

Construction of Prophylactic Human Papillomavirus Type 16 L1 Capsid Protein Vaccine Delivered by Live Attenuated *Shigella flexneri* Strain sh42

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Abstract To express human papillomavirus (HPV) L1 capsid protein in the recombinant strain of *Shigella* and study the potential of a live attenuated *Shigella*-based HPV prophylactic vaccine in preventing HPV infection, the *icsA/virG* fragment of *Shigella*-based prokaryotic expression plasmid pHS3199 was constructed. HPV type 16 L1 (HPV16L1) gene was inserted into plasmid pHS3199 to form the pHS3199-HPV16L1 construct, and pHS3199-HPV16L1 was electroporated into a live attenuated *Shigella* strain sh42. Western blotting analysis showed that HPV16L1 could be expressed stably in the recombinant strain sh42-HPV16L1. Sereny test results were negative, which showed that the sh42-HPV16L1 lost virulence. However, the attenuated recombinant strain partially maintained the invasive property as indicated by the HeLa cell infection assay. Specific IgG, IgA antibody against HPV16L1 virus-like particles (VLPs) were detected in the sera, intestinal lavage and vaginal lavage from animals immunized by sh42-HPV16L1. The number of antibody-secreting cells in the spleen and draining lymph nodes were increased significantly compared with the control group. Sera from immunized animals inhibited murine hemagglutination induced by HPV16L1 VLPs, which indicated that the candidate vaccine could stimulate an efficient immune response in guinea pig's mucosal sites. This may be an effective strategy for the development of an HPV prophylactic oral vaccine.

Key words human papillomavirus type 16; attenuated *Shigella flexneri*; vaccine; cervical cancer; prophylactic

Cervical cancer is the second most common cause of cancer-related deaths in women worldwide. More than 450,000 cases are diagnosed each year, resulting in nearly 250,000 deaths. It has been extensively confirmed that high-risk human papillomaviruses (HPVs), particularly types 16, 18, 33, 45 and 58, are the initiators of the vast majority of cervical cancers. HPV16 is the most prevalent, accounting for more than half of cervical cancer cases.

Moreover, HPV can induce malignant disease at other sites, such as the oral cavity, esophagus and lung [1,2]. It is therefore reasonable to assume that vaccines that protect against HPV infection would theoretically prevent women, especially those in developing countries, from developing cervical cancer and other HPV-related malignancies in later life.

HPVs can not be grown in the laboratory as a source of antigen for serological tests and conventional killed vaccine development, and they do not cause diseases in animals, so vaccine development is difficult. Therefore, HPV vaccines currently under development employ genetic engineering technology. The main requirement for prophylactic HPV vaccines is to induce neutralizing antibodies against natural structural viral capsid proteins to

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prevent virus entry into the host cell. The prerequisite to obtain this effect is that the immunogen should possess the natural structure to induce the conformation-dependent antibodies, and the route of immunization should favor the activation of mucosal immunity. A major breakthrough in HPV vaccine research came with the discovery that the capsid proteins L1 and L2 (or L1 alone) self-assemble into virus-like particles (VLPs) when expressed in appropriate host cells. VLPs closely resemble native HPV particles. They include the conformational epitopes that induce virus-neutralizing antibodies [3], and the phase I/II clinical trials have shown promising results. VLPs are not only immunogenic and safe, but also able to induce strong cell-mediated and humoral immune responses [4,5] in a controlled trial of HPV16 vaccine, and nearly 100% effectiveness was achieved [6]. However, the costly production and distribution of current VLP vaccines, for example, by the use of recombinant baculoviruses, will prevent their widespread application in developing countries. Moreover, we are not sure whether intramuscular injection is the optimal route, although the HPV VLP intramuscular administration can induce a stronger antibody response in the serum and can prevent the infection of HPV. A cheaper vaccine with a better delivery system in stimulating mucosal immunity is needed.

