8). In both viruses E4 proteins are largely cytoplasmic in location and in HPV-16-related lesions are invariably absent in areas of lesions where capsid proteins are absent as well (15). In short, E4 protein production appears to be a phenomenon of early infection in cervical precursor lesions, disappearing in the more advanced lesions in which the morphological features of HPV infection (koilocytotic atypia) disappear (20). However, continued production of these proteins may persist at levels below the threshold for detection. Whether the E4 proteins are truly early or late proteins is controversial, their role in viral maturation being unclear (29).

The HPV-6 E2 protein has been detected in genital lesions recently and has been localized in the nuclei of superficial

cells (109). Like E4, it appears to be produced as a function of lesional and viral maturation (109). Localization of the E5 proteins with antibodies has been attempted of late, and reports indicate that they are found in the nuclei of cells transformed by constructs containing the HPV-6 E5 ORF (111). The precise location in clinical tissues remains to be confirmed.

As might be expected, considerable effort has been expended to localize the E6 and E7 proteins, particularly of HPV-16 and other cancer-related viruses. Using antibodies, workers have successfully localized these proteins in cell extracts of cervical tumors and related cell lines (103). HPV-16 E7 has been previously characterized as a cytoplasmic phosphoprotein, but more recent reports suggest that both E7 and E6 are nuclear in location (104, 105, 108, 114). Immunohistochemical location of these proteins has not been demonstrated convincingly, leaving unclear their precise distribution and conditions under which they are expressed. RNA transcripts corresponding to these proteins have been identified in both cancer precursors and invasive cancers, suggesting that their expression is not limited to cells undergoing viral replication (20, 120). Thus, it is likely that these proteins are produced even under circumstances in which viral integration has silenced expression of the other proteins (111). Coupled with the potential requirement of these gene products for neoplastic transformation, these observations render the E7 gene product of particular interest to individuals seeking a tumor-specific marker (51).

Evidence for type-specific epitopes in HPV proteins. As mentioned above, one of the major limitations of traditional approaches to assessing the immune response has been the absence of reagents that would document a type-specific response. In particular, pooled virions expose proteins (or epitopes) that are derived from regions of the genome that share considerable relatedness among viruses (49). This portion of the review will focus primarily on efforts to identify regions of the genome which elicit type-specific antibody reactions in animals following immunization with viral proteins produced in vitro. As will be evident, such responses may or may not be duplicated by exposure to wild-type virus in vivo.

The principal studies of type-specific immune reagents focus on the late proteins, which are components of the viral capsid and which share considerable cross-homology among both animal papillomaviruses and HPVs (6, 49). Originally, Jensen et al. reported that antibodies to disrupted bovine papillomavirions cross-reacted with genital and cutaneous warts in immunohistochemical studies (49). More recently, the components of the L1 and L2 ORFs have been studied in greater detail with fusion protein technology, and regions of these ORFs that are more type specific have been identified. In an analysis of antibodies produced against the HPV-16 and HPV-6 L1 regions, Firzlaff et al. did not detect antibodies which reacted selectively to nuclei of cells infected by the respective type from which the antigens were produced (36). In contrast, they produced polyclonal antibodies to several clones derived from the HPV-16 and HPV-6 L2 ORFs which stained nuclei of lesions containing their respective viral DNA only (36). These observations suggest that epitopes in the L2 region are type specific for these two HPV types. Whether they will discriminate among multiple HPV types is unclear. It is important to emphasize that some L2 proteins were recognized serologically on Western blots (immunoblots) and not in tissue sections (36), suggesting that some targets identified on Western blot may be obscured in tissue sections by fixation or conformation differences. Moreover,

TABLE 2. Serological reactivity to genital papillomaviruses

Reference	Population studied ^a	Antigen used	% Cases	% Controls	Comment ^b
47	STD clinic	HPV-6b L1	66	57	— ^c
		HPV-16 L2	47	30	
		HPV-18 L2	25	26	
		HPV-16 E7	13	12	
		HPV-18 E7	3	1	
46	Cervical cancer	HPV-16 E4	9.5	8.3	NS
		HPV-16 E7	23.8	14.3	RR = 2.3
		HPV-16 L2	25.8	16.7	RR = 1.6
51	Colposcopy clinic	HPV-16 E4	32.6	11.4	P = 0.004
		HPV-16 E7	6.5	3.8	NS
	Cervical cancer	HPV-16 E4	15.9	7.8	P = 0.053
		HPV-16 E7	20.5	1.4	P = 0.00001
50	Transplant patients	HPV-16 E4	34.7		
		HPV-16 E7	19.8		
	Cervical cancer	HPV-16 E4	61.5		
		HPV-16 E7	19.2		
		HPV-18 E4	5.8		
		HPV-18 E7	7.7		
24	CIN and cancer	HPV-16 E2P ^b	78.0	21.0	
66	Cervical cancer	HPV-16 E2P ^b	45/25	20/5	IgG/IgA
25	Cervical cancer	HPV-16 L1P ^b	60	7	
33	Cervical cancer	HPV-16 E1P ^b	20/43	0/18	IgG/IgA
		HPV-16 E4P ^b	67	30	IgA
		HPV-16 E7P ^b	30	2	IgA
2	CIN (cone biopsy)	HPV-16 L1	21	5	
		HPV-16 L2	39	19	
		HPV-16 E4	25	9.5	
		HPV-16 E7	0	9.5	
		Any of above	57	33	

