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Immunogenic Properties of Recombinant and Synthetic Peptides of Human Papillomavirus

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Human papillomavirus (HPV) infection causes cervical cancer, one of the most dangerous malignant tumors of women worldwide. Epithelial dysplasia and carcinomas are usually associated with HPV genotypic variants HPV 16, 18, 31, 33, and 45 (in total, 80% of cases). The diseases are caused by the viral oncogenes that are viral regulatory proteins E6 and E7 activated after viral DNA integration into the cell genome [1, 2]. The infected cells contain no virus, as is customarily understood, and therefore, the antiviral agents fail to block the infection and eradicate cancer cells [2, 3]. Numerous laboratory experiments and clinical trials suggest that the use of vaccines that induce humoral and cell immunity is the most powerful method of preventing HPV infection.

Currently, most prophylactic vaccines are based on the recombinant viral capsid proteins L1 and L2 (the major and minor, respectively). The major protein L1 (55–60 kDa) is capable of self-assembly into virus-like particles (VLP) of 60 nm in size, which are strongly immunogenic and induce high levels of virus-neutralizing antibodies. Recently, Merck company developed on the basis of VLP a quadrivalent prophylactic vaccine Gardasil against seroptypes 6, 11, 16, and 18; this vaccine has been approved in the Unites States and Europe [4]. The prophylactic bivalent vaccine Cervarix against genotypes 16 and 18, which was developed by GlaxoSmithKline, has been also shown to possess a high type-specific efficiency.

Most therapeutic vaccines are prepared on the basis of either recombinant proteins E6 and E7 or synthetic peptides that simulate their T cell epitopes [5, 6]. The peptide vaccines have some advantages, such as the absence of foreign antigenic background, low allergenicity, possible bulk production, and conformity with the standard requirements accepted for the drugs. At the same time, low immunogenicity of these vaccines prevent their wide application; they should be applied in complex with adjuvants or using some other effective ways of peptide presentation. In principle, the therapeutic vaccines have been tested at the cellular level, in animal models, and even in clinical trials. However, the immunological and clinical results are still variable; no complete removal of the infection is guaranteed. Nevertheless, the studies in this field and tests of various preparations on the basis of recombinant proteins, chimeric constructs, synthetic peptides as such and in combination with various immunomodulators are continued [6, 7].

Here we describe the results obtained with the combined vaccine that is promising for prophylaxis and treatment of HPV infection of types 16, 18, and 31. The basic components of this vaccine are the recombinant proteins L1 of the above types as well as the synthetic peptides of the surface protein L1 and oncogenic protein E7. We have developed a system for expression of the full-length protein L1 in yeast *Saccharomyces cerevisiae*, strain Y618, where substantial amounts of the protein of interest in the VLP conformation were synthesized. The original gene sequences were obtained by PCR using the clinical material from the HPV-infected patients. These genes were transferred into special vectors, which were constructed on the basis of the pPDX1 plasmid (collection of GOSNIIgenetika, Moscow) (Fig. 1).

The expressed recombinant proteins L1 (rL1) were isolated from yeast transformants as described previously [8]. Their structure was verified by immunoblotting and enzyme immunoassay (EIA) using antipeptide polyclonal antibodies (PCAs) and conformation-dependent monoclonal antibodies (MCAs) against VLP. The set of MCA samples and baculovirus L1 VLP was kindly provided by Prof. N.D. Christensen (The

