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Vaginal DNA vaccination against infectious diseases transmitted through the vagina

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1. ABSTRACT

There is an urgent need for the development of vaccines against genital virus infections that are transmitted through heterosexual intercourse, including the HIV and HPV. In general, the surface of female genital mucosa, including vaginal mucosa, is the most common site of initiation of these infections. Thus, it is becoming clear that successful vaccines must induce both cellular and humoral immune responses in both the local genital tract and systemically. We believe that a strong vaginal immune response could be obtained by inducing strong gene expression of antigen-coding DNA in the local targeted tissue. In order to improve transfection efficiency in the vagina, it is important that methods allowing breakthrough of the various barriers, such as the epithelial layer, cellular and nuclear membrane, are developed. Therefore, systems providing less invasive and more effective delivery into the subepithelial layer are required. In this review, we will introduce our studies into efficient vaginal DNA vaccination methods, focusing on the effects of the menstrual cycle, utilization of the combination of functional peptides, and use of a needle-free injector.

2. INTRODUCTION

Human immunodeficiency virus (HIV) and human papillomavirus (HPV) are two of the most common sexually transmitted diseases in the world (1-5). The 2009 AIDS epidemic update states that an estimated 2.7 million new HIV infections occurred, and the number of people living with HIV worldwide continued to grow, reaching an estimated 33.4 million, in 2008. It is also estimated that 2 million deaths due to AIDS-related illness occurred worldwide (6). On the other hand, of the HPV-associated cancers, cervical cancer is the second largest cause of cancer deaths in women worldwide (4-7). Thus, there is an urgent need for the development of safe, easy-to-use, effective, stable and inexpensive vaccines against HIV and HPV.

Currently, there are two commercially available preventive HPV vaccines; Gardasil, developed by Merck, and Cervarix, developed by Glaxo Smith Kline (8-11). These vaccines are made up of HPV virus-like particles derived from the L1 major capsid protein. However, these preventive vaccines have not been shown to provide

therapeutic effects against pre-existing HPV infections. Furthermore, because of the considerable burden of HPV infections worldwide, it is estimated that it will take decades for "preventive" vaccines to significantly reduce the prevalence of cervical cancer (13). Thus, for current treatment of cervical cancer and its precursor lesions, it is important to focus on the development of "therapeutic" vaccines that can generate cellular immunity against HPV-infected cells, thus potentially eliminating preexisting lesions and malignant tumors (13). Among the various forms of therapeutic HPV vaccines, DNA vaccines have emerged as an attractive approach for antigen-specific immunology. Not only are DNA vaccines safe, stable and easy to produce, but they can also be used to sustain high levels of antigen expression in cells (115-116). Therefore, several therapeutic HPV DNA vaccine clinical trials have been completed or are currently ongoing (12-15). The application of DNA immunization as new generation vaccines has been well studied since its invention, and a variety of such vaccines have undergone clinical trials (16-18) or are used in veterinary practice (19, 20). DNA vaccines were first reported in the early 1990s as a novel method for vaccination (21-24). The DNA vaccines have several advantages, which include simplicity of manufacture, biological stability and cost-effectiveness. The safety of DNA vaccines is their most important advantage, as no live virus or viral fragments are utilized in the preparation of this type of vaccine. In addition, different genes can be combined simultaneously, resulting in multivalent vaccines. Another important benefit is the induction of not only humoral immunity but also cellular immunity.

The earliest Phase I clinical trial of a DNA vaccine was of an HIV-1 candidate tested in individuals infected with HIV-1, followed by studies in volunteers who were not infected with HIV-1 (25). Other prophylactic and therapeutic DNA vaccine trials followed, including trials that tested DNA vaccines against cancer, influenza, malaria, hepatitis B and HIV-1 (17, 26-29). Currently, clinical trials with a DNA HIV-1 vaccine have already started using an adenovirus-based prime-boost vaccine and vaccinia virus Ankara.

Presently, about 90 human clinical DNA vaccine trials are underway (18). Furthermore, in the past 3 years, four DNA products have been licensed for animal use: one against West Nile virus in horses (30), one against infectious hematopoietic necrosis virus in schooled salmon (31), one for treatment of melanoma in dogs (32), and the most recent licensure, growth hormone releasing hormone (GHRH) product for fetal loss in swine (33).

Several reports have established that mucosal transmission is the initial step towards systemic infection, such as HIV, and thus inhibition of viral mucosal transmission would appear to be the most efficient approach to preventing infection (3, 34-37). In general, the surface of female genital mucosa, including vaginal and cervical mucosa, is the most common site of initiation of HIV and HPV (1-5). Thus, a successful vaccine must induce both cellular and humoral immune responses in both the genital tract, which is the local entry site, and systemically. Thus, mucosal vaccination that induces strong immunity both at the local entry site and systemically is required to prevent these infections (3). Mucosal vaccines against HIV have been well investigated, including through the oral (38-41), nasal (42-47), rectal (48-52) and genital (53-58) routes. Furthermore, as we have noted previously, mucosal antibodies, and particularly secretory immunoglobulin (Ig) A, play an important role in protection against HIV-1 infection (59), and stimulation of interplay between innate and adaptive immunity seems to be crucial to elicit both mucosal and systemic responses. A few mucosal vaccination studies in mouse models have been performed to date, and provide proof-of-principal that mucosal immunization can induce IgA responses with neutralizing activity (60-62). However, more advanced antigen delivery systems for efficient mucosal vaccines are required, and immunization aimed to stimulate a mucosal virus-specific protective immune response in the female genital tract in humans still requires optimization, and should also include immunization strategies for natural protection against heterosexual infection (2, 63).

