

A human papillomavirus type 16 vaccine by oral delivery of L1 protein

Toshiyuki Sasagawa^{a,*,4}, Mayuko Tani^{a,4}, Walid Basha^a, Robert C. Rose^{c,2}, Hideki Tohda^{d,3}, Yuko Giga-Hama^{d,3}, Khadijeh K. Azar^a, Hideyo Yasuda^a, Akemi Sakai^a, Masaki Inoue^{b,1}

^a Health Science, School of Medicine, Kanazawa University, 5-11-80 Kodatsuno, Kanazawa, Ishikawa 920-0942, Japan

^b Molecular Transplantation, Postgraduate School, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8640, Japan

^c Infectious Disease Unit, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA

^d Central Laboratory, Asahi Glass Co, 1150 Hanezawa-cho, Kanagawa-ku, Yokohama 221-8755, Japan

Received 28 January 2005; accepted 2 February 2005

Available online 8 March 2005

Abstract

To establish an edible HPV16 vaccine, we constructed a recombinant HPV16 L1-expressing *Schizosaccharomyces pombe* yeast strain (HPV16L1 yeast). A preliminary study revealed that freeze-dried yeast cells could be delivered safely, and were digested in the mouse intestine. The freeze-dried HPV16 L1 yeast was administered orally as an edible vaccine, with or without the mucosal adjuvant heat-labile toxin LT (R192G), to 18 female BALB/c mice. After the third immunization, none of the mice that received the edible HPV16 vaccine showed specific antibody responses, whereas all of the positive controls that were administered intranasally with 5 µg of HPV16-virus-like particles (VLP) had serum IgG, and genital IgA and IgG that reacted with HPV16-VLP in enzyme-linked immunosorbent assays (ELISAs). When a suboptimal dose (1 µg) of HPV16-VLP was administered to all the mice, including the negative control mice, 50% of the mice that were pre-immunized with the edible HPV16 vaccine showed positive serum IgG responses, while none of the negative controls showed any response. Vaginal IgG and IgA antibodies were also elicited in 33 and 39%, respectively, of the mice that were given with the edible HPV16 vaccine and the intranasal boost. All of the antibodies reacted more strongly to intact HPV16-VLP than to denatured HPV16-L1 protein suggesting that the edible vaccine primes for antibody responses against conformation-dependent epitopes. The inclusion of adjuvant in the vaccine formulation marginally increased the genital IgA response ($P=0.06$). HPV16-L1 protein in the yeast might induce tolerance in the vaccinated animals that could be recovered by intranasal boosting with a suboptimal dose of HPV-VLP. This freeze-dried yeast system may be useful as an oral delivery of HPV 16 L1 protein.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Fission yeast; Mucosal immunity; Iga; Igg; Neutrolization

1. Introduction

Although mortality due to cervical cancer has recently decreased in developed countries, this type of cancer frequently occurs as a secondary cancer, and it is the fifth leading cause of death in women worldwide (Parkin et al., 2001). Certain

types of the human papillomavirus (HPV), which is sexually transmitted, represent the most important risk factors for cervical cancer (zur Hausen, 1991). Recent reports show that 30–50% of young women who have recently had sexual intercourse for the first time are infected with HPV in their cervixes (Ho et al., 1998; Franco et al., 1999; Molano et al., 2003). Surprisingly, most cervical HPV infections involve high-risk types that are likely to induce cancer. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, and possibly some other types, are considered to be high-risk types (Munoz et al., 2003). We have identified HPV types 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, and 67 as single infecting types in cervical cancer and precursor lesions in Japan

* Corresponding author. Tel.: +81 76 265 2500/2575; fax: +81 76 265 4363.

E-mail address: tsasa@med.kanazawa-u.ac.jp (T. Sasagawa).

¹ Tel.: +81 76 265 2000, fax: +81 76 234 4266.

² Tel.: +585 275 0263, fax: +585 442 9328.

³ Tel.: +81 45 374 8722, fax: +81 45 374 8872.

⁴ The first and second authors are equally contributed in this study.

(Sasagawa et al., 2001). Our recent report showed that more than 60% of women in their late teens, who visited outpatient obstetrics and gynecology clinics for various reasons, were infected with HPV, and that 50% of these women were infected with high-risk HPV types (Sasagawa et al., in press). This alarmingly high prevalence of HPV infection in young women suggests that education and social health programs aimed at preventing HPV infections may not be sufficiently effective in combating cervical cancer, especially in poor resource settings, in which cervical cancer prevention should be a priority (Yang et al., 2004). HPV testing and cytological screening may not be the best choices in these areas, due to the costs of screening tests and treatments. Thus, nationwide use of a prophylactic vaccine against the high-risk HPV types may prove advantageous in the prevention of cervical cancer.

The development of HPV vaccines has been hampered for many years due to a lack of animal systems for HPV propagation and of a methodology for the synthesis of HPV virions. Rose et al. (1993) showed that high-level production of HPV-11 L1 protein led to the assembly of virus-like particles (VLPs) in an insect cell system. Kirnbauer et al. (1993) succeeded in synthesizing HPV16-VLPs in this insect cell system. Subsequently, we succeeded in producing HPV6- and HPV16-derived VLPs in the yeast *Schizosaccharomyces pombe* (Sasagawa et al., 1995). Although the yield of VLPs from yeast is less than that obtained from the insect cell system, the yeast system confers advantages in terms of large-scale VLP production and safety of use in humans. Regardless of the method used for their production, HPV-VLPs are good candidates for a human vaccine, since VLPs have been shown to have the same antigenic properties as the native virions, and they do not carry any potentially oncogenic viral genes. Koutsky et al. (2002) were the first to demonstrate that parenteral immunization with a HPV16-VLP vaccine conferred 100% protection against HPV16 infection in women. It has also been reported that a HPV vaccine that targets HPV16 and HPV18 may reduce cervical cancer incidence by more than 50% (Kulasingam and Myers, 2003; Goldie et al., 2003).

Unfortunately, the parenteral HPV16-VLP vaccine is expensive, since it requires advanced techniques and special facilities for production and storage. In addition, repeated injections of the parenteral vaccine, which are usually required for efficacy, are impractical in poor resource settings, with limited numbers of trained clinical staff. Furthermore, it has been reported that parenteral immunization with VLPs is a poor inducer of secretory IgA, which plays a major role in mucosal immunity (Hagensee et al., 1995). Immunization of the mucosa-associated lymphoid tissue (MALT), which is located in the respiratory and digestive tracts, is important for the induction of effective mucosal responses against many viruses. Balmelli et al. (1998) have demonstrated that intranasal administration of HPV16-VLP elicits mucosal antibodies that neutralize HPV16. However, intranasal vaccination also requires the preparation of relatively large amounts of purified HPV-VLP. The gut-associated lym-

phoid tissue (GALT) constitutes an alternative site for immunization to induce strong mucosal immunity. Recently, two groups have produced edible HPV vaccines from tobacco and potato plants that express the HPV-11 (Warzecha et al., 2003) and HPV-16 (Biemelt et al., 2003) L1 genes, respectively, and they have tested them in animal model systems. Although edible vaccines alone do not induce HPV-specific antibodies, systemic or local booster immunizations with purified VLP elicit HPV-specific serum IgG.