The antigen delivery system of the vaccine is the key factor which determines the effectiveness of a given vaccine. The recombinant attenuated enteropathogenic bacteria, such as *Salmonella* or *Shigella*, may represent ideal antigen delivery systems, as they efficiently cross all mucosal surfaces to gain access to both mucosa-associated lymphoid tissue and draining lymph nodes. It has been demonstrated that the attenuated *Salmonella* can express HPV16L1 protein and stimulate strong neutralizing antibodies in mucosa sites [7,16]. Compared with *Salmonella*, several intrinsic advantages of *Shigella* strains make them ideal vehicles to deliver HPV L1 protein to mucosal sites. (1) *Shigella* bacilli are also enteropathogenic bacteria, only the ileum and colonic epithelium of humans and primates are their natural hosts. In principal, the invasion of HPV16L1 carried by the recombinant *Shigella* strain can cross the lumen of the gut by way of the M cells of Peyer's patches and then be taken up by macrophages and dendritic cells at local sites. Because of the establishment of a short-lived infection after their delivery, an innate immune response can be generated to promote the development of adaptive immune responses against HPV16L1 protein. These responses triggered by mucosal delivery can be effective at both mucosal and systemic sites [17,18]. (2) *Shigella* infection, unlike other attenuated live vectors such

as Bacille Calmette Guérin (BCG) and *Salmonella typhimurium* [19], is localized at the infection site and can not disseminate into circulation. Therefore, the attenuated *Shigella* can be used safely as a mucosa-tropic vaccine vehicle in non-immune compromised and immunocompromised hosts, such as those with HIV infection.

Therefore, in the present study, we expressed HPV16L1 in a strain of live recombinant attenuated *Shigella* strain sh42. The production of conformationally dependent and neutralizing antibodies in serum and body lavage fluid was assessed after immunization of guinea pigs with the live recombinant bacteria.

Materials and Methods

Bacterial strains and plasmids

Attenuated *Shigella flexneri* strain sh42 and its wild-type progenitor M90Ts (*S. flexneri* 5a serotype) were generously provided by Dr. Jun YU (Imperial College, London, UK). *S. flexneri* strains were routinely grown at 37 °C on Luria-Bertani (LB) agar plates containing 0.01% Congo red. Red colonies were implanted into LB broth and grown to an appropriate turbidity at 37 °C with vigorous shaking. *Escherichia coli* strain Top10 was purchased from Invitrogen (Carlsbad, USA) and routinely grown at 37 °C in LB medium (broth or plate containing 1.5% agar). Antibiotics were supplemented with the following final concentrations when needed: 100 µg/ml of streptomycin; 200 µg/ml of ampicillin and 50 µg/ml of gentamycin. Plasmid pBR322 was purchased from Invitrogen.

Construction of pHS3199-HPV16L1 plasmid

A 3.3 kb DNA fragment of gene *icsA/virG* of the *S. flexneri* strain was amplified by polymerase chain reaction (PCR) from M90Ts, the wild type of *Shigella flexneri* 5a strain, with forward primer 5'-GGGAATTCGCATGAATCAAATTCA-3' and reverse primer 5'-GCGGATCCTCAG-AAGGTATAT-3', which contained an *EcoRI* restriction site at the 5' end and a *BamHI* site at the 3' end. The *icsA/virG* gene was then directionally inserted into pBR322 to form a novel plasmid pHS3199 with ampicillin resistance. A fragment of HPV16L1 gene (5637–7154 nt, 1518 nt) was amplified by PCR from pFast-bacHPV16L1 with forward primer 5'-GCTCTAGACAGGAGCTATTTA-ATGTCTCTTTGGCTGCCT-3' (*XbaI* restriction site in italic and SD sequence underlined) and reverse primer 5'-CCCTTAAAGCTTAATTACAGCTTACGTTTTTT-

CGGTTTA-3' (*HindIII* restriction site in italic). An ATG start codon and a TTA stop codon were included in the forward and reverse primers, respectively. The HPV16L1 fragment and plasmid pHS3199 digested by *XbaI/HindIII* were ligated using T4 ligase (TaKaRa, Dalian, China) and the ligation product was designated pHS3199-HPV16L1 (Fig. 1).

