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MINIREVIEW

Cell-Mediated Immune Response to Human Papillomavirus Infection

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Acquisition of human papillomavirus (HPV) results in an infection of variable duration which may or may not be associated with clinically apparent lesions. Lesions caused by skin-tropic HPV types generally manifest as cutaneous warts and most often resolve over a period of months to years. On the other hand, anogenital infections are more likely to remain clinically inapparent. The development of DNA amplification-based tests has demonstrated that anogenital infections are quite common and generally self-limited. For example, we and others have shown by PCR testing of multiple samples collected at different points in time-that 70 to 90% of sexually active adolescents and young women who develop an incident cervical HPV infection will show clearance of infection within 12 to 30 months (47, 61, 94). The factors influencing the natural history of these infections are not well understood. Given that persistent anogenital infection with oncogenic HPV types is associated with increased risk of neoplasia and invasive cancers (22, 59, 70, 71, 94, 124) and that cervical carcinoma remains a leading cause of death among women in developing countries (129), understanding these factors is of considerable importance.

Substantial effort has been directed recently at understanding the role of the host's immune response in the natural history of HPV, and in particular, anti-viral, cell-mediated immunity. Empirical evidence for the importance of cell-mediated immunity in control of HPV infection comes from an extensive body of literature documenting the increased prevalence of HPV infection and associated disease among immunosuppressed populations, including those with iatrogenic immunosuppression such as renal transplant recipients and individuals with human immunodeficiency virus (HIV) infection. Sillman and coauthors (128) reported in 1984 on 20 immunosuppressed women with various causes of immunosuppression who had lower genital neoplasia, describing evidence of associated HPV infection in all 20. Penn, in 1986, reported a 100-fold increase in cancer of the vulva and anus in renal transplant recipients (111). In the 1990s, as molecular testing for HPV infection came into its own, several reports confirmed increased incidence of HPV infection (50) and associated morbidities, including warts (77) and cervical squamous intraepithelial lesions (SIL) (104), among immunosuppressed renal transplant recipients.

The most persuasive evidence for the association between cellular immune defects and HPV infection and related morbidities comes from persons with HIV infection. Such individuals show increased prevalence of anogenital HPV infection (31, 32, 60, 105–107) as well as longer periods of HPV persistence (31, 40, 46, 90, 136). In addition, infection with multiple HPV types and with oncogenic types are more common (9, 31, 46, 78, 90, 107, 136). Associations between markers of HIV disease status (e.g., viral load and CD4 lymphocyte count) and HPV infection have been inconsistent. Most studies have suggested that advanced disease and greater immunological deficit are associated with higher prevalence and persistence of HPV infection (8, 31, 60, 78, 107, 108). On the other hand, several studies have found that HIV status influences HPV infection and SIL independently of CD4 counts (93, 147). These differences may have been due to specific population characteristics, such as young age, or early stages of HIV infection (93). The risk of abnormal cervical or anal cytology as well as of HPV-related anogenital disease (cervical SIL or anal intraepithelial neoplasia) is also increased in HIV-infected individuals (9, 33, 46, 60, 73, 78, 93, 105, 106, 117, 147). While, as mentioned above, most studies have suggested that the association between HIV infection and increased prevalence of HPV infection and disease is related to the immunosuppression seen in HIV infection, there is also some evidence that mechanisms other than immunosuppression, such as direct molecular interactions between HIV and HPV viral genes, may influence the natural history of HPV (8, 43, 93, 146). Nonetheless, the bulk of evidence strongly supports the notion that altered cell-mediated immunity is associated with increased HPV infection and disease.

Studies of cell-mediated immune responses to HPV have been hampered by several factors. First, the life cycle and gene transcription patterns of HPV are dependent on the stages of differentiation of keratinocytes (138). Only recently have there been successful models that allow in vitro propagation of HPV. Second, interpretation of published studies is difficult due to the interlaboratory variability of target antigens and assays, inadequate assessment of HPV type specificity, and inadequate HPV characterization of the study population. Third, HPV infection is highly localized to squamous epithelial sites, without significant systemic manifestations. Consequently, demonstrating evidence of HPV-specific immune responses in peripheral blood has proved challenging. Nonetheless, the last decade has seen significant advances in unraveling the role of cellular immunity in the natural history of HPV infection.

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This review will cover current understanding and recent advances, starting with cellular aspects of innate immunity, and then covering the recognition and effector phases of the adaptive cellular response.

INNATE IMMUNITY

Direct antiviral and antiproliferative effects of cytokines. Epithelial cells, once viewed principally as a mechanical barrier, are now regarded as having much more complex roles in cellular immunity. Keratinocytes, including cervical keratinocytes, constitutively secrete low levels of a variety of cytokines (133, 151, 156), including proinflammatory cytokines, growth factors, and chemokines, and can be induced by various stimuli to produce significant amounts (10, 133, 151). In this section on innate immunity, the antiviral and antiproliferative effects of several cytokines will be considered. These include transforming growth factor β (TGF-β), tumor necrosis factor (TNF), and the type I interferons (alpha and beta interferon [IFN-α and -β, respectively]), which are products of, among other cell types, epithelial cells (133, 151). In the section on adaptive immunity, other functions of epithelial cell-derived cytokines will be reviewed as will the important areas of cytokine production by T cells and the roles of cytokines in the regulation of T-cell responses.

A number of studies have investigated the ability of cytokines, particularly TGF-β, TNF, and the interferons, to inhibit the proliferation in vitro of both normal and HPV-transformed keratinocytes, as well as inhibiting expression of HPV genes including the early genes *E6* and *E7*. Expression of the E6 and E7 proteins has been shown to be critical for malignant transformation of infected cells (34, 58). E6 has been shown to interact with the anti-oncogene protein p53 by enhancing its degradation through ubiquitin-mediated proteolysis (121) via a mechanism requiring E6-associated protein (13), while E7 interacts with the tumor-suppressing retinoblastoma gene protein (45). These examples oversimplify the roles of the E6 and E7 proteins in malignancy, since both have also been shown to interact with numerous other cell cycle functional genes.

