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Neutralization of Human Papillomavirus by Specific Nanobodies Against Major Capsid Protein L1

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The human papillomavirus (HPV) is the main cause of cervical cancer in developing countries. Rapid diagnosis and initiation of treatment of the HPV infection are critical. Various methods have been employed to reduce the immunogenicity of antibodies targeting HPV serotypes. Nanobodies are the smallest fragments of naturally occurring single-domain antibodies with their antigen-binding site compromised into a single domain. Nanobodies have remarkable properties such as high stability, solubility, and high homology to the human VH3 domain. In this study, a phagemid library was employed to enrich for nanobodies against the L1 protein of the human papilloma virus. Binding reactivity of the selected clones was evaluated using phage enzyme-linked immunosorbent assay (phage-ELISA). Finally, two nanobodies (sm5 and sm8) with the best reactivity against the Gardasil vaccine and the purified HPV-16 L1 protein were expressed and purified using a Ni^{+}-NTA column. The accuracy of expression and purification of the nanobodies was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting assays. *In vitro* studies demonstrated that neutralization was achieved by the selected nanobodies. The ease of generation and unique features of these molecules make nanobodies promising molecules for the new generation of HPV diagnosis and therapy.

Keywords: Nanobody, HPV-16, L1 protein, phage display

Despite the widespread performance of cytological screening in many countries, cervical cancer remains a major cause of mortality [32]. This disease is the second most common female cancer in the world, claiming around 270,000 lives annually [23]. More than 99% of cervical tumors are positive for DNA of the human papillomavirus (HPV), and a subset of HPV types are the causative agents of cervical cancer. Other tumors related to this virus, such as vaginal, vulvar, penile, anal, and oropharyngeal, represent an additional 0.7% of all cancer sites in both men and women, so that HPV is estimated to be responsible for 5.2% of all cancers and, as such, can also be considered as the most common known sexually transmitted agent [22]. The viral types that infect the genital tract fall into two categories: high-risk (associated with anogenital cancers) and low-risk (causing benign genital warts). Although over 115 genotypes of HPV have been identified, some are considered "high-risk", notably HPV-16 and HPV-18 [20].

HPV is a small, double-stranded DNA virus that is non-enveloped and has an icosahedral capsid [19]. The HPV genome is approximately 8 kb in length, which encodes 6 early (E1, E2, E4, E5, E6, and E7) and 2 late (L1 and L2) proteins. The late genes encode structural proteins that form the viral icosahedral capsid consisting of 72 capsomers [8]. It has been demonstrated that neutralization epitopes are present in the L1 major capsid protein. Both linear and conformational epitopes have been identified on the surface of HPV-16 L1 VLPs. More than three L1 regions (such as amino acids 111 to 130, 174 to 185, and 261 to 280) contain linear epitopes. The results suggest that conformational B-cell epitopes of HPV, VLPs, induce neutralizing antibodies. In contrast, cross-reactive epitopes are linear epitopes and mostly non-neutralizing. It has been suggested that such linear epitopes are not surface-exposed [2]. The HPV L1 proteins are capable of self-assembly into virus like particles (VLP) *in vitro* [14]. VLPs are immunogenic in humans and induce neutralizing antibodies [13, 31]. Gardasil is a prophylactic vaccine that is composed of VLPs formed by self-assembly of the recombinant L1 capsid proteins of

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HPV-6, HPV-11, HPV-16, and HPV-18 [12, 25]. There are several reports of monoclonal antibodies generated against HPV that bind to the L1 protein [3, 4, 18]. These monoclonal antibodies were analyzed for their binding affinities to L1-only VLPs and L1 linear epitopes [6].

Recombinant antibody technology is a rapidly developing field that specializes in developing recombinant fragments that can be used in therapy and diagnosis [11]. Recombinant antibodies are produced by expressing antibody fragments on the surface of filamentous phages, whereupon clones are selected based on their antigen reactivity [10]. Throughout the 1990s, to reduce the immunogenicity, innovative recombinant DNA technology dramatically reduced the human anti-mouse antibody (HAMA) response and enhanced the clinical efficiency of mouse monoclonal antibodies. The recombinant antibodies, partly owing to their ability to be highly expressed in prokaryotic and lower eukaryotic microorganisms, are currently in the late stages of clinical development. Various methods have been devised to improve the characteristics of the single-chain fragment variables (scFvs). By chance, in 1993, Hamers-Casterman and *et al*. [9] discovered a new type of antibody in Camelidae. This kind of antibody, contrary to conventional antibodies, consists of only heavy chains. Although it does not contain light chains and CH1 domains, it is functional and binds to its antigen like conventional antibodies. The antigen-binding site of this antibody consists of a single domain. This antibody was named as VHH or nanobody, and is less than 20 kDa in size and has a lot of potential applications in medical sciences [27]. Nanobodies have remarkable properties that can provide new opportunities for diagnostic and therapeutic purposes. This single-domain antibody remains functional even at high temperatures such as 90°C and is also readily soluble in aqueous solutions. Because of its small size, it can penetrate rapidly into the tissue and is an efficient tool for targeted tumor therapy. Specific binding with high affinity to its antigen and high homology to human VH3 are the other advantages of these molecules [21].

