

TITLE:

Effect of mucosal and systemic immunization with virus-like particles of severe acute respiratory syndrome coronavirus in mice

ABSTRACT:

Nasal administration has emerged as a promising and attractive route for vaccination, especially for the prophylaxis of respiratory diseases. Our previous studies have shown that severe acute respiratory syndrome coronavirus (SARS-CoV) virus-like particles (VLPs) can be assembled using a recombinant baculovirus (rBV) expression system and such VLPs induce specific humoral and cellular immune responses in mice after subcutaneous injection. Here, we investigated mucosal immune responses to SARS-CoV VLPs in a mouse model. Mice were immunized in parallel, intraperitoneally or intranasally, with VLPs alone or with VLPs plus cytosine-phosphate-guanosine (CpG). Immune responses, including the production of SARS-CoV-specific serum immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA), were determined in mucosal secretions and tissues. Both immunizations induced SARS-CoV-specific IgG, although the levels of IgG in groups immunized via the intraperitoneal (i.p.) route were higher. sIgA was detected in saliva in groups immunized intranasally but not in groups immunized intraperitoneally. CpG had an adjuvant effect on IgA production in genital tract washes when administered intranasally but only affected IgA production in faeces samples when administered intraperitoneally. In addition, IgA was also detected in mucosal tissues from the lung and intestine, while CpG induced an increased level of IgA in the intestine. Most importantly, neutralization antibodies were detected in sera after i.p. and intranasal (i.n.) immunizations. Secretions in genital tract washes from the i.n. group also showed neutralization activity. Furthermore, VLPs that were administered intraperitoneally elicited cellular immune responses as demonstrated by enzyme-linked immunospot (ELISPOT) assay analyses. In summary, our study indicates that mucosal immunization with rBV SARS-CoV VLPs represent an effective means for eliciting protective systemic and mucosal immune responses against SARS-CoV, providing important information for vaccine design.

Introduction:

Severe acute respiratory syndrome coronavirus (SARS-CoV) is a newly identified coronavirus that causes severe acute respiratory syndrome in humans through respiratory transmission.^{1,2} SARS-CoV is an enveloped, positive-stranded RNA virus, which has four major structural proteins, namely spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins.^{3–5} S protein contains important virus-neutralizing epitopes, and amino acid changes in the S protein can dramatically affect viral virulence. The M protein is the most abundant structural protein, spanning the membrane bilayer three times, and plays a key role in coronavirus assembly. The small E protein is a minor structural component, containing a hydrophobic region flanked by hydrophilic termini.

To prevent another SARS epidemic, continuous efforts have been made towards the development of a prophylactic vaccine. Most of the currently used antiviral vaccines are based on homologous inactivated or attenuated viral particles. However, reversion of an attenuated live vector to a virulent strain by genetic recombination cannot be excluded, precluding the use of this strategy for many pathogens.⁶ Virus-like particles (VLPs) represent a specific class of subunit vaccine that mimics the structure of authentic virus particles. VLPs are safer than inactivated or attenuated viral particles and are more likely to stimulate stronger immune responses than single protein-based vaccines.⁷ Systems for constructing VLPs have been well explored in human immunodeficiency virus (HIV), rotavirus, hepatitis C virus (HCV) and human papillomavirus (HPV).^{8–14} Currently, VLPs as an antigen presenting and delivery system are under investigation in preclinical studies or clinical trials against different human viruses. For example, an effective HPV VLP vaccine was developed and has been used in clinical trials.^{15–17}

Given the highly infectious nature of SARS-CoV, VLPs represent a potential option for SARS vaccine development. We and others have recently demonstrated that SARS-CoV VLPs can be assembled by coinfection with recombinant baculoviruses (rBVs).^{18,19} We also investigated the immunogenicity of SARS-CoV VLPs after administration via subcutaneous injection in mice and found that SARS-CoV VLPs activated immature dendritic cells and enhanced the expression of costimulatory molecules and the secretion of cytokines in vitro.^{20,21}

