Type-specific and cross-reactive antibodies induced by human papillomavirus 31 L1/L2 virus-like particles

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The aim of this study was to determine whether antibodies induced by human papillomavirus (HPV) type 31 L1/L2 virus-like particles (VLPs) could cross-react with VLPs of the closely related HPV-16 and distantly related HPV-11, and to investigate the potential role of the L2 protein in L1/L2 VLPs in inducing cross-neutralizing antibodies. Antisera were prepared from rabbits immunized with intact or denatured HPV-31 L1/L2 VLPs. Cross-reaction and cross-neutralization were analysed by Western blotting and ELISA, and by haemagglutination inhibition, respectively. Western blotting results showed that H31 L1/L2 (D) antiserum (antiserum from a rabbit immunized with denatured HPV-31 L1/L2 VLPs) could cross-react with the L1 protein of HPV-11 and -16. HPV-31 L1/L2 VLP antiserum showed strong cross-reaction with and cross-neutralization of HPV-16 VLPs, but this was significantly less with HPV-11 VLPs. In addition, the cross-neutralizing activity against HPV-16 L1/L2 VLPs was slightly higher than that against HPV-16 L1 VLPs, although the difference was not statistically significant. Epitope-blocking ELISA showed that mAb H16.V5 could partially inhibit the cross-reaction of HPV-31 L1/L2 VLP antiserum with HPV-16 L1/L2 VLPs. These results suggested that (i) H31 L1/L2 (D) antiserum could cross-react with L1 protein from both closely related and distantly related HPV types, but HPV-31 L1/L2 VLP antiserum could only cross-neutralize closely related HPV types, (ii) surface-exposed epitopes of the L2 protein in L1/L2 VLPs may induce only low titres of cross-neutralizing antibodies and (iii) certain epitopes that cross-reacted with HPV-31 L1/L2 VLP antiserum are located close to the epitopes recognized by mAb H16.V5. These findings may provide suggestions for the design of multivalent VLP vaccines.

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INTRODUCTION

Human papillomaviruses (HPVs) are small DNA viruses, and typically infect hosts via squamous or mucosal epithelia to cause both benign and malignant epithelial neoplasia. More than 100 types of HPV have been identified and they are divided into two groups: the low-risk types, such as HPV-6, -11, -34, -42 and -44, which are associated with the development of benign lesions, and the high-risk types, such as HPV-16, -18, -31, -33, -45 and -58, which can give rise to pre-cancerous lesions and cancer. HPV capsids consist of major (L1) and minor (L2) capsid proteins. The L1 protein alone or together with the L2 protein can self-assemble into virus-like particles (VLPs) when expressed in a recombinant expression system. As VLPs are structurally and immunologically similar to the native virions, VLPs are able to induce high titres of neutralizing antibodies (IgG and IgA) and effectively protect animals (Breitburd *et al.*, 1995; Kirnbauer *et al.*,

1996; Suzich *et al.*, 1995) and humans (Ault *et al.*, 2004; Brown *et al.*, 2004; Harper *et al.*, 2004; Koutsky *et al.*, 2002; Villa *et al.*, 2005) from papillomavirus infections. Specifically, the recently licensed quadrivalent HPV-6/11/16/18 L1 VLP vaccine 'Gardasil' (Merck) and the bivalent HPV-16/18 L1 VLP vaccine 'Cervarix' (GlaxoSmithKline), which is in phase III clinical trials, are both highly effective in protecting women against incident and persistent HPV infection (Harper *et al.*, 2004, 2006; Mao *et al.*, 2006). Therefore, VLPs are good prophylactic vaccines to prevent HPV infections and infection-associated tumours. Although the homology of the L1 protein among different mucosal HPV types can be up to 85 %, the neutralizing antibodies induced by L1 VLPs are predominantly genotype specific and cross-reactivity is only observed among closely related types, such as HPV-16 and HPV-31, HPV-6 and HPV-11, and HPV-18 and HPV-45 (Christensen *et al.*, 1994b; Combata *et al.*, 2002; Girolglou *et al.*, 2001). Therefore, current L1 VLP-based multivalent vaccines cannot prevent infection by all HPV types. As HPV capsids consist of L1 and L2 capsid proteins, it

Abbreviations: HAI, haemagglutination inhibition; HPV, human papillomavirus; VLP, virus-like particle.

seemed reasonable to explore the role of L2 proteins incorporated in L1/L2 VLPs in inducing consensus neutralizing antibodies.