We believe that a strong vaginal immune response can be obtained by inducing strong gene expression of antigen-coding DNA vaccines in local vaginal tissue. To this end, in order to improve transfection efficiency in antigen-presenting cells in the vaginal subepithelial layer, methods must be developed that enable breakthrough of the various barriers, such as the vaginal epithelial layer, cellular membrane and nuclear membrane. Therefore, less invasive and more effective delivery into the subepithelial layer is required.

Needle-free jet injection has been extensively investigated as a method to immunize laboratory animals, such as mice (105, 106), rabbits (107, 108), pigs and dogs (109) and monkeys, through the transdermal route. In addition, jet injection has been tested subcutaneously in several human clinical trials (110) and is already produced commercially for daily injection of insulin and h-growth hormone. The vast majority of studies in animals have demonstrated an enhancement in resulting immune responses with jet injection over conventional needle-syringe injection (111).

In this review, we will introduce our studies on efficient vaginal DNA vaccination methods for development of DNA vaccines that penetrate the vaginal membrane.

In females and heterosexual males, HPV and HIV infections currently occur through heterosexual intercourse and transmission through the vaginal mucosa. Thus, to prevent or treat these infections, strong vaginal immunity is required and needs to include cytotoxic T-lymphocytes (CTL) and IgA, which play an important role as the first line of defense in these infections. The local administration of vaccine antigens into the vagina of animals or human volunteers has been shown to predominantly result in the development of specific antibodies in local secretions, but in most cases the immune response is not disseminated to

remote mucosal sites or to the systemic component. Furthermore, local immunization via the male genital tract is unlikely to be practical (54-56). However, there are some reports indicating strong immune responses after vaginal administration of HIV vaccine candidates in animals (57). Therefore, we believe that specific systemic and mucosal immunity could be induced with the development of an efficient vaginal delivery system.

3. EFFECTS OF THE MENSTRUAL CYCLE

The vaginal mucosa is under constant exposure to infectious agents, and is consequently surveyed by a network of dendritic cells to induce mucosal immunity (64).

Unlike other mucosal tissues, the female reproductive tract undergoes dramatic hormone-dependent changes over the course of the menstrual cycle. One potential mechanism relates to the thickness and leakiness of the vaginal epithelial layer. With the increase in serum estrogen levels, the epithelial layer thickness increases during the estrous stage. Subsequently, during the metestrus stages, with the increase in serum progesterone levels and decrease in estrogen, the superficial layers of the vaginal epithelium are delaminated, and become maximally thin and leaky at the diestrus stage.

Vaginal absorption of relatively large and water soluble compounds, such as peptides and proteins, has been systemically determined in rats to be very poor and significantly influenced by the menstrual cycle (65-67). Studies have shown that after vaginal administration of an insulin suppository in rats, a slight decrease in the glucose level is observed during proestrus, whereas a marked decrease is observed during metestrus and diestrus. Furthermore, the absorption of water soluble peptides, such as insulin and leuproreline acetate, through the vaginal membrane is much higher during metestrus and diestrus than during proestrus and estrus (68).

We examined the transfection efficiency into vaginal mucosa during the estrous cycle, which has the four stages of proestrus, estrus, metestrus and diestrus, in mice (69). The estrous stage was assessed by daily morning microscopic observation of vaginal smears taken as a swab and stained with Giemsa solution, after which pCMV-Luc was electroporated at the vaginal surface at around 09:00 in the morning, following the smear check. The vaginal membrane was electroporated at its surface immediately after vaccine administration of PBS or one of the pDNAs, using an electroporator. A custom-designed needle electrode, consisting of two parallel needles (anode and cathode) 5 mm in length and 5 mm apart, each consisting of 3 platinum needles 1 mm in diameter, was used to apply 15 pulses of electricity at 250 V/cm for 5 ms. These electroporation parameters were established in our previous study as the optimal conditions for greatest gene transfection efficacy with minimal vaginal irritation (69).

The transfection efficiency was clearly affected by the estrous cycle. As shown in Figure 1a, at metestrus and diestrus, luciferase gene expression was 3-fold higher than at proestrus and estrus. The mucosa of the vagina consists of epithelial cell layers that form a barrier to absorption of water soluble and large molecules. Histological observation (Figure 1b) indicated that the difference in transfection efficiency during the four menstrual stages might be explained by a change in the membrane structure. At metestrus and diestrus, these epithelial cell layers are very thin compared with those at the other stages, and at diestrus they are extremely porous.