In this study, we used a freeze-dried preparation of a yeast strain that expresses HPV16 L1 protein as an edible vaccine in a mouse model system. Yeasts are good candidate edible vaccine vectors, since they are readily adaptable to large-scale production, may be administered safely to animals and humans, and they incorporate HPV16-VLPs into the nucleus. Indeed, all of the mice willingly ate this freeze-dried yeast; this represents an advantage, in terms of administration, over intranasally administered VLP vaccines, which require anesthesia. Our preliminary study demonstrated that freeze-dried yeast strains could be delivered safely, and that they were digested in the intestine, where GALT is located. In the present study, we investigated the efficacy of this edible yeast vaccine, and evaluated the optimal conditions for immunization, in terms of the dose of yeast and the addition of the *Escherichia coli*-derived mucosal adjuvant LT (R192G) (Freitag and Clements, 1999).

2. Materials and methods

2.1. Construction of a recombinant *S. pombe* strain that expresses the HPV16 L1 gene

We have previously reported that a strain of the fission yeast *S. pombe*, which expresses the HPV16 L1 gene (B27; wild-type HPV16) under the control of a thiamine-repressive promoter, was able to synthesize virus-like particles (VLPs) (Sasagawa et al., 1995). B27L1 has two amino acid changes at histidine 202 to aspartate and threonine 266 to alanine, and this HPV16 L1 gene could produce 68 times more virus-like particles than the prototype HPV16 L1 (Pushko et al., 1994). In order to increase L1 gene expression in yeast, we introduced B27L1 into a new vector, pTL2M, and expressed the HPV16 L1 protein (Tohda et al., 1994). This vector contains a cytomegalovirus promoter that expresses, constitutively, the introduced foreign gene. We confirmed by Western blot analysis that this recombinant yeast (pTL2-HPV16 L1) expressed the 55-kDa HPV16-L1 protein at the level of about 5% of total protein. The appropriate assembly of the purified HPV16 L1 protein was confirmed by the reactivity of this protein to two HPV16-monoclonal antibodies, Camvir-5 and Camvir-6, which recognized conformation-dependent epitopes in an enzyme-linked immunosorbent assay (ELISA) (kindly provided from Margaret Stanley, Cambridge University, Cambridge, UK). After culturing in YPD medium, the recombinant yeast was collected by centrifugation at $2000 \times g$ at

4 °C for 10 min. The yeast pellet was freeze-dried overnight at a maximum shelf temperature of (–)20 °C. The freeze-dried yeast samples were sealed in air-tight plastic tubes and stored at 4 °C until use.

2.2. Purification of HPV16-VLPs from recombinant yeast strains

HPV16-VLP was purified from the pTL2-HPV16 L1 yeast strain by cesium chloride gradient ultracentrifugation, as described previously (Sasagawa et al., 1996), but with some modifications. The yeast cells were cultured in 2 l of YPD medium, to allow the expression of the HPV16 L1 protein (Tohda et al., 1994). After centrifugation at $2000 \times g$ at 4 °C for 5 min, the cells were resuspended in 50 mM potassium phosphate buffer [50 mM KH_2PO_4 (pH 6.5), 20 mM ethylene diamine tetraacetic acid (EDTA) and kept on ice. After re-centrifugation, the pellet was resuspended in 10 ml of KKC buffer [20 mM KPO_4 (pH 6.5), 800 mM KCl, 0.1 mM CaCl_2 , 1.5 mM MgCl_2] that contained 5 mg/ml Novozyme (Sigma), and incubated at 32 °C for 30 min, to digest the yeast cell walls, and then sonicated gently for 1 min at 60 W. The cell extract was recovered by centrifugation at $7000 \times g$ at 4 °C for 10 min. The pellet was resuspended in 10 ml of VLP buffer that contained 0.5% NP-40, sonicated gently, and centrifuged as described above. The collected supernatants were layered onto 40% sucrose VLP buffer [10 mM HEPES, 10 mM KCl (pH 7.0)], and centrifuged at $27,000 \times g$ at 4 °C for 2 h in the Beckman SW28 rotor (Beckman Coulter, Fullerton, CA). The pellets were resuspended in VLP buffer and centrifuged to equilibrium in 27% (wt./wt.) CsCl-VLP buffer for 20 h at $27,000 \times g$ at 4 °C in the SW28 rotor. The appropriate fractions at a density of 1.29 g/ml were diluted in VLP buffer and centrifuged at $27,000 \times g$ at 4 °C for 2.5 h in the SW28 rotor. The pellet was resuspended in VLP buffer and stored at –30 °C. The yeast-derived HPV16-VLP protein was used for intranasal immunization.

2.3. Preparation of yeast and adjuvant for vaccination

The harvested yeast was divided into two stocks. One was stored at –80 °C until use as fresh-live yeast, and another one was freeze-dried at –80 °C over night, and was stored at –30 °C until use. Before vaccination, the freeze-dried yeast cells that expressed HPV16-L1 or the red fluorescence protein (RFP) were resuspended in more than 10 volumes of 80% ethanol in water, and incubated at 4 °C for 30 min, in order to inactivate the yeast cells. This procedure was recommended by the research committee of Kanazawa University to prevent propagation of the recombinant yeast in the animal facility. After centrifugation, the pellet was washed once in phosphate-buffered saline (PBS), and resuspended in PBS (150 mg wet weight per ml). The *E. coli* heat-labile toxin LT (R192G) (Freytag and Clements, 1999), which was kindly provided by Dr. John D. Clements (Tulane University Medical Center, New Orleans, LA, USA) was used as the mucosal

adjuvant in this study. The powder form of the adjuvant was suspended in PBS, and stored at –30 °C until use.

2.4. Yeast digestion in the mouse digestive tract

After being starved for 12 h, three BALB/c mice were given 20 mg (wet weight) of fresh-live or freeze-dried yeast that expressed RFP via the oral route. Each mouse was sacrificed 30 min, 1 h and 6 h after the administration, dissected, and examined for yeast digestion. The digestive tracts were removed and cut into pieces, and touch smears of the tissue section and stool were obtained on glass slides. Fluorescent yeast cells were observed on each slide using Axiovert S-100 Microscope (Carl Zeiss, Germany) and digital photographs were taken by Fuji 3CCD camera (Fuji-Film, Japan).

