Effects of Different Immunization Protocols and Adjuvant on Antibody Responses to Inactivated SARS-CoV Vaccine

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ABSTRACT

Severe acute respiratory syndrome (SARS) is a deadly and highly infectious disease caused by SARS Coronavirus (SARS-CoV). Inactivated SARS-CoV has been explored as a vaccine against SARS-CoV; however, current knowledge of inactivated SARS-CoV vaccine is quite limited. We attempted to investigate the effects of different immunization protocols and adjuvant on the antibody responses to inactivated SARS-CoV vaccine. With an intraperitoneal (IP) immunization protocol, inactivated SARS-CoV alone induced significant amounts of SARS-CoV–specific IgG antibodies in sera and a small quantity of SARS-CoV–specific IgA antibodies in the genital tract and feces, but failed to induce any detectable SARS-CoV–specific IgA antibodies in sera, saliva, lung, and intestine, and the addition of CpG ODN 2006 had only a marginal effect on antibody production. In contrast, with an intranasal (IN) immunization protocol, inactivated SARS-CoV alone failed to induce any detectable SARS-CoV–specific IgA antibodies in sera, saliva, lung, and intestine, except for a small quantity of IgA antibodies in fecal extracts and the genital tract, along with IgG antibodies in sera, but when given with adjuvant CpG ODN 2006, inactivated SARS-CoV induced significant amounts of SARS-CoV–specific IgG antibodies in sera, and a detectable amount of SARS-CoV–specific IgA antibodies in sera and all tested mucosal secretions and tissues (i.e., saliva, the genital tract, fecal extract, lung, and intestine). On a neutralization assay, neutralizing activity with the IP immunization protocol was detected in sera and mucosal secretions (from the saliva and genital tract), but sera from the IN protocol failed to show any neutralizing activity. Our study demonstrated that inactivated SARS-CoV vaccine is promising, and our data provide a sound foundation for the development of an effective inactivated SARS-CoV vaccine.

INTRODUCTION

Severe acute respiratory syndrome (SARS) caused by SARS Coronavirus (SARS-CoV) is a deadly disease that killed 775 persons during its first outbreak in 2003 (32). SARS-CoV is likely to strike again because it has been found in nonhuman hosts such as civets and bats (8,15,17). Therefore, an effective vaccine against SARS-CoV is needed to prevent future SARS outbreaks. Like many other mucosally-infecting microorganisms such as influenza, tuberculosis, cold viruses, sexually transmitted diseases, cholera, and diphtheria (23), SARS-CoV was found in many mucosal compartments including lung, intestine, distal convoluted renal tubules, sweat glands, and adrenal glands in SARS patients (12,13,31).
It also has been suggested that SARS-CoV might be transmitted through sexual exposure (16). Thus, an effective SARS-CoV vaccine should be able to initiate mucosal immunoresponses in multiple mucosal compartments simultaneously.

One prominent feature of mucosal immunoresponses is the production of secretory IgA (SIgA). The role of SIgA is supported by many carefully conducted studies in experimental animals and humans (1,2,11). For example, passive transfer of specific monoclonal SIgA antibodies against several kinds of viruses such as influenza virus, rotavirus, respiratory syncytial virus, and poliovirus was able to provide a high degree of protection against reinfection challenge (19,27). It has been suggested that serum antibodies specific to SARS-CoV could be crucial in the prevention of SARS (25,30). There were some attempts to develop an effective SARS-CoV vaccine using inactivated SARS-CoV (29); inactivated SARS-CoV has certain advantages, for instance it is relatively safe and unable to revert to its live form, and it is also relatively stable with no need for refrigeration. In animal studies, inactivated SARS-CoV vaccine yielded potent humoral and cell-mediated immunoresponses in mice and rhesus monkeys (26,28,36). However, in one detailed study of mucosal immunization with inactivated SARS-CoV, Qu et al. failed to detect any SARS-CoV–specific IgA production on ELISA after intranasal administration of inactivated SARS-CoV with CpG oligonucleotides or CTB, even though their results from indirect fluorescence (IF) suggested the presence of SARS-CoV–specific IgA in tracheobronchial lavage fluid (24). Thus more studies are warranted to develop an effective vaccine against SARS-CoV.

