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MINIREVIEW

Cross-Reactivity, Epitope Spreading, and *De Novo* Immune Stimulation Are Possible Mechanisms of Cross-Protection of Nonvaccine Human Papillomavirus (HPV) Types in Recipients of HPV Therapeutic Vaccines

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Numerous versions of human papillomavirus (HPV) therapeutic vaccines designed to treat individuals with established HPV infection, including those with cervical intraepithelial neoplasia (CIN), are in development because approved prophylactic vaccines are not effective once HPV infection is established. As human papillomavirus 16 (HPV-16) is the most commonly detected type worldwide, all versions of HPV therapeutic vaccines contain HPV-16, and some also contain HPV-18. While these two HPV types are responsible for approximately 70% of cervical cancer cases, there are other high-risk HPV types known to cause malignancy. Therefore, it would be of interest to assess whether these HPV therapeutic vaccines may confer cross-protection against other high-risk HPV types. Data available from a few clinical trials that enrolled subjects with CINs regardless of the HPV type(s) present demonstrated clinical responses, as measured by CIN regression, in subjects with both vaccine-matched and nonvaccine HPV types. The currently available evidence demonstrating cross-reactivity, epitope spreading, and *de novo* immune stimulation as possible mechanisms of cross-protection conferred by investigational HPV therapeutic vaccines is discussed.

Human papillomavirus (HPV) is best known as the causative agent of cervical cancer, the fourth most common cancer among women globally. This is the case despite advances in screening techniques and the availability of approved prophylactic vaccines. Every year in the United States, there are 12,360 new cases of cervical cancer and 4,020 deaths (1). High-risk HPV types associated with the development of malignancies have been linked to 90 to 93% of anal cancers, 12 to 63% of oropharyngeal cancers, 36 to 40% of penile cancers, 40 to 64% of vaginal cancers, and 40 to 51% of vulvar cancers (2). Overall, HPV is estimated to be responsible for 5.2% of the worldwide cancer burden (3). Of note, the incidence of HPV-associated anal and oropharyngeal cancers is increasing in the United States (4).

The designation of papillomaviruses as the family *Papillomaviridae* was created in the seventh report of the International Committee for the Taxonomy of Viruses (5). The papillomaviruses were further divided into genera by assigning Greek letters and into species by Roman numerals (6). For example, HPV-16, -31, -33, -35, -52, -58, and -67 belong to genus alpha, species 9 (α9) (6). The circular double-stranded-DNA genomes of papillomaviruses are approximately 8 kb in size and commonly encode 8 proteins (6). The L1 gene encodes a major capsid protein, while the L2 gene encodes a minor capsid protein. A more traditional designation of HPV types was based on the nucleotide sequence of the L1 gene. A designation of a new type was created whenever a full-length papillomavirus clone was described which was at least 10% dissimilar from any other known papillomavirus type (6).

Currently, three effective HPV prophylactic vaccines are commercially available, all of which contain HPV L1 proteins that are capable of forming viruslike particles (VLPs). Gardasil (Merck, Whitehouse Station, NJ, USA), a quadrivalent HPV VLP prophylactic vaccine containing the L1 proteins of HPV-16, -18, -6, and -11, was the first to be approved by the U.S. Food and Drug Administration (FDA), in 2006. Cervarix (GalaxoSmithKline Biologicals, Rixensart, Belgium), a bivalent version containing the L1 proteins of HPV-16 and -18, was approved 3 years later in the United States. Gardasil 9 (Merck), which includes L1 VLPs from HPV-16, -18, -31, -33, -45, -52, -59, -6, and -11, was approved by the FDA in late 2014. Gardasil and Cervarix were designed to prevent 70% of cervical, vulvar, vaginal, and anal cancer cases caused by HPV-16 and -18, while Gardasil 9 was designed to prevent approximately 90% of such cases. HPV types associated with the development of malignancy are regarded as high risk. On the other hand, HPV-6 and -11, which are included in Gardasil and Gardasil 9, are considered low risk and are associated with the development of genital warts. While Gardasil and Gardasil 9 include aluminum-containing adjuvant (amorphous aluminum hydroxyphosphate sulfate), Cervarix uses AS04, which is made of 3-*O*-desacyl-4′-monophosphoryl lipid A adsorbed onto aluminum as hydroxide salt. These three vaccines are called prophylactic vaccines, as they are designed to prevent HPV infection from occurring. In contrast, HPV therapeutic vaccines for individuals who have already acquired HPV are in development, and none are currently available on the market.

A common belief has been that HPV vaccines, both prophylactic and therapeutic, confer mostly HPV type-specific protection.