2.5. Mouse maintenance and vaccination protocols

Thirty female 6-week-old BALB/c mice were purchased from Charles River Co., Ltd. (Shiga, Japan). The mice were maintained in an air-conditioned room, and supplied with sterile chow and water in the animal facility of the School of Medicine, Kanazawa University. All animal procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University. Two mice were killed by accident, and another four mice were used in the preliminary experiment exploring yeast digestion.

The experiment was initiated when the mice reached 9 weeks of age. Intranasal administration of HPV16-VLP was performed under mild anesthesia with diethyl ether. After abstinence from water and chow for 12 h, all of the mice willingly ate freeze-dried yeast cells that were suspended in PBS. Twenty-four BALB/c mice were divided into six groups (Fig. 1). Three mice ingested yeast that contained the vector alone; these mice were selected as the negative controls (Group 1). Another three mice (Group 2) were administered intranasally with 5 µg of yeast-derived HPV16-VLP plus 10 µg of adjuvant LT (R192G); these mice were classified as the positive controls. The remaining mice were classified into four groups: two groups were administered orally with 50 mg (Group 3) or 150 mg (Group 4) of HPV16 L1-expressing yeast, and the other two groups received 50 mg (Group 5) or 150 mg (Group 6) of yeast plus 10 µg of LT (R192G). Three separate vaccinations were performed at 4-week intervals in the first experiment. Twelve weeks after the final oral immunization, a low dose of HPV16-VLP (1 µg) was administered intranasally as a booster immunization to all the mice, including the positive and negative controls.

2.6. ELISA for HPV16-specific antibodies in mouse sera and vaginal fluids

Blood samples were collected from the mouse-tail, and the vaginal fluid samples were obtained by repeated washing of the vagina with 100 µl of PBS using a micropipette. To avoid the influence of the mouse estrous cycle on antibody

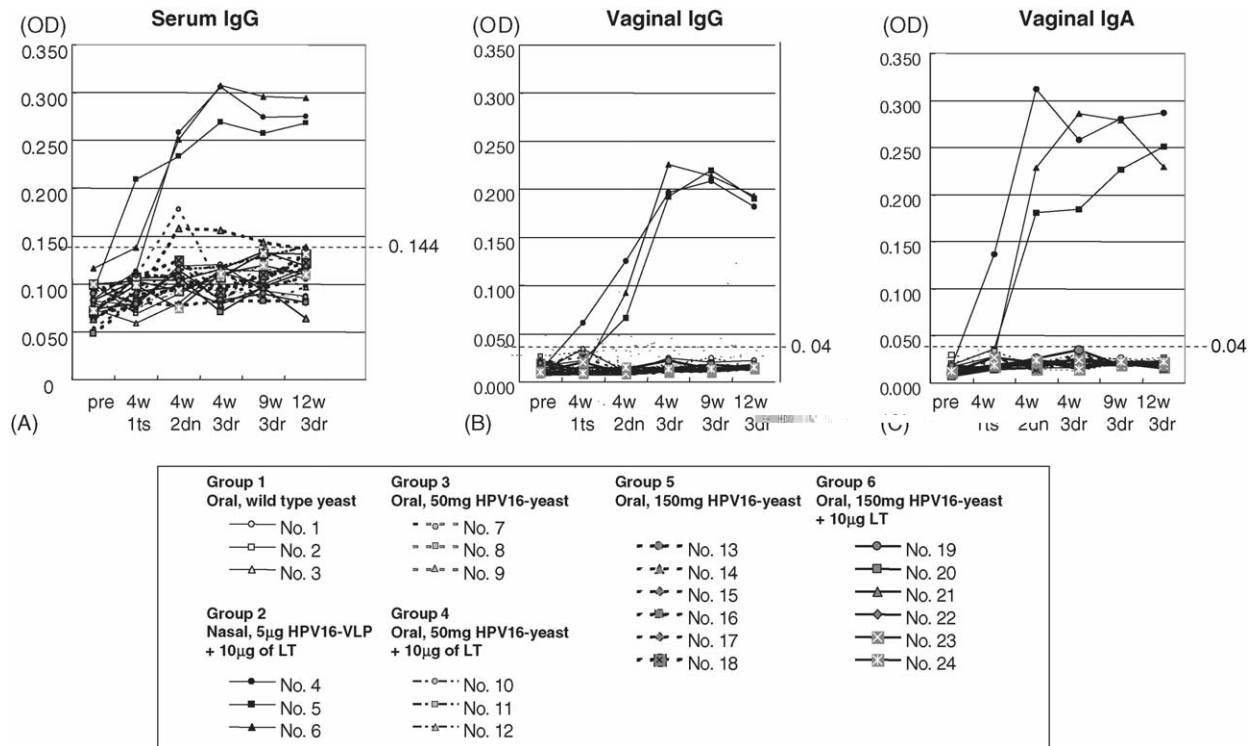


Fig. 1. Antibody responses by the primary immunization. The optical density (OD) values in ELISA for all mice are plotted in the graphs. The route, antigen and adjuvant for vaccination are shown below the graphs. A dot line indicates the cut-off point in each assay. A: IgG responses in sera, B: IgG responses in vaginal fluid, C: IgA responses in vaginal fluid.

production, vaginal secretions were collected twice at 5-day intervals, and the two samples were mixed and used for the analyses. The samples were collected a few days before the first oral immunization and 4 weeks after each immunization. All of the samples were divided into aliquots, to avoid repeated thawing, and were stored at -30°C until use.

HPV16-VLP protein that was purified from insect cells (Rose et al., 1994) was used for the ELISA. For antigen coating, 100 and 300 ng of HPV16-VLP for IgG and IgA detection, respectively, were incubated in PBS on the ELISA plate (NUNC Immunoplate Maxisorp; Nalgene Nunc International, Japan) at 4°C overnight. The coated plates were washed once with PBST (PBS, 0.1% Tween-20), and incubated with blocking buffer (3% albumin, 0.5% FCS in PBST) at room temperature (RT; $20\text{--}24^{\circ}\text{C}$) for 1 h. All of the subsequent washes were carried out with PBST. In the antibody reaction, 100 μl aliquots of samples that contained 1 μl of serum or 20 μl of vaginal wash were mixed with reaction buffer (1.5% bovine albumin, 0.25% FCS in PBST), added to the ELISA plate, and incubated at RT for 3 h. After three washes, 100 μl of the biotinylated anti-mouse IgA or IgG antibody (diluted 1:500 for IgA and 1:1000 for IgG in reaction buffer) was added to the plates, and incubated for 1 h at RT. After three washes, 100 μl of streptavidin–horseradish peroxidase conjugate (DAKO, Germany), which was diluted 1:5000 with PBST, was added to the plates, and incubated for 30 min. After three washes, 100 μl of 50 mM citrate buffer (pH 5.0; 0.0075% hydrogen peroxide), which contained

