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Intranasal immunization with inactivated SARS-CoV (SARS-associated coronavirus) induced local and serum antibodies in mice

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Abstract

SARS-CoV (severe acute respiratory syndrome-associated coronavirus) strain GZ50 was partially purified and inactivated with 1:2000 formaldehyde. In cell culture the inactivated virus blocked the replication of live virus by decreasing the $TCID_{5.0}$ of the live virus $10^{3.6}$ to $10^{4.6}$ times. Inactivated GZ50 was used to immunize mice intranasally either alone, or after precipitation with polyethylene glycol (PEG), or with CpG, or CTB as an adjuvant. The titer of serum neutralizing antibodies was up to 1:640. In mice immunized with adjuvants or PEG precipitated GZ50, specific IgA was detected in tracheal-lung wash fluid by immunofluorescence. Though serum antibodies were detected, no anti-SARS-IgA could be detected in mice immunized only with inactivated GZ50. The roles of adjuvants in intranasal immunization with inactivated. SARS-CoV is discussed.
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Keywords: SARS-CoV; Intranasal immunization; Inactivated vaccine

SARS (severe acute respiratory syndrome) is caused by a new coronavirus provisionally termed SARS-associated coronavirus (SARS-CoV). The etiological relationship and genomic sequences of SARS-CoV have been independently reported by various groups [1–5]. This indicates that this virus does not belong to any of the previously defined groups of the coronaviridae and should be assigned as a fourth group in the coronaviridae.

As SARS-CoV is highly infectious, and its origin is still not clearly identified, effective vaccines for protecting the population are urgently needed. Among all the possible approaches to developing vaccines against SARS, inactivated SARS-CoV vaccine ranks at the top of the list, because of the high replication competency of this virus in cell cultures [1], well-established inactivation processes with other coronaviruses and previous success in using the inactivated feline coronavirus vaccine for prevention of this disease [6]. To date, the pathogenesis of SARS has not yet been fully studied, however, the possible roles of host anti-SARS-CoV immune responses have been suggested in severe clinical cases [7]. In addition, antibody-mediated enhancement in feline coronavirus infection has been documented [8]. The risk of a SARS-CoV antibody enhancement phenomenon mediated by inactivated vaccine induced antibodies in vaccines need to be seriously considered. Intranasal immunization using an inactivated SARS-CoV vaccine could be effective both by blocking the live SARS-CoV at the site of entry and inducing antibodies in the respiratory tract and in serum. Besides, if virus infection can be blocked at the site of entry, there

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may be less risk for the vaccines to develop antibody enhancement phenomenon. Herein, we report the experimental immunization of mice by inactivated SARS-CoV in mice. Specific IgA was detected in tracheal-lung wash fluid and neutralizing antibodies in serum in intranasally immunized mice.

1. Methods and materials

1.1. Virus strains and inactivation of SARS-CoV

SARS-CoV strain GZ50 (GenBank accession number AY304495) was isolated from the nasopharyngeal wash fluid of a female patient who suffered from SARS in Guangzhou, late February 2003. The strain was first isolated using FRhK4 cell line and was further passaged in Vero cells. After inoculation of this virus at 10^5 $TCID_{50}$ per T25 flask (Greiner Labortechnik, Germany), CPE was detected as early as 24 h and peaked at 72 h. Serial passages of GZ50 strain in Vero cells consistently yielded CPE and the virus titer was between $10^{6.5}$ and 10^7 $TCID_{50}$. Full-length sequencing and phylogenetic analysis showed that GZ50 laid between the reported Hong Kong strains, the Canadian and US strains [9]. To study whether it shared antigenicity with virus strains from other cities in China, acetone fixed GZ50-infected cells were used to react with convalescent sera from Hong Kong, Guangdong and Shanghai patients. All convalescent sera showed a similar positive titer by indirect immunofluorescent assay (data not shown).