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However, some level of cross-protection against nonvaccine HPV types has been demonstrated for the prophylactic vaccines Gardasil and Cervarix (7–14). In the phase III randomized, double-blinded clinical trial examining the efficacy of Cervarix, Paavonen et al. observed vaccine efficacy not only against cervical intraepithelial neoplasias of grade 2 and worse (CIN2+) associated with HPV-16 and -18 but also against CIN2+ associated with HPV-31, -33, and -45 (11). In the study examining the effect of Gardasil on oncogenic nonvaccine HPV types, significant reduction of CIN2+ associated with 10 nonvaccine high-risk HPV types (HPV-31, -33, -35, -39, -45, -51, -52, -56, -58, and -59), most notably HPV-31, was observed (7). It is generally accepted that the bivalent prophylactic vaccine confers additional protection against HPV-31, -33, and -45, while the quadrivalent vaccine protects against HPV-31 (14). Furthermore, cross-neutralizing antibodies against HPV-31 and -45 have been demonstrated in vaccine recipients (15).

In order to gain insights into the mechanisms of cross-protection against nonvaccine HPV types, two groups studied serum samples from vaccine recipients using different approaches *in vitro* (16, 17). Serum samples from Cervarix recipients were incubated with HPV-16 or HPV-18 VLPs prior to a VLP-based multiplex immunoassay for antibodies against HPV-16, -18, -31, -33, -45, -52, and -58 L1 VLPs. The vaccine-derived antibodies were type specific and cross-reacted to a lesser degree with other HPV types within the species. For example, serum incubated with HPV-16 VLPs showed decreased antibody concentrations binding to HPV-16, -31, -33, -52, and -58 (α9 species) but not HPV-18. On the other hand, serum incubated with HPV-18 VLPs showed decreased antibody concentrations binding to HPV-18 and -45 (α7 species) but not HPV-16, -31, -33, -52, and -58 (17). Bissett and colleagues used L1 VLPs of nonvaccine HPV types (HPV-31, -33, -35, or -58) coupled to magnetic Sepharose beads to isolate antibodies from serum samples from Cervarix recipients (16). These purified antibodies were then tested for their ability to neutralize L1L2 pseudoviruses. The neutralization titers of HPV-16 L1L2 pseudoviruses, nonvaccine-HPV-type L1L2 pseudoviruses used for isolation, and other nonvaccine-HPV-type L1L2 pseudoviruses were compared. The titer against HPV-16 was greatly reduced after antibody depletion with nonvaccine VLPs. Increased neutralization of L1L2 pseudoviruses of nonvaccine HPV types not used for antibody isolation was not observed. The data appear to support the notion that cross-neutralization is due to a small fraction of antibodies exhibiting different but overlapping specificities rather than weak cross-recognition of nonvaccine types by vaccine-type-HPV-specific antibodies (16). Both of these studies examined a small number of subjects, and further work is needed to clarify exactly how prophylactic vaccines confer cross-protection. However, it is safe to conclude that some types of cross-reactivity are responsible for the observed cross-protection. The most recently FDA-approved HPV prophylactic vaccine, Gardasil 9, contains all the HPV types for which cross-protection by Gardasil and Cervarix has been shown. Therefore, further investigation in this area would be of academic interest.

Many clinical trials testing putative HPV therapeutic vaccines have selectively vaccinated subjects known to be positive for HPV-16 DNA (18–27) or for HPV-16 and/or -18 DNA (28–30). However, in some clinical trials, subjects with cervical intraepithelial lesions of grade 2 or 3 (CIN2/3) were enrolled regardless of the HPV type(s) detected (31–33). Therefore, cross-protection against nonvaccine HPV types could be assessed in these studies. An example is our phase I clinical trial of an HPV-16 E6 peptide-based HPV therapeutic vaccine (PepCan), which used a *Candida* skin test reagent (Candin; Nielsen Biosciences, San Diego, CA) as a novel vaccine adjuvant (33). Forty-four percent (4 of 9) of subjects with HPV-16 at entry and 57% (8 of 14) of subjects with nonvaccine HPV types showed histological regression. Nieminen and colleagues reported a regression rate of 20% (11 of 56) in vaccine (modified Vaccinia Ankara with modified HPV-16 E6 and E7 and human interleukin 2) recipients with HPV-16 monoinfection at entry, while the regression rate of all vaccine recipients was 31% (40 of 129) (32). In recipients of ZYC101a [plasmid DNA encoding regions of HPV-16 and -18 E6 and E7 proteins, encapsulated in biodegradable poly(D,L-lactide-co-glycolide) microparticles] who were less than 25 years old, the regression rate was 64% in those with HPV-16 or -18 at entry, 73% in subjects with other HPV types, and 23% in the placebo group (31). These data suggest that the cross-protection for CIN2/3 associated with nonvaccine HPV types is at least equal to that of CIN2/3 associated with vaccine HPV types. An obvious possible mechanism of cross-protection is cross-reactivity of T cells induced by the vaccines. Alternative possible mechanisms are epitope spreading and *de novo* immune stimulation.