Cross-neutralization by antibodies induced by HPV L2 protein or peptide has been observed among certain HPV types, i.e. HPV-6, -16 and -18 (Roden *et al.*, 2000), and HPV-6 and -16 (Kawana *et al.*, 1999, 2002). In particular, the peptide ¹⁰⁸LVEETSFIDAGAP¹²⁰ of the HPV-16 L2 protein has been investigated widely and identified as a common neutralizing epitope. Antisera from mice and volunteers immunized with this peptide were found to be able to neutralize pseudovirions of HPV-16, -6 and -52, and HPV-11 native virions, and also cross-reacted with HPV-18 L1/L2 VLPs. HPV-33 and -58 share higher homologies with HPV-16 in this L2 region than with HPV-6, so the antiserum induced by this peptide could probably also neutralize HPV-33 and -58 (Kawana *et al.*, 1999, 2001, 2003). Therefore, it is likely that aa 108–120 of HPV-16 L2 protein contains a consensus neutralizing epitope among genital HPVs. In addition, some studies have shown that vaccination with L2 proteins or peptides is able to provide immunity to homologous papillomavirus challenge in animal models and that this protection is mediated by neutralizing antibodies (Chandrachud *et al.*, 1995; Christensen *et al.*, 1991; Embers *et al.*, 2002; Kawana *et al.*, 2001). Pastrana *et al.* (2005) reported that antisera to aa 1–88 of bovine papillomavirus 1 L2 induced similar titres of neutralizing antibodies against HPV-16, HPV-18 and bovine papillomavirus 1 pseudovirions, and could also neutralize HPV-11 native virions and HPV-31, HPV-6 and cottontail rabbit papillomavirus pseudovirions, albeit with lower titres. They also showed that HPV-6, -16, -18, -31 and cottontail rabbit papillomavirus L2 antisera had some cross-neutralizing activity.

To date, cross-reactivity assays have focused mainly on L1 VLPs or L2 protein and peptide, but not on L1/L2 VLPs. To assess the importance of L2 protein in inducing cross-neutralizing antibodies, we selected HPV-31 L1/L2 VLPs as a model to investigate the cross-reactivity of HPV-31 L1/L2 VLP antiserum with L1 or L1/L2 VLPs of HPV-16 and -11. We compared the cross-neutralizing activity of HPV-31 L1/L2 VLP antiserum against L1/L2 VLPs versus L1 VLPs to elucidate further the potential role of the L2 protein in L1/L2 VLPs in inducing cross-neutralizing antibodies.

METHODS

Preparation of recombinant baculoviruses. HPV-31 L1 and L2 genes were amplified from pBR322-HPV-31 using L1 forward primer (5'-CGGGATCCGCCGCCACCATGTCTCTGTGGCGGCCTAG-3') and L1 reverse primer (5'-CCCAAGCTTTTACTTTTATGTTTT-TTACGTTTTGCTGG-3'), and L2 forward primer (5'-GGGTAC-CGCCGCCACCATGCGGTCCAAACGCTCTAC-3') and L2 reverse primer (5'-CCGCTCGAGTTAGGCCGCCACAGACATCTGTAA-AA-3'), respectively. Restriction enzyme sites are underlined. The HPV-31 L1 and L2 genes were subcloned into the baculovirus transfer vector pFastBacDual (Invitrogen) under the polyhedron promoter and p10 promoter, respectively. The final sequences were verified by

DNA sequencing. The recombinant baculovirus containing the HPV-31 L1 and L2 genes was generated using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen). The HPV-16 L1, HPV-16 L1/L2, HPV-11 L1 and HPV-11 L1/L2 recombinant baculoviruses have been generated by our laboratory using a similar method (Wang *et al.*, 2003; Zhang *et al.*, 2000).

Production and purification of VLPs. VLPs were produced and purified as described by Shi *et al.* (2001). Purified VLPs were dialysed extensively against 10 mM HEPES (pH 7.2), quantified using a bicinchoninic acid kit (Pierce) and analysed by 10 % SDS-PAGE with Coomassie blue staining, transmission electron microscopy (Kirnbauer *et al.*, 1992) and VLP-based ELISA (Christensen *et al.*, 1994a).