Since infection with certain HPV types, most commonly HPV type 16 (HPV-16), is associated with progression to high-grade dysplasia, in the present study we tried to isolate specific nanobodies against the HPV-16 major capsid protein L1. Camelid single-domain antibodies have several advantageous properties as compared with conventional antibodies, making them highly desirable molecules for the future development of novel diagnostic and therapeutic methods against human papillomavirus.

Materials and Methods

Materials

The purified HPV-16 L1 protein was kindly provided by Dr. Martin Müller (German Cancer Research Center, Germany). Gardasil vaccine manufactured by Merck & Co., Inc (West Point, PA, USA) was used as an antigen. Monoclonal anti-hemagglutinin (anti-HA) conjugated to horseradish peroxidase (HRP) was purchased from Roche (Mannheim, Germany). HRP-conjugated anti M13 antibody (anti-M13-HRP) was purchased from Amersham-Pharmacia-Biotech (Vienna, Austria). All other reagents used in this study were at least of analytical grade and purchased from Sigma Chemical Co. (St. Louise, MO, USA).

Cell Lines and Cell Culture

A431 cells (human vulvar squamous carcinoma cells) were cultured and infected in DMEM supplemented with 5% FBS. These cells were infected in DMEM (10% FBS) lacking both phenol red and hygromycin in the infection medium.

Strains and Media Composition

E. coli TG1 (Pharmacia, Uppsala, Sweden) was used as a host for phagemid manipulation and antibody high expression. The *E. coli* Rosetta gami 2 strain (Novagen, Madison, WI, USA) was used for soluble antibody production.

2×YT medium supplemented with 100 μg/ml ampicillin were used for the selection of transformants. Luria–Bertani (LB) medium supplemented with 0.01 M $MgCl_2$ and 0.02 M glucose was the medium of choice for electroporation, and 2×YT medium supplemented with ampicillin (150 μg/ml) and IPTG (1 mM) was used as expression medium.

Nanobody-Displayed Phage Libraries Amplification

The one-humped camel nanobody gene library (in pComb3x phagemid vector, containing HA- and His-tag) was prepared as described before [1]. The *E. coli* TG1 was grown in 2×YT medium at 37°C and inoculated in logarithmic phase with nanobody-displayed library phages. Infected cells were grown overnight at 37°C in 2×YT medium containing 100 μg/ml ampicillin, under rapid shaking. After overnight incubation at 37°C, phages were produced by rescue with helper phage M13KO7 (Amersham-Pharmacia-Biotech) and purified using PEG/NaCl (20 g/l and 2.5 M, respectively) precipitation. The phages were titrated and stored at 4°C.

Enrichment of Nanobody Gene Library

To obtain L1-specific phage displaying nanobodies, three consecutive rounds of biopanning were performed on a nanobody library. Counter selection was performed during each round of panning using bovine serum albumin (BSA) (1.5 μg/ml diluted in phosphate buffered saline, PBS)-coated 6-wells. In all of the experiments of this research, for immoblization of the conformational epitope of the L1 protein on the surface of the wells, the Gardasil was dialysed in PBS buffer, with the buffer exchanged every 5–7 h for at least 2 days, and then the dialysed solution (1.5 μg/ml in PBS) was incubated overnight at 4°C in 6-well plates (Nunc, Roskilde, Denmark). On the next day, the wells were washed twice with PBS and blocked by PBS containing 5% (w/v) ELISA-grade skimmed milk powder at 37°C for 1.5 h. After removing the blocking solution, phage particles (10^{12}/ml virion) in a total volume of 3 ml of PBS with 4% BSA were preincubated on the BSA-coated wells at 37°C for 1 h (to remove nonspecific binders). The unbound phages were subsequently loaded onto Gardasil (VLPs that contain a mixture of recombinant L1 capsid proteins of HPV-6, -11, -16, and -18) coated 6-well plates to recover the L1-specific nanobody-displayed phages. After incubation at 37°C for 1 h, the supernatants were discarded and the wells were

rinsed four times with 0.5% Tween 20 in PBS (PBST) and twice with distilled water. The bound phages were eluted by adding 1 ml of 100 mM triethylamine (TEA) (pH 10) and neutralized with 0.5 M Tris/HCl (pH 7.4). For three rounds of panning, the eluted phages were amplified by infecting log-phase *E. coli* TG1 cells, followed by their superinfection with the helper phage, as detailed above. In addition, the input and output phages were titrated. Three consecutive rounds of panning were performed and the stringency of selection was increased with each round by using increasing Tween concentrations (0.5–8%) in the washing steps.

Polyclonal Phage-ELISA

The 96-well plates were directly coated with 1.5 μg/ml L1 capsid proteins of HPV-6, -11, -16, and -18 (Gardasil) diluted in PBS overnight at 4°C. The same concentration of bovine serum albumin (BSA) was used as a negative control. The wells were washed with PBS and then blocked with a 5% solution of skimmed milk powder in phosphate buffer (MPBS) (10 mM, pH 7.2) for 1 h at 37°C. The blocking reagent was discarded and ~10^{12} transducing units of the library and output were pooled from each round of panning, transferred to appropriate wells, and incubated while shaking for 2 h at 37°C. The wells were washed four times with PBS containing 0.05% (v/v) Tween 20 and then four times with PBS. Mouse anti-M13 monoclonal antibody conjugated to HRP (1:2,500) was added to the well and incubated for 1 h at room temperature. At the end of the incubation time, wells were washed, added with 50 μl of substrate, 3,3',5,5'-tetramethyl benzidine (TMB), and incubated for 5–10 min. The enzyme reaction was terminated using 50 μl of 2 M H_2SO_4 solution and the color was measured at a wavelength of 450 nm [30].