Studies of the growth-inhibitory effects of cytokines and of the potential for HPV-infected cells to escape growth inhibition have, of necessity, relied mainly on cell lines and are highly dependent on experimental conditions. It is therefore not surprising that the results have been confusing. TGF-β provides a good example of the range of findings which have been reported. TGF-β1 has been shown to be both a product of and a growth inhibitor of nontumorigenic HPV type 16 (HPV-16)- and HPV-18-immortalized cells (17, 18). The growth-inhibitory effect in HPV-16- or -18-transformed cells appears to be associated with the inhibition of expression of *E6* and *E7* (17, 155). For HPV-16, this inhibition occurs at the level of transcription, and the effects of exogenous TGF-β1 can be blocked by cycloheximide, indicating a requirement for synthesis of negative regulatory proteins (17, 155). Woodworth and coauthors (155) concluded that TGF-β 1 may be an autocrine regulator of HPV gene expression in HPV-infected genital epithelial cells. It has also been shown that the TGF-β1-induced growth inhibition in HPV-16-immortalized cells is accompanied by suppression of steady-state levels of RNA for the proliferation-enhancing gene c-*myc* (18). In a pair of recent studies, the expression of several proliferation-enhancing genes known to be regulated by TGF-β1 was investigated. In the first of these studies (127), HPV-11-transformed xenografts showed overexpression of TGF-β1 and downregulation of the genes *bcl-2*, c-*myc*, c-*jun*, and c-Ha-*ras* and that encoding NFκB, suggesting a mechanistic association between TGF-β upregulation in HPV-transformed papillomas and growth inhibition. Further supporting this contention, the same group also reported that TGF-β 1 treatment of HPV-16-transformed, but not HPV-18-transformed, cells results in downregulation of *bcl-2* and NFκB gene expression (126). The authors postulated that while the different responses in the two cell lines may be virus type specific, they more likely derive from the different stages of tumor progression of the lines.

The complexities of interpretation of data derived from studies of cell lines are illustrated by various studies by Woodworth and coauthors. In contrast to the studies described above from their group and several others, they have more recently demonstrated (152) that when HPV-immortalized cells are grown in a medium that stimulates an early stage of squamous differentiation, TGF-β appears to stimulate, rather than inhibit, cell growth. This growth stimulation is seen only in HPV-immortalized, not normal (HPV-negative), cervical keratinocytes. The effect is indirect in that it involves increased expression of epidermal growth factor receptor and its ligand, amphiregulin, and the establishment of an autocrine growth-stimulatory loop. The fact that normal cervical cells grown under these same conditions exhibit the expected growth inhibition by TGF-β suggests the possibility that HPV-infected cells may escape this growth inhibition (as discussed in more detail below) and that this escape may occur early, even before malignant transformation. Culture conditions favoring early squamous differentiation could be argued to more accurately reflect in vivo physiologic conditions; it is nonetheless clear that conclusions drawn from cell line-based studies must be interpreted cautiously.

TNF is another cytokine product of keratinocytes which may have an antiproliferative effect on HPV-infected cells, but the findings have again been complex and are very similar to those for TGF-β. TNF appears to have an antiproliferative effect on HPV-16-harboring epithelial cells (84, 150), though not on HPV-18-immortalized cells (150). This effect involves growth arrest in G_0-G_1 (149). Malejczyk and coauthors (84) concluded, as it appears to be an autocrine mechanism, that it could provide self-regulatory growth control of HPV-associated neoplasia. Like TGF-β, TNF has been shown to repress HPV-16 *E6* and *E7* expression at the transcriptional level in an HPV-16-immortalized human keratinocyte cell line, sharing this ability with interleukin 1α (IL-1α) (72). Also like TGF-β, a growth-stimulatory effect involving an amphiregulin-mediated autocrine loop has been reported for both TNF and IL-1α in some, but not other, HPV-16- or -18-immortalized cells and cervical carcinoma lines (154). This likewise suggests the possibility of an early escape from growth inhibition in HPV-infected cells.

Interferons, including IFN-α and -β as well as the T-cell product IFN-γ, have also been investigated in regard to antiproliferative effects. IFN-α has been shown to inhibit proliferation of HPV-16-immortalized human keratinocytes at concentrations 10- to 100-fold lower than those needed to inhibit growth of normal human keratinocytes (68). The same low

dose inhibits transformation of normal human keratinocytes by HPV-16 DNA. IFN-α also inhibits HPV-16 E7 protein expression but not transcription and not E6 protein expression, suggesting that growth and transformation inhibition is mediated by an inhibition in E7 protein expression. Others (101, 112) have shown that IFN-α inhibits transcription of HPV-18 E6 and E7 genes in HeLa cells, a cervical carcinoma cell line containing integrated HPV-18 DNA. Interestingly, this effect appears to be shared with IFN-γ (101). The varied effects attributed to different interferons may be virus-type specific, cell-line specific, or dependent on other experimental variables. As a further example, one group (153) showed that IFN-γ but not IFN-α inhibits transcription of E6 and E7 genes in HPV-16-, -18-, and -33-immortalized keratinocytes, accompanied by growth inhibition, whereas another group (41) demonstrated that IFN-β reduces the transcription of E6 and E7 genes in an HPV-16-transformed keratinocyte line (HPK-IA). Neither IFN-α nor IFN-γ has this effect in HPK-IA cells (41). Most interestingly, this group (41, 42) also demonstrated a marked cytopathic effect on HPK-IA cells by IFN-β but not IFN-α, despite the sharing of a common receptor for these two cytokines. They suggested that the ability of this cell line to finely discriminate between the two cytokines may be secondary to as yet unrecognized receptor complexity and also concluded that interferon responsiveness differs among cell lines (41). These studies again highlight the notion that data collected from cell lines must be interpreted with caution. Nonetheless, most studies suggest a potential antiviral role for interferons in HPV-infected epithelium.