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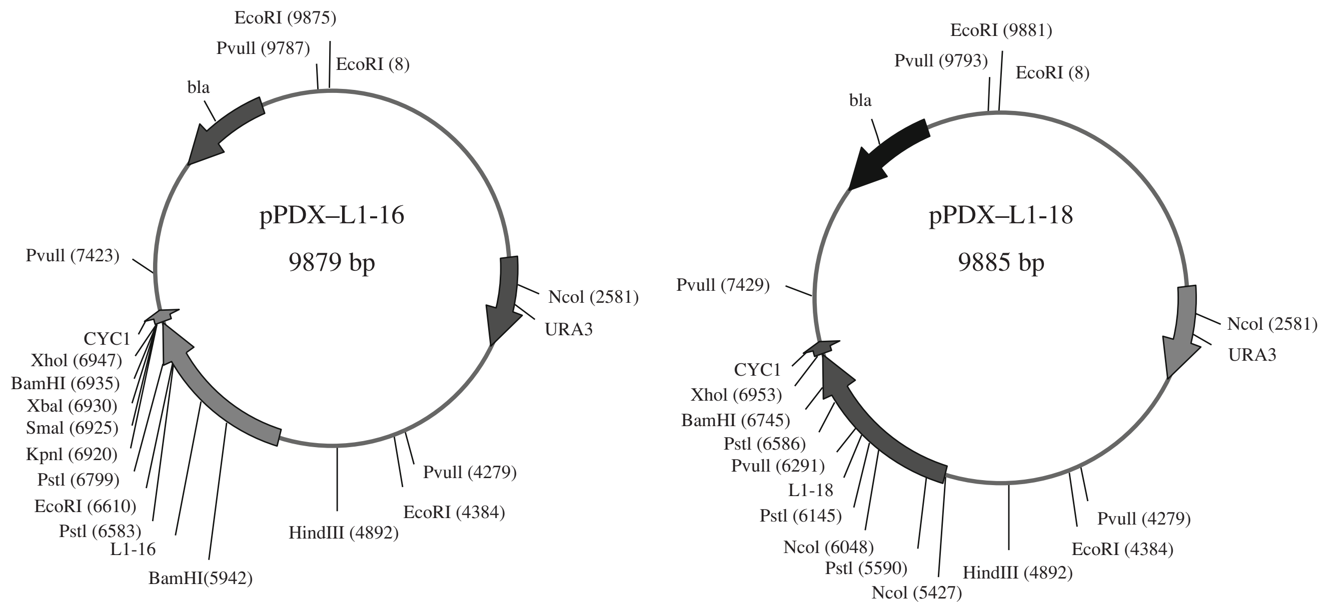

Fig. 1. Maps of yeast replicative plasmids used for expression of proteins L1 HPV 16 and 18 (pPDX2-L1-16 and pPDX2-L1-18).

Milton S. Hershey Medical Center, Hershy Pennsylvania); two other MCA (P3105-08 and P3105-32) were purchased from US Biological. Since MCAs against various type L1 proteins are hardly commercially available, we synthesized the peptides simulating B epitops of L1 proteins 16, 18, and 31 (Table 1) as well as the conjugates of the latter with keyhole limpet hemocyanin (KLH), an immunostimulating protein, to induce antipeptide antibodies.

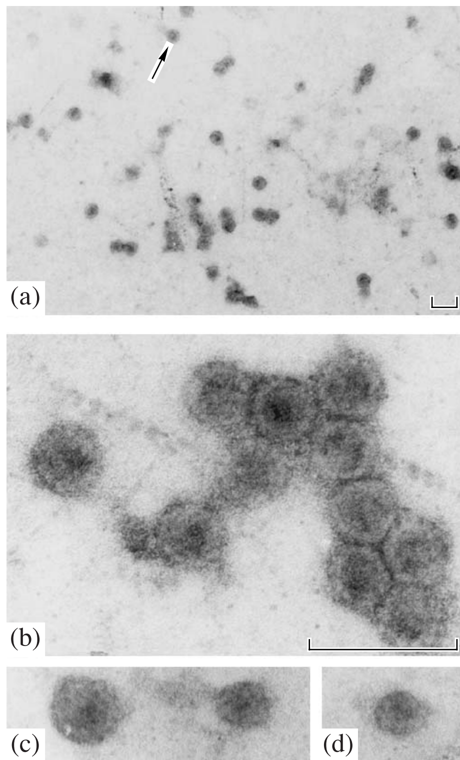

Fig. 2. Electron micrographs of the recombinant L1-16 (VLP): (a) magnification, 55000×; scale, 100 nm; (b–d) magnification, 350000×.