Antigen presentation is known to be most reduced at the estrus stage of the estrous cycle, at which time estrogen levels are most elevated and ovulation takes place (70), whereas the number of antigen presenting cells has been found to be maximal in the vagina, and the number of layers of epithelial cells lining the vagina of rodents decreases dramatically, at the diestrus stage, which would be expected to enhance uptake of luminal antigens. Indeed, uptake of proteins and the ability of vaginal immunization to induce specific antigen responses in mice are optimal when preparations are administered during diestrus (71) Thus, vaccine strategies for protection against sexually transmitted diseases must take into account that sex hormones affect immune responses. It has also been reported that the immune-associated cells in the vaginal submucosal membrane increase at diestrus (64).

These findings indicate that DNA vaccination at diestrus, the late luteal phase and early follicle phase in humans, would be most suitable for practical therapy.

4. VAGINAL AND SYSTEMIC IMMUNE RESPONSES BY VARIOUS INOCULATION ROUTES

Mucosal immunization is known to be a good method for vaccine delivery and has been commonly and successfully used in vaccination programs. The mucosal surfaces in our body are efficient immune producing organs, performing constant surveillance of foreign antigens. Several mucosal routes and surfaces, including oral, nasal, lung, rectal and vaginal mucosa, have been considered as potential vaccination sites (72).

To date, mucosal vaccines have been administered by either oral or nasal routes in order to induce immunity at multiple sites, including the female reproductive tract (73). On the other hand, there is evidence that local exposure to antigen can result in a much stronger immune response at the region of exposure than at distant sites (74), as recently demonstrated for CD8+ CTLs (75). Previous studies suggest that the vaginal immunization route can be used effectively for inducing local immune responses in the female genital tract (50, 76). However, only few studies have examined vaginal administration of DNA vaccines (77, 78).

We compared the vaginal local and systemic immune responses in mice after DNA immunization by the intradermal, nasal, and vaginal routes (79). We found that the IFN-γ levels from spleen cells from mice immunized

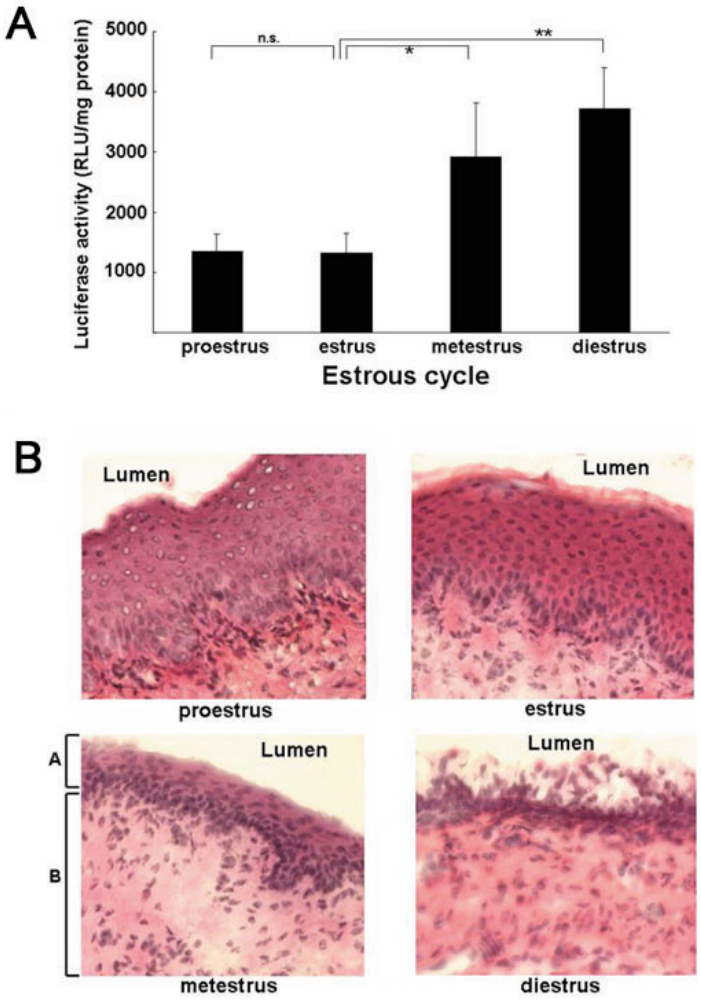

Figure 1. Effects of the estrus cycle on transfection of pCMV-Luc into vaginal mucosa in diestrous mice. (a) Luciferase activity in the vaginal mucosal membrane was determined 24 h after vaginal administration of pCMV-Luc (20 mg) at various estros stages via electroporation (250 V/cm, 5 ms, 15 pulses) in mice pretreated with 5% citric acid solution for 2 h. Each data point represents the mean ±S.E. (n=4). *: $p<0.05$, **: $p<0.01$, n.s.: $p>0.05$, (t-test). (b) Histological observation of a section of the vaginal mucosal membrane in mice during different stages of the estrous cycle. The stage of the estrus cycle of the mice was determined using a morning smear test. Vaginal tissue was collected and 10-μm frozen sections stained with hematoxylin and eosin. A: epithelium, B: stroma (subepithelium), Lumen: vaginal lumen.