A fair amount of evidence suggests that malignant transformation involves loss of responsiveness to the inhibitory effects of cytokines (18, 42, 85, 153, 155). TGF-β1, for example, has been shown to inhibit the growth and HPV gene transcription in nontumorigenic HPV-16-immortalized cells but not in HPV-16$^+$ cervical cancer lines (Ca Ski and SiHa) (18). Partial resistance to TGF-β1 can also be induced in HPV-16-immortalized cell lines by malignant transformation in vitro via transfection with the v-Ha-*ras* oncogene or the herpes simplex virus type 2 *Bgl*II N fragment (155). Resistance has likewise been demonstrated to interferon-mediated inhibition of growth and HPV early gene expression after malignant transformation (153). De Marco and coauthors (42) reported that the cytopathic effect noted above of IFN-β on HPV-16-immortalized cells is not induced in malignant, HPV-16-harboring SiHa cells. The authors suggested that a relatively conserved phenotype is required for the effect. As mentioned, more recent work suggests that resistance to the growth-inhibitory effects of several cytokines, accompanied by an indirect amphiregulin-mediated growth-stimulatory effect, may occur in HPV-immortalized cells even prior to malignant transformation (152, 154). The characterization of this growth-stimulatory effect has led to the suggestion that chronic inflammation, with production of proinflammatory cytokines, may actually provide a selective advantage for abnormal cells in vivo (154). These studies all support a possible escape of cytokine-mediated growth inhibition in HPV infection; the unanswered question is whether this is an early event or one associated with malignant transformation.

In addition to the amphiregulin-mediated loop described above, several other mechanisms by which HPV-infected cells may escape the growth-inhibitory effects of cytokines have been proposed. For example, Malejczyk and coauthors (85), comparing HPV-16-transformed cell sublines with different levels of tumorigenicity in nude (athymic) mice, showed a correlation between increased tumorigenicity, resistance to TNF-mediated inhibition of proliferation in vitro, and significantly decreased expression of TNF receptors. They also showed increased shedding of soluble type I TNF receptor in the more highly tumorigenic subline (83). Interestingly, levels of soluble type I and type II TNF receptors in serum have been shown to be significantly elevated in patients with HPV-16- or -18-associated cervical carcinomas or anogenital Bowen's carcinoma (87). The authors concluded that these soluble receptors may facilitate the growth of lesions (87).

Another possible mechanism of escape from antiproliferative cytokines was suggested by a recent report (131) that conversion of nontumorigenic HeLa-fibroblast hybrids to tumorigenic cells is accompanied by the development of resistance to the ability of TNF to suppress HPV-18 gene transcription, as well as changes in the composition of the activator protein 1 complex, which plays a role in expression of *E6* and *E7*. The authors proposed that loss of TNF-sensitivity may be causally related to alterations in the activator protein 1 complex.

A possible mechanism by which some HPV types may evade the effects of IFN-α was provided by a study (12) that showed that HPV-16 E7 protein inhibits the induction of IFN-α-inducible genes by inhibiting the translocation of p48, the DNA-binding component of the interferon-stimulated gene factor 3 transcription complex, to the nucleus upon IFN-α stimulation. A direct protein-protein interaction was identified between E7 and p48. This supports a previous report (4) that patients with condylomas that fail to respond to interferon (IFN-α and IFN-γ) treatment express higher levels of E7 mRNA than responders.

In summary, there appears to be evidence for the contention that various cytokines, notably TGF-β, TNF, IL-1, the type I interferons, and IFN-γ, may play roles in checking growth of HPV-infected cells and that viral persistence, disease progression, and/or malignant transformation could involve escape from those mechanisms. Since epithelial cells are capable of producing these cytokines (except IFN-γ), some authors have suggested that cytokines may play autocrine roles in keratinocyte growth regulation in HPV infection (84, 85, 155). These cytokines are all produced by multiple other cellular sources, of course, including macrophages, T cells, and NK cells, so many cell types probably contribute to the growth-inhibitory effects described above. Additional functions of cytokines will be covered below.

Role of NK cells. In patients with epidermodysplasia verruciformis, chronic infection with HPV leads to disseminated red plaques and verruca-like skin lesions. Reduced cytotoxicity of NK cells against keratinocytes (isolated from a premalignant lesion of a patient with epidermodysplasia verruciformis) in peripheral blood mononuclear cells (PBMC) from epidermodysplasia verruciformis patients (81) suggests the importance of NK cell activities in preventing lesions from developing.

Studies investigating the role of NK cells in the development of SIL also suggest protective effects of NK cells. Decreased NK cell lysis of HPV-16$^+$ keratinocytes in subjects with SIL or carcinomas has been reported (82). The same group has shown that decreased recognition of HPV-16$^+$ keratinocytes is re-

lated to unresponsiveness of PBMC to immunostimulatory cytokines such as IL-2 and IFN-α (86). Other groups have also shown similar importance of NK activity in regression of SIL (54, 134).

ADAPTIVE CELL-MEDIATED IMMUNITY

The various cellular components involved in the recognition and effector phases of adaptive epithelial immune responses have been demonstrated in both cutaneous and mucosal HPV-infected tissues (63, 89, 91). These include Langerhans cells, which capture antigens for transport to local draining lymph nodes and presentation to naive T cells; clonally expanded T cells that have homed back to infected epithelial tissues via mechanisms involving chemokines and adhesion molecules; and accessory cells such as macrophages. The adaptive immune response to HPV will be considered in terms of these two phases (recognition and effector), focusing on the cells involved and the soluble and membrane-bound molecules that mediate and regulate the responses.