Phage Titration

At the end of the panning rounds, the phage titers of input and output of selection were estimated by infection of logarithmically growing TG1 cells with serially diluted phages and plating on 2×YT ampicillin plates.

Monoclonal Phage-ELISA

After the third round of panning, 10^8 TG1 bacteria cells were grown to mid-logarithmic phase, infected by the output phagemids of the last round of panning, spread on LB agar plates containing ampicillin (100 μg/ml), and incubated overnight. At the end of the incubation time, more than 85 bacterial colonies were picked up and inoculated separately in 20 ml of 2×YT broth medium. Then phages were produced by rescue with the M13KO7 phage and purified by PEG-NaCl. The precipitated monoclonal nanobodies expressing phage particles were resuspended in sterile PBS (a titer of 10^{12}/ml) and screened by phage-ELISA using anti-M13-HRP as a tracer, as described above. The positive phage clones were sequenced to identify the unique nanobody genes.

Selection of HPV-16 L1-Specific Nanobodies

In order to produce and evaluate the soluble nanobody, 10^9 *E. coli* (Rosetta gami 2) bacteria cells (the non-suppressor strain) were grown to mid-logarithmic phase and the selected monoclonal phages were transformed into them. The Rosetta gami 2 cells harboring the recombinant phagamids were resuspended in 35 ml of 2×YT-ampicillin and the expression was induced by addition of IPTG to 1 mM at 37°C until the OD_{600} reached 0.7. Then bacteria cells were separated by centrifugation. The pellets were suspended in 800 μl of PBS. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. The cytoplasmic proteins were extracted by sonication and then clarified by centrifugation. To evaluate the binding activity of selected clones towards the conformational and linear epitopes of the L1 protein, two different concentrations (0.1 and 2 μg/ml) of the Gardasil and purified L1 protein, were coated onto the wells of microtiter plates. Briefly, for conformational epitopes, the dialyzed Gardasil was incubated overnight at 4°C, and for linear epitopes the recombinant L1 protein (prepared in transgenic plants, kindly provided by professor Martin Müller, German Cancer Research Center, Germany) diluted in bicarbonate buffer (pH 10.8) and incubated overnight at 37°C (denaturation of L1 protein and disruption of second and third structures) was used. The same concentration of BSA was used as a negative control. The plates were washed and blocked with MPBS (5%) for 1 h at 37°C. Then, wells were washed and added with diluted cytoplasmic proteins. The contents of the wells were incubated at 37°C for 2 h, washed, and monoclonal anti-HA conjugated to HRP (1:10,000) antibody added and incubated at 37°C for 1 h. The peroxidase enzyme activity was determined by adding 50 μl of TMB as a substrate. The reaction was stopped by the addition of 50 μl of 12 M H_2SO_4 and read out at 450 nm.

Expression and Purification

For production and purification of nanobodies, selected clones were cultured in 50 ml of LB medium at 37°C and 250 rpm until an OD_{600} of 0.7 was achieved and inoculated in 1 L of 2×YT containing casamino acids and ampicillin. The culture was induced with IPTG (1 mM). After 24 h, the cultures were further induced by adding 1 mM IPTG and incubated for a further 24 h at 37°C. Induced cultures were centrifuged and the cytoplasmic proteins were extracted by sonication. The nanobody present in these extracts was purified by immobilized metal affinity chromatography with 1 ml of nickel nitrilo-triacetic acid (Ni^+-NTA) resin (Qiagen) [28]. The purification was confirmed using SDS-PAGE and immunobloting analysis [16]. For immunoblotting, cytoplasmic proteins of selected clones were subjected to 12% SDS-PAGE and transferred from the gels onto nitrocellulose membranes using a buffer consisting of 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. The membranes were blocked for 1 h at room temperature in MPBS (5%). Subsequently, the membranes were incubated with anti-HA conjugated to HRP (1:1,000) for 90 min at room temperature. The membranes were washed two times with PBST (PBS containing 0.01% Tween 20) and two times with PBS. The immunoblot was visualized with DAB (3,3'-diaminobenzidine) substrate [29].

Analysis of Binding Specificity

The individual nanobodies were tested for their specificity and possible cross-reactivity with some cell surface receptors, mucosal glycoproteins, and serum proteins by ELISA. The antigens included MUC1 (DF3/Mucin 1, aberrantly glycosylated glycoprotein overexpressed in tumors of epithelial origin, (including the genital tract); endoglin (type I membrane glycoprotein located on cell surfaces and part of the TGF beta receptor complex); P24 (CA, the capsid protein of the HIV virus); skimmed milk; casein; and human serum albumin (HSA). For binding specificity, 1 μg/ml of each antigen was immoblized on 96-wells plate. The L1 protein (Gardasil) with the same concentration was used as a positive control. The selected nanobodies (400 ng/ml)

were added to the wells and incubated for 1 h at 37°C. At the end of the incubation time, the plate was washed, incubated with anti-HA conjugated to HRP (1:10,000), and the peroxidase activity was detected by adding TMB.