CROSS-REACTIVITY

Cross-protection and cross-reactivity of HPV prophylactic vaccine-induced antibodies have been demonstrated for HPV types closely phylogenetically related to the vaccine HPV types. Likewise, cross-reactivity of T cells induced by HPV therapeutic vaccines is expected to target epitopes of HPV types with high amino acid sequence homology. In Fig. 1, the amino acid sequences of HPV-16 E6 and E7 proteins (Papillomavirus Episteme, http://pave.niaid.nih.gov/#home), divided into overlapping 15-mer peptides, are compared (NCBI BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) with those of other high-risk and low-risk HPV types (34). Based on homology, peptides containing potentially cross-reactive T-cell epitopes are found most frequently in HPV-16-related types and to a lesser extent in other high-risk HPV types. In low-risk HPV types, such potentially cross-reactive peptides are only present in the E7 protein, not the E6 protein. Whether or not high amino acid homology results in cross-recognition has been addressed for a select number of T-cell epitopes described within the HPV-16 E6 and E7 proteins. Tables 1 and 2 list CD4 and CD8 HPV-16 T-cell epitopes described to date to our knowledge for which the amino acid sequences and the restricting HLA molecules have been identified. The homologous peptides used to study each of the marked epitopes in Tables 1 and 2 are listed in Tables 3 to 7.

Our group approached this question by isolating T-cell clones positive for an HPV-16 epitope and examining the recognition of homologous sequences from other high-risk HPV types using a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot assay (ELISPOT) (35–37). If the number of spot-forming units for a homologous sequence from another HPV type was equal to or greater than 50% of the number for HPV-16, then that HPV type was considered to be cross-reactive. Using this criterion, an example in which an HPV-16 E6 aa-52-to-62-epitope-specific CD4 T-cell clone was cross-reacting with a homologous peptide from HPV-45 has been shown (Table 3) (38). Other homologous pep-