In addition to the systemic immune responses, mucosal immune responses inducing local antibodies are critical because the mucosa is often the portal for inhaled antigens. To date,

intranasal (i.n.) immunization is emerging as possibly the most effective route for vaccination. Because the transmission route of SARS-CoV is through the mucosal membranes of the eyes, nose, or mouth,² a vaccine to achieve mucosal immunity should be considered as the primary goal for SARS vaccine development. In this study, we evaluated the immunogenicity of **SARS-CoV VLPs** when administered through the mucosal route. Our data demonstrate that **intraperitoneal (i.p.) and i.n. immunization with VLPs, with or without a cytosine-phosphate-guanosine (CpG) adjuvant**, **elicit both systemic and mucosal immune responses in mice.**

Adjuvants ::: Materials and methods:

The CpG oligodeoxynucleotide (ODN) 10104 (TCGTCGTTTCGTCGTTTTGTCGTT) and the non-CpG 2137 (TGCTGCTTTTGTGCTTTTGTGCTT) were purchased from Coley Pharmaceutical Canada (Ottawa, ON, Canada).

Production and purification of SARS-CoV VLPs ::: Materials and methods:

VLPs formed by the S, E and M proteins of SARS-CoV were successfully constructed in our previous study using a baculovirus system.²¹ Briefly, Sf21 insect cells were co-infected with two rBVs – one expressing the S protein and the other expressing the E and M proteins – at a multiplicity of infection of 5. At 4 days postinfection, the culture medium and the cells were collected and freeze-thawed twice to release VLPs. The sample was then centrifuged at 5000 g and the supernatant was filtered through a 0.45 µm pore-size filter. The lysates were pelleted at 150 000 g for 3 hr, placed on a 30–50% (w/w) sucrose density gradient and then centrifuged at 200 000 g for 3 hr. A visible band between the 30 and 40% sucrose layers was collected and pelleted by centrifugation at 150 000 g for 3 hr. The pellets were resuspended in phosphate-buffered saline (BS) and used for immunization. The total protein concentration of VLPs was determined using a Bio-Rad (Hercules, CA) protein assay. The incorporation of SARS-CoV VLPs was determined using electron microscopy and Western blotting.

Immunization protocols ::: Materials and methods:

Female BALB/c mice, 6–8 weeks of age, were purchased from Hubei CDC (Wuhan, China) and maintained in a specific pathogen-free (SPF) environment throughout the experiments. Mice (n=6 per group) were randomly divided into 10 groups. **Immunizations intranasally or intraperitoneally with SARS-CoV VLPs (20 µg), either alone or mixed with CpG ODN or the non-CpG control**, were performed at weeks 0, 2, 4 and 6 (Table 1). All reagents were suspended in 20 µl of PBS, and individual mice received 10 µl, twice, with a 30 min rest interval between each nasal administration of vaccine. Mice were anesthetized slightly with sodium pentobarbital and held in an inverted position with the nose down until droplets of vaccine that were applied to both external nares had been completely inhaled.

Sample collection ::: Materials and methods:

Samples of serum, saliva, vaginal lavage fluids, faeces, intestine and lung were collected 2 weeks after the final immunization from three mice in each group. Blood samples were collected by retro-orbital plexus puncture. Saliva was procured after i.p. injection of 20 µg of carbamylcholine chloride.²² Genital tract fluid was collected by washing with 20 µl of PBS five times per day for 3 days. Faecal extraction was obtained by adding 100 mg of faecal pellets to 1 ml of PBS containing 0.1% sodium azide. Lung and small intestine tissues were weighed, cut into small pieces (2–3 mm long), suspended in extraction buffer (2% saponin–0.1% NaN₃ in PBS), rocked overnight (100 mg of lung in 400 µl of extraction buffer and 100 mg of small intestine in 200 µl of extraction buffer) and supernatants were collected after centrifugation.²³ All samples were stored at –20° before to antibody titration.

Enzyme-linked immunosorbent assay analysis ::: Materials and methods:

Enzyme-linked immunosorbent assay (ELISA) was used to determine the titres of specific antibodies, as previously described with modifications.²⁴ Briefly, 5 µg/ml of inactivated SARS-CoV in 0.1 M carbonate buffer (pH 9.6) was used to coat 96-well microtitre plates (Corning Costar, Acton, MA) at 4° overnight. After the plates were blocked with 1% bovine serum albumin (BSA), a series of diluted samples was added and incubated at 37° for 1 hr, then washed three times with PBS containing 0.05% Tween-20. Specific antibodies were detected by incubation with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) or immunoglobulin A (IgA) (Sigma, St. Louis, MO) at 37° for 1 hr, followed by three washes. The reaction was visualized by addition of the substrate para-nitrophenyl phosphate, and the absorbance at 405 nm was

measured using an ELISA plate reader (Bio-RAD, Hercules, CA). Antibody-positive cut-off values were set as means \pm 2 \times standard deviation (SD) of non-immunized mice (i.e. PBS-immunized mice) and the titre was expressed as the highest serum dilution giving a positive reaction. Values were presented as means \pm SD of three mice of each group.