In this paper, we report our investigation of the effects of different immunization protocols with inactivated SARS-CoV and stimulatory CpG oligonucleotides on the antibody responses to SARS-CoV. Our ELISA data have shown that use of an intraperitoneal (IP) immunization protocol induced SARS-CoV–specific SIgA in sera, but not in any of the tested mucosal compartments, but that an intranasal (IN) immunization protocol induced SARS-CoV–specific SIgA in both sera and all of the tested mucosal compartments. Furthermore, our neutralization data demonstrated that SIgA from multiple mucosal compartments induced by the IN immunization protocol showed neutralizing activity. Finally, ways to optimize protocols for using inactivated SARS-CoV vaccine are discussed.

MATERIALS AND METHODS

Adjuvant

The adjuvant used in all experiments was the stimulatory CpG oligodeoxyribonucleotide (ODN) 2006 (TGCTGCTTTTGTGCTTTTGTGCTTT) and non-CpG ODN 2006-GC (TGCTGCTTTTGTGCTTTTGTGCTTT) was included as a negative control. Both ODNs were purchased from Coley Pharmaceutical Canada, Ottawa, Ontario, Canada.

Inactivated SARS-CoV

The inactivated SARS-CoV was made from SARS-CoV Z-1 strain virus, isolated from the blood of the first SARS patient from Zhejiang Province, China, in 2003. The inactivated SARS-CoV was provided by Wuhan Institute of Biological Products (Wuhan, China) (14). The methods of virus propagation, virus inactivation, virus purification, and virus quantification are briefly described here. Healthy Vero E6 cells were inoculated by SARS-CoV Z-1 strain and incubated at 37°C for 48 h until the virus titer reached 10^7 TCID50. The culture was harvested after three freeze-thaw cycles and β-Propiolactone was added at 1:2000 (v/v) for inactivation. The mixture was kept at 4°C for 24 h, then the process was repeated to inactivate the virus completely, and the mixture was then passed through an 0.8-μm filter to remove cell debris. The inactivated virus was concentrated with Centriplus YM-100 (Millipore Corp., Billerica, MA), and purified by Sepharose 4 Fast Flow (FF) column chromatography (Amersham Biosciences, Sweden). The total protein content of the sample before and after chromatography was measured using the Lowry method (14).

Immunization protocols and sample collection

Female BALB/c mice, 6–8 weeks of age, were purchased from Hubei CDC (Wuhan, China) and maintained in a specific pathogen-free environment throughout the experiments. The mice were divided into 12 groups with 6 animals per group (n = 6). Six groups of mice were intranasally (IN) immunized with one of these six protocols: PBS (“PBS”), inactivated SARS-CoV (10 μg/mouse) alone (“Ag”), inactivated SARS-CoV (10 μg/mouse) plus CpG ODN 2006 (10 μg/mouse) (“10 μg CpG”), inactivated SARS-CoV (10 μg/mouse) plus CpG ODN 2006 (20 μg/mouse) (“20 μg CpG”), inactivated SARS-CoV (10 μg/mouse) plus non-CpG ODN 2006-GC (20 μg/mouse) (“N CpG”). The remaining six groups were intraperitoneally (IP) immunized using the same protocols: PBS, Ag, 10 μg CpG, 20 μg CpG, 50 μg CpG, and N CpG. For the IN immunization protocol, each mouse was anesthetized slightly with sodium pentobarbital and held with its nose up and the drops of vaccine were applied to both nostrils until they were completely inhaled (9). All intranasally-delivered agents were suspended in PBS, and individual mice each received 10 μL five times with a 30-min resting interval between doses (total volume = 50 μL). For the IP immunization protocol, the...
agent was diluted with PBS to a total of 200 μL. In both protocols, each mouse was immunized three times, at day 0, day 14, and day 28. Details of the immunization protocols are summarized in Table 1.