Hybrid CBA/lacxC57BL/6)F1 mice were intraperitoneally immunized with these conjugates three to four times at two-week intervals. The antisera against the conjugates with the NC-723 and NC-724 peptides displayed the highest reactivity with type-16 and type-18 rL1 proteins (Table 2). Unlike MCA P3105-32, both the antipeptide sera and MCA P3105-08 readily recognized rL1-16 and rL1-18. Thus, the antipeptide antibodies proved to be helpful tools for analyzing L1 expression.

It should be noted that MCAs (H16 U4 S/N 9 and H18 J4 S/N), provided by Christensen in the form of supernatants and exhibiting virus-neutralizing activity, also readily interacted with rL1-16 rL1-18 (data not shown). Since these MCA were specific for the conformational VLP epitops, our results confirmed indirectly the accuracy of assembly of yeast rL1 proteins. The electron microscopic analysis provided the direct evidence, because the isolated rL1 were spherical particles of 60 nm in diameter (Fig. 2) and were completely identical in morphology to VLP obtained previously and the viral capsid [9].

The immunogenicity of the obtained rL1 proteins (rL1-16 and rL1-18) was tested on the hybrid F1 mice in the presence and absence of immunostimulating agents, such as polyoxydonium (PO) and polymuramyl (PM). PO is a copolymer of *N*-oxy-1.4-ethylenepiperazine and *N*-carboxyethyl-1.4-ethylenepiperazinium bromide; it stimulates cytokine synthesis, phagocyto-

Table 1. Synthesized peptides of the HPV proteins L1 and E7

Sequence	Amino acid numeration	Peptide code
L1 HPV16		
C-QPLGVGISGHPLLNKLDDTE-C	C–97–116–C	NC-724
C-LNKLDDTENASAYAANAGVDNRE-C	109–132	NC-723
AGVDNRECI	165–173	NC-732
FNRAGTVGENVPDDLYIKGSGS	247–268	C-12
DWNFGLQPPPGGTL	387–400	C-13
QPPGGTLEDTYRFVTQAIAC	393–413	C-14
L1 HPV18		
NVFPIFLQM	54–62	NC-731
GVNHQHLPARRAEPQRHTMLCMCCKCEARIKL	43–74	NC-737
L1 HPV31		
YYSIPKSDNPKKIVVPK	35–51	NC-743
ENSNRYAGGPGTDNR	117–131	NC-745
KGSPCSNNAITPGDCPPL	158–175	AG-39
IANSDTTFKSSNFK	335–348	NC-744
DWNFGLTTPPSGS	388–400	AG-38
E7 HPV16		
GQAEPDRAHYNIVTFCCK	43–60	NC-725
E7 HPV31		
VLDLQPEATD	12–21	NC-727
GQAEPDTSNYNIVTFCCQ	43–60	P-1
GQAEPDTSNYNIVTFCCQCKSTLRLCVQSTQVDIR	43–77	P-236
DTSNYNIVTF	48–57	NC-730
TSNYNIVTF	49–57	NC-729
ELLMGSFGIVCPNA	81–93A	NC-728

Table 2. Reactivity of rL1 types 16 and 18 (optical density data recorded at 450 nm)

Recombinant yeast proteins	Antipeptide antisera to peptides					MCAs	
	C-12	C-13	C-14	NC-723	NC-724	P3105-08 anti-L1-16	P3105-32 anti-L1-18
PBS* (control)	0.093	0.089	0.112	0.079	0.157	0.049	0.083
rL1-16	0.738	0.322	0.566	0.868	1.035	0.981	0.176
rL1-18	0.683	0.392	0.543	0.402	1.348	1.211	1.015

* , PBS, phosphate-buffered saline

sis, natural killer cytotoxicity, and antibody formation. PM is a bacterial peptidoglycan that stimulates expression of antigen-presenting and costimulatory molecules as well as secretion of IL-12 and other compounds. Both PO and PM were developed in the Institute of Immunology. The animals were immunized intraperitoneally three times at two-week intervals, and the samples were obtained immediately before each injection.