Haemagglutination and haemagglutination inhibition (HAI) assays. Haemagglutination and HAI assays were performed as described by Roden *et al.* (1995, 1996).

Animals and immunizations. Adult female New Zealand White rabbits were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, P. R. China, and kept in the animal facility of the Institute of Basic Medical Science, Chinese Academy of Medical Sciences. Rabbits were immunized subcutaneously three times at 2 week intervals with approximately 50 µg intact or denatured HPV-31 L1/L2 VLPs mixed 1 : 1 with complete (first immunization) or incomplete (second and third immunizations) Freund's adjuvant in a total volume of 1 ml. Two weeks after the third immunization, rabbits were sacrificed and antisera were collected. The antiserum from the rabbit immunized with denatured HPV-31 L1/L2 VLPs was named H31 L1/L2 (D) antiserum. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Western blotting. One microgram of HPV-16 L1/L2 VLPs, HPV-11 L1/L2 VLPs or HPV-31 L1/L2 VLPs was denatured by boiling for 5 min in the presence of DTT. The denatured samples were separated by 8 % SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was blocked and incubated with H31 L1/L2 (D) antiserum (1 : 2000 dilution) at 4 °C overnight. After washing, the membrane was incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed Laboratories) at a 1 : 5000 dilution. The reaction was detected using a SuperSignal West Pico trial kit (Pierce).

ELISA. Ninety-six-well ELISA plates (Costar) were coated with 100 ng of the respective VLPs in 100 µl PBS (pH 7.4) at 4 °C overnight. Wells were blocked for 2 h at room temperature, and serum or mAb was serially diluted and added to the wells (100 µl per well) for 2 h at room temperature. The wells were washed and incubated at 37 °C for 1 h with peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Zymed Laboratories) at a 1 : 3000 dilution. After extensive washing, orthophenylenediamine (Sigma) was added (100 µl per well) and incubated for 5–10 min at room temperature in the dark and the reaction was stopped by the addition of 1 M H₂SO₄ (50 µl per well). Absorbance of the samples in the plates was read in an automated ELISA microplate reader at 490 nm. The end-point titres were calculated as the reciprocal of the highest serum dilution with an absolute absorbance value greater than 0.2 and at least twice the background absorbance value.

Epitope-blocking ELISA. An epitope-blocking ELISA was performed as described by Girolglou *et al.* (2001). Briefly, HPV-31 L1/L2 VLPs or HPV-16 L1/L2 VLPs were added to 96-well plates (0.1 µg per well) and incubated overnight at 4 °C. Plates were washed and blocked with 5 % BSA in PBS with 0.05 % Tween 20 at room temperature for 2 h. mAbs H31.A6, H16.V5 and H16.E70, kindly

provided by Professor Neil D. Christensen, Gittlen Cancer Research Foundation, Pennsylvania State University, Hershey, PA, USA (Christensen *et al.*, 1996a), were serially diluted and added in parallel rows of the plate. Plates were incubated for 2 h at room temperature. The wells were washed and pre-titrated HPV-31 L1/L2 VLP antiserum was diluted to a pre-determined point and added at a constant dilution (1 : 150 000, 1 : 50 000 and 1 : 50 000 dilution for H31.A6, H16.V5 and H16.E70 epitope-blocking ELISA, respectively) to wells containing pre-formed VLP-mAb complexes. After a 2 h incubation at room temperature, wells were washed and bound rabbit antiserum or mAb was detected by adding peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Zymed Laboratories) in alternative parallel rows of the plate.

Statistical analysis. Statistical analysis was performed by one-way analysis of variance or Student's *t*-test using spss software. Values were compared between different groups. *P* values <0.05 were considered to be statistically significant.