Construction of recombinant *Shigella* strain sh42-HPV16L1

The pHS3199-HPV16L1 construct was transferred into the attenuated sh42 competent cell by electroporation (Multiporator, Eppendorf, Germany) at 2500 V, one pulse, for 5 μ s. The ampicillin-resistant colonies were picked up from the agar plate and grown in LB broth to the mid-logarithmic phase. The cells were collected by centrifugation and resuspended in phosphate-buffered saline (PBS). The cell lysates were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane (Invitrogen) for Western blotting. The HPV16L1 protein was identified with a mouse monoclonal antibody against HPV16L1 (DAKO A/S, Glostrup, Denmark), and horseradish peroxidase (HRP)-conjugated

rabbit anti-mouse polyclonal antibody as the second antibody. Color development was carried out by the addition of the diaminobenzidine (DAB) substrate-chromogen solution.

Genetic stability of recombinant strain sh42-HPV16L1

The genetic stability of the recombinant strain sh42-HPV16L1 was determined by consecutive passage culture. The single sh42-HPV16L1 colony picked up from the LB agar plate containing 50 μ g/ml ampicillin was incubated in LB broth containing ampicillin at 37 °C overnight, then 10^{-4} – 10^{-6} folds dilution was made in antibiotic-free LB broth. The appropriate volume of the diluted bacterial suspension was incubated overnight and further diluted in the same folds as above with antibiotic-free LB broth. This procedure was repeated until the 140th generation was obtained.

For each generation, part of the remaining diluted bacterial suspension was used for detecting the expression of HPV16L1 protein by Western blot; the rest of the diluted bacterial suspension was used for counting the frequency of ampicillin-resistant colonies. For the frequency analysis, the diluted bacterial suspension of each generation was transferred onto non-ampicillin LB agar plates at 37 °C