Recognition phase. A number of studies have addressed the potential role for Langerhans cells in viral diseases with cutaneous manifestations including HPV infection. A reduced number of epidermal Langerhans cells has been documented in lesions due to herpes simplex virus and HIV as well as HPV (89). One report (148) documented drastic reductions in $CD1^+$ Langerhans cells in cutaneous plantar and hand warts compared with normal epithelium, but interestingly no reductions were found in mucosal genital condylomas and laryngeal papillomas. On the other hand, several investigators have described decreases in Langerhans cell density in genital HPV infection, condylomas, or SIL (91, 92, 140). It is possible that a decrease in Langerhans cell density in the epidermis of HPV-infected tissues simply represents normal egress of antigen-carrying Langerhans cells from the epidermis to draining lymph nodes for antigen presentation to naïve T cells (89).

The possibility certainly exists that reductions in Langerhans cells, whether due to normal egress or due to other as yet unidentified mechanisms, may contribute to impaired immune surveillance. Some suggest that the depletion of intraepithelial Langerhans cells associated with HPV infection may, along with other local immune deficiencies, contribute to prolonged infection or possibly malignancy (89, 140). One study found pretreatment biopsy specimens from patients whose genital condylomas failed to respond to interferon therapy to be depleted of Langerhans cells, compared with pretreatment biopsy specimens from responding patients (7). Several studies have suggested that Langerhans cells in HPV lesions may be functionally impaired, which again may contribute to persistence of infection. For example, abnormal morphology of Langerhans cells in genital condylomas has been reported (25, 91), with blunting of the dendritic architecture. One group (28), using paired biopsies and two different antibodies (S-100 and CD1) to stain Langerhans cells, recently demonstrated that $S\text{-}100^+$ cells are significantly reduced in SIL compared with normal cervical epithelium, in agreement with an earlier report (140), whereas $CD1^+$ cells are not, suggesting that there is a defect in S-100 protein expression. Since S-100 stains a family of related calcium-binding proteins, they suggested that these proteins may be important in the function of Langerhans cells in HPV infection. In contrast to reports suggesting Langerhans cell functional deficits, Cooper and coauthors (30), studying lesions from patients with the HPV-related cutaneous disease epidermodysplasia verruciformis, reported that despite slight reductions in Langerhans cell number in lesions, as well as abnormal Langerhans cell morphology, isolated Langerhans cells appear to be functionally intact in their ability to present alloantigens to T cells.

Once Langerhans cells have captured antigen, the recognition phase continues with their migration to draining lymph nodes. As recently reviewed by Wang and coauthors (151), cytokines, elaborated principally by keratinocytes with some contribution from the Langerhans cells themselves, appear to be crucial mediators of this process. Particularly important cytokines are thought to include IL-1α and TNF (mainly produced by keratinocytes) and IL-1β (mainly produced by Langerhans cells), all of which promote Langerhans cell migration, and IL-10, produced by keratinocytes, which acts as an inhibitor of Langerhans cell migration (151). Other cytokines, for example granulocyte-macrophage colony-stimulating factor, also produced by keratinocytes, promote the initiation of Langerhans cell maturation into mature dendritic cells (74). A possible association between deficits in production of these cytokines and persistent HPV infections has been postulated (156), based on the findings of reduced cytokine levels (IL-1α and 1β, TNF, and granulocyte-macrophage colony-stimulating factor, among others) in several HPV-immortalized cervical cell lines and several cervical cancer lines, compared with normal cervical cells. Given that lower levels of cytokine production were seen with more prolonged periods of subculture, the results from the HPV-immortalized cell lines may not be accurate reflections of in vivo conditions. Nonetheless, the findings provide preliminary evidence for an association between diminished production by keratinocytes of various cytokines and persistent HPV infection. Support also comes from in vivo evidence recently reported by Mota and coauthors (96), who found that TNF expression by keratinocytes was absent in some SIL biopsy specimens studied but present in all biopsy specimens of normal cervical squamous epithelium. Furthermore, fewer high-grade SIL (HSIL) than low-grade SIL (LSIL) biopsy specimens were positive for TNF expression. Conversely, IL-10 expression by keratinocytes was present in many SIL biopsy specimens but absent in normal epithelium. Noting the importance of these two cytokines in regulating Langerhans cell functions, they suggested that these alterations may contribute to an altered antigen-presenting environment in premalignant cervical lesions. The studies described above suggest the possibility that HPV may evade immunosurveillance by modulating the production of cytokines by infected keratinocytes or, alternatively, that individuals with abnormal responses in this regard may be at increased risk of HPV infection or associated malignancy.

Effector phase. (i) Proliferation and related studies of T-cell responsiveness to HPV. A large number of studies have investigated the role of helper T lymphocytes in providing protection against the development of HPV-associated lesions by measuring T-cell proliferative responses (36, 39, 55, 64, 65, 79, 80, 97, 125) or IL-2 release (14, 38, 143). Unlike studies addressing the role of NK cells which support their protective

role (see above), the results from proliferation assay studies are inconsistent.

Studies to date have focused on helper T-lymphocyte responses to HPV-16 antigens, as HPV-16 is the most prevalent among the oncogenic HPV types and is the type most commonly associated with invasive cervical cancers (16). Because of the roles of the E6 and E7 gene products in cell transformation described above, responses to HPV-16 E6 and E7 proteins and peptides have been widely studied (36, 38, 39, 64, 65, 80, 97, 143). In three of the five studies which were cross-sectional in design, more-frequent responses to these antigens were observed in subjects who were cytologically normal compared with subjects who had developed SIL (80, 97, 143), suggesting that these antigens are important in SIL prevention. In one study, more-frequent responses to HPV 16 E7 peptides were observed in SIL subjects with HPV-16, -31, or -33 infections compared with controls without SIL (HPV status unknown) (65), and in another study no correlation between responses and the presence of SIL was found (36). Although clinical correlation to in vitro assays is essential, interpretation of cross-sectional studies remains limited.