^a STD, Sexually transmitted disease; CIN, cervical intraepithelial neoplasia.

^b NS, Not stated; RR, relative risk; IgA, immunoglobulin A.

^c —, Controls are listed here as hospitalized children from this study.

anti-L2 antibodies that cross-reacted with more than one HPV fusion protein type on Western blot appeared type specific in immunohistochemical analyses (36). The reasons for this disparity in reactivity are unclear, but these observations indicate the importance of the method used and target preparation.

Efforts to identify specific epitopes in the L1 ORF appear to have been partially successful. Cason et al. produced monoclonal antibodies to a fusion protein containing a portion of the HPV-16 L1 ORF and identified five monoclonal antibodies that reacted preferentially to HPV-16-related lesions in tissue sections (9). Similar to the study by Firzlaff et al., these authors did not observe consistent reactivity of the sera with proteins examined by enzyme-linked immunosorbent assay (ELISA) and by immunohistochemical staining (9).

Unlike the L1 ORF, the HPV-16 E4 ORF encodes a protein with considerably less predicted relatedness with other HPV types (29). Doorbar et al. noted considerable sequence divergence even in the cutaneous HPV types 1, 2, and 4 (29). Crum et al. also observed no immunohistochemical cross-reactivity with antibodies to a portion of the HPV-16 E4 protein and when tested on lesions containing a variety of HPV types (15) (Fig. 8).

Using murine monoclonal antibody, Tindle et al. have also identified specific epitopes in the HPV-17 E7 protein. The MAbs immunoprecipitated the E7 proteins in lysates of HPV-16-related carcinoma cell lines (123). Analysis of the DNA sequence corresponding to the epitopes confirmed that they were unique to HPV-16 E7. The authors concluded that

the immunogenicity of these epitopes may make them candidates for eliciting protective antibodies in vaccines (123).

On the basis of above studies, it would appear that a variety of late ORFs might encode proteins that are type specific. These proteins might allow serological distinction between patient exposures to different HPV types, as well as the production of vaccines. However, one important caveat concerns the ability to extrapolate data obtained by testing immune sera from animals to humans. Christensen et al. observed considerable disparity in antibody reactivity when serum was tested with denatured proteins on Western blots, with intact virions by ELISA, and by an antibody neutralization test (14). Even in immunohistochemical assays, results obtained on formalin-fixed tissue were inferior to tests on frozen sections, questioning the validity of a negative immunohistochemical assay on fixed material. Likewise, denatured proteins identified on Western blot analysis may be invisible when sera are tested with whole virions in ELISAs. The authors questioned the value of the fusion protein or synthetic peptide technology for detecting the in vivo immune response, especially to particular antigens that might aid in developing vaccines (14).

Serological reactivity to HPV. With the applications of fusion protein technology to express portions of, or all, ORFs of HPVs and the widespread availability of sequence data from which linear epitopes could be predicted, a multitude of studies have attempted to characterize the immune response to genital papillomaviruses (Table 2, Fig. 9). The reader should note that these studies involve a variety of case and control populations and use a variety of reagents.

FIG. 9. Serological reactivity of human sera to portions of the HPV-16 E4 and L1 fusion proteins by Western blot analysis. Lanes 1 and 3 illustrate the reactivity of serum samples from two different patients to these respective fusion proteins. Lanes 2 and 4 contain pATH vector proteins alone reacted with the respective sera as controls (2).

They can be conveniently condensed to three groups of studies examining (i) the general populations, (ii) high-risk populations (i.e., in sexually transmitted disease clinics), and (iii) patients with HPV-related disease. For convenience and brevity, we will review eight studies that are summarized in Table 2. This discussion will be incomplete because of rapidly accumulating evidence in the field; nevertheless, it will describe a variety of methods, strategies, and populations and the difficulties which may be expected.