one tablet of ABT [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma) was added to the plates. After 1 h of color development at RT, the optical density (OD) values were read at dual wavelengths (405/540 nm) using an automated plate reader (iEMS Reader MS; Labsystems, Japan). The final OD values were calculated by subtraction of the OD values at 540 nm from those at 405 nm. The cut-off points for IgA and IgG in the serum and vaginal samples were assigned as the average OD values plus 2.5 standard deviations from the OD values of 10 serum and vaginal samples that were obtained from non-immunized mice. The ELISA was repeated more than twice for each sample, and the mean OD value was used for the evaluation. Two sets of positive and negative control samples were included in each experiment. The OD values of the samples were adjusted with the mean values of the positive controls, to compensate for inter-assay variation, and the OD values of the negative controls were used for quality control in each assay. Additional tests were performed when very high background reactions were observed in the negative controls, or when samples showed ambiguous results in different tests. In the latter case, the third test was performed and the mean OD value of three tests was adopted for the evaluation.

2.7. Characterization of antibodies induced in mice

Antibody response to the denatured HPV16 L1 protein was tested with the ELISA coated with denatured

HPV16-VLP. Aliquates (100 ng for IgG, and 300 ng for IgA) of HPV16-VLP in bicarbonate buffer were boiled for 10 min, and were used as antigen in the ELISA. The reactivity to intact and denatured HPV16-VLP was examined on the same plate with those of the positive and negative control samples. The titer of antibody was determined by reactivity of serially diluted serum and vaginal washing fluids, and the cut-off point of OD values were the same as those described above.

2.8. Statistical analysis

The Mann–Whitney test was used to compare OD values between two groups, and Fisher's exact probability test was used for comparing the positive rates between two groups. All of the analyses were performed using the Excel Statistics for Windows software (SSRI, Tokyo, Japan).

3. Results

3.1. Preliminary experiment of an edible vaccine

In a preliminary experiment, fresh-live or inactivated freeze-dried yeast that expressed red fluorescence protein (RFP) were administered orally to six mice, and the numbers of fluorescent yeast were counted at different sites in the digestive tracts of the mice. The fresh-live yeast cells were not digested, since intact yeast cells were seen in any parts of the gut and excreted in the stools. In contrast, the freeze-dried yeast cells were scarcely disrupted in the stomach or jejunum, but disrupted in the ileum and the large intestine, and few intact yeast was seen in stool, suggesting that freeze-dried yeast cells are digested in the intestine. Therefore, we used inactivated, freeze-dried HPV16 L1-expressing yeast as the edible vaccine.

At the first experiment, 10 or 20 mg of freeze-dried yeast that contained the HPV16 L1 protein (HPV16 L1-yeast) in the presence of the mucosal adjuvant LT (R192G) were administered in 20 mice. However, no antibody was produced in any of these mice. Then, we intranasally administered 5 μ g of purified HPV-16 VLP to these mice. After this boosting immunization by intranasal route, 67% of the mice produced anti-HPV16 serum IgG and vaginal IgA antibodies (data not shown).

In the second experiment, 18 mice were fed with 50 or 150 mg of freeze-dried HPV16 L1-yeast in the presence (Groups 2, 4, and 6) or absence (Groups 1, 3, and 5) of the mucosal adjuvant LT (R192G) (Fig. 1). As a positive control, three mice (Group 2) were administered intranasally with 5 μ g of purified VLP and LT (R192G), and another three mice were administered orally with freeze-dried yeast that contained the vector alone, as negative controls (Group 1). After three immunizations, the levels of HPV16-specific immunoglobulins (IgG and IgA) were evaluated by ELISA (Fig. 1).

All of the positive control mice showed high OD values for serum IgG, vaginal IgG, and vaginal IgA, although serum IgA responses were not detected (data not shown). In contrast, none of the mice that ingested HPV16 L1-yeast showed positive responses for serum IgG, genital IgA, and IgG. We found conflicting results in two tests for serum IgG in some of the mice that were fed HPV16 L1-yeast. An additional test was performed in these mice, and the mean OD values from three tests were adopted. Finally, two mice (No. 7 in Group 3, and No. 14 in Group 5) out of eighteen (11%) had weak and transient serum IgG responses, respectively, after the second immunization (Fig. 1(A)). In contrast, no vaginal IgG or IgA responses were observed in any of the mice that underwent oral vaccination (Fig. 1(B and C)).

3.2. Boosting immune responses by intranasal administration of purified HPV16-VLP

In order to confirm priming by HPV16 L1 antigen in the mice that underwent oral vaccination of HPV16 L1-yeast, a suboptimal dose (1 μ g) of purified HPV16-VLP was administered as a booster to all 24 mice *via* the intra-nasal route. Four weeks later, all three positive controls (100%), the nine mice (50%) that received the oral vaccination, and none of the negative controls showed positive reactions for serum IgG (Fig. 2(A)). In terms of vaginal IgG, the three positive controls (100%), six of the mice (33%) that received the oral vaccination, and none of the negative controls showed positive (Fig. 2(B)). With respect to vaginal IgA, the three positive controls (100%), and seven of the mice (39%) received the oral vaccination; none of the negative controls showed positive (Fig. 2(C)). Unexpectedly, two mice (Nos 7 and 14) that received the oral vaccination and had shown transiently positive before the intranasal boosting were still negative in the final test.

Following intranasal boosting with purified HPV16-VLP, no antibodies were elicited in the negative controls, whereas some mice that were given HPV16 L1-yeast showed positive antibody responses, which suggests that HPV16-specific responses can be primed by oral vaccination. When the antibody levels were compared, all of the mice that received oral vaccination with HPV16 L1-yeast showed lower OD values than did the positive control mice ($P < 0.05$; Mann–Whitney test).