Few studies have investigated T-cell proliferative responses to HPV-16 E6 or E7 gene products using longitudinal designs. One such study reported that the subjects with positive responses to an E6 peptide (amino acids 1 to 30), an E7 peptide (amino acids 72 to 97), or both were likely to have cleared their HPV infection and SIL at the following visit (64). In contrast, another group has reported that positive T-cell proliferative responses (39) and IL-2 release (38) are seen more frequently in subjects with progressing SIL than subjects with regressing disease.

The results from studies focusing on HPV-16 proteins other than E6 and E7 have been equally confusing. T-cell proliferative responses to HPV-16 L1 are more frequently observed in subjects with SIL than in healthy age-matched controls (79, 125). A cross-sectional analysis (14) of helper T-cell responses to HPV-16 E2 protein in women with SIL showed an association between responses to the C-terminal domain and previous or present HPV-16 infection but no association with disease outcome. However, a longitudinal analysis of the same study revealed that these responses frequently occur at the time of viral clearance. Therefore, such responses may correlate with clearance of viral infection but not necessarily with the resolution of SIL. A study of proliferative responses to HPV-16 E5 (55) showed that these were more frequently seen in subjects with HPV-16$^+$ LSIL (43%) than subjects with HPV-16$^+$ HSIL (22%) or HPV-16$^+$ subjects without SIL (20%). A longitudinal analysis of this study population would be of great interest to determine whether higher responses in subjects with LSIL are associated with its resolution.

The inconsistencies reported among these studies can be explained by several factors. The first factor is differences in antigens: studies have differed in their choice of peptide versus protein antigens and in the specific sequences used. Some antigens are surely more antigenic than others. The second factor is differences in subject populations: cross-sectional studies are limited since the important events such as clearance or regression may not yet have occurred. Even longitudinal studies rarely have sufficient information on the women's entire history of HPV exposure (that, is HPV history since first intercourse), making the interpretation of positive responses in current HPV$^-$ women difficult. The third factor is a lack of a simple correlation between helper T-cell responses and the natural history of infection: while helper T lymphocytes have been shown to be necessary in eventual production of antibodies by B lymphocytes (37, 102) and in aiding the development of cytotoxic lymphocytes (CTL) (3, 56), the activities of helper T cells themselves may not correlate directly with clearance of virus and virus-associated lesions since they are not themselves the killers.

(ii) Studies of CTL-mediated killing. CD8-positive, major histocompatibility complex (MHC) class I-restricted CTL are known to be responsible for recognizing and killing virus-infected host cells and virus-induced tumors (57). Immunohistochemical analysis of SIL and cervical cancer specimens has demonstrated the presence of activated CTL in lesions (15). Studies using mouse models have demonstrated that immunization with HPV-16 *E6*- or *E7*-transfected nontumorigenic fibroblasts can lead to regression of tumors expressing *E6* or *E7*, respectively, and that the regression is mediated by CD8$^+$ CTL (20, 21).

In humans, HPV-16 E6- and/or E7-specific CTL have been demonstrated in women with cervical cancer and women with SIL. Alexander and coauthors (1) stimulated PBMC from cervical cancer patients with a human leukocyte antigen A2 (HLA-A2)-restricted HPV-16 E7 peptide [E7 amino acids 11 to 20, or E7 (11–20)] and showed that CTL were capable of lysing HLA-matched, HPV-16 E7 (11–20)-pulsed targets in two of three patients. Another group has identified HPV-specific CTL in lymph nodes and tumors of cervical cancer patients (49). CTL to HPV-16 E6 and E7 have also been demonstrated in PBMC stimulated in vitro with the cervical carcinoma line Ca Ski in some patients with SIL (48).

HPV-16 E6- and E7-specific CTL have also been demonstrated in subjects who had evidence of HPV-16 infection but who had not developed SIL (98–100). In a small cross-sectional study (98), the percentage of subjects who demonstrated HPV-16 E6- and/or E7-specific CTL was higher in a group of women with HPV-16 infection who had not developed SIL than in a group of women with HPV-16 infection who had developed SIL. The effector cell phenotypes in these assays have been shown to include both CD4$^+$ and CD8$^+$ T lymphocytes (99).

In a similar subject population, we examined the association between HPV-16 E6- and E7-specific CTL and HPV-16 persistence, in a longitudinal study design involving multiple CTL assays in women with evidence of cervical HPV-16 infection as detected by PCR (100). Lack of CTL response to the HPV-16 E6 protein, but not the E7 protein, was correlated with persistent HPV-16 infection, suggesting that a CTL response to E6 is important in the clearance of HPV-16 infection. Notably, CTL responses in this study were shown to be frequently transient. Most women had several repeated CTL assays performed at 4-month intervals, of which only some were positive. This most likely reflects the low sensitivity of current the assay to detect circulating T cells that are specific to localized infections. A similar study looking at women with SIL associated with HPV-16 would be important in elucidating the role of CTL in the regression of SIL.

Recently, a novel technique for identifying antigen-specific

T lymphocytes, using peptide-MHC tetramers, has been used to identify and isolate T lymphocytes specific for HPV. Youde and coauthors (158) reported positive tetramer responses to HPV 16 E7 (11–20) in patients with cervical cancer and carcinoma in situ. However, the interpretation of positive responses in cancer patients is unknown since, by definition, these patients have failed immune responses in that infection persisted and the development of neoplasia was not prevented.