via all inoculation routes were nearly identical, indicating that these local mucosal immunization routes are effective for inducing systemic cellular immunity as well as intradermal immunization. Intradermal administration of pCMV-OVA resulted in the highest induction of IgG2a, possibly because the intradermal environment is particularly primed for producing immune responses due to the presence of large numbers of dendritic cells, which are powerful antigen presenting cells. Dermal dendritic cells are readily transfected in vivo by cutaneous immunization with foreign genes, and then migrate to the lymph nodes (80), where stimulation of T- and B-cells occurs. The nasal and vaginal inoculation routes of pCMV-OVA also exhibited considerably higher induction than the control. These findings suggest that by vaginal immunization using our delivery systems, the dendritic cells in the vaginal mucosa may uptake a number of foreign genes and then migrate to the lymph nodes, similar to the response seen by intradermal immunization, resulting in strong immune responses being induced both locally and systemically.

In the local vaginal immune response, in which the vaginal tract was washed by flushing with 150 μL PBS and the resultant PBS wash solution used to determine the level of local vaginal IgA production, vaginal administration of pCMV-OVA clearly induced the highest vaginal IgA production among the inoculation routes (Figure 2). This result is consistent with previous studies that have demonstrated that the greatest levels of antigen-specific IgA antibodies may be generated in regions that are closest to the site of antigen exposure, such as the oral, rectal and vaginal sites (74, 81, 82).

These findings clearly indicate that the vaginal mucosa may be the most suitable site for administration of a mucosal DNA vaccine against HIV

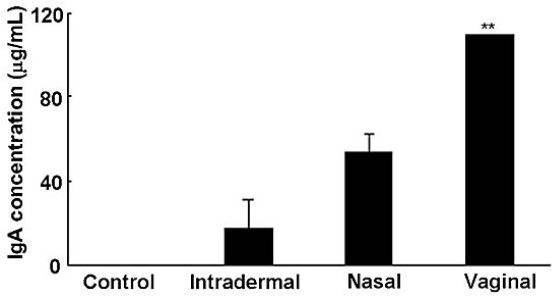

Figure 2. Vaginal mucosal OVA-specific IgA secretion in mice after immunization by various routes. Mice were immunized with pOVA 3 times via the intradermal, nasal or vaginal routes at 2-week intervals. Control mice were not treated. Mice were sacrificed 1 week after final immunization. Vaginal IgA levels as O.D. in rinsed vaginal fluids were analyzed by ELISA. The concentrations of IgA were calculated using a standard curve established using serially diluted mouse immunoglobulin reference serum (Bethyl Laboratories, Inc, Montgomery, TX, USA). Each bar represents the mean ± S.E. (n=5). **P<0.01 compared with control, intradermal or nasal immunization.

5. UTILIZATION OF CPP AND NLS FOR VAGINAL DNA VACCINATION

5.1. CPP and NLS for gene delivery

Dendritic cells, which originate in the bone marrow, are professional antigen-capturing cells and antigen-presenting cells, and these processes initiate the primary immune responses in our body. This central role in cell-mediated immunity has made them an attractive target for immunotherapy (83-85). To generate strongly effective dendritic cells, technologies are needed that produce high antigen expression as a result of delivering DNA encoding antigen into the nucleus of dendritic cells, which are non-dividing cells.

In order to promote the gene expression of pDNA in dendritic cells, the numerous barriers to gene delivery into the cells and nucleus must be overcome. These barriers include (i) cellular adhesion and uptake, (ii) escape from endosomes to the cytoplasm prior to delivery to fusion by lysosomes, (iii) trafficking to the nucleus, and (iv) uptake to the nucleus (86). In particular, large foreign molecular substances such as proteins and genes are mostly unable to enter the nucleus of non-dividing cells, in which the nuclear membrane does not disappear upon cell division, since nuclear transfer is strictly controlled by precise machinery.

Gene transfer systems based on fusogenic liposomes, cationic liposomes or polycations have gained wide acceptance over the last decade as gene transfer vectors. In addition, practical transfer systems using electroporation or sonoporation have been utilized. However, at present, the usefulness and application of gene transfer systems as therapeutic devices are limited by the transient, low levels of gene expression observed *in vivo*. One of the limiting steps responsible for the low gene expression achieved with these non-viral vectors is inefficient intracellular trafficking of DNA. To overcome cytoplasmic degradation of the gene, via effective transport into the nucleus, and to improve the efficiency of gene expression, the use of cell penetrating peptides (CPPs) or nuclear localization signal (NLS) peptides for non-viral gene transfer has been widely investigated (87-94).

Recently, a cellular internalization method using short peptides derived from protein-transduction domains has attracted much attention. Several CPPs, such as HIV-1 Tat fragments, less than 30 amino acid residues in length, are capable of crossing a plasma membrane (95-97). In addition, they can deliver their associated molecules into cells. The Tat peptide has been reported to be capable of delivering b-galactosidase (120 kDa) to various organs when administered intraperitoneally to mice.