Formaldehyde (37%, Sigma) at 1:2000 concentration at 4 °C for 72 h completely inactivated GZ50. Crude inactivated virus solution was spun at 38,000 rpm for16 h with 20% sucrose cushion, and the precipitate was resuspended in PBS. Inactivation of the virus was confirmed by using 100 times concentrated formaldehyde treated virus (viral copy number was 2.3×10^9/ml) to inoculate Vero cells. When no CPE was detected, cell supernatants were blindly passaged for three passages. Cell cultures were fixed with cold acetone and stained with SARS antibody positive convalescent serum by indirect immunofluorescent assay and no positively stained cells were found.

Inactivated influenza type A/panama/2007 virus strain **(**H3N2), the licensed vaccine currently used in human in China, (provided without adjuvant by Shanghai Institute of Biological Products) served as the negative control for the blocking assay of live SARS-CoV replication.

1.2. Blocking of inactivated virus versus live virus in cell culture

To study whether formaldehyde-inactivated GZ50 retains its binding sites versus cell receptors, we examined the blocking effect of inactivated SARS-CoV against the replication of live SARS-CoV viruses in cell culture. Vero cells were cultured in 96-well plates and treated with 100 μl per well of inactivated GZ50 virus solution at 1:10, 1:100, 1:1000 and 1:10,000 dilutions in culture medium. The neat concentration of the virus pool used was 10^7 copies of viral genome/ml (assayed by a real-time PCR, diagnostic kit provided by DaAn Co, Guangzhou). Wells were treated with inactivated viruses in triplicates for 1 h at 36.5 °C, while controls were cells treated only with the culture medium. One hundred microliters of live GHGZ virus (10^7 copies of viral genome/ml) at 10^{-1} to 10^{-7}dilutions were added to the inactivated virus treated wells and the control wells, and CPE was recorded for up to 72 h after the inoculation of live virus. At 72 h, culture medium was decanted and treated with 100 μl of 10% formaldehyde for 30 min, followed by staining with Coomasie Blue for 2 h. All manipulations were done in a BSL-3 hood in the BSL-3 laboratory. The blocking effect of inactivated virus was judged visually. Intact living cells should be stained blue, while cells with CPE could not be stained.

1.3. Inactivated virus and adjuvants used for immunization

The quantity of protein in the inactivated virus was determined at 260 nm by spectrophotometry. Eighty micrograms of inactivated virus was used per mouse for subcutaneous (s.c.) immunization with alum as the adjuvant. For intranasal (i.n.) immunization, 50 μg of inactivated viruses in 30 μl of phosphate buffer saline (PBS) was used per mouse with or without adjuvant. The adjuvant used for i.n. immunization was either phosphorothiate-modified CPG oligonucleotide #1668 (5′-TCCATGACGTTCTGAGCTTCCTGATGCT 3′) [10] (**s**ynthesized and purified by SBS Gentech Co. LTD Shanghai, China) 1 μg/mouse, or cholera toxin B (CTB, Sigma) 10 μg/mouse.

The polyethylene glycol (PEG mw 6000) was used to precipitate the inactivated virus, and was adjusted to 25 μg in 20 μl of PBS for intranasal immunization.

1.4. Intranasal immunization of mice

Balb/c mice (18–20 g, male) were used in all experiments. Mice underwent light ether anesthesia were immunized intranasally with 15 μl of inactivated virus or with 15 μl of inactivated virus containing adjuvant to each nostril. For PEG-precipitated inactivated virus, 10 μl was delivered into each nostril. Groups of mice were immunized totally four times and serum anti-SARS-CoV was measured by neutralization tests. Two weeks after the last boosting, mice were sacrificed and tracheal-lung wash fluid was collected by infusion of the tracheal-lung tract with 1 ml of PBS per mouse, diluted at 1:5 and checked for anti-SARS-CoV IgA by indirect immunfluorscence (IF). Mice, which were not immunized, served as controls and non-infected Vero cell controls were included in all IF studies. Groups of mice immunized with different adjuvants and protocols for immunization are listed in Fig. 1.