	Percent Homology to HPV16 Sequences	HPV-16 related						High-risk types													Low-risk types												CP6108
	(sequence)	31	33	35	52	58	67	18	39	45	51	53	56	59	66	68	69	70	73	82	6	11	40	42	54	61	62	71	72	81	83	84	
E6 1-15	MHQKRTAMFQDPQER	33	33	47	27	33	0	0	0	0	33	0	33	0	0	13	47	0	0	33	0	0	0	0	0	0	0	0	0	0	0	0	0
E6 6-20	TAMFQDPQERPRKLP	60	53	67	53	53	53	60	60	60	53	40	53	60	47	60	60	60	40	53	0	0	0	0	0	0	0	0	0	0	0	0	0
E6 11-25	DPQERPRKLPQLCTE	53	47	67	53	47	47	73	67	73	47	53	53	60	47	60	53	67	53	47	0	0	0	47	33	0	40	0	0	0	0	0	0
E6 16-30	PRKLPQLCTELQTTI	40	60	40	33	47	53	60	60	60	33	40	27	0	0	53	40	67	40	33	0	0	0	53	33	0	0	0	0	0	0	0	0
E6 21-35	QLCTELQTTIHDIIL	0	60	47	40	47	53	53	53	47	33	0	27	0	0	47	0	60	53	20	0	0	0	0	0	0	0	0	0	0	0	33	0
E6 26-40	LQTTIHDIILECVYC	33	67	53	40	53	60	53	60	47	40	27	40	40	53	60	40	67	53	27	20	20	27	20	27	33	27	27	0	27	0	0	33
E6 31-45	HDIILECVYCKQQLL	53	53	67	53	47	53	47	40	40	53	40	53	53	53	53	40	67	47	40	27	20	33	27	33	47	40	40	33	40	0	40	40
E6 36-50	ECVYCKQQLLRREVY	47	60	73	67	60	60	33	53	67	60	53	67	53	53	67	53	67	53	60	27	20	47	40	33	40	33	40	33	33	0	40	40
E6 41-55	KQQLLRREVYDFAFR	40	67	67	60	53	60	33	40	47	33	47	53	53	0	60	40	60	53	33	0	0	0	0	0	0	0	0	0	0	0	0	0
E6 46-60	RREVYDFAFRDLCIV	67	73	47	73	73	73	47	60	80	33	53	40	67	0	60	53	67	87	53	0	0	53	0	40	0	27	27	0	0	47	0	0
E6 51-65	DFAFRDLCIVYRDGN	73	73	53	67	80	73	60	67	73	40	60	27	67	0	67	53	67	73	53	0	0	53	33	40	0	0	40	0	40	33	0	0
E6 56-70	DLCIVYRDGNPYAVC	67	60	80	80	87	80	60	67	73	73	73	60	73	60	67	0	73	73	67	0	0	47	53	53	47	0	47	53	40	40	47	0
E6 61-75	YRDGNPYAVCDKCLK	60	53	73	67	73	67	53	60	53	73	67	53	60	60	60	40	67	60	67	53	47	47	47	47	40	30	47	47	40	33	47	33
E6 66-80	PYAVCDKCLKFYSKI	67	53	87	67	60	53	60	60	60	80	67	73	53	73	60	60	60	80	73	60	47	40	67	40	33	33	40	40	40	40	47	40
E6 71-85	DKCLKFYSKISEYRH	67	73	87	73	67	53	60	53	53	67	47	53	20	53	53	67	53	73	67	53	33	33	53	33	40	0	0	0	0	40	47	40
E6 76-90	FYSKISEYRHYCYSL	67	80	80	87	80	53	53	47	0	60	40	60	0	60	47	67	0	60	53	40	33	0	47	0	0	13	0	0	0	0	0	0
E6 81-95	SEYRHYCYSLYGTTL	73	80	73	87	87	67	60	0	53	67	40	60	33	60	0	60	60	67	60	47	40	0	0	0	47	13	0	0	0	47	0	60
E6 86-100	YCYSLYGTTLEQQYN	60	53	67	53	60	47	40	40	47	47	0	47	40	53	40	47	53	47	40	0	0	0	0	0	40	0	0	47	0	33	0	60
E6 91-105	YGTTLEQQYNKPLCD	67	60	67	53	40	53	33	33	33	40	0	60	47	53	33	47	53	67	53	*	27	0	33	40	40	33	27	47	33	40	33	60
E6 96-110	EQQYNKPLCDLLIRC	60	60	73	40	27	47	40	40	40	47	0	60	47	0	40	53	0	53	53	33	27	0	20	53	0	40	27	53	40	47	40	53
E6 101-115	KPLCDLLIRCINCQK	67	67	80	60	47	60	60	40	60	60	60	73	47	60	40	60	53	67	60	47	40	33	47	53	47	40	47	47	47	53	40	40
E6 106-120	LLIRCINCQKPLCPE	87	73	87	73	73	67	67	73	67	60	67	73	73	67	53	73	67	73	73	60	60	53	53	53	53	0	60	53	47	60	53	47
E6 111-125	INCQKPLCPEEKQRH	87	73	87	80	73	80	67	67	67	60	67	67	53	67	60	67	67	73	60	53	53	0	53	67	40	0	53	40	40	47	47	40
E6 116-130	PLCPEEKQRHLDKKQ	93	60	67	60	60	73	60	67	53	53	67	53	40	60	60	67	60	60	47	40	53	0	0	60	0	0	40	0	0	33	33	0
E6 121-135	EKQRHLDKKQRFHNI	93	67	80	60	67	80	73	67	67	60	67	67	53	60	67	67	60	67	60	40	47	0	47	60	33	33	47	33	20	40	33	40
E6 126-140	LDKKQRFHNIRGRWT	87	67*	73	60	60	67	47	27	0	67	53	53	40	47	0	53	47	53	53	0	33*	0	40	47	40	40	40	40	33	47	40	47
E6 131-145	RFHNIRGRWTGRCMS	80	73	93	80	80	73	33	53	0	67	53	33	60	53	53	53	53	60	67	27	27	33	53	60	60	53	53	60	47	67	53	60
E6 136-150	RGRWTGRCMSCCRSS	53	47	67	53	47	53	0	0	0	47	40	33	0	40	0	40	33	40	47	33	33	33	40	47	47	40	33	47	33	67	40	40
E6 141-155	GRCMSCCRSSRTRRE	47	47	40	0	0	47	0	27	13	0	27	27	33	27	13	40	33	0	0	27	27	0	0	0	0	27	20	27	0	40	27	27
E6 146-158	CCRSSRTRRETQL	0	0	38	0	27	27	38	40	40	0	0	31	39	0	46	40	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E71-15	MHGDTPTLHEYMLDL	67	67	60	53	60	60	47	60	33	13	53	53	53	60	53	53	67	53	13	53	53	53	40	47	0	*	0	0	60*	0	40	47
E7 6-20	PTLHEYMLDLQPETT	73	73	60	60	67	67	0	47	0	0	47	0	40	0	33	0	60*	40	0	47	47	47	0	33	0	27	47	0	27	0	0	33
E7 11-25	YMLDLQPETTDLYCY	80	80	80	87	67	87	0	13	27	53	0	33	33	0	0	67	27	27	27	53	53	33	47	60	33	13	60	13	27	33	0	13
E7 16-30	QPETTDLYCYEQLND	80	80	73	87	67	87	47	40	47	53	60	53	53	53	53	67	60*	40	40	40	47	33	67	67	60	53	60	53	53	47	47	13
E7 21-35	DLYCYEQLNDSSEEE	80	80	93	80	73	87	47	40	47	40	67	40	73*	67	60	80*	60	40	40	73	73	0	80	73	80	80	67	73	80	80	80	47
E7 26-40	EQLNDSSEEEDEIDG	73	53	60	60	67	67	80	33	67	60	67	47	33	80*	33	73*	53	47	53	80	67	53	0	53	60	73	47	87*	67	67	67	53
E7 31-45	SSEEEDEIDGPAGQA	80	53	53	33	40	73*	33	47	27	53	40	40	27	53*	33	40	0	40	47	53	0	27	0	0	33	47	73*	47	40	47	40	40
E7 36-50	DEIDGPAGQAEPDRA	80*	0	73	47	47	60*	40	27	40	27	0	0	33	27	27	0	0	0	33	27	0	0	0	0	0	0	53*	27	0	0	0	0
E7 41-55	PAGQAEPDRAHYNIV	80	60	73	53	60	60	40	0	40	27	0	0	0	0	0	0	0	0	33	20	0	0	0	0	0	0	27	0	0	0	0	0
E7 46-60	EPDRAHYNIVTFCCK	73	0	67	33	0	47	0	0	0	0	0	0	40	0	0	0	0	0	0	40	40	0	0	0	0	0	40*	0	0	0	0	0
E7 51-65	HYNIVTFCCKCDSTL	80	53*	73	67	0	60*	27	40	33	0	27	0	53	27	33	27	40	53	0	60	60	0	0	0	0	27	0	0	0	0	0	0
E7 56-70	TFCCKCDSTLRLCVQ	87	53*	80	67	33	40	27	53	33	0	47	0	47	47*	47	40	53	0	0	60	53	0	0	53	47	40*	33*	0	0	0	0	0
E7 61-75	CDSTLRLCVQSTHVD	87	53	80	67	47	53	0	33	13	0	40	0	0	20	0	0	33	0	0	53	47	0	0	53	27	27	0	0	0	0	0	0
E7 66-80	RLCVQSTHVDIRTLE	80	60	87	60	53	53	0	0	0	0	0	0	0	0	0	0	0	0	0	53	53	33	53	67	27	0	53	0	0	0	0	0
E7 71-85	STHVDIRTLEDLLMG	73	47	87	53	60	47	0	0	0	0	0	53	0	47	0	40	0	47	0	40	53	40	0	53	20	20	47	20	0	0	0	0
E7 76-90	IRTLEDLLMGTLGIV	67	60	87	53	47	60	0	0	47	0	40	40	0	40	0	40	0	73	0	60	73	53	60	33	47	47	67	40	53	40	33*	47
E7 81-95	DLLMGTLGIVCPICS	73	67	87	47	60	67	40	47	40	53	53	53	53	53	0	40	47	87	40	73	80	73	60	33	53	47	73	53	53	47	27	47
E7 86-100	TLGIVCPICSQKP	54	54	69	38	0	69	38	33	33	0	0	0	46	0	0	0	33	69	8	60	77	47	47	38	33	33	62	46	46	31	31	38