All samples were collected at the second week after the final immunization. Serum samples were collected by retro-orbital plexus puncture. Fecal extraction was performed by adding 0.1 g of fecal pellets to 1 mL of PBS containing 0.1% sodium azide, followed by vortex mixing and centrifugation at 7000g for 15 min, then the supernatant was collected. Saliva was procured after IP injection of 20 μg of carbachol chloride (34). Genital tract lavage was obtained by washing the genital tract with 40 mL of PBS three times, after which the fluid was combined and centrifuged at 7000g for 15 min, then the supernatant was collected. Small intestines and lungs were obtained on day 42, when all the mice were sacrificed. Small intestines were collected after removal of feces. The lungs were washed with large volumes of PBS three times to remove blood. Then the lung and small intestinal tissues were weighed, cut into small pieces, mixed with a special extraction buffer (2% saponin and 0.1% NaN₃ in PBS), and rocked overnight (100 mg of lung in 400 μL of washing buffer, and 100 mg of small intestine in 200 μL of washing buffer); after that the supernatants were collected. The antibody titer was defined as the highest sample dilution that resulted in an absorbance value at least 2.1 times that of non-immunized mice (PBS-immunized mice). The antibody titer of each group was expressed as the arithmetic mean titer ± standard error (SE) (20).

**In vitro neutralization assay**

Neutralization testing was carried out with pseudotyped SARS-CoV produced by transfecting 293T cells with three expression plasmids. We prepared pseudotyped SARS-CoV as previously described with slight modification (7). Briefly, subconfluently grown 293T cells in one 10-cm-diameter dish were transfected with 15 μg of pMP71-eGFP-pre, 25 μg of pSV-Mo-MLVgagpol, and 8 μg of S protein expression plasmids by using a calcium phosphate transfection method. The medium was replaced 8 h after transfection. Pseudotype vector-containing supernatants were harvested after incubation for an additional 39 h, filtered through a 0.22-μm filter, and concentrated by ultracentrifugation.

**Table 1. Protocols Used to Immunize Mice with Inactivated SARS-CoV**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization route</th>
<th>Inactivated SARS-CoV (μg)</th>
<th>CpG ODN 2006 (μg)</th>
<th>non-CpG ODN 2006-GC (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PBS</td>
<td>IN</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Ag</td>
<td>IN</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. 10 μg CpG</td>
<td>IN</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>4. 20 μg CpG</td>
<td>IN</td>
<td>10</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>5. 50 μg CpG</td>
<td>IN</td>
<td>10</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>6. N CpG</td>
<td>IN</td>
<td>10</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>7. PBS</td>
<td>IP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8. Ag</td>
<td>IP</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9. 10 μg CpG</td>
<td>IP</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>10. 20 μg CpG</td>
<td>IP</td>
<td>10</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>11. 50 μg CpG</td>
<td>IP</td>
<td>10</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>12. N CpG</td>
<td>IP</td>
<td>10</td>
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<td>20</td>
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</table>

Vaccines were diluted to 50 μL for intranasal (IN) immunization and 200 μL for intraperitoneal (IP) immunization. Each mouse was immunized three times, on day 0, day 14, and day 28.


\[ \mu m \text{ pore Millex-HV filter (Millipore, Schwalbach, Germany), then stored at } -70^\circ C. \]

For neutralizing testing, Vero-E6 cells (10^4 cells/well) were added to 96-well plates 18 h before infection. Samples including serum, saliva, and genital tract lavage fluid were pooled and heat-inactivated (56°C for 30 min), then serially twofold diluted with Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, and a final volume of 50 μL was mixed with 50 μL of diluted supernatant of pseudotyped SARS-CoV that contained about 100 infectious particles. The virus-serum mixtures were incubated at 37°C for 2 h and then added to the Vero-E6 cells. Twenty hours later, 100 μL of fresh medium was added after washing twice with DMEM. At day 2 post-infection, infectivity was determined as the number of cells that express GFP. The neutralizing antibody titer was defined as the highest dilution of tested samples that can reduce virus infectivity by 50% compared with negative control samples. Pre-immune samples and samples from mice immunized with PBS were used as negative controls. Human antiserum from convalescent SARS patients was used as a positive control.

\textbf{Statistical analysis}

The statistical significance of the difference between groups was calculated by Student's t-test for two groups, or by Tukey's test using analysis of variance (ANOVA) for three or more groups. Differences were considered significant when \( p < 0.05 \).