3.3. Effects of oral HPV16 vaccine dose and adjuvant on immune responses

No differences were observed in the antibody-positive rates between mice that were fed HPV16 L1-yeast at low doses (50 mg; Groups 3 and 5) or high doses (150 mg; Groups 4 and 6). We used the mucosal adjuvant LT (R192G) to enhance the antibody responses in mice that were fed the HPV16 L1-yeast vaccine (Groups 5 and 6). No serious adverse effects were seen in the mice that received the adjuvant. Although no differences in serum IgG responses were ob-

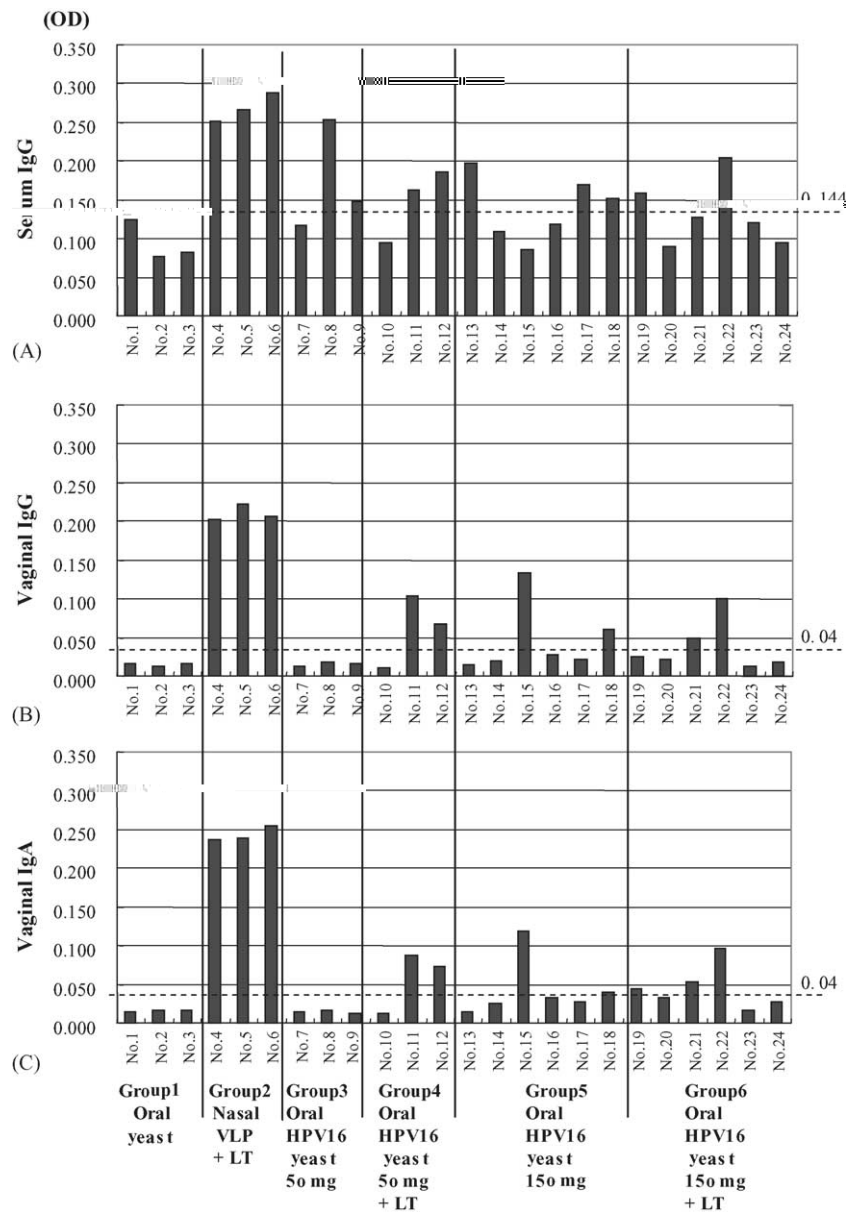


Fig. 2. Antibody responses after the intranasal boosting immunization using a suboptimal dose (1 μ g) of purified HPV16-VLP. The optical density (OD) values in ELISA for all mice were plotted in the graphs. A dot line indicates the cut-off point in each antibody response. Samples exceeded the cut-off point were accounted as positive. A: IgG responses in sera, B: IgG responses in vaginal fluid, C: IgA responses in vaginal fluid.

served between the groups that received the vaccine with adjuvant (Groups 4 and 6) and without adjuvant (Group 3 and 5) (Fig. 2(A)), the positivity rates were two-fold higher for vaginal IgG and five-fold higher for vaginal IgA in the adjuvant groups (Groups 4 and 6) than in the non-adjuvant groups (Groups 3 and 5) (Fig. 2(B) and (C)). However, the statistical difference was only marginal for the vaginal IgA responses, and was not valid for the vaginal IgG responses ($P=0.066$ for IgA, and $P>0.1$ for IgG; Fisher's exact test). The OD values for vaginal IgA in the mice that were given HPV16 L1-yeast plus LT were also slightly higher than those in mice that received HPV16 L1-yeast without adjuvant ($P=0.085$; Mann-Whitney test).

3.4. Characterization of the antibodies elicited by oral and intranasal vaccination

Previous studies have clearly demonstrated that neutralizing antibodies react with conformation-dependent epitopes on HPV-VLPs. We investigated whether the antibodies that were induced in this study reacted against the assembled HPV16 L1 (intact VLP) rather than the denatured L1 protein (denatured VLP). After the second immunization, the induced serum IgG antibodies reacted more strongly with intact VLP than with denatured VLP in all of the positive controls that received intranasal vaccination with HPV16-VLP, although two of these mice (Nos. 4 and 6)