Neutralization assay :: Materials and methods:

For the neutralization assay, HIV-based pseudoviruses were prepared as previously described.²⁵ In brief, 12 μ g each of pHIV-Luc (pNL4.3.Luc.R-E--Luc) and the S protein-expressing plasmids were cotransfected into 2×10^6 293T cells in 10-cm dishes. The medium was replaced with fresh medium 8 hr after transfection. Pseudotype vector-containing supernatants were harvested at 48 hr post-transfection, clarified from cell debris by centrifugation at 3000 g and filtered through a 0.45 μ m pore-size filter (Millipore, Billerica, MA) before storage at -70° in aliquots for the neutralizing test.

A detailed neutralization assay has been described previously.²⁶ In brief, HeLa-hACE2 cells (2×10^4 cells/well) were seeded into 96-well plates 18 hr before infection. The next day, serum samples were heat inactivated at 56° for 30 min and serially diluted twofold in Dulbecco's modified Eagle's minimal essential medium. A final volume of 30 μ l of the heat-inactivated diluted serum was mixed with 10 ng of pseudoviruses suspended in 30 μ l of Dulbecco's modified Eagle's minimal essential medium, and incubated at 37° for 1 hr. After incubation, 40 μ l of medium containing 16 ng of polybrene was added to 96-well microtitre plates. Following 3 hr of incubation at 37° , serum/virus mixtures were replaced with cell culture medium. The plates were incubated at 37° in the presence of 5% CO₂ for 2 days, and the infection was monitored by measuring luciferase activity, expressed from the reporter gene carried by the pseudovirus, using a luciferase assay system (Promega, Madison, WI). The neutralizing antibody titre was defined as the highest dilution of tested samples that reduced virus infectivity by 50% compared with negative control samples.

Enzyme-linked immunospot assay :: Materials and methods:

Nitrocellulose membranes of 96-well enzyme-linked immunospot (ELISPOT) plates (Millipore, Molsheim, France) were pre-wet with 15 μ l of 70% ethanol, then coated overnight at 4° with 100 μ l of anti-mouse interferon- γ (IFN- γ) or 15 μ g/ml of interleukin (IL)-4 monoclonal antibody (mAb) (Mabtech, Stockholm, Sweden). The antibody-coated plates were blocked with RPMI-1640 containing 10% fetal bovine serum (FBS) for at least 2 hr at room temperature, then 1×10^6 splenocytes in 100 μ l of medium (RPMI-1640 containing 10% FBS, 10 mm glutamine, 100 U/ml of penicillin and 100 μ g/ml of streptomycin) also containing 10 μ g/ml of purified recombinant S protein were incubated for 20 hr at 37° . All stimulation conditions were tested in triplicate, and cell viability was confirmed by adding 4 μ g/ml of concanavalin A (Con A) (Sigma). The plates were washed five times with PBS containing 0.05% Tween, then incubated with 100 μ l of biotinylated anti-mouse IFN- γ or IL-4 mAb (1 μ g/ml in PBS containing 0.5% FBS; Mabtech) for 2 hr at room temperature. After five washes, 100 μ l of streptavidin-horseradish peroxidase reagent was added. Following 1 hr of incubation at room temperature and five subsequent washes, 100 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added for 15 min. The reaction was terminated by discarding the substrate solution and washing the plates under running tap water. After drying, the spots were scanned and counted using ELISPOT image analysis (Biosys, Karben, Germany). The number of spot-forming cells (SFC) per 10^6 splenocytes was calculated.

Statistical analysis :: Materials and methods:

All data are presented as the mean \pm SD. spss 13.0 for Windows was used for statistical analysis. Statistical analysis was assessed using the Student's t-test. A P-value of < 0.05 was considered statistically significant.