FIG 1 Amino acid sequence homologies between peptides (15 amino acids in length) of HPV-16 and other HPV types. The peptides with ≥70% homology are highlighted in yellow. *, amino acid insertion(s) is present.

tides (≥70% amino acid homology) from HPV-31, -33, -58, and -73 were not recognized. Therefore, cross-recognition with HPV-45, which belongs to the α7 species (HPV-18 related), was observed, while cross-recognition with other α9 species (HPV-16-related types HPV-31, -33, and -58) was not.

A similar conclusion can be drawn regarding cross-recognition of homologous CD8 HPV T-cell epitopes. A CD8 T-cell clone specific for the HPV-16 E6 aa-52-to-61 epitope restricted by the HLA class I B57 molecule has been shown to cross-recognize HPV-35 (α9), -39 (α7), -45 (α7), -51 (α5), and -73 (α11) (35) (Table 4), while another CD8 T-cell clone specific for the same peptide but restricted by another HLA class I molecule, B58, has been shown to cross-recognize HPV-31 (α9), -33 (α9), -35 (α9), -39 (α7), -45 (α7), -51 (α5), -58 (α9), and -73 (α11) (36) (Table 5). On the other hand, a CD8 T-cell clone recognizing the HPV-16 E6 aa-75-to-83 epitope restricted by the HLA class I B62 molecule (Table 6) and another clone recognizing the E6 aa-133-to-142 epitope restricted by the HLA class I A6801 molecule (Table 7) did not recognize any homologous peptides tested (37). Therefore, the presence of amino acid homology does not automatically lead to cross-recognition and it is not species specific when present. Whether or not such cross-recognition is present in natural infec-

TABLE 1 CD4 T-cell epitopes of HPV-16 E6 and E7 proteins described, with HLA restriction elements identified