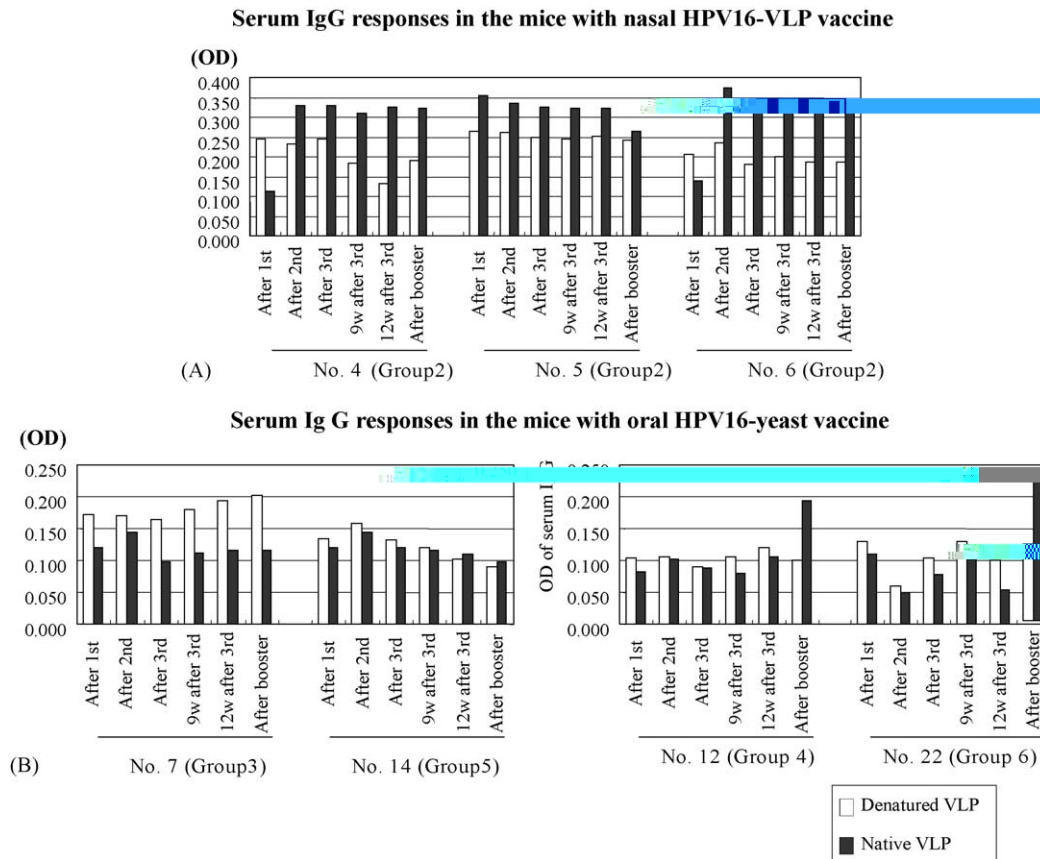


Fig. 3. Antibody responses to denatured HPV16 L1 protein and intact HPV16-virus-like particles. Antibody responses to denatured HPV16 L1 and the intact HPV16-VLP were examined in some mice at different time points after vaccination to know when the antibody responding to HPV16 were elicited. (A) Positive control mice, (B) some of the mice undertook the edible HPV16-yeast and the intranasal HPV16-VLP boosting. White bars: responses to denatured HPV16 L1 protein, black bars: responses to intact HPV16-VLP.

had shown stronger responses to denatured VLP at the first intranasal immunization (Fig. 3(A)). Seroconversion from a non-specific type of reaction to a specific type of reaction appeared to occur after the second immunization. In the mice that were given the edible vaccine, stronger responses were elicited to the intact VLP after the intranasal booster immunization (Fig. 3(B)). In contrast, of the two mice that received the oral vaccine, and that were transiently positive but finally negative after boosting, one mouse (No. 7) reacted more strongly to denatured VLP, and the other mouse (No. 14) showed equivalent reactivities to both types of VLP throughout the time course of the experiment (Fig. 3(B)).

The results of the assays of the vaginal samples from the mice that were given the edible HPV16 vaccine showed that the IgG antibodies elicited in six mice (Fig. 4(A)) and the IgA antibodies elicited in seven mice (Fig. 4(B)) reacted more strongly to intact VLP than to denatured VLP. These results suggest that the serum and vaginal antibodies elicited by the edible HPV16-yeast vaccine recognize conformation-dependent epitopes in HPV16-VLP and, therefore, are probably neutralizing antibodies.

Antibody titer was examined in some available samples (Table 1). The positive control mice which were adminis-

tered with purified VLP by intranasal route showed high titers (6400–25,600 for serum IgG, and 1600–3200 for vaginal IgA). In contrast, mice which were orally administered with HPV16-yeast showed that lower titers (1600–3200 for serum IgG, and 100–800 for vaginal IgA) than those in the positive controls. Four samples which were accounted as negative in this study showed zero.

4. Discussion

Koutsky et al. (2002) have recently shown 100% protection against HPV16 infection following injections of HPV16-VLP. It has been proposed that prophylactic HPV16 vaccination could reduce the incidence of cervical cancer by about 50% (Kulasingam and Myers, 2003; Goldie et al., 2003). It has been speculated that the anti-HPV antibodies induced by vaccination persist for only a couple of years. Repeated injections might be necessary to maintain life-long immunity against HPV. Mucosal immunity is a major route for the induction of protection against the high-risk HPV types that are responsible for cervical cancer. Intranasal immunization with HPV16-VLP induces neutralizing antibodies

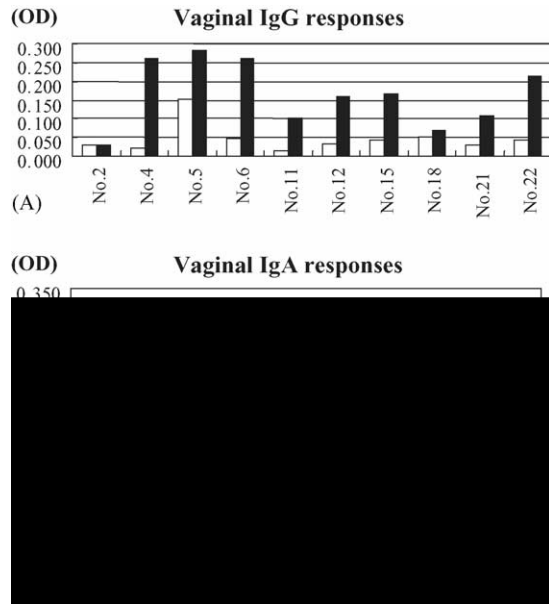


Fig. 4. Antibody responses to intact HPV16-particles in the vaginal fluid of responding and non-responding mice by vaccination. Antibody responses to denatured HPV16 L1 protein and intact VLP in vaginal fluid were examined in some of the mice treated with different vaccines. The response to the intact HPV16-VLP may represent a neutralizing ability to native HPV16 virions. (A) Vaginal IgG responses in one negative control (No. 2), three positive controls (Nos. 4, 5, 6), and six mice (Nos. 11, 12, 15, 18, 21, 22) that were administered with an edible vaccine and were responding to HPV16. (B) Vaginal IgA responses in one negative control (No. 2), three positive controls (Nos. 3, 4, 5), and responding (Nos. 11, 12, 15, 18, 19, 21, 22) and non-responding (Nos. 8, 9, 16) mice administered with an edible yeast vaccine. White bars: responses to denatured HPV16 L1 protein, black bars: responses to intact HPV16-VLP.

in mucosal secretions (Balmelli et al., 1998). It has been reported that mucosal rather than parenteral immunization with purified HPV16-VLP induces neutralizing titers of antibodies throughout the estrous cycle of mice (Nardelli-Haeffliger et al., 1999), which suggests that mucosal immunization may be more efficacious than parenteral immunization in inducing continuous protection of the female genital tract. Previously, we tested the serum samples of patients with cervical HPV infections; 11 of 14 patients who were infected with HPV31 or HPV58 had IgA antibodies against HPV16-VLP, whereas only two of the patients had IgG reacted to HPV16-VLP (Sasagawa et al., 1998). Cross-reactivities against different HPV types are observed more frequently for IgM antibodies (Sasagawa et al., 1996). The polymeric structures of secretory IgA and IgM may lead to more cross-reactivity between different HPV types than IgG, which has a monomeric form. However, we were unable to confirm this in the present system, due to the limited volume of the vaginal samples.