Two of the first studies on the immune response to HPV type 6, 16, and 18 fusion proteins were performed by Jenison et al. They focused on serological responses of healthy adults, patients from sexually transmitted disease clinics, and hospitalized children to HPV type 6, 16, and 18 L1, L2, E2, and E7 proteins (45–48). They relied principally on Western blots for analysis of sera, using ELISA to analyze seroreactivity to an epitope of HPV-6B. The highest percentage of serological positive responses were to HPV-6b L1. However, 47% demonstrated reactivity to the HPV-16 L2 protein. Importantly, 57 and 30% of children were seropositive to the HPV-6b L1 and HPV-16 L2 proteins, respectively. In a subsequent study which included women with cervical cancer, strong (2+ or 3+) seroreactivity to HPV-16 E7 was observed in 24% of cases versus 14% of controls and, for HPV-16 L2, 26 versus 17% of controls (46). Crude and adjusted relative risks for seroreactivity to these reagents were as high as 2.0 and 2.3, respectively (46).

Other research groups have focused on the E7 and E4 ORFs of HPV-16, including work by Jochmus-Kudielka et al. and Koechel et al. (50, 51, 58). In the study by Jochmus-Kudielka et al., the authors evaluated populations with and without HPV-related neoplasia, the latter including patients with high-grade precursor lesions and invasive carcinomas. Targets included fusion proteins containing the HPV-16 E4 and E7 ORFs. In this study, approximately 21% of cases were seropositive for anti-E7 antibodies, versus only 1.6% of controls, indicating a 14-fold crude "relative risk" for seropositivity to the E7 protein (51; Table 2). In contrast, antibodies to the E4 protein were distributed nearly equally in both children and healthy adults as well as in patients. The authors concluded that antibodies to the E4 protein were

indicative of "infection" in contrast to antibodies to the E7 protein, which correlated more strongly with neoplasia (51). In a follow-up study of 52 serum samples from cervical cancer patients, the same authors found seroreactivity to HPV-16 E4 and E7 in 61.5 and 19.2%, respectively, versus 5.8 and 7.7% for the HPV-18 E4 and E7 proteins (50). This is consistent with the greater prevalence rate of HPV-16 (approximately fivefold) versus HPV-18 in cervical cancers. However, in the same study, these authors found seroreactivity to the HPV-16 E4 and E7 to be 34.7 and 19.8%, respectively, in 121 renal transplant patients, suggesting that phenomena other than clinical disease may be associated with high proportions of patients seropositive to these proteins (50).

In a similar study, Koechel et al. contrasted seroreactivity in HPV-16 and HPV-18 L1, L2, E4, and E7 proteins in patients with cervical cancer and controls (58). For the E4 and E7 proteins, 63, 6.5, and 9.8% of cervical cancer cases, noncervical cancer cases, and cancer-free controls, respectively, were positive. In contrast, 57, 42, and 44%, respectively, reacted to late proteins from these two viral types (58).

In an effort to more closely define specific epitopes that will be disease specific, Dillner et al. performed a series of studies designed to identify linear epitopes that would preferentially react with sera from patients with cervical neoplasia (23–25). In their first report, an epitope in the region of the E2 ORF of HPV-16 E2 was investigated by constructing synthetic peptides and incubating them with patients' sera in an ELISA. They reported that 78% of patients with cervical neoplasia (either precancers or invasive cancer) and 21% of controls had values above an arbitrary limit of three times background (24). Moreover, they found that the antibodies were predominately immunoglobulin A (24). This study was subsequently repeated by Lehtinen et al., who reported that 25% of cancer cases and 5% of controls possessed immunoglobulin A antibodies to the peptide from the E2 ORF (67). They inferred that testing for this epitope alone was useful in only a portion of cases, but that it may reflect the natural history of the disease.

In a subsequent report, Dillner et al. described extensive studies of the HPV-16 L1 and L2 ORFs to detect antibodies to type-specific epitopes in patients who were documented to have HPV-16-related cervical carcinomas (25). By ELISA, they noted that, in as many as 60% of their patients, epitopes were more likely to be found in the L1 region, in contrast to 7% of controls (25). They subsequently expanded their study with a detailed analysis of the entire coding region of the HPV-16 genome and summarized their findings with linear epitopes as follows: (i) most of the immune reactivity was in the immunoglobulin A class; (ii) the L1, E7, and E2 proteins contained epitopes that were strongly associated with cervical cancer, and the E2 region reacted in up to 87% of cases; (iii) reactivity to the carboxy terminus of L1 and L2 was seen in healthy individuals; and (iv) the E1, E5, and E6 epitopes were virtually nonreactive (23).

UNANSWERED QUESTIONS

Discrepancies between Studies

From the above data, there is strong evidence that HPV-related neoplasms are associated with immune responses to a variety of HPV-derived proteins or peptides. The combined studies may explain in part certain discrepancies. For example, the elegant studies by Jenison et al. failed to

identify a strong relationship between seroreactivity to L2 and disease (46, 48). Subsequent studies by Dillner et al. and Koechel offer an explanation for this; this is, this region does not contain disease-specific epitopes (23–25, 58). Somewhat less clear are the conflicting data over the E7 ORF. Whereas Koechel and Jochmus-Kudielka reported a strong relationship between HPV-16 E7 seroreactivity and invasive carcinoma, Jenison et al. did not, a reflection, perhaps, of different technology, patients, and control populations (46, 48, 50, 58). The finding by Dillner et al. of infrequent seroreactivity to HPV-16 E7 versus that of Koechel and Jochmus-Kudielka may reflect different assay conditions or sensitivities between ELISA and Western blot (23–25, 50, 58).