Specific IgG response in sera induced by i.n. and i.p. immunization :: Results:

Two weeks after the final immunization, sera from **i.n. and i.p. immunization groups** were collected to detect SARS-CoV-specific IgG induced by VLPs. **Specific IgG levels were greatly enhanced in all groups of mice.** As shown in Fig. 1, **both immunization protocols with VLPs alone or with CpG adjuvant induced SARS-CoV-specific IgG. The IgG level was higher in the i.p. immunization group than in the i.n. immunization group, as expected** ($P < 0.01$). CpG failed to enhance immune responses in the serum samples when administered by either route.

Antibody responses in mucosal secretions :: Results:

Mucosal surface secretory IgA (sIgA) may play an important role in protecting against viral infection, and therefore the specific sIgA levels in different mucosal secretions were assayed using ELISA. In the i.p. immunization group the following results were obtained: no detectable specific sIgA was found in saliva (Fig. 2a); low, but detectable, specific sIgA was found in genital tract washes when the mice were immunized with VLPs alone (Fig. 2b); and VLPs administered with 10 µg of CpG induced a specific sIgA response in faeces (Fig. 2c) ($P < 0.05$). In the i.n. immunization group the following results were obtained: specific sIgA was induced in saliva but there was no significant difference between groups immunized with or without CpG (Fig. 2a); in the absence of adjuvant, VLPs induced specific sIgA in the genital tract ($P < 0.01$ compared with PBS group) and the titres were enhanced ($P < 0.05$) when VLPs were coadministered with 10 µg of CpG (Fig. 2b); specific sIgA levels in faeces, with or without CpG, were lower than those in other mucosal secretions (Fig. 2c).

Antibody responses in mucosal tissues ::: Results:

After boosting twice, SARS-CoV-specific sIgA were determined in mucosal tissues, including lung and intestine. As shown in Fig. 3, sIgA were detectable in lung and intestine from i.p. groups, with or without CpG, and the co-administration of 10 µg of CpG did not enhance the lung response. Following the i.n. administration of VLPs, specific sIgA responses were detected in the lung, and the co-administration of VLPs with 10 µg of CpG enhanced the immune response but it was not statistically significant ($P > 0.05$); there was no significant sIgA response in intestine when VLPs were administered alone ($P > 0.05$) and the sIgA levels were increased when 10 µg of adjuvant was used ($P < 0.05$).

Neutralization antibodies in sera and mucosal secretions ::: Results:

The level of neutralizing antibodies is critical for protection against SARS-CoV. Here we used HIV-based pseudoviruses to determine the anti-SARS-CoV neutralizing activities in serum and in the genital tract. As shown in Fig. 4, sera from intraperitoneally and intranasally immunized mouse groups demonstrated potent anti-SARS-CoV pseudovirus neutralizing activities. Surprisingly, the neutralization titre from i.n. groups that received VLPs alone was five times higher than that from i.p. groups, and was 25 times higher when co-administered with 10 µg of CpG. Additionally, genital tract washes demonstrated a low, but detectable, neutralizing antibody titre, and CpG contributed to the enhanced production of antibodies.

Cellular immune responses in SARS-CoV VLPs-vaccinated mice ::: Results:

The type of cellular immune response that was elicited by mice vaccinated intraperitoneally was evaluated using the ELISPOT assay. As shown in Fig. 5, specific IFN-γ secretions were induced in all experimental groups compared with the control groups ($P < 0.01$). Compared to immunization with VLPs alone, the number of cells secreting IL-4 was 1.5 times higher in mice that were co-administered CpG.

Discussion:

Given the significant health and economic impact of a SARS outbreak, an effective vaccine against epidemic and zoonotic strains of the virus is urgently needed. To date, a variety of candidate vaccines, such as DNA,²⁷ adenovirus-mediated,²⁸ a combination of whole killed virus and DNA,²⁹ inactive virus³⁰ and recombinant proteins or their fragments are under preclinical or clinical study. In addition, VLPs, morphologically mimicking the native virus and capable of eliciting strong immune responses, represent a safe option for vaccine design. We previously reported that SARS-CoV S and N DNA vaccines evoked humoral and cellular immune responses when administered through different vaccination routes.³¹ We also documented that SARS-CoV VLPs could be formed using a baculovirus expression system, and specific SARS-CoV IgG and T helper type 1 (Th1)-based cellular immune responses were achieved after subcutaneous injection of high doses (100 µg) of VLPs into mice.²¹ Because the route of administration of an immunogen, and other variables, such as frequency, dose and timing, could influence the immune response,³² in this study we evaluated the immunogenicity of non-replicating VLPs administered at a low dose (20 µg) by mucosal routes and investigated the effects of CpG as an adjuvant. Our results indicate that VLPs administered via i.p. or i.n. routes induced specific serum IgG, although the antibody titre was lower in the i.n. immunization group (Fig 1), while CpG had little effect in terms of enhancing the IgG response. Additionally, sIgA was detected in mucosal secretions and tissues. A detectable sIgA response existed in genital tract washes and in faeces samples from animals in the i.p. groups immunized with VLPs plus 10 µg of