As determined by ELISA, the rL1-16 and rL1-18 preparations were very immunogenic even in the absence of adjuvants. After the second or third immunization, the IgG-antibody titer reached 1 : 25000 to 1 : 50000, and the level of antibodies depended on the number of immunizations. The immunostimulating agents, PO and PM, as well as Freund's adjuvant, had no effect on the level of IgG response to rL1 (Fig. 3). The antisera against rL1-16 and rL1-18 were also tested

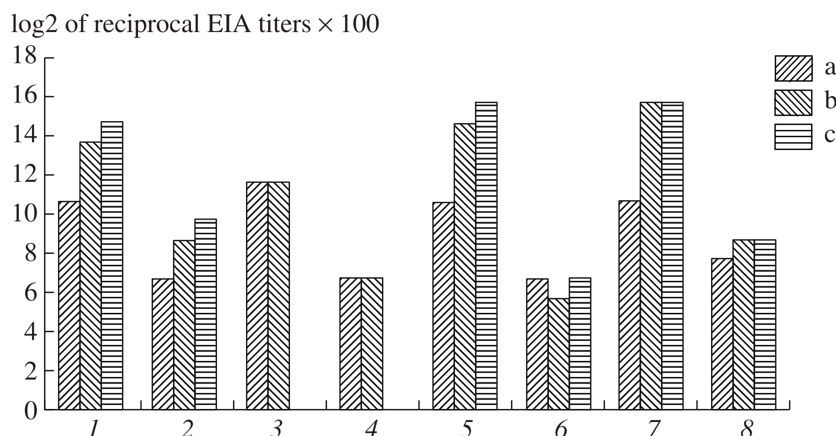

Fig. 3. Dynamics of the immune responses of mice (CBA × C57BL/6)F1 immunized with rL1 preparations: (a) primary immunization, (b) secondary immunization, and (c) tertiary immunization. Immunogens: *1*, *2*, rL1-16; *3, 4*, rL1-16 + PO; *5, 6*, rL1-18; *7, 8*, rL1-18 + PM. Antigens on the plate: *1,3*, rL1-16; *2, 4, 6, 8*, NC-723; *5, 7*, rL1-18. (a) primary immunization; (b) secondary immunization; (c) tertiary immunization.

for the reactivity against the L1 peptide fragments. The results showed that only peptide NC-723 interacted effectively with antisera against rL1-16 and rL1-18. The antibodies against peptide NC-723 also readily interacted with rL1-16 and rL1-18. This phenomenon can be explained by the fact that the homology between the respective regions of L1-16 and L1-18 reached 75%, whereas homology for the N-terminus of the peptide was 100%; the peptide effectively simulated this fragment of the protein. Note that this region was mapped as the virus-neutralizing epitope [9].

Since we aimed at developing a vaccine on the basis of peptide preparations, peptide–PO and peptide–KLH conjugates were synthesized. The peptides were bound to PO by means of azide condensation. The animals were immunized (1) with free peptides emulsified in a Freund's complete adjuvant (FCA) (peptide + FCA); (2) with conjugates of peptide and PO (peptide + PO); (3) with conjugates of peptide and both KLH and FCA (peptide + KLH + FCA); (4) with a mixture of a peptide and polymuramyl adjuvant (peptide + PM) (Table 2 and Table 3). Synthesis of IgG antibodies was assessed

Table 3. Reciprocal antibody titers (EIA) in mice immunized with peptides that were either mixed with the Freund's complete adjuvant (FCA) or conjugated with polyoxidonium (PO)

Immunogen form	Antigen in ELISA				
	C-12	C-13	C-14	NC-723	NC-724
C-12 + FCA	50				
C-12-PO	800				
C-12-KLH + FCA	≥6400				
C-13 + FCA		≥50			
C-13-PO		50			
C-13-KLH + FCA		≥102400			
C-14 + FCA			⩾6400		
C-14-PO			⩾6400		
NC-723 + FCA				≥800	
NC-723-PO				≥100	
NC-723-KLH + FCA				51200	
NC-724 + FCA					100
NC-724-PO					50
NC-724-KLH + FCA					1600
N	50	50	50	50	50

N, serum of intact mice ($n = 4$).