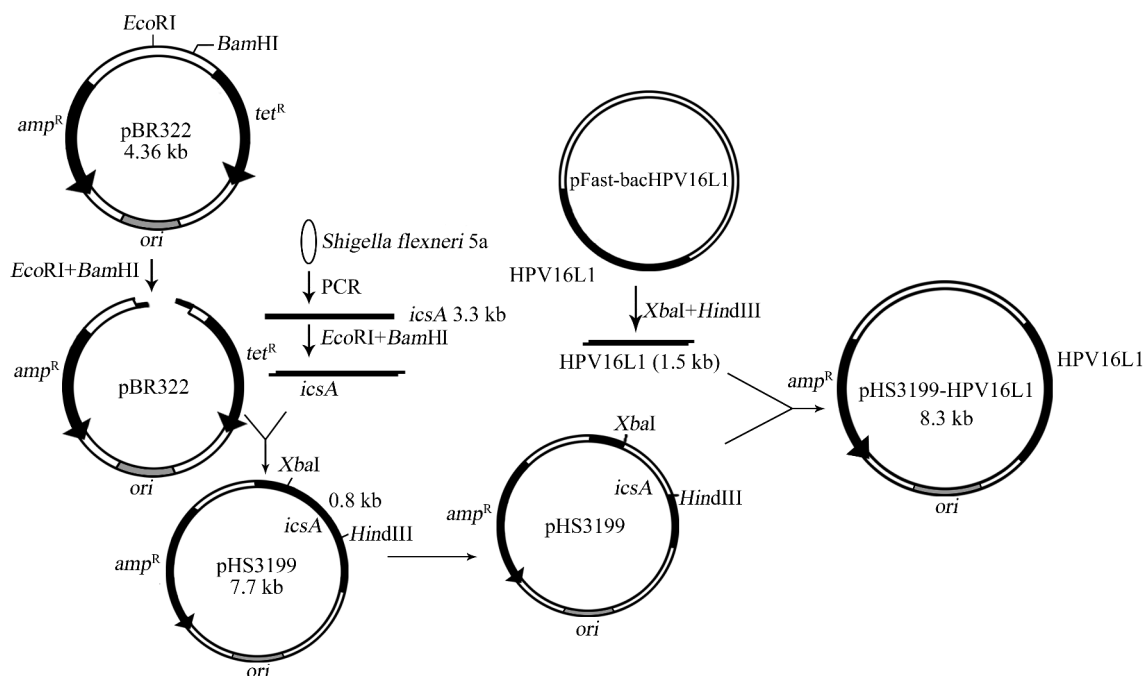


Fig. 1 Schematic diagram of recombinant plasmid pHS3199-HPV16L1

The *icsA/virG* gene of *Shigella flexneri* 5a strain was inserted into pBR322, forming the prokaryotic expression plasmid pHS3199, then the HPV16L1 gene was cloned into pHS3199, resulting in pHS3199-HPV16L1.

overnight. One hundred colonies were picked up randomly and seeded on ampicillin-containing LB agar plates at 37 °C overnight, then the number of colonies was counted and the frequency was analyzed.

Safety test of recombinant strain sh42-HPV16L1

The virulence of recombinant strains was tested with the classical Sereny test [8]. Briefly, 6–8-week-old outbred female Hartley guinea pigs were challenged by the recombinant strain sh42-HPV16L1: 25 µl PBS containing 5×10^8 CFU sh42-HPV16L1 was inoculated into each eye through the conjunctival sac, and the eyes were observed for 7 d for the development of keratoconjunctivitis. Development of disease was rated as follows: grade 0, no disease or mild irritation; grade 1, mild conjunctivitis or late development and/or rapid clearing of symptoms; grade 2, keratoconjunctivitis without purulence; grade 3, fully developed keratoconjunctivitis with purulence.

Invasion ability of recombinant strain sh42-HPV16L1

HeLa cell infection assay was performed to test the invasion ability of the recombinant strain sh42-HPV16L1. In brief, HeLa cell monolayers were incubated on 35 mm plates in 5% CO₂ at 37 °C to half-confluence in antibiotic-free Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum, then 25 µl of mid-logarithmic phase bacteria was overlaid, spun down to adhere the HeLa cells at 1500 rpm for 10 min, and incubated together in humidified 5% CO₂ at 37 °C for 30 min. The HeLa cells were then washed six times with DMEM, and treated with DMEM containing 50 µg/ml gentamicin for 90 min to kill the extracellular bacteria. After washing with PBS, the HeLa cells were fixed with 4% formalin and Giemsa staining was carried out. The number of intracellular bacteria was counted under microscopy [9].

Immunogenicity of recombinant strain sh42-HPV16L1

The efficacy of sh42-HPV16L1 to evoke mucosal immunity against HPV16L1 was tested by immunization through mucosal routes as previously described [10]. The red colony of sh42-HPV16L1 was picked from the LB plate containing 0.01% Congo red and cultured to the mid-logarithmic phase. Twenty-five microliters of the bacterial culture harvested in 1×PBS were inoculated into the guinea pigs' conjunctival sac ($2.5\text{--}5 \times 10^8$ CFU per eye) on day 0, 2, 4, 14 and 15. Animals inoculated with PBS or sh42-pHS3199 were used as the control group (6–8 guinea pigs in each group). Two weeks after the last immunization, the animals were killed and their blood samples were drawn. Vaginal and intestinal fluids were collected in lavage buffer

composed of phenylmethylsulfonyl fluoride (PMSF) inoptine and NaN₃, and the spleens and draining lymph nodes were harvested.

Enzyme-linked immunosorbent assay (ELISA) was used to measure antibodies against HPV16 VLP in the serum, and vaginal and intestinal lavage fluid. Each well of the polyvinyl microtiter plates was coated with 100 µl 50 mM carbonate buffer (pH 9.6) with or without 1 µg HPV16L1 VLP [11]. Guinea pigs' serum (1:10 dilution), vaginal and intestinal lavage fluid without dilution, HRP-conjugated anti-guinea pig IgG (DaKo) and IgA (1:1200; Bethyl, Montgomery, USA) were added consecutively. Absorbance was read at 450 nm.