Except during mitosis, macromolecules such as proteins or nucleic acids cannot enter the nucleus through the nuclear pores. The intranuclear transfer of cellular and viral proteins, DNA and/or RNA occurs by means of an energy-dependent mechanism which involves peptidic NLS sequences that bind to structures called the nuclear pore complex (NPC) via transport receptors such as importins α/β (98). To date, the literature indicates that the NLS approach has potential for improving DNA nuclear delivery and expression with non-viral vectors (99, 100).

Recent studies have shown that some signaling molecules are transported into the nucleus by NLS- and importin-independent processes by associating directly with proteins of NPCs (101). Importin a molecules bind to the previously identified NLSs of NF-κB p50, and NF-κB p50 is bound by the N-terminal NLS binding site of importin α3 (102). In addition, we reported that a NF-κB p50 analog (Table 1), a nuclear localizing signal, synthesized in our laboratory, significantly promoted the nuclear transport and gene expression of pEGFP in dendritic cells, and we described that the NF-κB p50 analog induces highly efficient intranuclear transport of pDNA following the elevation of intracellular uptake (103).

5.2. Vaginal gene expression upon vaginal DNA delivery using CPP and NLS

We also examined the effects of the CPP and NLS analog peptides (Table 1) on vaginal gene transfection (69). Peptide carriers/pCMV-Luc (weight ratio: 10/1) complexes were prepared by mixing pCMV-Luc with both peptides and incubating at 4°C for 30 min. These peptides complexed with pCMV-Luc were then administered into vaginal mucosa that had been pretreated with 5% citric acid solution for 2 h and electroporated (250 V/cm, 5 ms, 15 pulses) during diestrus. Luciferase activity of the vaginal mucosa was then measured 24 h later. As shown in Figure 3, the luciferase activity of mucosa treated with the Tat analog/pCMV-Luc complex was 3-fold higher than that treated with pCMV-Luc alone. Furthermore, a Tat analog/NF-κB analog/pCMV-Luc complex induced significantly greater luciferase gene expression in the vaginal mucosa than the Tat analog/pDNA complex. This level of expression was also statistically higher than those obtained by the widely used non-viral vectors,

Table 1. Structure of synthetic Tat and NF-κB analogs

Peptides	Sequence
Tat analog	Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-*Cys*-*Gly*-NH_2
NF-κB analog	*Gly*-Gln-Arg-Lys-Arg-Gln-Lys-*Cys*-*Gly*-NH_2

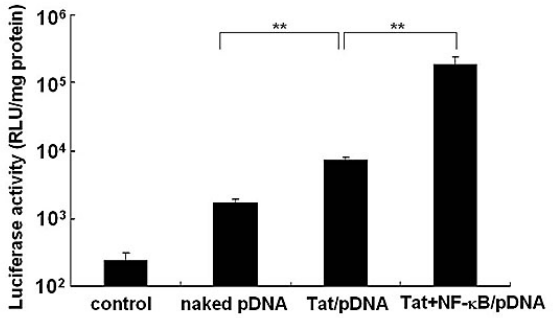

Figure 3. Luciferase activity in the vaginal membrane after vaginal administration of pCMV-Luc with Tat and NF-κB analogs. Mice at diestrus received intravaginal administration of pCMV-Luc (20 mg) and complexes with Tat analog (200 mg) or NF-κB analog (200 mg), using electroporation (250 V/cm, 5 ms, 15 pulses). Mouse vaginas had been pretreated with 5% citric acid solution for 2 h followed by electroporation. Each data point represents the mean ±S.E. (n=4). **: $p<0.01$, (t-test).

polyethyleneimine (PEI, Branched, Mw 76 kDa; Sigma-Aldrich Co., USA) and Lipofectamine® (Invitrogen Japan K.K., Japan) (69). These results suggest that the Tat and NF-κB analogs facilitate the transfection of pDNA possibly by increasing both cell penetration and translocation into the nucleus. Thus, these CPP and NLS analogs are promising potentiators for DNA vaccines.

5.3. Immune responses at both the local and systemic levels upon vaginal DNA immunization using CPP and NLS

We also examined the immune responses induced by intravaginal pCMV-OVA immunization by electroporation, with or without the Tat/NF-κB analogs, in mice at diestrus (79). No increase was observed in the serum IgG2a response in non-treated control, PBS-transfected or naked pCMV-OVA alone-transfected mice. Although the female genital tract contains diffuse mucosa-associated lymphoid tissue typical of an immune effector site, it differs from the intestinal mucosa in that a larger fraction of the total Ig in associated secretions is of the IgG isotype (104). The serum IgG2a concentration in mice immunized with naked pCMV-OVA increased only a little, as gene expression in the vagina was not sufficiently strong to induce systemic antibodies under these immunization conditions. On the other hand, an increased level of anti-OVA IgG2a was obtained in mice immunized with the Tat and NF-κB analogs/pCMV-OVA complexes. This is because the Tat and NF-κB analogs also significantly promoted vaginal transfection of the pDNA of the marker gene, pLuc. These results suggest that the elevation of antigen-specific systemic antibodies in mice immunized with the Tat and NF-κB analogs/pCMV-OVA complex was probably caused by strong expression of antigen (OVA) in the vaginal mucosa, including in antigen presenting cells, such as dendritic cells.