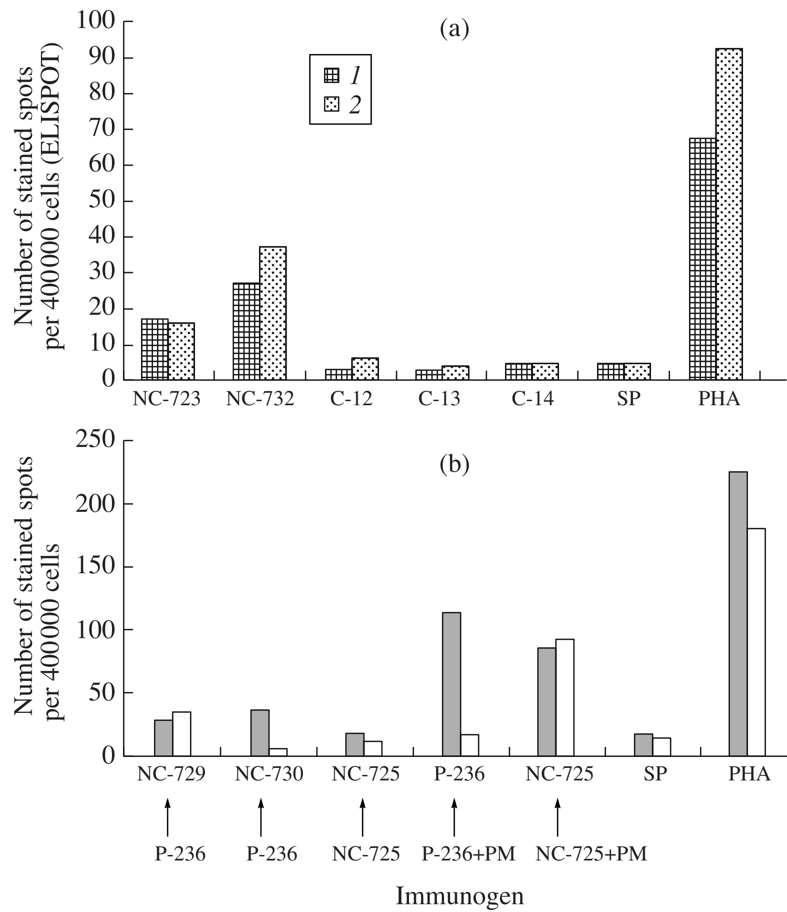

Fig. 4. (a) T-cell reactivity of the combined population ($CD4^+$, $CD8^+$) and of $CD8^+$-enriched splenocyte population of CBA × C57BL/6)F1 mice immunized three times with the recombinant VLP16 (without adjuvant). The abscissa axis shows the peptides and PHA used for specific cell activation. Designations: *1*, total splenocyte population ($CD4^+$, $CD8^+$); *2*, $CD8^+$-enriched splenocytes. (b) T-cell reactivity of the total splenocyte population after triple immunization with peptides P-236 (E7 HPV31) and NC-725 (E7 HPV16) in the absence and presence of the PM adjuvant. The abscissa axis shows the peptides and PHA used for specific cell activation (the upper line) and the immunogens used (the lower line).

using solid-phase ELISA. Table 3 shows the titers of serum antibodies determined after double immunization of mice with various forms of peptides, which were L1 HPV16 fragments. It can be seen that free peptides emulsified in FCA were non-immunogenic, except for peptide C-14.

The covalent conjugates peptides–polyoxydonium displayed a higher immunogenicity. For example, free peptide C-12 caused no antibody production even in the presence of FCA, whereas the same peptide conjugated to PO (FCA-free) induced generation of the antibody at a titer of 1 : 800 after the second immunization. Peptides C-13 and NC-724 did not induce antibody production in both variants, whereas the NC-723 peptide displayed a moderate activity in the presence of FCA. Of interest is the fact that peptide C-14 was extremely immunogenic in all forms.