Nanobody-Mediated Neutralization

HPV-16 virions (1.0×10^8 particles, 100 particles/cell) were incubated with dilutions (1, 10, and 50 nM) of purified nanobodies for 1 h at 37°C. The mixture was then added to monolayers of A431 cells. The medium was replaced again after 6 h. Infection was assayed 4 days post-infection by quantitative real-time RT-PCR (QRT-PCR). Control cultures contained either no virus or virus-infected cultures without nanobodies. After 4 days, cells were harvested and total RNA was isolated using a Qiagen RNA purification kit (Qiagen, Valencia, CA, USA) and used as a template for cDNA synthesis (Roche). QRT-PCR for E1^E4 and β-actin (as an internal control) transcripts was performed as described by Culp and Christensen [5].

Statistical Analysis

Different experimental groups within the study were compared by using the Kruskal–Wallis test. Comparisons between the pairs of groups were performed with the Mann–Whitney test. A probability of less than 0.05 ($p<0.05$) was used for statistical significance.

RESULTS

Phage Biopanning

The nanobody gene library was selected against Gardasil through three rounds of panning. In phage panning, we eluted 3.1×10^2, 4.3×10^4, and 3.7×10^5 phages after the first, second, and third rounds, respectively. Titration experiments showed a gradual increase in the output/input ratio of phage particles after each round of selection, representing the progressive enrichment of the L1-specific clones during these successive rounds of panning. This ratio increased about 13-fold after the second round of biopanning and 8.6-fold after the third round of cell panning. The binding absorption (OD_{450}) results of Gardasil in the first, second, and third rounds of panning were 0.384 ± 0.05, 0.628 ± 0.031, and 1.133 ± 0.012, respectively. These increases in titers of output phages and the difference of OD_{450} in consecutive rounds of panning compared with negative control (BSA) in polyclonal phage-ELISA (0.323 ± 0.014, 0.354 ± 0.0169, and 0.354 ± 0.007 after the first, second, and third rounds, respectively) shows the accuracy of the enrichment process.

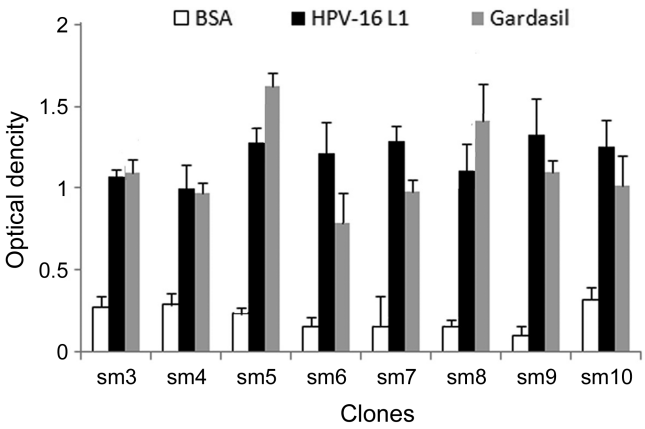

Fig. 1. Monoclonal phage-ELISA results in OD_{450} using anti-M13-HRP as tracer.
Among the 8 selected clones, sm5 and sm8 showed significant reaction against both Gardasil and L1 protein and also did not cross-react with the BSA as negative control. All data were expressed as mean ± SD. All assays were performed in triplicates. Error bars represent standard deviation of a triplicate determination.

Selection of Specific Nanobodies Against the L1 Protein

After the third round of panning, more than 85 nanobody-expressing phages were screened using a monoclonal phage-ELISA by using anti-M13-HRP as a tracer. Eight clones specifically recognized recombinant linear epitopes of the L1 protein (purified recombinant L1 protein) and conformation-dependent epitopes (Gardasil) and did not cross-react with the negative control. Fig. 1 shows monoclonal phage-ELISA results using the anti-M13-HRP as tracer. Among the selected nanobodies, sm5 and sm8 showed the best reactivity against the L1 epitopes. The identity of the selected nanobodies was confirmed by sequencing of the selected clones genome using nanobody-specific primers.

The Rosetta gami 2 bacterial cells were infected with the selected phagemids that had specificity against the major capsid protein L1 (sm3, sm4, sm5, sm6, sm7, sm8, sm9, sm10). The bacterial cells harboring these phagemids were induced by IPTG. The cytoplasmic extract of the transformants was evaluated by ELISA. Two transformants (sm5 and sm8) showed higher reactivity towards the HPV-16 L1 protein. Table 1 shows the reactivities of these two clones against two different concentrations of Gardasil and the purified L1 protein.

Table 1. Reactivity of selected nanobodies against different concentrations of antigen in OD_{450} using anti-HA conjugated to HRP.

Nanobodies	Gardasil (μg/ml)		HPV-16 L1 (μg/ml)		BSA (μg/ml)	
	2	0.1	2	0.1	2	0.1
sm5	2.048 ± 0.372	0.498 ± 0.242	1.603 ± 0.282	0.691 ± 0.172	0.632 ± 0.114	0.312 ± 0.107
sm8	1.686 ± 0.150	0.392 ± 0.143	1.392 ± 0.211	0.421 ± 0.125	0.482 ± 0.157	0.292 ± 0.121

The results are the average of reactivity (OD_{450}) for each nanobody and presented as mean ± SD. All assays were performed in triplicates.