The frequency of HPV16L1-specific antibody-secreting cells (ASCs) in the immunized animals was determined using a modified enzyme-linked immunospot (ELISPOT) assay based on the method of Czerkinsky [13]. The splenocytes and lymphocytes from draining lymph nodes were prepared for ELISPOT as described previously [13]. Briefly, each well of U-bottomed 96-well microtiter plates was coated with 1 µg HPV16L1 VLP, and antigen-specific ASCs were visualized as blue spots. The number of ASCs was counted under stereomicroscopy and the data were recorded as ASCs per 10⁶ cells.

Murine erythrocyte inhibition hemagglutination assay

HPV16 VLP causes hemagglutination of murine erythrocytes, which can be inhibited by conformation-dependent neutralizing antibodies against VLP [14]. Hence, we tested whether the serum from candidate vaccine-immunized mice could inhibit VLP-induced hemagglutination of murine erythrocytes (HAI). The whole assay was performed as described previously [15].

Results

Identification of recombinant plasmid pHS3199-HPV16L1

The PCR of HPV16L1 gene with specific primers generated a 1.5 kb product (sequencing confirmed, data not shown). After having been cleaved by *Xba*I/*Hind*III, the fragment was inserted into the plasmid pHS3199 cleaved with the same enzymes. The insertion of the HPV16L1 fragment into pHS3199 plasmid was confirmed by *Xba*I/*Hind*III digestion and 1% agarose electrophoresis. The results showed the HPV16L1 fragment was successfully cloned into pHS3199 plasmid (**Fig. 2**). The construct was designated pHS3199-HPV16L1.

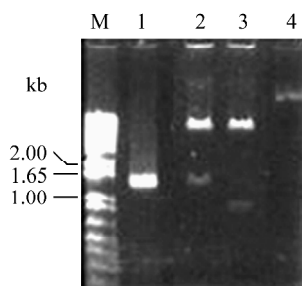


Fig. 2 Identification of the pHS3199-HPV16L1 construct

M, DNA marker (1 kb plus DNA ladder; Gibco Invitrogen Corporation, New York, USA); 1, 1.5 kb polymerase chain reaction product; 2, recombinant pHS3199-HPV16L1 digested by *Xba*I and *Hind*III; 3, plasmid pHS3199 digested by *Xba*I and *Hind*III; 4, plasmid pHS3199-HPV16L1.

Identification of the expression of HPV16L1 protein in recombinant strain sh42-HPV16L1

After the plasmid pHS3199-HPV16L1 was transferred into attenuated sh42 by electroporation, the expression of HPV16L1 protein in the recombinant strain sh42-HPV16L1 was analyzed by SDS-PAGE and Western blotting. The SDS-PAGE result showed a single protein band with a molecular weight of 58 kDa corresponding to that of HPV16L1 protein, which reacted specifically with the anti-HPV16L1 monoclonal antibody as proved by Western blotting (Fig. 3). The recombinant strain was designated sh42-HPV16L1.

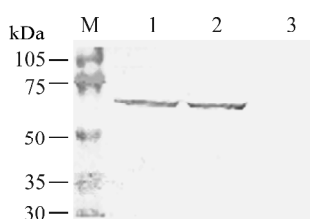


Fig. 3 Expression of human papillomavirus type 16 L1 capsid protein (HPV16L1) in recombinant strain sh42-HPV16L1, analyzed by Western blotting

M, rainbow protein molecular weight marker (RPN800; Amersham Life Science, Buckinghamshire, England); 1, recombinant strain sh42-HPV16L1; 2, HPV16 virus-like particles; 3, sh42-pHS3199.

Genetic and expression stability of recombinant strain sh42-HPV16L1

The genetic stability of the recombinant strain sh42-

HPV16L1 was measured by its ampicillin resistance. The level of resistance was maintained in the 140th generation, and the growth rate of colonies was up to 100%. The target protein HPV16L1 was expressed stably in the recombinant strain in the 140th generation, as demonstrated by Western blotting (Fig. 4), and there was no apparent difference in the level of protein expression.