The secretory IgA levels against OVA in the vaginal secretions (Figure 4) in mice administered naked pCMV-OVA and Tat and NF-κB analogs/pCMV-OVA complexes were found to be significantly increased compared to the control or PBS group, indicating that vaginal DNA immunization under conditions of high transfection efficiency of pDNA strongly induces local vaginal immune responses.

It is important that direct vaginal immunization protocols having strongly vaginal gene expression induce significantly higher production of vaginal IgA than serum IgG2a. These data are consistent with previous studies that demonstrated that the greatest levels of antigen-specific IgA may be generated in regions that are closest to the site of antigen exposure (55). These findings confirm that this phenomenon is also true in DNA immunization. Furthermore, these findings determined that vaginal DNA vaccination by inducing high gene expression in local vaginal tissue can induce the production of both systemic and local antibodies, in particular marked induction of local IgA antibody secretion into the vaginal tract.

Next we determined the OVA-specific IFN-γ (Th1-type cytokine) production from inguinal lymph node (local) and spleen (systemic) cells following co-culture with OVA protein. The levels of IFN-γ from inguinal lymph node cells (Figure 5a) can be used to indicate induction of vaginal Th1-dependent immunity because vaginal mucosal immune tissue belongs to the inguinal lymph nodes. The levels of IFN-γ in mice immunized with naked pCMV-OVA were higher than in control or PBS-administered mice. Furthermore, the peptide carriers Tat and NF-κB significantly enhanced cellular immune activity in the vaginal mucosal membrane. The levels of IFN-γ in the spleen cells were similar to those in the inguinal lymph node cells, indicating induction of systemic immunity (Figure 5b). These findings suggest that local lymph nodes regulate the vaginal tract, and that the systemic Th1-dependent immune responses can be promoted by intravaginal direct immunization with a pDNA vector coupled with peptide carriers such as cell penetrating peptides and nuclear localizing signals.

6. NEEDLE-FREE INJECTORS FOR VAGINAL DNA VACCINATION

We believe that a strong vaginal immune response can be obtained by inducing strong gene expression of antigen-coding DNA vaccines in local vaginal tissue. In order to improve transfection efficiency in antigen-presenting cells in the vaginal subepithelial layer, it is important to breakthrough the various barriers, such as the vaginal epithelial layer, cellular membrane and nuclear membrane. This requires the development of less invasive and more effective delivery methods into the subepithelial layer

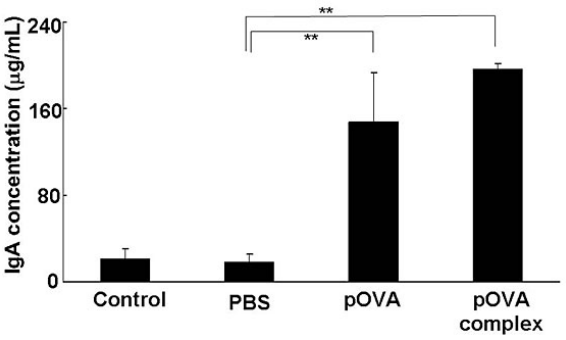

Figure 4. OVA-specific vaginal IgA production after immunization with pCMV-OVA through vaginal mucosa using electroporation. Mice were immunized 3 times at 1-week intervals through the vaginal mucosa at diestrus with PBS, naked pCMV-OVA (pOVA), and Tat and NF-κB analogs/pOVA complexes (denoted pOVA complex) using electroporation (250 V/cm, 5 ms, 15 pulses) with 5% citric acid as an absorption enhancer. Control mice were not treated. Mice were sacrificed 1 week after the final immunization and IgA in rinsed vaginal fluid responses as O.D. were analyzed by ELISA. The concentrations of IgA were calculated using a standard curve established using serially diluted mouse immunoglobulin reference serum (Bethyl Laboratories, Inc, Montgomery, TX, USA). Each bar represents the mean ± S.E. (n=5). ** P <0.01.