\section*{RESULTS}

\textbf{Both IN and IP immunization protocols induced SARS-CoV–specific IgG response in sera, but with differing profiles}

IP immunization is a common method for inducing antigen-specific IgG responses in sera. We first detected SARS-CoV–specific IgG antibodies in the sera from the IP immunized mice by ELISA. As expected, all IP immunized groups (Ag, 10 μg CpG, 20 μg CpG, 50 μg CpG, and N CpG) produced significant SARS-CoV–specific IgG antibodies in their sera as shown in Fig. 1b. In addition, inactivated SARS-CoV alone was capable of inducing serum SARS-CoV–specific IgG responses, and the adjuvant effect of CpG ODN 2006 was marginal (i.e., only a twofold increase of the titer was seen with 50 μg CpG over that of Ag alone [\( p = 0.038 \)]) (Fig. 1b). These data verified that the inactivated SARS-CoV was immunogenic.

An IN immunization protocol is not a common means for effectively inducing IgG responses in sera. As shown in Fig. 1a, the IN immunization protocol with inactivated SARS-CoV alone induced only minimal serum SARS-CoV–specific IgG antibodies, and it was strikingly lower than that seen with the IP immunization protocol (\( p = 0.021 \)). However, with the IN immunization protocol, when inactivated SARS-CoV was given with adjunct CpG ODN 2006, the production of SARS-CoV–specific IgG antibodies was comparable to that seen with the IP immunization protocol, especially for the 50-μg CpG group (\( p = 0.817 \)) (Fig. 1a and b). In addition, the serum IgG titer of the 50-μg CpG group was nearly 50 times that of Ag-alone group (\( p = 0.011 \)) (Fig. 1a). These data suggest that a suitable adjuvant may be required for an IN immunization protocol to elicit serum IgG antibodies effectively.

\textbf{IN but not IP immunization induced a SARS-CoV–specific IgA response in sera}

We next determined the serum levels of SARS-CoV–specific IgA antibodies in all groups. With the IN immunization protocol, inactivated SARS-CoV alone (Ag) or inactivated SARS-CoV plus non-CpG ODN 2006-GC (N CpG) did not induce any detectable SARS-CoV–specific IgA antibodies, but when the inactivated SARS-CoV was given with adjuvant CpG ODN 2006 (10 μg CpG, 20 μg CpG, and 50 μg CpG), significant amounts of SARS-CoV–specific IgA antibodies were detected compared to those seen with antigen alone (\( p < 0.05 \)) (Fig. 1c). In contrast, with the IP immunization protocol, no serum SARS-CoV–specific IgA antibodies could be detected in most of the study groups, while only small amounts of SARS-CoV–specific IgA antibodies were detected in the 10-μg CpG and N CpG groups (Fig. 1d).

\textbf{IN but not IP immunization protocol induced significant SARS-CoV–specific SIgA responses in mucosal secretions}

Secretory IgA (SIgA) antibodies on mucosal surfaces contribute to protection against viral infection via immune exclusion, intracellular neutralization, and immune excretion (11,18,21,35). Using ELISA we next determined the titers of SARS-CoV–specific SIgA antibodies in three mucosal secretions (i.e., saliva, genital tract lavage fluid, and fecal extraction). With the IN immunization protocol, inactivated SARS-CoV alone (Ag) or inactivated SARS-CoV plus non-CpG ODN 2006-GC (N CpG) did not induce significant detectable SARS-CoV–specific IgA antibodies, but when the inactivated SARS-CoV was given with adjuvant CpG ODN 2006 (10 μg CpG, 20 μg CpG, and 50 μg CpG), significant amounts of SARS-CoV–specific IgA antibodies were detected in saliva (Fig. 2a) (\( p < 0.05 \)), genital tract lavage fluid (Fig. 2c) (\( p < 0.05 \)), and fecal extracts (Fig. 2e) (\( p < 0.05 \)). In contrast, with the IP immunization protocol, no SARS-CoV–specific SIgA antibodies were de-
ANTIBODY RESPONSES TO INACTIVATED SARS-COV VACCINE

Intranasal (IN) but not IP immunization protocol induced SARS-CoV-specific IgA response in mucosal tissues