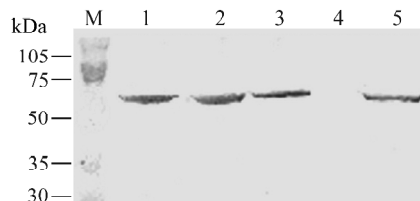


Fig. 4 Human papillomavirus type 16 L1 capsid protein (HPV16L1) expression in recombinant strain sh42-HPV16L1, monitored by Western blotting

M, standard protein molecular weight marker; 1, 2, 3 and 5, the L1 protein expression level of different generations of the recombinant strain sh42-HPV16L1: original generation, 20th generation, 60th generation and 140th generation, respectively; 4, sh42-pHS3199. The recombinant strain sh42-HPV16L1 could express HPV16L1 protein stably from the original generation to the 140th generation.

Safety of recombinant strain sh42-HPV16L1

The virulence of the recombinant strain sh42-HPV16L1 was determined by the Sereny test. None of the eyes inoculated with sh42-HPV16L1 developed keratoconjunctivitis. The Sereny test results also indicated that sh42-pHS3199 and sh42-HPV16L1 had lost virulence compared with the wild-type strain M90Ts (Table 1).

Table 1 Virulence of wild-type *Shigella flexneri* strain M90Ts, sh42-pHS3199 and recombinant strain of human papillomavirus sh42-HPV16L1, evaluated in guinea pigs by the Sereny test

Strain	Number of eyes				Total
	Grade 0	Grade 1	Grade 2	Grade 3	
M90Ts	0	0	0	8	8
sh42-HPV16L1	12	0	0	0	12
sh42-pHS3199	6	0	0	0	6

The severity of keratoconjunctivitis was rated as follows: grade 0, no disease or mild irritation; grade 1, mild conjunctivitis or late development and/or rapid clearing of symptoms; grade 2, keratoconjunctivitis without purulence; grade 3, fully developed keratoconjunctivitis with purulence.

Invasion ability of recombinant strain sh42-HPV16L1

HeLa cells were incubated with sh42-HPV16L1 and the wild-type strain M90Ts, and the number of bacilli intruding into HeLa cells was enumerated. The results of HeLa cell infection assay showed that the intracellular bacterial number in the recombinant strain was less compared with the wild-type strain M90Ts, which reflected that the invasion ability of the recombinant strain sh42-HPV16L1 was diminished but not completely abolished. This is a prerequisite for a candidate vaccine (Fig. 5).

Immunogenicity of sh42-HPV16L1

To evaluate the immunogenicity of sh42-HPV16L1, specific IgG and IgA levels in the serum and body fluid (vaginal and intestinal lavage) of immunized animals were tested by ELISA. Antibody secreted cells in the spleen and draining lymph nodes specifically against HPV16 VLP were detected by ELISPOT. Compared with the control group (immunized by PBS and sh42-pHS3199), the IgG level was much higher than that of IgA in the serum of immunized animals ($P < 0.05$). The IgG and IgA levels in the lavages of both the intestine and vagina showed no apparent differences ($P > 0.05$); the IgA level in the lavages of the intestine and vagina were slightly higher than that in serum, but the difference was not significant. More significantly, after immunization through the mucosal route, the immune response in other mucosal sites, such as intestine and vagina, reached a similar level (Fig. 6).

The frequency of HPV16L1-specific IgG/IgA ASCs in the spleen, mandibular lymph nodes (MDLN), mesenteric lymph nodes (MSLN), peyer's patches (PP) and superficial ventral cervical lymph nodes (SCVLN) of immunized

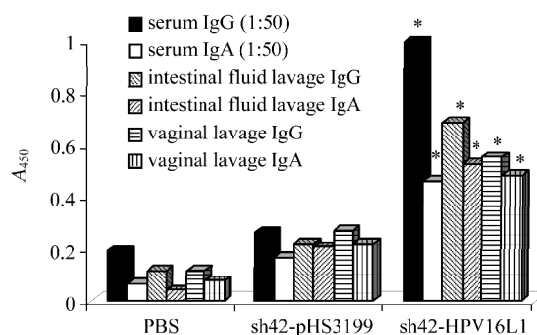


Fig. 6 Antibody titers (IgG and IgA) against human papillomavirus type 16 L1 capsid protein (HPV16L1) virus-like particles (VLP) in sera (diluted in 1:50), and intestinal and vaginal lavages of guinea pigs, tested by enzyme-linked immunosorbent assay