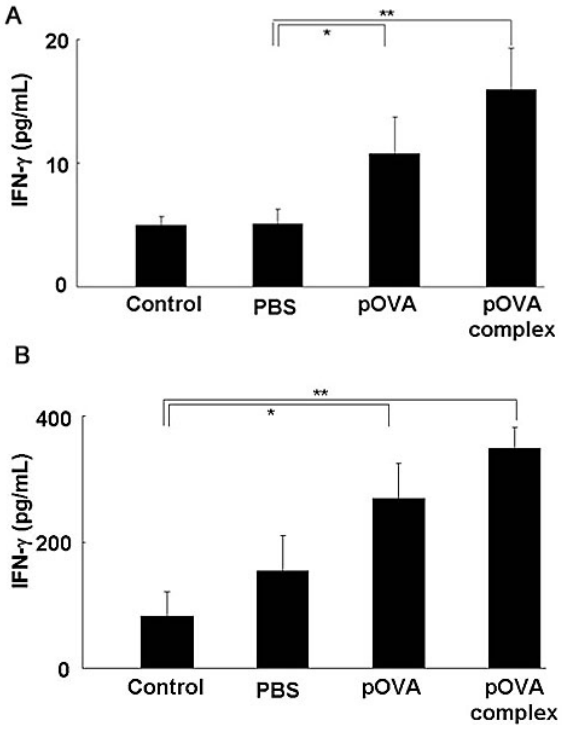

Figure 5. OVA-specific IFN-γ production from inguinal lymph node and spleen cells after immunization with pCMV-OVA through vaginal mucosa using electroporation. Mice were immunized 3 times at 1-week intervals through the vaginal mucosa at diestrus with PBS, naked pCMV-OVA (pOVA), and Tat and NF-κB analogs/pOVA complexes (denoted pOVA complex) using electroporation (250 V/cm, 5 ms, 15 pulses) with 5% citric acid as an absorption enhancer. Control mice were not treated. Mice were sacrificed 1 week after the final immunization. The spleen (a) and inguinal lymph node (b) cells (5 × 105 cells/mL) were prepared and co-cultured with OVA (2 mg/mL) for 72 h. IFN-γ in the conditioned-medium was analyzed by ELISA. Each bar represents the mean ± S.E. (n=5). * P <0.05, ** P <0.01.

Needle-free jet injection has been extensively investigated as a method to immunize laboratory animals, such as mice (105, 106), rabbits (107, 108), pigs and dogs (109) and monkeys, through the transdermal route.

We also confirmed that the luciferase activity in rat skin inoculated with the needle-free jet injector was 300-fold greater than that by needle-syringe injection (112). As shown in Figure 6, the pCMV-Luc solution via the needle-syringe typically forms a sphere of fluid at the injection spot of the tissue, whereas the pCMV-Luc solution through the needle-free jet injector disperses more widely into the dermal tissue, likely due to the high pressure of the fluid stream. This wide distribution by the needle-free injector possibly achieves markedly higher luciferase activity in rat skin. In addition, the luciferase activity following injection by needle-syringe injection with electroporation was higher than that by needle-syringe injection alone, whereas the luciferase activity following administration by the needle-free injector with electroporation did not differ from that by the needle-free injector alone. Thus, needle-free injection provides a similarly wide and effective delivery of pDNA into local tissue cells to electroporation. These results indicate that the needle-free injector can deliver pDNA widely in dermal tissue and might deliver to a number of antigen-presenting cells, which induce immune responses. OVA-specific IFN-γ production as well as OVA-specific IgG2a production levels in mice immunized with the needle-free injector were also significantly greater than those by conventional needle-syringe injection. This was due to a wider distribution of pDNA solution in the dermal tissue injected through the needle-free jet injector, resulting in a higher contact incidence between the pOVA and antigen-presenting cells, such as antigen presenting cells and lymphocytes found in dermal tissue.

However, needle-free dermal vaccination cannot induce local mucosal immunization. Therefore, we next investigated the utility of the needle-free injector as a vaginal vaccination tool using rabbits (112) because we expected that needle-free mucosal vaccination can induce strong mucosal immune responses This injection device, which has an injector angle of 45°, was designed for use in the human vagina from the entrance to inject in a right-angled direction into the middle site of the tract. Gene expression in rabbit vagina using the needle-free injector was significantly greater than that using conventional needle-syringe injection (Figure 7). Moreover, intravaginal vaccination using the needle-free injector significantly promoted vaginal IgA secretion and IFN-γ mRNA

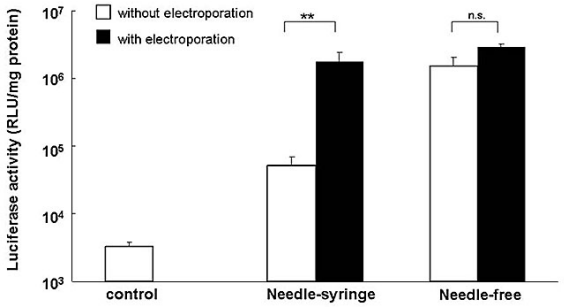

Figure 6. Luciferase activity in rat skin after intradermal injection of pLuc solution by conventional needle-syringe injection or the needle-free injector with electroporation. Luciferase activity in rat skin was determined after intradermal administration of pLuc (20 mg) by conventional needle-syringe injection or needle-free injector, both with electroporation (200 V/cm, 5 ms, 8 pulses). Each bar represents the mean ± S.E. (n=5). **P<0.01, n.s.P>0.05