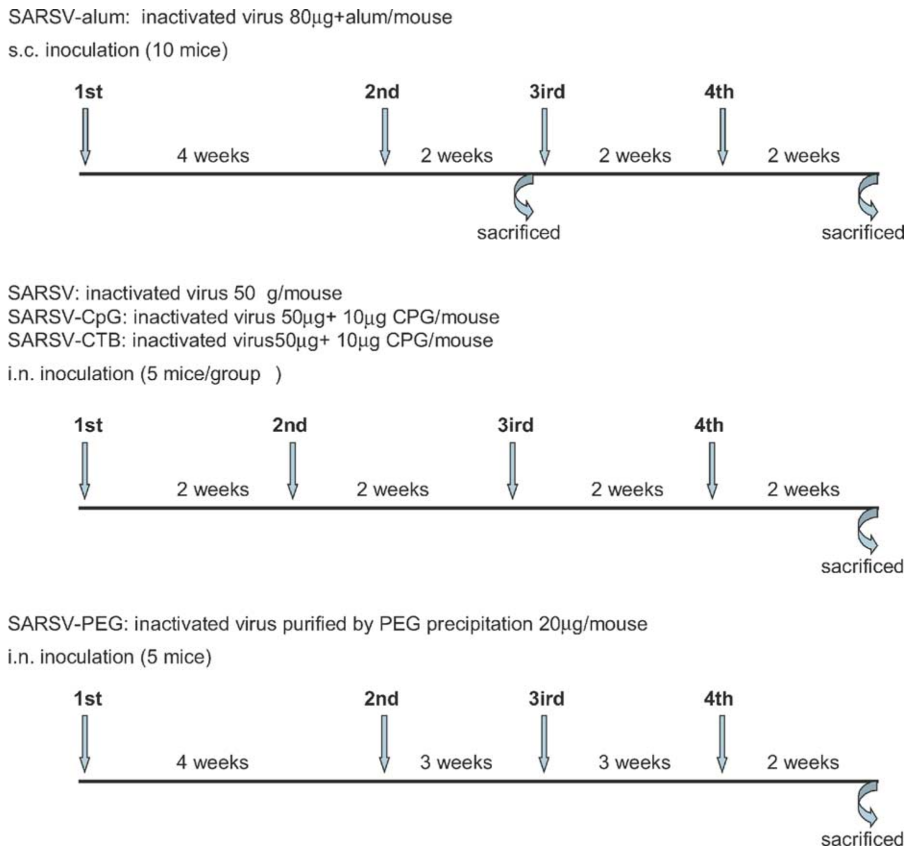


Fig. 1. The immunization protocols of inactivated SARS-CoV The protocols of inactivated SARS-CoV immunization of different groups of mice (five mice in each group) are shown in this diagram. All groups were immunized four times, at 2–4 weeks intervals. s.c. represents immunized subcutaneously. i.n. represents immunized intranasally. Adjuvants used were CPG ODN, cholera toxin B subunit (CTB). PEG-SARS-CoV represents polyethylene glycol-precipitated inactivated SARS-CoV.

1.5. Assays for SARS-CoV antibodies

Three different methods were used, namely indirect immunofluoresence, neutralization test and ELISA. For indirect IF, SARS-CoV infected cells were fixed on slides, mouse sera or tracheal-lung wash fluids were added to the slides, incubated at 37 °C for 30 min and FITC-labeled anti-mouse IgG or IgA was used as the second antibodies. A convalescent serum from SARS patient, confirmed by neutralization test was used as the positive control, while uninfected cells and non-immunized mouse serum, tracheal-lung wash fluid served as the negative controls. Some serum samples were absorbed with packed Vero cells prior to IF staining in order to decrease the nonspecific background staining. Positive staining was judged by the intensity of fluorescence of the cells and was graded 3,2,1 ± and − (negative) accordingly.