It was shown that antibody titer against HPV16L1 VLP in sera, and intestinal fluid and vaginal lavage from animals immunized using sh42-HPV16L1 was significantly higher than that of control animals immunized using phosphate-buffered saline (PBS) or sh42-pHS3199 ($*P < 0.05$).

animals were inspected by ELISPOT (Fig. 7). Both the specific IgA and IgG ASCs in spleen cells were much higher than those in other tested tissues. MDLN, the nearest local draining lymph nodes to the conjunctival sac, also contained higher frequencies of the specific ASCs, which were slightly lower than that in the spleen. The other lymph nodes tested also showed increased levels of ASCs compared with the control sh42-pHS3199 or PBS.

HAI induced by sera of immunized animals

HAI assay demonstrated that the sera from immunized animals could significantly inhibit the hemagglutination activity induced by HPV16L1 VLPs, which indicated that

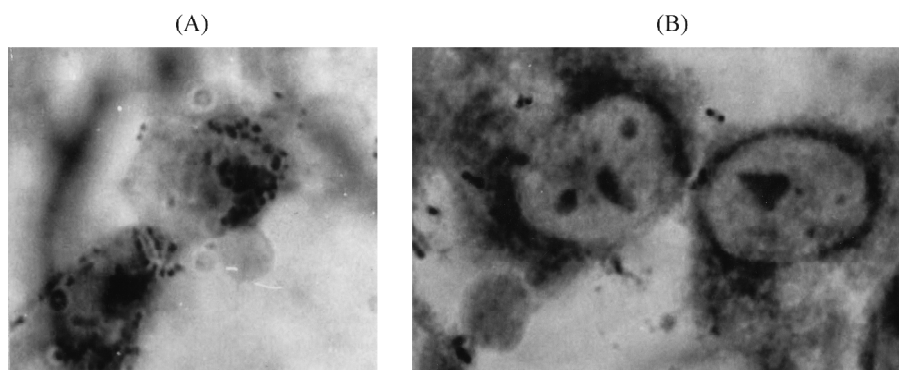


Fig. 5 Intracellular bacteria in infected HeLa cells as shown by Giemsa staining

(A) Wild-type *Shigella flexneri* strain M90Ts invasion of HeLa cells. (B) Human papillomavirus recombinant strain sh42-HPV16L1 invasion of HeLa cells. Intracellular bacterial number in (B) is less compared with (A). Magnification, 1000 \times .

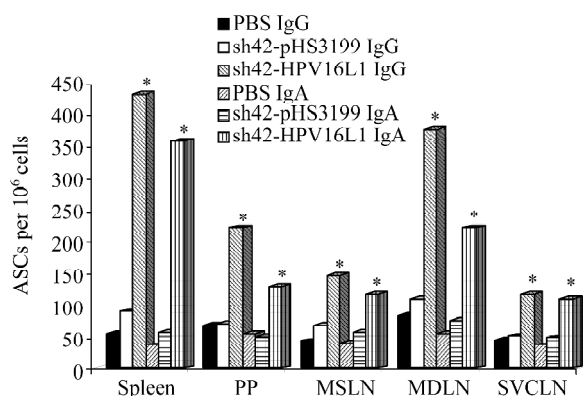


Fig. 7 Frequency of antibody-secreting cells (ASCs) of anti-human papillomavirus type 16 L1 capsid protein (HPV16L1) virus-like particles (VLP) IgG and IgA in spleen and lymph nodes, inspected by enzyme-linked immunospot

It was shown that the frequency of ASCs secreting anti-HPV16L1 VLP IgG and IgA in the spleen and draining lymph nodes in animals immunized by sh42-HPV16L1 were significantly higher than in animals immunized by phosphate-buffered saline (PBS) or sh42-pHS3199 (* $P < 0.05$). MDLN, mandibular lymph nodes; MSLN, mesenteric lymph nodes; PP, peyer's patches; SVCLN, superficial ventral cervical lymph nodes.

the anti-sera were conformation-dependent (Fig. 8).