RESULTS

Production of HPV-31 L1/L2 VLPs

The purified HPV-31 L1/L2 VLPs were analysed by SDS-PAGE with Coomassie blue staining, as shown in Fig. 1(a). They were found to be composed of L1 (~55 kDa) and L2 (~75 kDa) protein. There was also a minor polypeptide with a molecular mass of ~45 kDa. As described by Cook *et al.* (1999), the p45 band was present regardless of sample preparation conditions and is a C-terminally truncated form of L1. The ratio of L1 (comprising p55 and p45) to L2 molecules was approximately 30 : 1, as analysed by the

SynGene genomic and proteomic gel doc systems (data not shown), which is similar to that found in HPV-16 L1/L2 VLPs (Kirnbauer *et al.*, 1993). Transmission electron microscopy showed that the HPV-31 L1/L2 VLPs were regular spherical particles with a diameter of ~55 nm (Fig. 1c). Using a bicinchoninic acid kit, the yield of HPV-31 L1/L2 VLPs was calculated: 6–8 mg HPV-31 L1/L2 VLPs was obtained from a 1 l culture of Sf-9 insect cells.

mAb H31.A6, an HPV-31 type-specific, conformation-dependent neutralizing mAb, was found to bind efficiently to the purified HPV-31 L1/L2 VLPs (Fig. 1b). In addition, HPV-31 L1/L2 VLPs could interact effectively with the receptors for papillomaviruses on mouse erythrocytes and haemagglutinate mouse erythrocytes (Fig. 1d). Thus, the HPV-31 L1/L2 VLPs prepared in this study were structurally and immunologically similar to the native virions.

Cross-reactivity of H31 L1/L2 (D) antiserum

H31 L1/L2 (D) antiserum was found to cross-react with denatured HPV-11 (a distantly related HPV type) and HPV-16 (a closely related type) L1 protein. The cross-reaction with HPV-16 L1 was slightly stronger than that with HPV-11 L1 (Fig. 2). As a positive control, H31 L1/L2 (D) antiserum bound to denatured HPV-31 L1 with the highest efficiency. However, there was only a weak HPV-31 L2 band with a molecular mass of ~75 kDa, and no HPV-11 or -16 L2 bands could be detected using H31 L1/L2 (D) antiserum (Fig. 2).

Fig. 1. Analysis of purified HPV-31 L1/L2 VLPs. (a) Following gradient centrifugation, the VLP band (at a density of ~1.29 g ml⁻¹) was harvested and analysed by 10 % SDS-PAGE followed by Coomassie blue staining. (b) Binding of mAb H31.A6 to HPV-31 L1/L2 VLPs, determined by ELISA. (c) Transmission electron microscopy of purified VLPs. Bar, 50 nm. (d) Haemagglutination of mouse erythrocytes by HPV-31 L1/L2 VLPs.

Fig. 2. Western blot showing the cross-reaction of H31 L1/L2 (D) antiserum with denatured HPV-16 L1/L2 VLPs (lane 1), denatured HPV-11 L1/L2 VLPs (lane 2) and denatured HPV-31 L1/L2 VLPs (lane 3).

Cross-reactivity of HPV-31 L1/L2 VLP antiserum

The purified HPV-31 L1/L2 VLPs in this study were highly immunogenic and induced high titres of antibodies. The HPV-31-specific ELISA titre of HPV-31 L1/L2 VLP antiserum was as high as $1 : 5 \times 10^7$ (Fig. 3a). The A_{490} values of HPV-11 L1/L2 VLPs with twofold serially diluted HPV-31 L1/L2 VLP antiserum were higher than those of HPV-11 L1 VLPs. Similarly, the A_{490} values of HPV-16 L1/L2 VLPs were slightly higher than those of HPV-16 L1 VLPs (Fig. 3b). The cross-reacting ELISA titres with HPV-16 L1 and L1/L2 VLPs were greater than $1 : 4 \times 10^5$, whilst the ELISA titre with HPV-11 L1 or L1/L2 VLPs was approximately $1 : 5.8 \times 10^4$ and $1 : 8.5 \times 10^4$, respectively. However, the difference between the cross-reacting activity of HPV-11 or 16 L1 VLPs and the respective L1/L2 VLPs was not statistically significant ($P > 0.05$). Therefore, the presence of L2 protein in HPV-31 L1/L2 VLPs induced only very low titres of L2-specific antibodies, if any. In addition, the cross-reacting activity of HPV-31 L1/L2 VLP antiserum with HPV-16 VLPs was significantly higher than that with HPV-11 VLPs ($P < 0.05$).