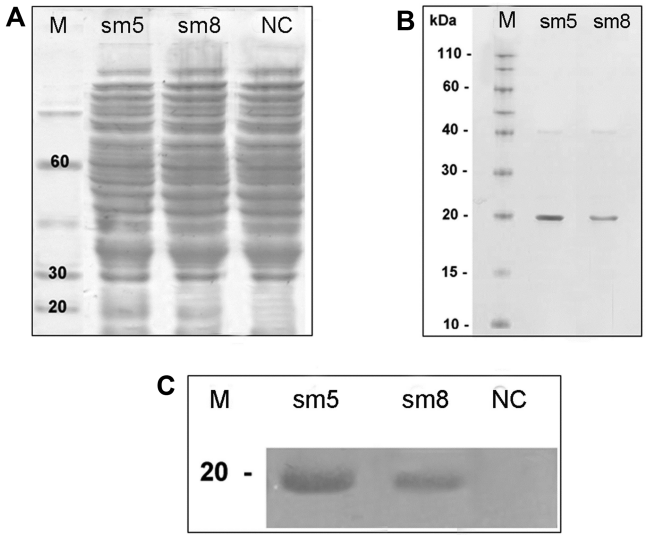

Fig. 2. Expression analysis.
(**A**) SDS-PAGE analysis of cytoplasmic extracts; (**B**) Coomassie-stained SDS-PAGE gel after affinity chromatography purification; (**C**) Immunoblot analysis of purified nanobodies. (sm5) and (sm8), the selected nanobodies; (M), molecular weight marker; (NC), negative control (non-transformed bacteria).

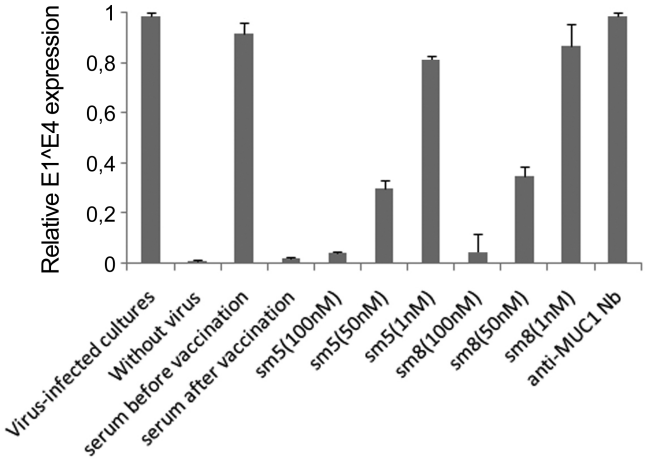

Fig. 3. Neutralization of HPV-16 virions by sm5 and sm8 nanobodies.
Control cultures contained either no virus (negative control) or virus-infected cultures without nanobodies (positive culture). Anti-MUC1 Nb refers to a nanobody produced against MUC1 peptide that is found to be an important epitope expressed in cancerous tissue. All data were expressed as mean ± SD. All assays were performed in triplicates. Error bars represent standard deviation of a triplicate determination.

Expression and Purification of Selected Nanobodies

For expression analysis of the nanobody fragments, soluble nanobodies were extracted from the bacterial cytoplasm, and visualized by Coomassie blue staining of the SDS-PAGE gel. Expression analysis by SDS-PAGE indicated that the optimal IPTG concentration to be used was 1 mM. The expression of the sm5 and sm8 nanobodies was found to be 0.5% and 0.3%, respectively, of the total soluble proteins of the bacterial cell lysates following the gel scanning procedure. Recombinant nanobodies were purified from the lysate extracts using (Ni^{+}-NTA) resin affinity chromatography. Elution fractions containing the pure recombinant protein of each transformants were separated and combined as a pool for neutralization assays. The molecular masses of these two fusion proteins containing the nanobody and tags were around 20 kDa. The purification was confirmed using SDS-PAGE and immunobloting analysis. As showed in Fig. 2, the protein bonds with an apparent molecular mass of around 20 kDa were clearly present in the sm5 and sm8 nanobodies, which were not observed in the IPTG-induced Rosetta gami 2 cells without the plasmid. The yield of expression of the sm5 and sm8 nanobodies was between 0.3 and 0.1 mg/l of the bacterial cultures, respectively.

Neutralization of the Virions by HPV-16 L1 Reactive Nanobodies

To test whether neutralization might be accomplished by monovalent nanobodies against the L1 protein, purified sm5 and sm8 nanobodies were incubated with HPV-16 virions and after 1 h layered over rinsed A431 monolayers. Four days post-infection, total RNA was assayed for the presence of E1^E4 transcripts using quantitative RT-PCR. Neutralization experiments by quantitative RT-PCR revealed that neutralization could be attained with these recombinant proteins (Fig. 3). Relative E1^E4 expression of the sm5 and sm8 nanobodies at the 50 nM concentration showed 75% and 60% neutralization of HPV-16 virions, respectively. A431 cell culture without infection by virions was used as a negative control (no expression of E1^E4). A431 cell culture infected by HPV and without nanobodies or with nonspecific nanobody did not show any significant neutralization reaction.

Table 2. Sensitivity of selected nanobodies.