CpG adjuvant. Mucosal immunity is mainly mediated by sIgA and has been shown to be effectively induced by i.n. immunization. CpGs are potent systemic and mucosal adjuvants in mice and were shown to be potent adjuvants for antigens delivered intranasally.^{33–35} Interestingly, we found that CpG induced lower IgA production compared with antigen alone in the genital tract after immunization via the i.p. route. However, the difference was not statistically significant.

Moderate increases in sIgA levels were detected in saliva, genital tract washes and faeces from i.n. groups. CpG was an effective adjuvant at a dose of 10 µg for the genital tract wash sample. SARS-CoV-specific IgA were also detected in lung and intestine after both i.p and i.n. administration. High IgA levels in lung upon non-mucosal immunization contradict the results of Bessa et al.³⁶ but our results were supported by two studies which suggested that the i.p. route might be effective as an alternative IgA induction route in lung.^{37,38} The inconsistency between the results of Bessa et al. and others may be attributed to differences in the characteristics of the antigens used. Interestingly, in i.n. groups, VLPs alone induced specific sIgA in all samples except for those from the intestine. Given that inactivated SARS-CoV alone through the i.n. route failed to induce any detectable specific IgA in sera, saliva, lung and intestine,³⁸ our results provide evidence that SARS-CoV VLPs are more immunogenic than inactivated viral particles. Although VLPs administered alone can produce immune responses, the co-administration of VLPs with mucosal adjuvants may improve the efficacy of a vaccine candidate. CpG ODN has been explored as a mucosal adjuvant in recent years and may represent a promising adjuvant applicable to humans.^{23,39,40} CpG ODN 10104, containing five CpG islands, has the potential to enhance a specific antibody response. Indeed, in the current study, CpG enhanced mucosal immune responses in genital tract washes and intestine.

The induction of neutralizing antibody is a key criterion of vaccine efficacy evaluation. We observed relatively high titres of neutralizing antibody activity in sera from mice immunized via the i.n.- and i.p. routes and in genital tract washes from the i.n. group. Surprisingly, the serum neutralization antibody induced by VLPs alone from the i.n. group was five times higher than that in the i.p. group, and 25 times higher when CpG adjuvant was used, suggesting that different delivery routes play an important role in antigen uptake. Of interest, we observed higher neutralizing titres in the presence of CpGs, while the titres obtained following ELISA were similar in the presence or absence of CpGs. The detection methodology may account for such differences. ELISA was performed by coating the plates with inactivated SARS-CoV as capture antigen, while HIV-based pseudoviruses were used in the neutralization assay. Therefore, ELISA detected the total antibodies induced by VLPs, whereas only the specific S antibody contributed to the neutralization activity.

In addition to antibody responses, T-cell immune responses were also demonstrated in our study. IFN-γ, a marker for Th1 responses, and IL-4, a marker for T helper type 2 (Th2) responses, were determined using the ELISPOT assay. Our results showed that SARS-CoV VLPs induced a Th1-based cellular immune response in mice immunized intraperitoneally but not in mice immunized intranasally (data not shown). The weak cellular immune responses induced through the i.n. route in the current study are in agreement with the report by Bessa et al. that intranasal immunization with Qβ-VLP stimulated a poor cytotoxic T-cell response.

In summary, the findings in the current study have indicated that SARS-CoV VLPs are capable of inducing systemic and mucosal immune responses after i.n. and i.p. administration in mice and that CpG ODN is an effective adjuvant in some local apparatus, providing a promising strategy for SARS-CoV vaccine development. However, the effects in mice may be more dramatic than those in humans and the way in which the antigen is taken up may be different as a result of the nature of the antigen. Before the development of VLPs as an effective vaccine against SARS-CoV in humans, it needs to be addressed whether the i.n. immunization in mice corresponds to the i.n. immunization in humans.