The lungs are the major target for infection of SARS-CoV, and the gastrointestinal tract is also a possible site for harboring SARS-CoV (12,13,16,31). Using ELISA we next determined the SARS-CoV–specific IgA antibody titers in two types of mucosal tissue, the lung and intestine. With the IN immunization protocol, inactivated SARS-CoV alone (Ag) or inactivated SARS-CoV plus non-CpG ODN 2006-GC (N CpG) did not induce significant detectable SARS-CoV–specific IgA antibodies, but when the inactivated SARS-CoV was given with adjuvant CpG ODN 2006 (10 μg CpG, 20 μg CpG, and 50 μg CpG), significant amounts of SARS-CoV–specific IgA antibodies were detected in lung (Fig. 3a) and intestinal tissues (Fig. 3c). Interestingly, the 50-μg dose of CpG ODN 2006 dramatically enhanced the production of SARS-CoV–specific IgA antibodies in lung and intestine. In contrast, with the IP immunization protocol, no significant SARS-CoV–specific IgA antibodies were detected in lung (Fig. 3b) and intestine (Fig. 3d).

The IN immunization protocol induced neutralizing activities in mucosal secretions, but the IP immunization protocol induced neutralizing activities in sera

Neutralizing activities are key components in the protective immune responses to viral infections. We next...
FIG. 2. SARS-CoV–specific SIgA responses in mucosal secretions. BALB/c mice were immunized using the same protocols as those described in Fig. 1. Mucosal secretions (i.e., saliva, genital tract lavage fluid, and fecal extracts) were collected at day 42 post-inoculation. SARS-CoV–specific IgA titers were determined using ELISA. Each bar represents the arithmetic mean titer ± SE of each group for SARS-CoV–specific IgA. (a) SARS-CoV–specific IgA in saliva from animals receiving the IN immunization protocol. (b) SARS-CoV–specific IgA in saliva from animals receiving the IP immunization protocol. (c) SARS-CoV–specific IgA in genital tract lavage fluid from animals receiving the IN immunization protocol. (d) SARS-CoV–specific IgA in genital tract lavage fluid from animals receiving the IP immunization protocol. (e) SARS-CoV–specific IgA in fecal extracts from animals receiving the IN immunization protocol. (f) SARS-CoV–specific IgA in fecal extractions from animals receiving the IP immunization protocol.
tried to assay the neutralizing activities of sera and mucosal secretions. At the time we were carrying out this study, the use of live SARS-CoV for neutralization assay testing was prohibited in China; thus the neutralization experiments were carried out by an in vitro neutralization assay based on a SARS-CoV pseudovirus system (7).

With the IP immunization protocol, only sera were used for the neutralizing assay, and mucosal secretions and samples from lung and intestine were not included because IgA, the only antibodies found in mucosal samples, was not detected. As shown in Fig. 4a, sera from the Ag and 50-μg CpG groups had neutralizing titers of about 2000 and 6000, respectively. Sera from convalescent human patients showed a neutralizing titer of about 1000, which is in accordance with previous reports (22). As mentioned above, the IP immunization protocol induced SARS-CoV–specific IgG, but not IgA antibodies in sera. This suggests that the neutralizing activities result from these IgG antibodies.

With the IN immunization protocol, sera, mucosal secretions (i.e., saliva, genital tract lavage fluid, and fecal extracts), and samples from mucosal tissues (i.e., lung and intestine) were tested for their neutralizing activities, but fecal extracts and samples from lung and intestine were detrimental to the cells, so the neutralizing assay could not be carried out. As shown in Fig. 4b, saliva and genital tract lavage fluid demonstrated low but reproducible neutralizing titers. However, sera from the Ag and 50-μg CpG groups had no detectable neutralizing activity; this is puzzling because we have shown that the IN immunization protocol induced significant SARS-CoV–specific IgG and IgA antibodies in the sera of 50-μg CpG group.

**DISCUSSION**

In the present study we attempted to explore two aspects associated with the development of an effective in-