(iii) Defining antigenic epitopes of HPV. Considerable effort has been devoted by many investigators to identifying antigenic epitopes of HPV, using both mouse model systems (51, 52, 116, 118, 120) and human systems (62, 66, 67, 76, 116, 135, 139). Kast and coauthors (67) identified potential CTL epitopes of HPV-16 E6 and E7 proteins for five common HLA types by measuring binding of each of 150 nonamer peptides. Immunogenicity of nine of these potential antigenic epitopes for HLA-2.1 was tested (116). Using HLA-2.1 transgenic mice four immunogenic peptides were identified in vivo [E6 (29–38), E7 (11–20), E7 (82–90), and E7 (86–93)]. In addition, CTL induction of PBMC from humans confirmed immunogenicity, in vitro, of three of the four peptides [E7 (11–20), E7 (82–90), and E7 (86–93)]. CTL to one of these peptides [E7 (11–20)] have been demonstrated in SIL and cancer patients, in studies mentioned above (1, 49, 158). Whether this epitope plays an important role in the elimination of HPV-16 infection and of HPV-associated lesions has not been investigated.

In a recent report (62), one group has succeeded in isolating and expanding a TNF-secreting $CD4^+$ tumor infiltrating lymphocyte line, from a cervical cancer patient, which recognizes autologous cervical cancer cells. The cell line was shown to define an HLA-DR4-presented epitope provided by the E7 genes of HPV-59 and HPV-68.

(iv) Antigen presentation in the effector phase. (a) MHC class II-restricted antigen presentation. A number of studies have examined the expression of MHC class II antigen in HPV infection. IFN-γ treatment of HPV-16-, -18-, and -33-immortalized keratinocytes has been shown to enhance transcription of class II MHC molecules (153). Viac and coauthors (148) demonstrated epithelial cells expressing the MHC class II antigen HLA-DR in genital condylomas and laryngeal papillomas. They described a close spatial association between HLA-DR^+ epithelial cells, T-cell infiltrates, and high densities of Langerhans cells and postulated that the HLA-DR upregulation may be due to IFN-γ secretion by activated T cells and that the HLA-DR^+ keratinocytes may participate in immune reactions. Interestingly, they reported that HLA-DR expression on keratinocytes was absent in cutaneous warts, highlighting possible differences between cutaneous and mucosal anti-HPV immune responses. Another group (25) has also shown HLA-DR upregulation on keratinocytes in genital condylomas, although they emphasized that the functional significance of this remains an area of controversy.

Another area of controversy is the role of MHC class II expression in persistence of HPV infection or progression of HPV-associated disease. Several studies report evidence of impaired MHC class II expression (6, 25, 156), whereas other studies offer a more confusing picture, showing more frequent expression of HLA-DR in HSIL than in LSIL (26, 96). The hypothesis that HPV may evade cellular immune responses by, among other mechanisms, inhibiting upregulation of MHC class II expression, is suggested by a study (6) comparing patients whose genital condylomas responded to interferon treatment with those who did not. Impaired upregulation of this antigen in the nonresponders, compared with the responders, was associated with overexpression of the HPV E7 gene, suggesting a possible causal link between high *E7* expression in the nonresponders and the decreased inducibility of HLA-DR expression.

(b) MHC class I-restricted antigen presentation. Normal human keratinocytes constitutively express MHC class I molecules and are susceptible to class I-mediated lysis by alloantigen-primed CTL (137). In addition, exogenous IFN-γ increases this susceptibility (137). Losses of MHC class I expression have been reported in HPV-infected tissues, with some possible differences between cutaneous and mucosal tissues. For example, one group (148) has reported a drastic reduction in MHC class I expression in cutaneous warts, but only mild reductions in condylomas and laryngeal papillomas. More advanced mucosal lesions, however, do exhibit significant losses of MHC class I expression, as illustrated by a report (29) showing that specimens from at least 30% of cervical cancer biopsies studied demonstrated some alteration in MHC class I expression, varying from complete loss of expression to loss of particular allelic products. While that study found no correlation with tumor type, disease stage, or detection of HPV-16 or -18 DNA in the biopsy specimens, Torres and coauthors (142) did find that loss of MHC class I expression in cervical cancer biopsy specimens correlates with tumor invasiveness and more aggressive histology as assessed by the Glanz histoprognostic index of malignancy. An interesting recent study (145) correlated downregulation of MHC class I expression with a more aggressive course of the HPV-related disease recurrent respiratory papillomatosis (RRP). They examined the expression of the transporter associated with antigen presentation (TAP-1) and MHC class I proteins in laryngeal papilloma biopsies from patients with RRP and showed that downregulation of both is associated with more frequent disease recurrences. They concluded that HPV may evade immunological recognition by downregulating TAP-1. Others have cautioned, however, that MHC class I downregulation may be the result of altered levels of or sensitivity to cytokines, such as IFN-γ and TNF, rather than direct effects of viral infection (88). Regardless of the underlying reasons for changes in MHC class I expression, the data suggest that in persistent HPV infection or HPV-associated malignancy, loss or downregulation of MHC class I expression may contribute significantly to diminished recognition and removal of infected cells by CTL.

(v) Regulation of T-cell responses. (a) Chemokines and adhesion molecules. An important component of the effector phase of cutaneous immune responses is the recruitment and retention of activated lymphocytes at sites of inflammation, which reflect processes involving both soluble chemokines and membrane-bound adhesion molecules. Schröder (122) reviewed the roles of chemokines in cutaneous immunity, with particular emphasis on the importance of epithelial cell IL-8 production in the recruitment of neutrophils and T cells. Cervical keratinocytes constitutively produce IL-8 (156) and, as with other keratinocytes (122), secretion is enhanced by activation with IL-1 or TNF (156). The T-cell product IFN-γ has

been shown to synergize with TNF in enhancement of keratinocyte IL-8 production (11), which may provide a positive feedback loop by which T cells participate in their own enrichment at sites of inflammation.