Antibodies	Antigens (1μg/ml)							
	Gardasil	BSA	MUC1	Skimmed milk	Casein	Endogline	HSA	P24
SM5	2.142 ± 0.12	0.626 ± 0.01	0.650 ± 0.03	0.707 ± 0.02	0.774 ± 0.11	0.552 ± 0.04	0.728 ± 0.07	0.781 ± 0.06
SM8	1.708 ± 0.07	0.484 ± 0.02	0.506 ± 0.14	0.596 ± 0.04	0.622 ± 0.01	0.504 ± 0.12	0.612 ± 0.09	0.594 ± 0.02

The results are the average of reactivity (OD_{450}) for each nanobody and presented as mean ± SD. All assays were performed in triplicates. HSA refers to human serum albumin, which is an aboundant plasma protein. MUC1 is a human tumor antigen, which is overexpressed in tumors of epithelial origin (including genital tract). P24 is a capsid protein of the HIV.

Results of the Binding Specificity

To ensure the specificity of the nanobodies, direct binding of the anti-L1 nanobodies to the L1 protein and irrelevant antigens was investigated by ELISA. Based on the results, the selected nanobodies exhibited significant binding to the L1 protein but also revealed slightly cross-reaction with irrelevant antigens. Table 2 lists the results of the nanobody binding specificities.

DISCUSSION

Infection with the high-risk types of HPV is now considered to be the most significant risk factor in the development of cervical cancer [14]. The HPV major capsid protein L1 is an efficient target protein for the human humoral immune response and also a useful marker for revealing the status of productive and active HPV infections [13]. This is due to the high prevalence of HPV malignancies and HPV-associated lesions, and the application limitations of mouse-originated monoclonal antibodies such as, expensive production methods (due to them being based on mammalian expression systems) and their potential immunogenicity. The phage display technique has proven to be a powerful technique for the selection of specific peptides or proteins against a specific target. One of the most successful applications of phage display has been in the isolation of monoclonal recombinant antibodies from a large antibody gene library [35]. The discovery of nanobodies has now opened unprecedented opportunities to obtain soluble antigen-binding fragments of minimal size by phage display technology. Nanobodies are a unique form of monoclonal antibodies, characterized by a single antigen binding domain. They are small molecules and strictly monomeric. These molecules bind their target with nM affinity as well as being pH, solvent, and temperature stable (*e.g.*, stable for one week in serum in 37°C). This variable fragment with a typical immunoglobulin fold and prolate shape (4.4 nm high; 2.8 nm diameter) shares a large sequence identity with the human VH3 gene family, but with four amino acid substitutions in framework 2. These substitutions render the surface more hydrophilic and explain the soluble behavior and concomitant higher functional expression levels of nanobodies [21]. The single domain nature of nanobodies also makes molecular manipulation easy and facilitates the production of multivalent formats of monoclonal antibodies as compared with conventional recombinant antibodies, which is problematic owing to aggregation and reduced affinity due to mispairing of variable H and L domains, in addition to being well expressed in bacterial expression systems and thus they are cheaper and easier to produce in all kind of formats than standard monoclonal antibodies. Moreover, nanobodies often bind to epitopes that prove to be less immunogenic for conventional antibodies. Collectively, these features make nanobodies as ideal small-sized candidates for immunotherapy, and thus opening the path to new and sophisticated design solutions for the treatment of cancer.

Improved VLP vaccines (such as Gardasil) would promote the production of neutralizing antibodies targeting multiple HPV types [6, 17, 18, 26, 34]. Here, we enriched a one-humped camel nanobody gene library against Gardasil as a source of conformation-dependent epitopes of human papillomavirus in phage display technology. The enrichment was performed after three rounds of panning. For evaluation of the biopanning process, phage titration and polyclonal phage-ELISA were used and the increase in titer of the output phages and OD_{450} in the consecutive rounds of panning demonstrated the accuracy of processing.

Several studies have demonstrated that HPV capsids contain neutralizing epitopes that are generally conformation-dependent and involve the hypervariable loops. Although neutralizing epitopes exist on the surface of the capsid, immunodominance can be maintained in the context of the VLPs [3, 24]. The antibodies that directly interact with the hypervariable loops of L1 (dense in neutralization-sensitive epitopes) are likely to be neutralizing antibodies [6]. Based on these findings, for selection of the neutralizing and specific nanobodies, we chose the clones that showed significant reaction against both Gardasil and the L1 protein (purified non-VLP protein) among the 85 evaluated clones. The selected clones were re-infected in a non-suppressor strain of *Escherichia coli* (Rosetta gami 2), which was unable to suppress the amber stop codon between the gIII and the nanobody inserted gene, and subsequently the expression of the soluble protein was induced by IPTG. Our findings indicate that the selected nanobodies are capable of specific recognition of the L1 epitopes and have low cross-reaction with irrelevant antigens, perhaps by virtue of their smaller non-antigen binding surface areas, minimizing unwanted cross-reactivities as opposed to larger multidomain immunoglobulins. The sm5 and sm8 nanobodies were selected by ELISA and the results demonstrate that these soluble nanobodies bound to the Gardasil vaccine and purified L1 protein effectively and neutralized virions in cell culture experiments. They were also capable of acting as both captors and detectors for HPV-16 particles, indicating their potential as a diagnostic and therapeutic agent. Since our goal in this work was to prepare nanobodies with high binding activity to the L1 protein, especially HPV-16, we chose the nanobodies with highest reactivity against the Gardasil (which is a mixture of recombinant L1 capsid proteins of HPV-6, -11, -16, and -18) and the purified L1 protein. By selecting antibodies that show some degree of cross-reactivity for other types of HPV, this enhances their ability to be utilized to neutralize virions in the cervix immediately at the time of infection.