In previous studies, booster immunization with purified VLPs by oral gavage with the HPV11-potato vaccine or by injection of the HPV16-potato vaccine induced HPV-type-specific serum IgG antibodies (Warzecha et al., 2003; Biemelt et al., 2003). The present study is the first to demonstrate that the edible HPV16 L1-yeast vaccine elicits HPV16-specific vaginal IgA and IgG antibodies, as well as serum IgG. As was seen in previous studies, although orally administered freeze-dried HPV16 L1-yeast alone did not induce anti-HPV16 antibodies, co-administration of a suboptimal dose of the vaccine *via* the nasal route elicited serum IgG, vaginal IgG, and vaginal IgA in 50, 33, and 39% of the mice, respectively. These responses were not induced directly by intranasal boosting with purified HPV16-VLP, since no antibodies were elicited in the negative control mice that received the same booster

Table 1
Titers of serum IgG and vaginal IgA antibodies elicited in mice

Mouse ID No.	Routes of vaccination 1st–3rd/4th	Antigen and dose			Titers (dilution)	
		Yeast	HPV16VLP	Adjuvant	Serum IgG	Vaginal IgA
1	Oral/nasal	Wt. ^a 50 mg	1 mg		0	0
2	Oral/nasal	Wt. 50 mg	1 mg		0	0
4	Nasal/nasal	0	5 mg + 1 mg	10 mg	6400	1600
5	Nasal/nasal	0	5 mg + 1 mg	10 mg	25600	3200
6	Nasal/nasal	0	5 mg + 1 mg	10 mg	NA	NA
7	Oral/nasal	HPV16 ^b 50 mg	1 mg	–	NA ^c	0
8	Oral/nasal	HPV16 50 mg	1 mg	–	3200	NA
9	Oral/nasal	HPV16 50 mg	1 mg	–	1600	NA
10	Oral/nasal	HPV16 50 mg	1 mg	10 mg	NA	0
11	Oral/nasal	HPV16 50 mg	1 mg	10 mg	1600	400
12	Oral/nasal	HPV16 50 mg	1 mg	10 mg	1600	800
13	Oral/nasal	HPV16 150 mg	1 mg	–	1600	NA
15	Oral/nasal	HPV16 150 mg	1 mg	–	1600	800
17	Oral/nasal	HPV16 150 mg	1 mg	–	3200	NA
19	Oral/nasal	HPV16 150 mg	1 mg	10 mg	1600	100
21	Oral/nasal	HPV16 150 mg	1 mg	10 mg	NA	100
22	Oral/nasal	HPV16 150 mg	1 mg	10 mg	3200	800

^a Wildtype yeast.

^b HPV16L1-yeast.

^c Not applicable.

immunization. Intranasally administered HPV-VLP probably enhances the activity of primed B cells that recognize the HPV16 L1 protein. Interestingly, all of the induced antibodies reacted more strongly to intact HPV16-VLP than to denatured L1, which suggests that these antibodies recognize conformation-dependent HPV16 L1 epitopes, and that they have neutralizing ability (Harro et al., 2001).

It is unclear why the edible vaccine alone does not induce HPV-specific antibodies. One possibility is that the immune cells are exposed to a low level of assembled L1 protein in the yeast-protein expression system, as has been noted for the plant system (Warzecha et al., 2003). The finding that neutralizing antibodies are induced by oral immunization with purified HPV16-VLP (Gerber et al., 2001) supports this possibility. Another study demonstrated that oral administration of a soluble antigen or an enzymatic digest induced immune tolerance, whereas exposure of the ileum to the intact antigen using an encapsulated antigen delivery system induced immune responses (Barone et al., 2000). The immune tolerance was abrogated when the ileum was re-stimulated with the intact antigen or with cholera toxin. We postulate that intranasal boosting with purified VLP abrogates the tolerance induced by oral HPV16 L1-yeast administration in some of the mice. When we followed the serum IgG responses in two mice that were transiently positive for IgG before boosting, one of these mice (No. 14) showed a decreased response to the intact VLP after intranasal boosting (Fig. 3(B)), which may reflect oral tolerance against HPV16 L1 that was not abrogated by nasal boosting. The fact that more than half of the mice that received the oral vaccine did not respond to intranasal boosting suggests oral tolerance. The different doses of freeze-dried HPV16 L1-yeast had no amplifying effect on antibody production, whereas co-administration of the vaccine with adjuvant LT (R192G) marginally enhanced the mucosal IgA responses ($P=0.066$). It has been reported that immune tolerance is induced when inflammation is not induced at the time of antigen recognition (Tindle, 2002). The low efficacy of the edible HPV16 vaccine, in terms of induced immune responses, might be overcome by optimization of the LT dosage, administration of a co-stimulatory factor, or modification of the antigen (Kim et al., 2003).

Acknowledgement

We thank Prof. Margaret Stanley (Cambridge University, Cambridge, UK) for providing anti-HPV16 antibodies and Dr. John D. Clements (Tulane University Medical Center, New Orleans, LA, USA) for providing the *Escherichia coli* heat-labile toxin LT (R192G).

References

Balmelli, C., Roden, R., Potts, A., Schiller, J., De Grandi, P., Nardelli-Haefliger, D., 1998. Nasal immunization of mice with human papillo-

- mavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. *J. Virol.* 72, 8220–8229.
- Barone, K.S., Reilly, M.R., Flanagan, M.P., Michael, J.G., 2000. Abrogation of oral tolerance by feeding encapsulated antigen. *Cell. Immunol.* 199, 65–72.
- Biemelt, S., Sonnewald, U., Galmbacher, P., Willmitzer, L., Muller, M., 2003. Production of human papillomavirus type 16 virus-like particles in transgenic plants. *J. Virol.* 77, 9211–9220.
- Franco, E.L., Villa, L.L., Sobrinho, J.P., Prado, J.M., Rousseau, M.C., Desy, M., Rohan, T.E., 1999. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. *J. Infect. Dis.* 180, 1415–1423.
- Freytag, L.C., Clements, J.D., 1999. Bacterial toxins as mucosal adjuvants. *Curr. Top. Microbiol. Immunol.* 236, 215–236.
- Gerber, S., Lane, C., Brown, D.M., Lord, E., DiLorenzo, M., Clements, J.D., Rybicki, E., Williamson, A.L., Rose, R.C., 2001. Human papillomavirus virus-like particles are efficient oral immunogens when coadministered with *Escherichia coli* heat-labile enterotoxin mutant R192G or CpG DNA. *J. Virol.* 75, 4752–4760.
- Goldie, S.J., Grima, D., Kohli, M., Wright, T.C., Weinstein, M., Franco, E., 2003. A comprehensive natural history model of HPV infection and cervical cancer to estimate the clinical impact of a prophylactic HPV-16/18 vaccine. *Int. J. Cancer* 106, 896–904.
- Hagensee, M.E., Carter, J.J., Wipf, G.C., Galloway, D.A., 1995. Immunization of mice with HPV vaccinia virus recombinants generates serum IgG, IgM, and mucosal IgA antibodies. *Virology* 206, 174–182.
- Harro, C.D., Pang, Y.Y., Roden, R.B., Hildesheim, A., Wang, Z., Reynolds, M.J., Mast, T.C., Robinson, R., Murphy, B.R., Karron, R.A., Dillner, J., Schiller, J.T., Lowy, D.R., 2001. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. *J. Natl. Cancer Inst.* 93, 284–292.
- Ho, G.Y., Bierman, R., Beardsley, L., Chang, C.J., Burk, R.D., 1998. Natural history of cervicovaginal papillomavirus infection in young women. *N. Engl. J. Med.* 338, 423–428.
- Kim, S.J., Lee, C., Lee, S.Y., Kim, I., Park, J.S., Sasagawa, T., Ko, J.J., Park, S.E., Oh, Y.K., 2003. Enhanced immunogenicity of human papillomavirus 16 L1 genetic vaccines fused to an ER-targeting secretory signal peptide and RANTES. *Gene Ther.* 10, 1268–1273.
- Kirnbauer, R., Taub, J., Greenstone, H., Roden, R., Durst, M., Gissmann, L., Lowy, D.R., Schiller, J.T., 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1–L2 into virus-like particles. *J. Virol.* 67, 6929–6936.
- Koutsky, L.A., Ault, K.A., Wheeler, C.M., Brown, D.R., Barr, E., Alvarez, F.B., Chiacchierini, L.M., Jansen, K.U., Proof of Principle Study Investigators, 2002. A controlled trial of a human papillomavirus type 16 vaccine. *N. Engl. J. Med.* 347, 1645–1651.
- Kulasingam, S.L., Myers, E.R., 2003. Potential health and economic impact of adding a human papillomavirus vaccine to screening programs. *JAMA* 290, 781–789.
- Molano, M., Van den Brule, A., Plummer, M., Weiderpass, E., Posso, H., Arslan, A., Meijer, C.J., Munoz, N., Franceschi, S., HPV Study Group, 2003. Determinants of clearance of human papillomavirus infections in Colombian women with normal cytology: a population-based 5-year follow-up study. *Am. J. Epidemiol.* 158, 486–494.
- Munoz, N., Bosch, F.X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K.V., Snijders, P.J., Meijer, C.J., International Agency for Research on Cancer Multicenter Cervical Cancer Study Group, 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N. Engl. J. Med.* 348, 518–527.
- Nardelli-Haefliger, D., Roden, R., Balmelli, C., Potts, A., Schiller, J., De Grandi, P., 1999. Mucosal but not parenteral immunization with purified human papillomavirus type 16 virus-like particles induces neutralizing titers of antibodies throughout the estrous cycle of mice. *J. Virol.* 73, 9609–9613.
- Parkin, D.M., Bray, F.I., Devesa, S.S., 2001. Cancer burden in the year 2000, 2001. The global picture. *Eur. J. Cancer* 37, 54–66.

- Pushko, P., Sasagawa, T., Cuzik, J., Crawford, L., 1994. Sequence variation in the capsid protein genes of HPV16. *J. Gen. Virol.* 75, 911–916.
- Rose, R.C., Bonne, W., Reichman, R.C., Garcea, R.L., 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.
- Rose, R.C., Bonne, W., DaRin, C., McCance, D.J., Reichman, R.C., 1994. Serological differentiation of human papillomavirus types 11, 16 and 18 using recombinant virus-like particles. *J. Gen. Virol.* 75, 2445–2449.
- Sasagawa, T., Pushko, P., Steers, G., Gschmeissner, S.E., Hajibagheri, M.A., Finch, J., Crawford, L., Tommasino, M., 1995. Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast *Schizosaccharomyces pombe*. *Virology* 206, 126–135.
- Sasagawa, T., Inoue, M., Lehtinen, M., Zhang, W., Gschmeissner, S.E., Hajibagheri, M.A., Finch, J., Crawford, L., 1996. Serological responses to human papillomavirus type 6 and 16 virus-like particles in patients with cervical neoplastic lesions. *Clin. Diagn. Lab. Immunol.* 3, 403–410.
- Sasagawa, T., Yamazaki, H., Dong, Y.Z., Satake, S., Tateno, M., Inoue, M., 1998. Immunoglobulin-A and -G responses against virus-like particles (VLP) of human papillomavirus type 16 in women with cervical cancer and cervical intra-epithelial lesions. *Int. J. Cancer* 75, 529–535.
- Sasagawa, T., Basha, W., Yamazaki, H., Inoue, M., 2001. High-risk and multiple human papillomavirus infections associated with cervical abnormalities in Japanese women. *Cancer Epidemiol. Biomark. Prev.* 10, 45–52.
- Sasagawa, T., Tani, M., Yasuda, H., Azar, K.K., Fukae, T., Ukita, T., Yamazaki, H., Satake, S., Nakano, T., Inoue, M., in press. Sexual behavior and high-risk human papillomavirus (HPV) infections in Japanese women. *Sex. Trans. Inf.*
- Tindle, R.W., 2002. Immune evasion in human papillomavirus-associated cervical cancer. *Nat. Rev. Cancer* 2, 59–65.
- Tohda, H., Okada, H., Giga-Hama, Y., Okayama, H., Kumagai, H., 1994. A copy-number-controlled expression vector for the fission yeast *Schizosaccharomyces pombe*. *Gene* 150, 275–280.
- Warzecha, H., Mason, H.S., Lane, C., Tryggvesson, A., Rybicki, E., Williamson, A.L., Clements, J.D., Rose, R.C., 2003. Oral immunogenicity of human papillomavirus-like particles expressed in potato. *J. Virol.* 77, 8702–8711.
- Yang, B.H., Bray, F.I., Parkin, D.M., Sellors, J.W., Zhang, Z.F., 2004. Cervical cancer as a priority for prevention in different world regions: an evaluation using years of life lost. *Int. J. Cancer* 109, 418–424.
- zur Hausen, H., 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* 184, 9–13.