Studies of the association between chemokine production and the natural history of HPV infection have been few. In a recent study of chemokine levels in cervicovaginal lavage samples from HIV-seropositive women, Spear and coauthors (132) demonstrated significantly higher IL-8 levels in women with genital tract dysplasia or cytologic or histologic evidence of HPV infection than in women without, whereas the levels of two other chemokines studied (RANTES [regulated-on-activation normal T-expressed and secreted factor] and MIP-1α [macrophage inflammatory protein 1α]) were not significantly different. They noted, however, that comparisons of cervicovaginal lavage samples are confounded by uncertainties about dilutional artifact which may be introduced during lavage. Furthermore, interpretation of the apparent induction of IL-8 in HPV infection is difficult in the setting of HIV infection and associated immune imbalances. In contrast, other authors (156) have reported significantly diminished constitutive production of IL-8 in HPV-16- or -18-immortalized cell lines and in some cervical carcinoma cell lines. Treatment with IL-1 or TNF failed to upregulate IL-8 production to the same degree as in normal cervical cells.

The chemokine macrophage chemoattractant protein 1 (MCP-1) may also be important in HPV control. It has been reported that HPV-18$^+$ HeLa cells transfected with an expression vector containing cDNA for MCP-1 show significant growth retardation accompanied by a marked macrophage infiltrate when inoculated into nude mice, whereas, HeLa cells without the vector lead to rapidly growing tumors and no macrophage infiltration (69). Keratinocytes are capable of producing MCP-1 (122) and the endogenous gene is also present in HeLa cells and not structurally rearranged (69). However, its expression appears to be suppressed in HeLa cells and is only marginally upregulated by TNF (69). This suggests another potential method of escape from host defenses in HPV-associated malignancy.

A larger number of studies have addressed the roles of adhesion molecules, with particular focus on keratinocyte expression of intercellular adhesion molecule 1 (ICAM-1) and its ligand, leukocyte function-associated antigen 1 (LFA-1). In addition to its role in cell migration, the LFA-1–ICAM-1 interaction appears to be involved in specific antigen recognition and is critical for CTL-mediated killing (103). Constitutive keratinocyte expression of ICAM-1 is very low but is readily and markedly increased by stimulation with the cytokines TNF and IFN-γ (103). Morelli and coauthors (91) showed that in vulvar condylomas with mononuclear infiltrates, LFA-1$^+$ T cells could be seen forming small clusters in the lower half of the epithelium around ICAM-1$^+$ keratinocytes. ICAM-1 expression overlapped with HLA-DR expression, supporting the importance of this adhesion molecule in T-cell–keratinocyte interactions. Similar findings of foci of epithelial cells expressing ICAM-1 and LFA$^+$ leukocytes (mainly CD8$^+$) have been described in condylomas and laryngeal papillomas (148). In a study comparing regressing and nonregressing genital condylomas (25), there was significant induction in the regressing lesions of ICAM-1 and two other adhesion molecules, E-selectin and vascular cell adhesion molecule 1. The regressing lesions also contained significantly more T cells and macrophages than the nonregressing controls. Malignant transformation may be accompanied by diminished expression of adhesion molecules, although, to date, studies are not clear on this. Some investigators (156) have reported evidence that malignant transformation of cervical keratinocytes is associated with impaired ICAM-1 inducibility, whereas others (27) have reported upregulation of ICAM-1, vascular cell adhesion molecule 1, and E-selectin in HSIL, compared with LSIL or normal cervical epithelium. The latter study's findings suggest a role for adhesion molecules in antineoplastic immune responses in HSIL but do not support the contention that impaired expression of adhesion molecules is associated with progression of HPV-induced disease.

(b) Cytokines involved in regulating T-cell responses. In recent years, studies of immunoregulation have focused on an apparent functional dichotomy of cytokines, between those that support cellular immune responses and those that support humoral responses, as well as a parallel dichotomy of cytokine-producing helper T cells. The phenotypic classification of activated helper T cells into IFN-γ-, TNF-, and IL-2-producing T helper type 1 (Th1) cells, which stimulate cellular responses, and IL-4-, IL-5-, IL-10-, and IL-13-producing T helper type 2 (Th2) cells, which stimulate humoral responses, has been reviewed elsewhere (95, 110). Accessory cell-derived cytokines, such as IL-12, which promotes Th1 cell development, are also important regulators of T-cell functions. Additionally, accessory cells, and even keratinocytes, contribute to production of various Th1 and Th2 cytokines, such as TNF and IL-10. The existence of Th1 and Th2 cells in humans has been confirmed by several elegant studies (113, 115). The hypothesis that the Th1-Th2 model of immunoregulation may play a role in the natural history of HPV infection is suggested by two other infections involving infection of epithelial tissues by intracellular pathogens: cutaneous leishmaniasis (19, 114) and leprosy (119, 157).

Some studies suggest that HPV infection normally elicits a Th1 response. Immune responses to HPV vaccination have been shown in a mouse model to be characterized by the secretion of the Th1 cytokines IFN-γ and IL-2 (44). Tsukui and coauthors (143) have demonstrated IL-2 production by peripheral blood lymphocytes, in response to HPV-16 peptides, in women with SIL or cervical cancer, and in cytologically normal women with histories of HPV-16 infection.