Epitope (length)	Sequence	HLA	Reference
E6 11–32 (22)	DPQERPRKLPQLCTELQTTIHD	DP17	59
E6 11–32 (22)	DPQERPRKLPQLCTELQTTIHD	DP1401	59
E6 37–68 (32)	CVYCKQQLLRREVYDFAFRDLCIVYRDGNPYA	DP0201	59
E6 52–62 (11)[a]	FAFRDLCIVYR	DR11	38
E6 52–61 (10)	FAFRDLCIVY	DP0201	59
E6 61–82 (22)	YRDGNPYAVCDKCLKFYSKISE	DP0101	59
E6 61–82 (22)	YRDGNPYAVCDKCLKFYSKISE	DP1401	59
E6 71–92 (22)	DKCLKFYSKISEYRHYCYSLYG	DP0101	59
E6 73–105 (33)	CLKFYSKISEYRHYCYSLYGTTLEQQYNKPLCD	DP0401	59
E6 74–83 (10)[b]	LKFYSKISEY	DP	39
E6 91–112 (22)	YGTTLEQQYNKPLCDLLIRCIN	DR15 or DQ05	59
E6 101–122 (22)	KPLCDLLIRCINCQKPLCPEEK	DQ06	59
E6 121–142 (22)	EKQRHLDKKQRFHNIRGRWTGR	DP0201 or DQ05	59
E6 127–141 (15)	DKKQRFHNIRGRWTG	DR01	60
E6 129–138 (10)	KQRFHNIRGR	DR7	59
E7 21–42 (22)	DLYCYEQLNDSSEEEDEIDGPA	DR4	59
E7 35–50 (16)	EDEIDGPAGQAEPDRA	DQ2	61
E7 43–77 (35)	GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR	DR3	61
E7 50–62 (13)	AHYNIVTFCCKCD	DR15	61
E7 51–72 (22)	HYNIVTFCCKCDSTLRLCVQST	DP1901	59
E7 58–68 (11)	CCKCDSTLRLC	DR17	62
E7 61–80 (20)	CDSTLRLCVQSTHVDIRTLE	DR0901	63
E7 71–85 (15)	STHVDIRTLEDLLMG	DQ0201	64
E7 76–86 (11)	IRTLEDLLMGT	DR12	59

[a] Cross-reactivity to homologous sequences from other HPV types has been tested and demonstrated (Table 3).
[b] Cross-recognition of homologous peptides from HPV types 31 and 35 has been demonstrated (39).

tion is not known, since the generation of homologous epitopes from native protein by endogenous antigen processing has not been investigated. On the other hand, the possibility of cross-recognition can be ruled out in the absence of peptide recognition.

Another group has taken the investigation of cross-recognition of HPV-16 CD4 T-cell epitopes one step further. van den Hende and colleagues investigated the cross-recognition of five closely related members of the α9 species (HPV-31, -33, -35, -52, and -58)

TABLE 2 CD8 T-cell epitopes of HPV-16 E6 and E7 proteins described, with HLA restriction elements identified

Epitope (length)	Sequence	HLA	Reference(s)
E6 13–22 (10)	QERPRKLPQL	B7	59
E6 29–37 (9)	TIHDIILEC	B48	65
E6 29–38 (10)	TIHDIILECV	A02, A0201	59, 65, 66
E6 31–38 (8)	HDIILECV	B4002	65
E6 52–61 (10)[a]	FAFRDLCIVY	B57	35, 59, 67
E6 52–61 (10)	FAFRDLCIVY	B35	65
E6 52–61 (10)[b]	FAFRDLCIVY	B58	36
E6 75–83 (9)[c]	KFYSKISEY	B62	37
E6 80–88 (9)	ISEYRHYCY	B18	68
E6 133–142 (10)[d]	HNIRGRWTGR	A6801	37
E6 137–146 (10)	GRWTGRCMSC	B27	59
E6 149–158 (10)	SSRTRRETQL	B14	59
E7 7–15 (9)	TLHEYMLDL	B8	69
E7 7–15 (9)	TLHEYMLDL	B48	67
E7 11–19 (9)	YMLDLQPET	A02, A0201	59, 70
E7 11–20 (10)	YMLDLQPETT	A0201	66
E7 44–52 (9)	QAEPDRAHY	B18	68
E7 61–69 (9)	CDSTLRLCV	A2402	71
E7 67–76 (10)	LCVQSTHVDI	A2402	71
E7 79–87 (9)	LEDLLMGTL	B60	67
E7 82–90 (9)	LLMGTLGIV	A0201	66
E7 86–93 (8)	TLGIVCPI	A0201	66

[a] Cross-reactivity to homologous sequences from other HPV types has been tested and demonstrated (Table 4).
[b] Cross-reactivity to homologous sequences from other HPV types has been tested and demonstrated (Table 5).
[c] Cross-reactivity to homologous sequences from other HPV types has been tested but not demonstrated (Table 6).
[d] Cross-reactivity to homologous sequences from other HPV types has been tested but not demonstrated (Table 7).

TABLE 3 Peptides from high-risk HPV types homologous to HPV-16 E6 aa-51-to-65 epitope for assessment of cross-reactive CD4 epitopes[a]

HPV type	Species	Epitope (length)	Sequence
16	α9	E6 51–65 (15)	DFAFRDLCIVYRDGN
31	α9	E6 44–58 (15)	DFAF**T**DL**T**IVYRD**DT**
33	α9	E6 44–58 (15)	DFAFA**D**L**TV**VYR**E**GN
45	α7	E6 46–60 (15)	**Q**FAF**K**DLCIVYRD**CI**
58	α9	E6 44–58 (15)	DF**V**FA**D**LR**I**VYRDGN
73	α11	E6 45–59 (15)	DFAF**S**DLCIVYRD**KP**

[a] Amino acids different from those of HPV-16 are shown in bold, and peptides recognized in ELISPOTs are highlighted in gray.