Microtiter plates were used in the neutralization assay. Serial 2-fold dilutions of serum samples were separately mixed with 100 $TCID_{50}$ of virus (GZ50), incubated at 37 ° for 1 h and added to Vero E6 cells. Sera from non–immunized mice were used as the negative control. In each assay a virus back-titration (virus in serials 2-fold dilution with medium), virus positive control (100 $TCID_{50}$) and negative cell controls with medium in parallel with the neutralization test were included. Each dilution of serum or virus control was tested in quadruplicates. Results were observed daily and CPE endpoints were read and recorded up to 3 days after virus inoculation. The $TCID_{50}$ was calculated by the Reed-Muench method. The titer of neutralization antibody was determined based on the highest dilution of each serum, which completely suppressed CPE induced by the virus in at least 2- of 4-wells.

For ELISA, a commercial available ELISA kit manufactured by HuaDa Co. (Beijing, China) was used. The antigen used in this kit was a crude lysate of SARS-CoV infected Vero cells and labeled anti-mouse Ig was used as the second antibodies in these assays. All procedures were carried out according to the directions recommended by the manufacturer.

2. Results

2.1. Blocking effects of inactivated GZ50 on live virus replication

The blocking effects of inactivated viruses versus live virus replication are shown in Table 1. Compared to the control live virus infected cells, inactivated virus (at 1:1000 di-

Table 1
Blocking effects of SARS-CoV strain GHGZ versus live virus replication in Vero cells

Experiment 1 dilutions of virus	Dilution of inactivated virus												Virus back titration					
	1:10			1:100			1:1000			1:10000								
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
10^{-3}	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
10^{-4}	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
10^{-5}	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
10^{-6}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
10^{-7}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Uninfected	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Experiment 2																		
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+
10^{-4}	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
10^{-5}	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
10^{-6}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
10^{-7}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Uninfected	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+: Cytopathic effects observed, −: no cytopathic effects detected.

lution, 10^6 $TCID_{50}$) treated cells resulted in a $10^{3.6}$ to $10^{4.6}$ times fall in $TCID_{50}$. In contrast, inactivated influenza type A virus showed no blocking effect on the replication of live GZ50 virus in cells. The $TCID_{50}$ of GZ50 in influenza type A virus treated Vero cells was the same as that of the control non-treated cells. In formaldehyde (diluted according to the preparation of inactivated virus) mock-treated cells, no blocking effect on live virus replication was observed.

2.2. *Antibody responses in immunized mouse sera and lung-wash fluid*

After two subcutaneous injections of 80 μg of inactivated virus, only low titer of ELISA antibody (1:8) was detected in three out of five mice of the s.c. group, while sera from the i.n. groups were all negative. However, when sera from the s.c. and from the i.n. groups were assayed by neutralization test (NT), all showed positive results. Since NT was more sensitive that ELISA, sera from all groups of mice after the fourth immunizaation were compared in one NT assay and the results are shown in Fig. 2. All s.c. mice developed high titers of neutralizing antibodies, the highest being 1:1280. After four doses of i.n. immunization of inactivated virus with and without adjuvant, substantial levels of serum neutralizing antibodies were detected in all the mice. Due to the viscosity of PEG precipitated inactivated SARS-CoV, although a lower dosage of virus was used for i.n. immunization, the serum neutralizing antibody titer was 1:160 in all mice of this group.

When tracheal- lung-wash fluid was tested for anti-SARS IgA by IF, no positive staining was detected in the group immunized only with inactivated virus. However, strong IF staining at 1:5 dilution was shown in all groups of mice immunized with the virus plus the adjuvants, and in mice immunized with PEG- inactivated virus (Fig. 3).

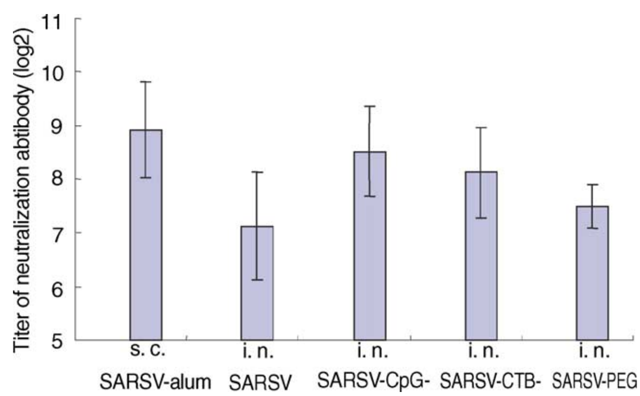


Fig. 2. The titer of anti-SARS-CoV neutralization antibody in sera from immunized mice The bars represent the neutralizing titer and standard deviation of each group of immunized mice. s.c., i.n., CPG, CTB are similar to those as stated in Fig. 1.