Discussion

Although the attenuated *Shigella* is theoretically an ideal

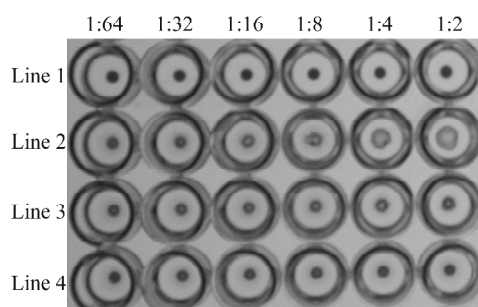


Fig. 8 Hemagglutination inhibition assay with sera from immunized guinea pigs

Line 1, negative control, phosphate-buffered saline and 1% (V/V) mouse red blood cells; line 2, positive control, virus-like particles (VLPs) and mouse red blood cells, from left to right, VLP concentrations are 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 μg per well; line 3, red blood cells+1.2 μg VLP and sera from guinea pigs immunized by human papillomavirus recombinant strain sh42-HPV16L1 (dilution ratios from left to right are 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64); line 4, red blood cells+0.6 μg VLP and sera from guinea pigs immunized by sh42-HPV16L1 (dilution ratios from left to right are 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64). It is shown that the sera could inhibit the mouse erythrocyte hemagglutination activity induced by HPV16L1 VLPs.

vehicle for a mucosal predominant vaccine, the key point was to confirm that HPV16L1 protein can be expressed in *Shigella* strains. We used a new prokaryotic expression plasmid based on the invasive plasmid *icsA/virG* of *Shigella*, in which the HPV16L1 gene fragment was inserted. The new construct carrying the HPV16L1 coding fragment was introduced into the attenuated *S. flexneri* strain sh42. It was demonstrated that HPV16L1 protein could be stably expressed in sh42. Recombinant sh42-HPV16L1 lost virulence completely, but retained its partially invasive ability, which warranted that HPV16L1 protein could be presented to a mucosal site, mimicking the process of natural infection. The results of animal tests showed that sh42-HPV16L1 exhibited good immunogenicity, and it could elicit specific antibodies against HPV16 VLP in systemic (serum) as well as at mucosal sites (intestinal and vaginal lavage fluids). The IgA level, which is more important to evaluate the efficacy of a vaccine against a mucosal pathogen, was significantly higher than in control groups. The frequency of VLP-specific ASCs in the regional draining lymph nodes and spleen were measured by ELISPOT assay, and the results indicated that the number of ASCs at these sites was significantly higher than that in control groups.

The HPV neutralizing antibody is conformation-dependent, and only the immunogen with the natural conformational epitopes can stimulate such an antibody. HPV VLP cause hemagglutination of murine erythrocytes, which can be inhibited by specific neutralizing antibodies, referred to as HAI, which can well reflect the properties of functional neutralizing antibodies [20]. The result of HAI in the experiment showed that IgG in the sera of immunized animals could block VLP-induced hemagglutination of murine erythrocytes. This demonstrated that sera from immunized animals with the candidate vaccine were conformation-dependent.

In conclusion, the current study provides evidence that an attenuated *Shigella* strain expressing the major capsid protein of HPV16 represents a promising vaccine candidate against HPV16 infection. Conformational-dependent VLP-specific antibodies, which correlate with protection from experimental challenge in animal virus models, were generated both systemically and locally at genital and intestinal mucosal sites. The vaccine could be cheaply produced, be given non-invasively and potentially induce long-lasting protection after a single inoculation. These characteristics are particularly desirable in improving recipients' compliancy in vaccination and in a vaccine targeted for use in developing countries, where cervical cancer is the leading cause of cancer-related deaths in women.

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