(39). Using overlapping peptides, approximately half of the responding subjects displayed recognition of more than two other HPV types, suggesting that cross-recognition may be relatively common. However, further investigation using enriched and clonal T-cell populations and naturally processed epitopes derived from whole proteins demonstrated only one example of an HPV-16-specific CD4 T-cell clone capable of cross-recognizing homologous peptides of other HPV types (Table 1). Therefore, they concluded that the HPV-16 E6-specific CD4 T-cell responses are unlikely to cross-recognize and so unlikely to cross-protect against other highly related HPV types. Overall, cross-recognition can be demonstrated, but rarely. Thus, cross-recognition is unlikely to be the main mechanism of cross-protection seen in the recipients of HPV therapeutic vaccines.

EPITOPE SPREADING

Epitope spreading is a process in which antigenic epitopes distinct from and non-cross-reactive with an inducing epitope become additional targets of an ongoing immune response (40). It can be beneficial to the host by resulting in protection from other pathogens, or it can be harmful to the host in the setting of autoimmunity (41). In our phase I clinical trial of a peptide-based HPV therapeutic vaccine, statistically significant increases in CD3 T-cell responses to HPV-16 E7 protein, which was not included in the vaccine, were demonstrated in two vaccine recipients (33).

TABLE 4 Peptides from high-risk HPV types homologous to HPV-16 E6 aa-52-to-61 (HLA B57 restricted) epitope for assessment of cross-reactive CD8 epitopes[a]

HPV type	Species	Epitope (length)	Sequence
16	α9	52–61 (10)	FAFRDLCIVY
18	α7	47–56 (10)	FAF**K**DL**FV**VY
31	α9	45–54 (10)	FAF**T**DL**T**IVY
33	α9	45–54 (10)	FAFA**D**L**TV**VY
35	α9	45–54 (10)	FA**CY**DLCIVY
39	α7	47–56 (10)	FAF**S**DL**YV**VY
45	α7	47–56 (10)	FAF**K**DLCIVY
51	α5	45–54 (10)	**V**AF**TEIK**IVY
52	α9	45–54 (10)	F**L**F**T**DL**R**IVY
56	α6	48–57 (10)	FA**CTE**L**KL**VY
58	α9	45–54 (10)	F**V**FA**D**LR**I**VY
59	α7	47–56 (10)	FAF**N**DL**F**IVY
68	α7	47–56 (10)	FAF**G**DL**NV**VY
73	α11	45–54 (10)	FAF**S**DLCIVY

[a] Amino acids different from those in HPV 16 are shown in bold, and peptides recognized in ELISPOTs are highlighted in gray.

TABLE 5 Peptides from high-risk HPV types homologous to HPV 16 E6 aa-52-to-61 (HLA B58 restricted) epitope for assessment of cross-reactive CD8 epitopes[a]

HPV type	Species	Epitope (length)	Sequence
16	α9	52–61 (10)	FAFRDLCIVY
18	α7	47–56 (10)	FAF**K**DL**FV**VY
31	α9	45–54 (10)	FAF**T**DL**T**IVY
33	α9	45–54 (10)	FAFA**D**L**TV**VY
35	α9	45–54 (10)	FA**CY**DLCIVY
39	α7	47–56 (10)	FAF**S**DL**YV**VY
45	α7	47–56 (10)	FAF**K**DLCIVY
51	α5	45–54 (10)	**V**AF**TEIK**IVY
52	α9	45–54 (10)	F**L**F**T**DL**R**IVY
56	α6	48–57 (10)	FA**CTE**L**KL**VY
58	α9	45–54 (10)	F**V**FA**D**LR**I**VY
59	α7	47–56 (10)	FAF**N**DL**F**IVY
68	α7	47–56 (10)	FAF**G**DL**NV**VY
73	α11	45–54 (10)	FAF**S**DLCIVY

[a] Amino acids different from those in HPV-16 are shown in bold, and peptides recognized in ELISPOTs are highlighted in gray.

Both subjects also had significantly increased responses to the E6 protein, which is included in the vaccine. One subject had HPV-16 DNA detected before and after vaccination, while the other subject had HPV-45 detected prior to and after vaccination. These may be the first examples of epitope spreading in recipients of an HPV therapeutic vaccine (33). For the subject with HPV-45, cross-recognition would be unlikely, as the HPV-45 E7 aa-1-to-15 region only has 33% amino acid homology with the HPV-16 sequences of the same region (Fig. 1). The presence of latent HPV-16 infection no longer detectable with the current method of HPV detection may be more likely. Epitope spreading has been shown to correlate with tumor regression in peptide-based cancer immunotherapy (42–47) and may be quite beneficial in enhancing the therapeutic effects of the treatment. Therefore, future investigations to uncover additional evidence of epitope spreading and to elucidate underlying mechanisms should be pursued.