To induce E7-specific CTL-response, the peptides of E7 proteins of HPV16 and HPV31 were synthesized (Table 1), which simulated the dominant T epitopes predicted with well-known algorithms (www.expasy.org). Although the therapeutic vaccine implicates induction of cytotoxic cellular response, activation of T helper cells (THL) is also important for induction of virus-neutralizing antibodies (IgG) in response to rL1 administration. Secretion of gamma-interferon (IFN-γ) by CTL/$CD8^+$ and THL/$CD4^+$ serves as marker for activation of these cells. We studied the proliferative response of splenocytes of mice immunized with rL1-16 and with synthetic peptides, which were administered together with the immunostimulating agents PO and PM. The antigen-specific IFN-γ-producing cells were detected and counted using the commercial kits from BD Biosciences for ELISPOT. The cells obtained either from mice immunized three times or from control nonimmunized mice were washed and placed into a medium containing fetal serum. $CD8^+$ cells were separated from $CD4^+$ cells using magnetic beads with immobilized anti-CD4 antibodies (Dynalbeads Mouse CD4). By this method, the cells with the desired phenotype can be selected almost completely

(98% efficacy). The cells were specifically activated with the peptides comprised of 9–35 amino acid residues, which were used at concentrations of 2–10 μg/ml. Each experiment was run in triplicate. The average numbers of the stained spots per well containing about 400000 cells can be seen in Fig. 4a. Spontaneous proliferation (SP) of cells in the absence of peptides as well as proliferation of intact mouse cells in the presence of peptides served as negative control. Cell proliferation in the presence of a strong mitogen, phytohemagglutinin (FHA), served as a positive control. It can be seen on the diagram that immunization of mice with rL1-16 induced a noticeable T-cell response directed, in particular, against the epitopes for NC-732 and NC-723 peptides. The experiments with $CD8^+$-enriched populations (CTL phenotype) confirmed the development of the cytotoxic response to rL1-16.

After the administration of peptides from E7 proteins of HPV31 and HPV16 genotypes, which was repeated three times, the T cell response developed against P-236 and NC-725 peptides when the latter were administered in combinations with the PM adjuvant (Fig. 4b). A multivalent immunogen, such as the mixture of three peptides (P-236 + NC-725 + NC-728) combined with PM, also induced specific T-cell response. Note that PM itself is capable of T-cell activating and this activity grows up with the enrichment of the population with CD8 lymphocytes; i.e., PM possibly activates specific killers (data not shown).

It seems that the vaccination with peptides representing multiple epitopes is more efficient than the use of short peptides representing only the CTL epitopes [10]. The long peptides are likely to undergo more effective endocytosis and processing within the antigen-presenting cells (APC), which are followed by surface expression in the complex with MHC-I molecules. For example, unlike the peptides comprised of nine or ten amino acid residues (NC-727, NC-729, and NC-730), which were previously identified as T epitopes, the 35-aa peptide P-236 induced a significantly stronger cell response.

The antigen delivery system is important for antitumor vaccine development. This system should deliver the antigen to the specialized APC of the lymphatic tissue. For example, the antigen delivery to the dendrite cells (DCs) is expected to increase the time of antigen presentation and to enhance the cytokine production and T-cell memory. This strategy is rarely used so far in developing the peptide vaccines, although some adjuvants, for example CpG, are capable of interacting with DCs.

As expected, the experiments in mice showed that the free peptides of the L1 protein were weakly immunogenic, except for the C-14 peptide. The peptide conjugation with KLH enabled obtaining the high-titer antipeptide sera suitable for analytical purposes. In addition, when conjugated with PM, the peptides were capable of inducing strong antigen-specific B- and T-cell responses. PM was shown to induce IL-12 secretion from DC [11], suggesting that specific interaction occurs between PM and these cells, which explains the high adjuvant activity of PM with respect to peptides. Although the developed synthetic constructs proved to possess a high immunogenicity, their protective potential should be verified in an adequate biological model.

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