Culp *et al*. [6] utilized some HPV-16-specific monoclonal antibodies, Fab fragments, and scFv fragments to be able to compare the effectiveness of monovalent and divalent binding events on interrupting viral infection. Data derived with whole monoclonal antibodies and their Fab fragments showed that equivalent saturation of each corresponding epitope results in comparable neutralization, regardless of the valency of the binding molecule. Their finding suggests that the parental antibodies, each targeting a different loop of L1, can neutralize virions by a mechanism independent of particle aggregation and L1 cross-linking [6]. Our results confirmed these findings and showed that monovalent binding events by nanobodies can also neutralize HPV virions, as has been shown by scFv and Fab fragments.

In this work, our goal was not so much to compete with the existing diagnostic and therapeutic antibodies for papillomavirus but rather to begin exploring the capacity of a novel type of antibody (nanobody) to recognize the L1 protein epitopes and neutralizing activities. Virus neutralization is defined as the abrogation of virus infectiousness by the association of an antibody with the viral particle. In principle, neutralization can inhibit infectiousness by several mechanisms. Prevention of virus attachment would inhibit the entry process, and many neutralizing antibodies act by abrogating virion attachment to the host cells. Antibodies can also potentially interfere with post-attachment interactions of a virus with its receptors or co-receptors or with the cellular endocytic machinery [15]. Neutralizing antibodies have also been described that act after viral endocytosis by negatively affecting trafficking, membrane penetration, uncoating, nuclear import, or viral transcription [7, 33, 36]. Since the host infection site of HPV-16 is the genital mucosa, development of effective mucosal captors with negligible cross-reactivity towards irrelevant proteins and glycoproteins of that environment is of interest to neutralize the primary infection by the virus. In the present work, we produced two nanobodies that are stable and sensitive L1-specific targeting agents, which could be used in vaginal gels or washing solutions for the prevention of viral infection. The unique features of the nanobodies make them valuable tools for the prevention and therapy of cervical cancer. Development of such new ligands and improved understanding of tumor biology will allow HPV-specific nanobodies to be used in combinational therapies in a synergistic manner in the future.

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References

1. Ahmadvand, D., M. J. Rasaee, F. Rahbarizadeh, R. E. Kontermann, and F. Sheikholislami. 2009. Cell selection and characterization of a novel human endothelial cell specific nanobody. *Mol. Immunol.* **46:** 1814–1823.
2. Chen, X. S., R. L. Garcea, I. Goldberg, G. Casini, and S. C. Harrison. 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol. Cell.* **5:** 557–567.
3. Christensen, N. D., J. W. Kreider, N. M. Cladel, S. D. Patrick, and P. A. Welsh. 1990. Monoclonal antibody-mediated neutralization of infectious human papillomavirus type 11. *J. Virol.* **64:** 5678–5681.
4. Christensen, N. D., C. A. Reed, N. M. Cladel, K. Hall, and G. S. Leiserowitz. 1996. Monoclonal antibodies to HPV-6 L1 virus-like particles identify conformational and linear neutralizing epitopes on HPV-11 in addition to type-specific epitopes on HPV-6. *Virology* **224:** 477–486.
5. Culp, T. D. and N. D. Christensen. 2003. Quantitative RT-PCR assay for HPV infection in cultured cells. *J. Virol. Methods* **111:** 135–144.
6. Culp, T. D., C. M. Spatz, C. A. Reed, and N. D. Christensen. 2007. Binding and neutralization efficiencies of monoclonal antibodies, Fab fragments, and scFv specific for L1 epitopes on the capsid of infectious HPV particles. *Virology* **361:** 435–446.
7. Day, P. M., C. D. Thompson, C. B. Buck, Y. Y. Pang, D. R. Lowy, and J. T. Schiller. 2007. Neutralization of human papillomavirus with monoclonal antibodies reveals different mechanisms of inhibition. *J. Virol.* **81:** 8784–8792.
8. Ganguly, N. and S. P. Parihar. 2009. Human papillomavirus E6 and E7 oncoproteins as risk factors for tumorigenesis. *J. Biosci.* **34:** 113–123.
9. Hamers-Casterman, C., T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. B. Songa, *et al*. 1993. Naturally occurring antibodies devoid of light chains. *Nature* **363:** 446–448.
10. Harrison, J. L., S. C. Williams, G. Winter, and A. Nissim. 1996. Screening of phage antibody libraries. *Methods Enzymol.* **267:** 83–109.
11. Holliger, P. and P. J. Hudson. 2005. Engineered antibody fragments and the rise of single domains. *Nat. Biotechnol.* **23:** 1126–1136.
12. Kemp, T. J., A. Hildesheim, M. Safaeian, J. G. Dauner, Y. Pan, C. Porras, *et al*. 2011. HPV16/18 L1 VLP vaccine induces cross-neutralizing antibodies that may mediate cross-protection. *Vaccine* **29:** 2011–2014.
13. Kirnbauer, R., F. Booy, N. Cheng, D. R. Lowy, and J. T. Schiller. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* **89:** 12180–12184.
14. Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Durst, L. Gissmann, *et al*. 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J. Virol.* **67:** 6929–6936.