To determine whether the cross-reacting antibodies could neutralize the respective VLPs, HAI assays were performed. As shown in Table 1, the HAI titre of HPV-31 L1/L2 VLP antiserum against HPV-16 L1 or L1/L2 VLPs was significantly higher than those against HPV-11 L1 and L1/L2 VLPs ($P < 0.05$), and the HAI titre against HPV-31 L1/L2 VLPs was the highest. The HAI titre against HPV-16 L1/L2 VLPs was 3493 ± 877.6 , which was slightly higher than that against HPV-16 L1 VLPs (2746 ± 482.2), but the difference was not statistically significant ($P > 0.05$).

Epitope-blocking ELISA of HPV-31 L1/L2 VLPs antiserum

To investigate whether the epitopes recognized by HPV-31 L1/L2 VLP antiserum might be related to the epitopes

Fig. 3. Cross-reactivity of H31 L1/L2 VLP antiserum. The type-specific (a) and cross-reacting (b) activities of HPV-31 L1/L2 VLP antiserum were determined by ELISA. All results were expressed as the mean $\pm$ SD of three individual experiments.

defined by genotype-specific neutralizing mAbs, three well-known, type-specific neutralizing mAbs, H31.A6, H16.V5 and H16.E70, were used in an epitope-blocking ELISA. The results indicated that mAb H31.A6 could inhibit the binding of HPV-31 L1/L2 VLP antiserum to HPV-31 L1/L2 VLPs (Fig. 4a) and that mAb H16.V5 could partially inhibit the cross-reaction of HPV-31 L1/L2 VLP antiserum with HPV-16 L1/L2 VLPs (Fig. 4b), whilst the inhibitory effect of H16.E70 mAb was very weak (Fig. 4c). High concentrations of H31.A6 or H16.V5 could not completely block the

Table 1. HAI assay

Type of HPV VLP	HAI titre*
HPV-11 L1 VLPs	<50†
HPV-11 L1/L2 VLPs	<50†
HPV-16 L1 VLPs	$2\,746 \pm 482.2\ddagger$
HPV-16 L1/L2 VLPs	$3\,493 \pm 877.6$
HPV-31 L1/L2 VLPs	$2\,839\,893 \pm 500\,539.5$

*HAI titres were expressed as the mean $\pm$ SD of three individual experiments.

† $P < 0.05$ using one-way analysis of variance compared with HPV-16 L1 or L1/L2 VLPs.

‡ $P > 0.05$ using Student's *t*-test compared with HPV-16 L1/L2 VLP.

binding of HPV-31 L1/L2 VLP antiserum to HPV-31 L1/L2 VLPs or HPV-16 L1/L2 VLPs, respectively, which is consistent with the fact that HPV-31 L1/L2 VLP antiserum was induced by multiple epitopes.

DISCUSSION

Previous cross-neutralizing assays have mainly focused on antisera to HPV L1 VLPs. In this study, we investigated whether the L2 protein in HPV-31 L1/L2 VLPs could induce cross-neutralizing antibodies against HPV-11 and -16 L1 or L1/L2 VLPs. Our results showed that HPV-31 L1/

L2 VLP antiserum had slightly higher, but not statistically significantly different, cross-reacting activity with HPV-11 or -16 L1/L2 VLPs than with HPV-11 or -16 L1 VLPs, respectively. Similarly, the HAI titre against HPV-16 L1/L2 VLPs was slightly higher than, but not statistically significantly different to, that against HPV-16 L1 VLPs. As the presence of L2 proteins was the unique difference between HPV L1/L2 VLPs and HPV L1 VLPs, the slight differences in these cross-reacting or cross-neutralizing activities were most likely caused by the cross-reaction or cross-neutralization of HPV-31 L2-specific antibodies with the surface-exposed parts of the L2 proteins in HPV-11 or -16 L1/L2 VLPs. Therefore, we suggest that the L2 proteins incorporated in HPV L1/L2 VLPs may be able to induce cross-neutralizing activity, albeit with very low titre. However, the differences between the cross-reacting and cross-neutralizing activities of HPV-31 L1/L2 VLP antiserum with L1 VLPs and L1/L2 VLPs were not statistically significant. As the ratio of L1 to L2 molecules in HPV-31 L1/L2 VLPs was about 30 : 1, only 12 L2 molecules were incorporated in each VLP, whilst the number of L1 molecules was up to 360. In addition, only a few parts of L2 are exposed at the surface of L1/L2 VLPs. Thus, the relative number of surface-exposed L2 epitopes in HPV-31 L1/L2 VLPs was very low, which might be the reason why the level of cross-neutralizing antibodies induced by the L2 protein in L1/L2 VLPs was very low. Similarly, the low number of L2 protein molecules relative to L1 in the denatured HPV-31 L1/L2 VLPs might explain why only a weak HPV-31 L2 band and no HPV-11 or -16 L2 bands were detected using H31 L1/L2 (D) antiserum (Fig. 2).