DE NOVO IMMUNE STIMULATION

The idea of using *Candida* skin testing reagent as a novel vaccine adjuvant came about from observations that intralesional injections of recall antigens result in common wart regression (48–54). Traditionally, recall antigens, which typically include a panel derived from *Candida*, mumps virus, and *Trichophyton*, were used as a control to indicate intact cell-mediated immunity in patients

TABLE 6 Peptides from high-risk HPV types homologous to HPV-16 E6 aa-75-to-83 (HLA B62 restricted) epitope for assessment of cross-reactive CD8 epitopes[a]

HPV type	Species	Epitope (length)	Sequence
16	α9	75–83 (9)	KFYSKISEY
33	α9	68–76 (9)	**RFL**SKISEY
51	α5	68–76 (9)	**L**FYSKI**R**EY
52	α9	68–76 (9)	**RFL**SKISEY
56	α6	71–79 (9)	**L**FYSK**VRK**Y
73	α11	69–77 (9)	KFYSKI**R**EY

[a] Amino acids different from those in HPV-16 are shown in bold, and peptides recognized in ELISPOTs are highlighted in gray.

TABLE 7 Peptides from high-risk HPV types homologous to HPV-16 E6 aa-133-to-142 (HLA A6801 restricted) epitope for assessment of cross-reactive CD8 epitopes[a]

HPV type	Species	Epitope (length)	Sequence
16	α9	133–142 (10)	HNIRGRWTGR
31	α9	126–135 (10)	HNI**G**GRWTGR
33	α9	126–135 (10)	HNI**S**GRW**A**GR
51	α5	137–146 (10)	**AN**C**W**Q**R**T**RQ**R
52	α9	126–135 (10)	HNI**M**GRWTGR
58	α9	126–135 (10)	HNI**S**GRWTGR

[a] Amino acids different from those in HPV-16 are shown in bold, and peptides recognized in ELISPOTs are highlighted in gray.

TABLE 8 Defining cross-protection by HPV clearance

HPV type(s)	No. (%) of subjects ($n = 23$) who had indicated HPV type(s): Prior to vaccination	Cleared
16	9	3 (33)
16 related[a]	15	5 (33)
Non-16-related high risk[b]	16	4 (25)
Low risk[c]	13	8 (62)

[a] HPV-16-related HPV types include HPV-31, -33, -35, -52, and -58.
[b] Other high-risk HPV types include HPV-39, -45, -51, -53, -56, -59, -66, -73, and -82.
[c] Low-risk HPV types include HPV-6, -61, -62, -72, -81, -83, -84, and -CP6108.

being tested for tuberculosis by placing purified protein derivative (PPD; an extract of *Mycobacterium tuberculosis*); T-cell-mediated inflammation would emerge within 24 to 48 h (55). Many studies have shown that recall antigens were effective not only in regressing injected warts, but also in regressing untreated distant warts (48–52, 54). These studies suggested that T cells may have a role in wart regression. In a recently completed phase I investigational new drug study in which the largest wart was treated with Candin, our group reported complete resolution of the treated warts in 82% (9 of 11) of the subjects and complete resolution of distant untreated warts in 75% (6 of 8) of the subjects (52). Furthermore, T-cell responses to the HPV-57 L1 peptide were detected in 67% (6 of 9) of the complete responders. Therefore, intralesional injection of *Candida* may have resulted in *de novo* generation of anti-HPV T-cell responses. *In vitro* experiments have demonstrated that *Candida* has a proliferative effect on T-cells and that the cytokine most frequently produced by Langerhans cells exposed to *Candida* was interleukin 12, which promotes T-cell response (56, 57). Intriguingly, injecting the wart, which is the site of active infection, may not be necessary to induce T-cell responses, as one group reported that weekly intradermal injections of PPD in the forearms was effective in treating anogenital warts in pregnant women (58).

Additional evidence of *de novo* immune stimulation was demonstrated in our clinical trial of the HPV therapeutic vaccine mentioned above (33). HPV-DNA testing was performed prior to vaccination and 20 weeks after initiation of vaccination. The rate of HPV clearance was higher for low-risk HPV types (62%) than for HPV-16 (33%), HPV-16-related types (33%), and other high-risk types (25%) (Table 8), although the vaccine only contained HPV-16 E6 peptides. Since there is no amino acid sequence homology equal to or greater than 70% between the E6 protein of HPV-16 and the E6 proteins of low-risk HPV types (Fig. 1), *de novo* immune stimulation is likely responsible for the low-risk HPV types becoming undetectable. One should keep in mind that the subjects' own immunity may account for some degree of HPV clearance. Nevertheless, it is possible that our HPV therapeutic vaccine, which consists of HPV-16 E6 peptides and *Candida* skin test reagent, may work through a nonspecific immune stimulatory effect of the *Candida* skin test reagent in addition to the HPV-specific effects induced by the HPV-16 E6 peptides (33).

The three potential mechanisms discussed here, cross-recognition, epitope spreading, and *de novo* immune stimulation, need not be mutually exclusive. Further investigation of the mechanisms of cross-protection conferred by HPV therapeutic vaccines should yield interesting findings, and they may be quite different from the mechanisms of cross-protection conferred by the HPV prophylactic vaccines.

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M.N. is one of the inventors named in patents and patent applications describing an HPV therapeutic vaccine named PepCan.

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