3. Discussion

To date, several approaches for developing SARS vaccines have been described, including subcutaneous immunization with inactivated vaccines [11], expression of recombinant spike protein in adenovirus [12], and by use of synthetic oligonucleotides coding for the spike proteins for producing recombinant immunogen. In this study, formaldehyde inactivated SARS-CoV was used to immunize mice intranasally. Though the inactivated virus was only partially purified, experimental immunization in mice yielded interesting results. Since SARS-CoV is a newly described virus and intranasal delivery of this inactivated virus has not been explored; the aim of this study was to investigate whether this approach of immunizatioin could induce serum antibodies and local antibodies. To ensure that enough stimulus was given to the mice, four doses of immunization were used prior to sacrific-

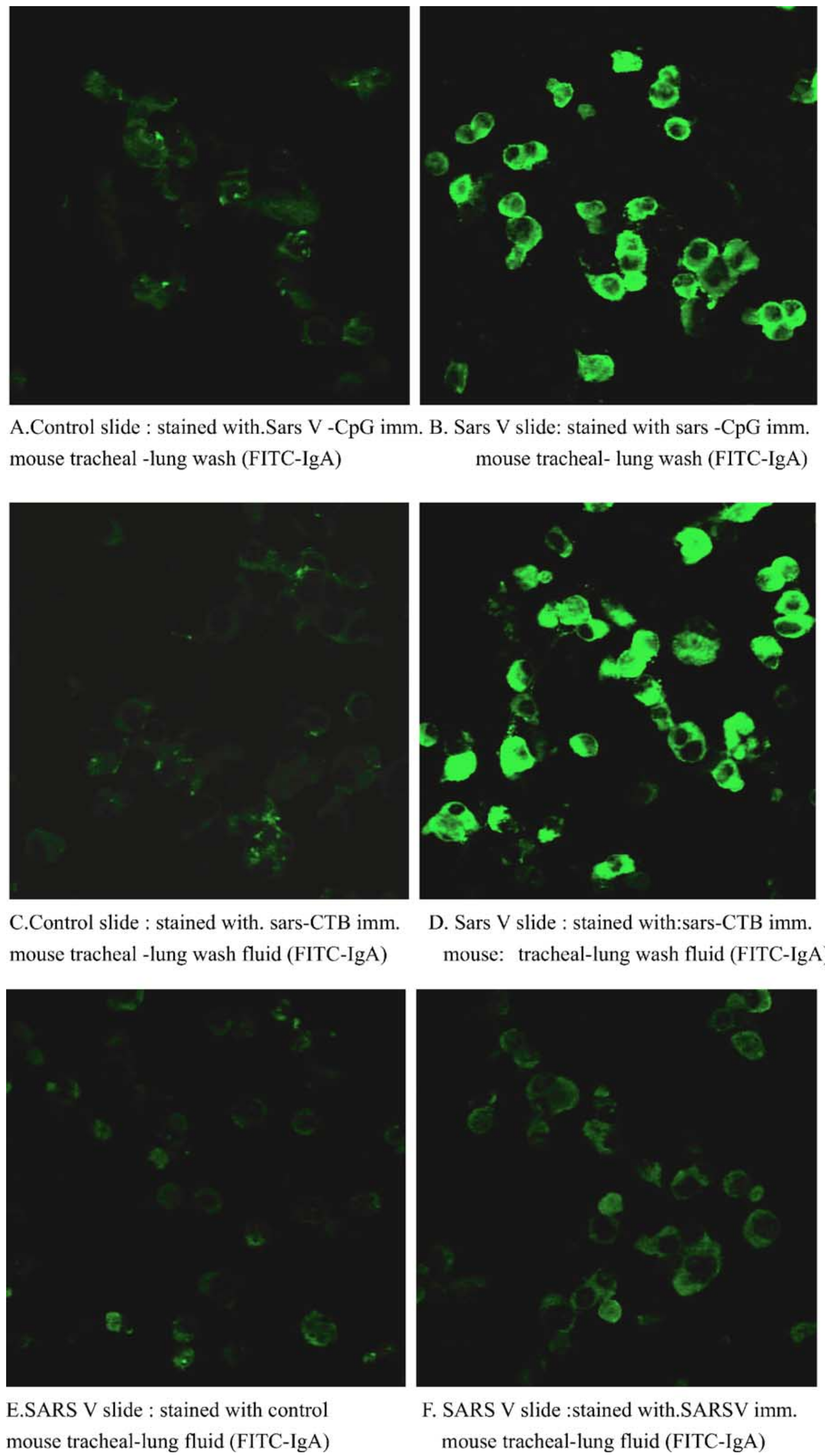
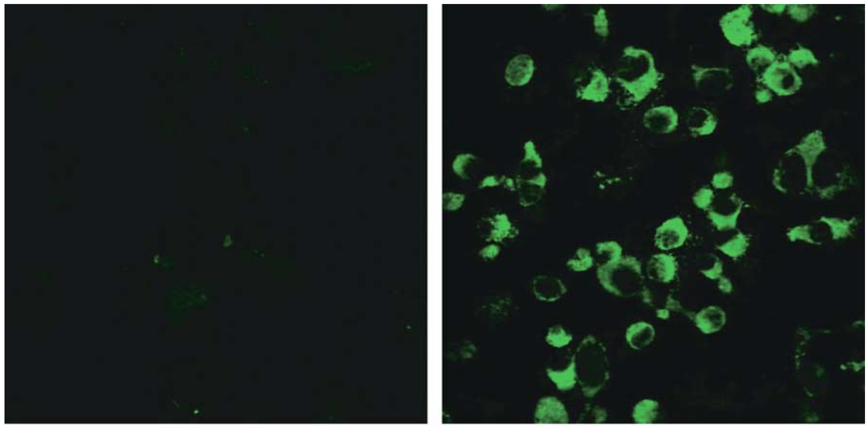