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**FIG. 3.** SARS-CoV–specific SIgA response in mucosal tissues. BALB/c mice were immunized as described in Fig. 1. Mucosal tissues (i.e., lung and intestine) were collected on day 42 post-inoculation. SARS-CoV–specific IgA antibody titers were determined using ELISA. Each bar represents the arithmetic mean titer ± SE of each study group for SARS-CoV–specific IgA. (a) SARS-CoV–specific IgA antibodies in lung from animals in the IN immunization protocol. (b) SARS-CoV–specific IgA antibodies in lung from animals in the IP immunization protocol. (c) SARS-CoV–specific IgA antibodies in intestine from animals in the IN immunization protocol. (d) SARS-CoV–specific IgA antibodies in intestine from animals in the IP immunization protocol.
activated SARS-CoV vaccine. One aspect was how different immunization protocols affected the induced antibody response profiles. Our data have definitively shown that intranasal and intraperitoneal immunization protocols induced different antibody responses; the IP immunization protocol induced SARS-CoV–specific IgG antibodies in sera and SARS-CoV–specific IgA antibodies in sera, saliva, genital tract lavage fluid, fecal extracts, lung, and intestine. Furthermore, neutralizing activities were detected in sera from animals in the IP immunization protocol, and saliva and genital tract lavage fluid from animals in the IN immunization protocol. Another aspect we examined was whether CpG ODN was a suitable adjuvant for inactivated SARS-CoV vaccine. Our data demonstrated that CpG ODN 2006 was effective in enhancing IgG and IgA production in animals in both immunization protocols, and that the adjuvant effects of CpG ODN 2006 were more prominent in animals in the IN immunization protocol.

Since SARS-CoV is being spread among non-human hosts, the lurking danger of another SARS outbreak is real and imminent; thus an effective vaccine against SARS-CoV is badly needed (2). Among the candidates for SARS vaccine development, inactivated SARS-CoV vaccine is the most promising one because it is relatively safe and stable. An inactivated SARS-CoV vaccine with alum adjuvant is scheduled to soon enter phase 2 human clinical trials in China, and so far no clinical complications have been observed (33). Nonetheless, many aspects of an inactivated SARS-CoV vaccine needed to be explored.

CpG ODN is one type of promising mucosal adjuvant. The adjuvant activity of CpG ODN has different effects on innate and adaptive immune responses, including stimulation of B cells to proliferate and secrete immunoglobulin, improvement of antigen presentation by upregulating co-stimulatory and MHC class II molecule expression, and activation of monocytes, macrophages, and dendritic cells to secrete IFN-α and IFN-β, IL-6, IL-12, GM-CSF, chemokines, and TNF-α. CpG ODN has been shown to be an effective adjuvant for vaccines against a variety of bacteria, viruses, fungi, and parasites (4).

In view of CpG ODN’s mucosal adjuvant activity, we chose CpG ODN 2006 as the adjuvant for our experiments for the following reasons. First, CpG ODN 2006 has strong stimulatory activities due to the four CpG islands in its sequence. This is in contrast to the CpG ODN 1668 used in the study by Qu et al. (24), which contains only one CpG island. Second, more and more studies demonstrate that CpG ODN is a potential mucosal adjuvant. It can significantly increase the specific antibody response at different sites when co-immunized mucosally (6,9,10). Finally, CpG ODNs might be the next adjuvants used in humans, so the results from their study should be more relevant to the development of an inactivated SARS-CoV vaccine for humans.
It is now widely believed that the mucosal immune system can only be activated by direct antigenic challenge at a mucosal surface, whereas an IP immunization protocol induces only a serum, but not a mucosal, antibody response. Our ELISA data showed that high titers of serum IgG could be induced by an IP immunization protocol with inactivated SARS-CoV alone (Fig. 1b). This is in accordance with data from other studies (14, 28). In contrast, an IN immunization protocol with inactivated SARS-CoV alone failed to induce serum IgG or IgA (Fig. 1a and 1c). With the addition of CpG ODN 2006, the IN immunization protocol induced SARS-CoV–specific IgA antibodies in sera, saliva, genital tract lavage fluid, fecal extracts, lung, and intestine that were detectable by ELISA. This is contrary to the results of Qu et al., who failed to detect any SARS-CoV–specific IgA antibodies in their experiments by ELISA (24). This discrepancy may be caused by several factors. For example, the antigen preparations used in the two ELISA protocols were different (inactivated SARS-CoV in our experiments versus the crude lysates of SARS-CoV–infected Vero cells in the experiments of Qu et al.). More importantly, the CpG ODN 2006 used in our study has four CpGs, meaning it has high potency in inducing immune responses. However, whether more CpGs in an oligodeoxyribonucleotide increase its potency needs further study. Our results have also apparently demonstrated that a suitable adjuvant is necessary for an inactivated virus vaccine to effectively elicit systemic and mucosal antibodies via an IN immunization protocol. While CpG ODN 2006 showed excellent adjuvant activity in our experiments, future experiments exploring other adjuvants would be helpful in providing effective alternatives.