Another area that deserves scrutiny is the evaluation of linear epitopes. The degree to which these predicted sequences are valid remain unclear (66). In particular, immune response for linear molecules on ELISA should be corroborated by similar reactivity with intact proteins.

Are These Findings Specific for HPV Type?

One unanswered question is whether the increased (and presumably type-specific) seroreactivity identified in cancer cases can be linked to the respective viruses with which they are associated. Although the data presented by Dillner et al. are based on women with HPV-16-related neoplasms, conspicuously absent from these studies are cases of cancer associated with other HPV viruses (25). If the hypothesis that HPV E7/E2 seropositivity is related to cancer is true, then patients with HPV-16-positive lesions should preferentially react with HPV-16-derived proteins versus patients with neoplasms associated with other HPV types. This possibility remains unconfirmed. Until cancers associated with other types are included in the analysis, it will be impossible to determine whether reaction to these proteins or epitopes is type specific or simply disease specific. Although demonstrating this phenomenon may be difficult when overall exposure to the virus is high, it would provide a much stronger link among the virus, its associated neoplasm, and the host immune response.

Is the Control Population HPV Negative?

No study has conducted a rigorous analysis of the control population to determine whether these persons carry HPV DNA in their genital tract. Notwithstanding the questionable importance of a single analysis for HPV-16 nucleic acids, such studies are important to determine further whether negative control patients who are seropositive are more likely to carry HPV DNA in their genital tract. While such studies may not shed light on the relationship between seroreactivity and cancer, it may shed light on the immunological significance of occult HPV infection.

Does Seroreactivity Identify the Route of Exposure?

This remains an important caveat in the dialogue seeking to link seroreactivity to cancer-associated viruses with actual risk of cervical cancer. Inasmuch as exposure to genital HPVs may occur at other sites (as described previously), the validity of serological studies is obscured. Again, however, the studies uncovering HPV nucleic acids in sites such as the oropharynx may or may not be detecting evidence of infection that has produced an immune response. One approach to this question is to investigate the local immune response

in the genital tract. This area of research remains undeveloped, given the technical limitations of obtaining satisfactory cervical mucus samples and the cyclic variability in the amounts of immunoglobulin present (43, 100). Nevertheless, this remains the principal site of disease and may provide data which may be more directly linked to disease.

Can Serological Studies Explain the Nature of HPV Gene Expression in Genital Neoplasia?

The observations that E4 and late (capsid antigen) proteins are less specific for cancer and found in patients either with no disease or with precursor lesions are consistent with their association with infection or the early (and possibly self-limiting) phases of HPV-related disease. In contrast, E7 reactivity is associated preferentially with cancer. It is tempting to speculate that E7 is consistently expressed in both the early and later phases of neoplasia, being the one HPV protein that is produced in the absence of viral replication. If so, this would support its potential value as a marker for more advanced disease. Unfortunately, this hypothesis must coexist with the knowledge that a high proportion of renal transplant patients and, in some populations, healthy controls harbor antibodies to this protein. Again, data linking seroreactivity to HPV-16 E7 with HPV-16-related neoplasia vis-à-vis other HPV-related neoplasms are needed to support this concept.

Can Serological Studies Be Used to Prevent Invasive Cancer?

The above questions are particularly important with respect to the concept of preventing cancer, in that it is critical to establish that seroreactivity is a marker for disease before addressing the role of serological studies in the detection of cancer. If indeed seroreactivity to early proteins such as E7 and E4 is a marker for disease, and if tests are sensitive and specific enough to identify women at risk, the next problem is to define the frequency of seroreactivity in women with precursor lesions before the onset of cervical cancer. The analysis of women with high-grade precursor lesions must produce fruitful information if this technique is to be useful as an initial screening test in large populations. If patients with precursor lesions cannot be distinguished reliably, the test becomes valuable only to detect cancer in its early stages.

One area of HPV immunology just recently explored is delayed hypersensitivity. A recent study of delayed hypersensitivity to HPV-derived proteins has indicated that women with cervical precancers may be more likely to have a cutaneous reaction to HPV-16 late (L1) proteins (44a). If this proves to be true, alternatives to serologic testing may be more productive for diagnosing the presence of HPV-related diseases. Further exploration of the use of these reagents that generate immune responses may yield strategies for protection from infection by these cancer-associated papillomaviruses (13).

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