The contention that a Th1 response is important for clearance of HPV infection, or, conversely, that lack of such a response may be associated with persistent infection or the development of HPV-related neoplasia, has support from several studies. In a study of cytokine mRNA patterns in exfoliated cervical cells (123), we identified seven subjects who were HPV$^+$ and who subsequently had cleared their infections on examination 4 months later. All seven showed a Th1 pattern (expression of IFN-γ mRNA and absence of IL-4 mRNA) preceding clearance, compared with highly variable patterns in HPV$^-$ women with previous histories of HPV infection. Another group (109), using cervical biopsy tissue, showed decreased IFN-γ mRNA expression in women with SIL or cervical cancer, compared with normal cervical tissue. The normal cervical tissue was obtained from HPV 16- and 18-negative

women, although it is not clear if they were positive for any other HPV types. Another recent study (2) examined cervical biopsies by immunohistochemistry and found that SIL, particularly HSIL, biopsies contained fewer Th1 (IL-2^+) cells and an increased density of Th2 (IL-4^+) cells, compared with normal cervical epithelium. Two studies have used peripheral blood lymphocytes. In the study by Tsukui and coauthors described above (143), the fraction showing IL-2 production when stimulated in vitro with HPV-16 peptides correlated with cervical disease: highest levels were found in cytologically normal HPV-16^+ women, intermediate levels were found in women with SIL, and the lowest levels were reported in women with cervical cancer. Another group (23) examined antigen- and mitogen-stimulated cytokine production by PBMC in women with SIL and reported a shift from Th1 cytokines to Th2 cytokines in women whose HPV infection extended beyond the cervix to other sites of the lower genital tract, as evaluated by colposcopic, cytologic, and histologic methods and by HPV detection via molecular hybridization. The authors speculated that augmented IL-10 production may decrease immune recognition of HPV-associated tumors by downregulating expression of MHC class I and/or class II molecules. As described above, both of these molecules have been reported to show diminished expression in persistent or progressing HPV-associated lesions, although, as noted, the evidence for loss of MHC class II expression is confusing at best.

A couple of recent studies have investigated cytokine production in women coinfected with HPV and HIV. One study (75) assessed peripheral blood T-cell cytokine profiles by flow cytometry. A shift away from Th1 (IL-2^+, IFN-γ^+, and/or TNF^+) and toward Th2 (IL-10^+) phenotypes was described in women with either abnormal cervical cytology or SIL, compared with healthy controls. The shift occurs both in women with and without HIV infection, but the decrease in peripheral blood Th1 cells is greatest in coinfected women. Another study (35) examined cytokine protein production in cervical secretions collected from adolescents. The investigators showed that HPV infection is associated with decreased IL-10 levels in HIV-seronegative women but with increased levels in HIV-seropositive women. The decreased IL-10 levels in HPV^+ but HIV^- women supports the contention that an anti-HPV immune response is normally associated with a switch away from Th2 cytokines. On the other hand, the finding that coinfected (HPV^+ and HIV^+) subjects have significantly higher IL-10 levels than women with either infection alone is harder to explain. A Th1-to-Th2 shift has been described in HIV infection (24). Therefore, it may be that the T cells that have homed to the cervical mucosa, due to the presence of HPV, have been biased to a Th2 profile by the patient's concomitant HIV infection. As IL-10 is also a product of macrophages, another possibility is that these cells were a source of the higher IL-10 levels seen in the coinfected subjects. Further support for this hypothesis comes from their finding that coinfection with HIV and HPV is also associated with higher levels of the accessory cell product IL-12 (especially in women with a third sexually transmitted infection), compared with women with only HIV or HPV infection (35). As cautioned previously, interpretation of HPV-associated immune responses is difficult in the setting of HIV infection. Nonetheless, these studies do lend support to the notions that HPV infection elicits a Th1 type of response (35) and that a shift toward a Th2 response is associated with the development of HPV-associated cervical pathology (75).

EVASION OF CELL-MEDIATED IMMUNITY BY HPV

The possibility that HPV may have evolved mechanisms to evade effective cellular immune responses has been suggested at several points in the preceding discussion. This topic has recently been reviewed, and several potential mechanisms of escape were proposed (53). First, HPV may have evolved mechanisms to limit the extent to which viral antigens are exposed to immunologic recognition. HPV delays expression of abundant viral proteins (the capsid proteins encoded by the late viral genes) until the terminal differentiation stage of squamous epithelium, an anatomically superficial location where immunocompetent cells would have less access to them. In contrast, in the basal epithelium, where the early genes (such as *E6* and *E7*) are expressed, the level of protein expression is low and confined to a nuclear location, thus potentially limiting an effective immune response against the cells in which the virus is actively replicating. The authors proposed that the delayed expression of capsid-encoding genes may be due to a pattern of codon usage that inhibits their expression in basal epithelial cells. Second, as suggested earlier in this review, viral proteins such as E7 may modulate immune responses. For example, the authors (53) suggested that overexpression of E7 protein may inhibit the antigen presenting function of dendritic cells in the epithelium. Third, keratinocytes may be relatively less susceptible to CTL-mediated lysis than other infected cells and may even present antigen in a tolerogenic manner. As emphasized by the authors, the role that these mechanisms play in affecting the natural history of HPV infections remains speculative at this point.

IMMUNE MODULATION OF HPV: CLINICAL IMPLICATIONS

An increased understanding of the importance of cellular immunity in clearance of HPV infections has led to therapeutic trials, in patients with anogenital condylomas, with interferons (5–7) and immune response modifiers (130, 144). Patients who respond to interferon (IFN-α and IFN-γ) treatment show reduced HPV copy number in biopsy specimens (5). Comparisons of patients who do or do not respond to interferon treatment has, in turn, contributed further to our understanding of effective antipapillomavirus cellular responses (4, 6, 7). Likewise, use of the immune response modifier drug imiquimod has resulted in condyloma clearance, associated with tissue production of IFN-α, -β, and -γ and TNF, as well as decreases in DNA and mRNA for viral genes (144). Advances in therapeutic methods will no doubt continue to both benefit from and contribute to a fuller picture of the host's response to HPV infection.

Similarly, work toward an effective vaccine continues apace (141). Given the worldwide morbidity and mortality resulting from anogenital infection by HPV, this approach is likely to eventually reap the greatest rewards in preventing HPV-derived disease. As the present review suggests, we are beginning to gain a better understanding of what constitues an effective host response to HPV infection, as well as what deficiencies in that response may be associated with viral persistence and the

development of premalignant lesions and carcinoma. Still, much remains to be understood in the area of immunity and HPV.

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