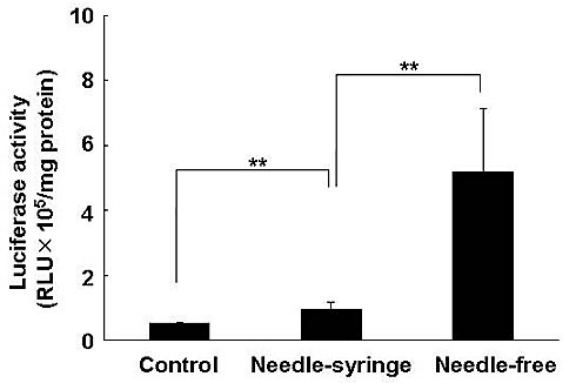

Figure 7. Luciferase activity in rabbit vagina after intravaginal injection of pLuc solution by conventional needle-syringe injection or a needle-free injector. Luciferase activity in rabbit vagina was determined after intravaginal administration of pLuc (100 mg) by conventional needle-syringe injection or a needle-free injector. Each bar represents the mean ± S.E. (n=3). **P<0.01

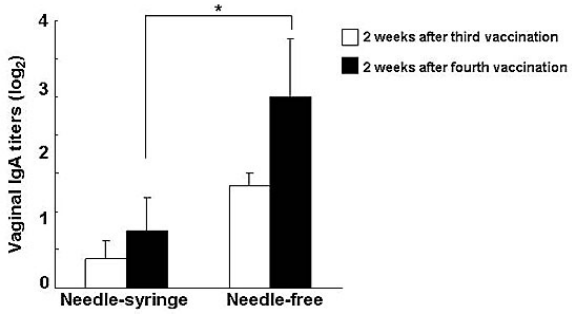

Figure 8. OVA-specific vaginal IgA titer in rabbits after intravaginal injection of pOVA by conventional needle-syringe injection or a needle-free injector. Rabbits were intravaginally immunized three or four times by needle-syringe injection or needle-free injector injection of pOVA solution at 2-week intervals. Vaginal washes were collected 2 weeks after the third or fourth vaccinations and vaginal IgA titers were analyzed by ELISA. Data are expressed as geometric means (log2) of reciprocal dilutions of OVA-specific IgA in vaginal secretions. Each bar represents the mean ± S.E. (n=3-4). *P<0.05

expression in lymphocytes compared to conventional needle-syringe injection. These results demonstrate that the needle-free injector can be used not only as an intradermal vaccination device but also as a mucosal vaccination device. This study has demonstrated for the first time that a needle-free injector can be used for effective local mucosal vaccination. Although we used naked pDNA without a gene vector in this study, the combination of a needle-free injector and effective gene carriers might further promote the effects of vaginal vaccination. Importantly, needle-free vaccine delivery can avoid the risk of transmission of infectious disease between patients or between patients and healthcare providers (113). In conclusion, local vaginal DNA vaccination using a needle-free jet injector is potentially a useful, safe, easy and potent method for the prevention and treatment of mucosal infectious diseases.

7. CONCLUSION

Currently, several treatment methods using protein or DNA vaccination, neutralizing human monoclonal antibody for HIV-1 (115), or stem cells transplantation (116) exist. Of these, a vaccine could be the most attractive therapy against HIV-1 infection. A successful vaccine against HIV and HPV requires strong local CTL activity at the site of viral entry, the vaginal mucosa and draining lymph node, as well as systemically. Even the most promising vaccine formulations may fail to establish protective immunity, if the route of vaccine administration is not optimal for induction of local immune responses in the local mucosa, such as the rectum or vagina. In this review, we introduced our studies into efficient vaginal DNA vaccination methods, focusing on the effects of the menstrual cycle, utilization of the combination of cell penetrating peptide (CPP) and nuclear localization signal (NLS), and use of the needle-free injector. We first described in this review that the transfection efficiency of plasmid DNA into vaginal mucosa is strongly influenced by the estrous cycle with higher luciferase gene expression observed during diestrus. Then, the Tat analog and NF-κB analog synthesized by our laboratory showed statistically significant promotion of the transfection of pDNA, possibly through facilitating cell penetration and nuclear localization. Recently, we developed a new peptide-based gene vector, using arginine, histidine and cysteine, that performs multiple functions, including cellular uptake, endosomal escape and nucleic acids condensation and decondensation by disulfide cross linkage in the cytosol for more effective DNA vaccination. In addition, this vector promotes siRNA silencing effects in vitro (114). In the near future, we will utilize this vector for a vaginal DNA vaccine. We also described here that a strong vaginal immune response will be obtained as a result of strong gene expression of an antigen-coding DNA

vaccine in antigen-presenting cells, including dendritic cells and macrophages, in vaginal tissue, and in order to promote gene expression in the vagina, the menstrual cycle phase, utilization of CPP and NLS and needle-free injection should be considered. DNA vaccination using direct vaginal immunization with functional peptide carriers may be effective in vaginal DNA vaccination. Furthermore, local vaginal DNA vaccination using a needle-free jet injector is potentially a useful, safe, easy and potent method for the prevention and treatment of mucosal infectious diseases.

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