SIgA has long been known to act as a mucosal barrier to infection by preventing attachment of viruses to epithelial cells, and in vivo experimental studies have demonstrated that virus-specific IgA antibodies can protect the host from infection and help resolve chronic infection (35). Our study demonstrated for the first time that inactivated SARS-CoV with CpG ODN 2006 as adjuvant, when given via an IN immunization protocol induced distant humoral immune responses in different mucosal compartments, such as the mouth (saliva), small intestine, and genital tract, but the IP immunization protocol failed to induce such immune responses in mucosal compartments. The mucosal immune response with specific IgAs at different mucosal sites can prevent virus colonization and attachment to mucosal surfaces during the initial phase of infection, and in all mucosal compartments. Our data also support the concept of the existence of a “common mucosal immune system,” meaning that administration at a single mucosal site can induce an immune response at other mucosal surfaces and systemic tissues (5), via the communication pathways among the various compartments of the immune system.

Whether the effective induction of mucosal IgA antibodies in diverse mucosal compartments is useful depends on the neutralizing activities of the IgA antibodies induced. Our data showed that neutralizing activity was detected in sera from groups receiving the IP immunization protocol, and saliva and genital tract lavage fluid from groups receiving the IN immunization protocol. The neutralizing assay for fecal extracts and samples from lung and intestine needs to be optimized, because we failed to produce any meaningful results with the protocols we used for this study. However, our findings did include one surprise, namely that we failed to detect neutralizing activity in the sera from groups receiving the IN immunization protocol, even though the sera contained significant amount of SARS-CoV–specific IgG and IgA antibodies based on our ELISA results. It raises an interesting possibility, that perhaps the antibodies in sera and mucosal compartments when using an IN immunization protocol might have different antigenic epitope profiles. Our data suggest that the antibodies from mucosal compartments have epitope specificity against pseudotyped SARS-CoV, but the antibodies from sera do not. In addition, this is in contrast to the results from Qu et al., namely that neutralizing activity was detected in the sera when an IN immunization protocol was used with inactivated SARS-CoV alone. This apparent discord may be due to the different neutralizing assays used in the two sets of experiments (live SARS-CoV was used in the experiments of Qu et al., versus the pseudotyped SARS-CoV we used). It should be noted that the ELISA experiments of Qu et al. failed to detect any SARS-CoV–specific IgA or IgG antibodies in the sera from groups receiving IN immunization. Thus the neutralizing activity detected in their assays was more likely caused by factors other than IgG or IgA. Our data on neutralizing activity suggest that for an inactivated SARS-CoV vaccine to be effective, more than one immunization protocol should be used; a combination of two or more immunization protocols should be explored to improve the effectiveness of the vaccination. On the other hand, although neutralization of SARS-CoV via antibodies in serum and secretory IgA in mucosal surfaces would likely protect the body from infection, it is important to note that anti-virus IgA responses may contribute to protection against infection in the absence of classical neutralizing activity via immune exclusion, intracellular virus neutralization, and transepithelial transport of immune complexes. The mucosal immune response, with or without traditional neutralization activity by mucosal immunization, may play a much more important role in early protection from virus entry via the mucosal epithelial barrier.
CONCLUSION

In summary, our study is another advance toward the development of an effective inactivated SARS-CoV vaccine, providing a better understanding of two critical aspects of vaccine development. First, our data suggest that a combination of intranasal and intraperitoneal immunization protocols might be desirable for providing serum and mucosal neutralizing activity against SARS-CoV, because the IP protocol induced neutralizing activity in sera, and the IN protocol induced neutralizing activity in mucosal compartments. This finding suggests that other immunization protocols such as intravenous immunization and subcutaneous immunization should be investigated, so the optimal combination of these different protocols can be developed. Second, our data demonstrated that CpG ODN 2006 was an effective adjuvant, especially when used in the IN immunization protocol. Because of the diverse population of CpG ODNs, future studies using different CpG ODNs or combinations should be carried out. Our data suggest that CpG ODNs with strong stimulatory activities are more effective. Our study has thus laid a solid foundation and provided a clear direction for the development of an effective inactivated SARS-CoV vaccine.

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