15. Klasse, P. J. and Q. J. Sattentau. 2001. Mechanisms of virus neutralization by antibody. *Curr. Top. Microbiol. Immunol.* **260:** 87–108.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
17. Lin, K., K. Doolan, C. F. Hung, and T. C. Wu. 2010. Perspectives for preventive and therapeutic HPV vaccines. *J. Formos. Med. Assoc*. **109:** 4–24.
18. Ludmerer, S. W., W. L. McClements, X. M. Wang, J. C. Ling, K. U. Jansen, and N. D. Christensen. 2000. HPV11 mutant virus-like particles elicit immune responses that neutralize virus and delineate a novel neutralizing domain. *Virology* **266:** 237–245.
19. Modis, Y., B. L. Trus, and S. C. Harrison. 2002. Atomic model of the papillomavirus capsid. *EMBO J.* **21:** 4754–4762.
20. Motoyama, S., C. A. Ladines-Llave, S. Luis Villanueva, and T. Maruo. 2004. The role of human papilloma virus in the molecular biology of cervical carcinogenesis. *Kobe J. Med. Sci.* **50:** 9–19.
21. Muyldermans, S. 2001. Single domain camel antibodies: Current status. *J. Biotechnol*. **74:** 277–302.
22. Parkin, D. M., M. Almonte, L. Bruni, G. Clifford, M. P. Curado, and M. Pineros. 2008. Burden and trends of type-specific human papillomavirus infections and related diseases in the Latin America and Caribbean region. *Vaccine* **26 (Suppl 11):** L1–15.
23. Parkin, D. M., F. Bray, J. Ferlay, and P. Pisani. 2005. Global cancer statistics, 2002. *CA Cancer J. Clin.* **55:** 74–108.
24. Pastrana, D. V., C. B. Buck, Y. Y. Pang, C. D. Thompson, P. E. Castle, P. C. FitzGerald, *et al*. 2004. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology* **321:** 205–216.
25. Pathirana, D., P. Hillemanns, K. U. Petry, N. Becker, N. H. Brockmeyer, R. Erdmann, *et al*. 2009. Short version of the German evidence-based guidelines for prophylactic vaccination against HPV-associated neoplasia. *Vaccine* **27:** 4551–4559.
26. Pomfret, T. C., J. M. Gagnon Jr., and A. T. Gilchrist. 2011. Quadrivalent human papillomavirus (HPV) vaccine: A review of safety, efficacy, and pharmacoeconomics. *J. Clin. Pharm. Ther.* **36:** 1–9.
27. Rahbarizadeh, F., D. Ahmadvand, and Z. Sharifzadeh. 2011. Nanobody; an old concept and new vehicle for immunotargeting. *Immunol. Invest*. **40:** 299–338.
28. Rahbarizadeh, F., M. J. Rasaee, M. Forouzandeh-Moghadam, and A. A. Allameh. 2005. High expression and purification of the recombinant camelid anti-MUC1 single domain antibodies in *Escherichia coli*. *Protein Expr. Purif.* **44:** 32–38.
29. Rahbarizadeh, F., M. J. Rasaee, M. Forouzandeh, and A. A. Allameh. 2006. Over expression of anti-MUC1 single-domain antibody fragments in the yeast *Pichia pastoris*. *Mol. Immunol.* **43:** 426–435.
30. Rahbarizadeh, F., M. J. Rasaee, M. Forouzandeh Moghadam, A. A. Allameh, and E. Sadroddiny. 2004. Production of novel recombinant single-domain antibodies against tandem repeat region of MUC1 mucin. *Hybrid. Hybridomics* **23:** 151–159.
31. Rose, R. C., W. Bonnez, R. C. Reichman, and R. L. Garcea. 1993. Expression of human papillomavirus type 11 L1 protein in insect cells: *In vivo* and *in vitro* assembly of viruslike particles. *J. Virol.* **67:** 1936–1944.
32. Sawaya, G. F. 2009. Cervical-cancer screening – new guidelines and the balance between benefits and harms. *N. Engl. J. Med.* **361:** 2503–2505.
33. Virgin, H. W., M. A. Mann, and K. L. Tyler. 1994. Protective antibodies inhibit reovirus internalization and uncoating by intracellular proteases. *J. Virol.* **68:** 6719–6729.
34. White, W. I., S. D. Wilson, W. Bonnez, R. C. Rose, S. Koenig, and J. A. Suzich. 1998. *In vitro* infection and type-restricted antibody-mediated neutralization of authentic human papillomavirus type 16. *J. Virol.* **72:** 959–964.
35. Yang, T., L. Yang, W. Chai, R. Li, J. Xie, and B. Niu. 2011. A strategy for high-level expression of a single-chain variable fragment against TNFalpha by subcloning antibody variable regions from the phage display vector pCANTAB 5E into pBV220. *Protein Expr. Purif.* **76:** 109–114.
36. Zhuge, W., F. Jia, G. Mackay, A. Kumar, and O. Narayan. 2001. Antibodies that neutralize SIV(mac)251 in T lymphocytes cause interruption of the viral life cycle in macrophages by preventing nuclear import of viral DNA. *Virology* **287:** 436–445.