It is known that VLP-based ELISAs can measure a combination of neutralizing and non-neutralizing antibodies, as well as antibodies to immunogenic insect cells or baculovirus proteins common to the vaccine and ELISA antigen. In addition, it had been demonstrated that protection from virus infection correlates better with HAI titres than ELISA titres (Roden *et al.*, 1995, 1996). In this study, although HPV-31 L1/L2 VLP antiserum could cross-react with HPV-11 VLPs in ELISA, it could not cross-neutralize HPV-11 VLPs in an HAI assay. However, the HPV-31 L1/L2 VLP antiserum could cross-neutralize the closely related HPV-16 VLPs. Therefore, our results suggest that, for the low level of cross-neutralizing antibodies induced by L2 proteins in L1/L2 VLPs, the cross-neutralizing activity of HPV L1/L2 VLP antiserum was still mainly dependent on the homology of the L1 protein among different types. It has been demonstrated that antibodies induced by one type of L1 VLPs can only cross-neutralize closely related HPV types and not distantly related types (Christensen *et al.*, 1996b; Combata *et al.*, 2002; Fleury *et al.*, 2006). However, previous studies have shown that antibodies induced by HPV L2 peptide or protein have good cross-neutralizing activity, even against distantly related types (Kawana *et al.*, 1999, 2001, 2003; Pastrana *et al.*, 2005; Roden *et al.*, 2000). Thus, L2-induced cross-neutralizing antibodies may be important in

Fig. 4. Epitope-blocking ELISA. The inhibitory effects of HPV-31-specific mAb H31.A6 (▲) (a), and HPV-16-specific mAbs H16.V5 (□) (b) and H16.E70 (■) (c) on the binding of HPV-31 L1/L2 VLP antiserum (●) to HPV-31 L1/L2 VLPs and HPV-16 L1/L2 VLPs, respectively, were determined using an epitope-blocking ELISA.

preventing infection of multiple types of HPV. As the level of L2-induced cross-neutralizing antibodies was very low as a result of the relatively low number of surface-exposed L2 epitopes in L1/L2 VLPs, it would be reasonable to improve the level of L2-induced cross-neutralizing antibodies by enhancing the relative number of outer-surface-exposed L2 epitopes in VLPs, for example by fusing important L2 epitopes into certain L1 surface-exposed regions to generate L1–L2 chimeric VLPs.

Our results also demonstrated that binding of mAb H16.V5 could partly inhibit the cross-reaction of HPV-31 L1/L2 VLP antiserum with HPV-16 L1/L2 VLPs, suggesting that the epitopes of HPV-16 L1/L2 VLPs that cross-react with HPV-31 L1/L2 VLP antiserum are located near to the epitopes recognized by mAb H16.V5. As mAb H16.V5 recognizes epitopes located mainly on the FG loop of HPV-16 L1, which is important for recognition by both type-specific neutralizing antibodies and cross-reactive antibodies (Carpentier *et al.*, 2005), essential attention should be paid to the FG loop of L1 in the development of L1–L2 chimeric VLPs.

In conclusion, our data demonstrated that the L2 protein in HPV L1/L2 VLPs may induce cross-neutralizing antibodies, albeit with very low titres, and that certain epitopes in HPV-16 L1/L2 VLPs that cross-react with HPV-31 L1/L2 VLP antiserum are located close to the H16.V5-recognized epitopes. These findings may provide suggestions for the design of effective multivalent VLP vaccines for the prevention of infection with multiple types of HPV.

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