Fig. 3. Immunofluorescent study of anti-SARS-CoV IgA in tracheal-lung wash fluid from different groups of intranasal immunized mice SARS-CoV infected cells and non-infected control cells were stained separately with tracheal-lung wash fluid from different groups of immunized mice. FITC-labeled anti-mouse IgA was used as second antibodies. Immunofluorescence was observed and graded as stated in Table 2.



G. Control slide: stained with SARS-PEG imm. mouse tracheal-lung fluid (FITC-IgA)

H.SARS V slide: stained with SARS-PEG imm. mouse tracheal-lung wash fluid (FITC-(IgA)

Fig. 3. (*Continued*).

ing the animals for studying the antibodies in tracheal- lung wash fluid.

Intranasal immunization with inactivated viruses has not succeeded in inducing effective antibodies in other studies, while several approaches to increase the efficacy of intranasal or other mucosal immunization by inactivated viruses, inactivated bacteria or constructs containing viral or bacterial proteins have been presented [13–18]. In this study we used CPG-ODN and CTB as adjuvants and PEG-precipitated inactivated virus was used to potentiate the uptake of the inactivated viruses by antigen presenting cells, and to maintain inactivated viruses at the site of administration. After four intranasal doses of the inactivated virus, serum anti-SARS-CoV neutralizing antibodies were detected, but no anti-SARS-IgA was found in the tracheal-lung wash fluid. In contrast, when the inactivated virus was co-administered intranasally with adjuvant (either CPG or CTB), both serum anti-SARS-CoV neutralizing antibodies and specific IgA in tracheal-lung wash fluid were detected (Table 2, Fig. 2). These results indicate that local IgA antibodies could only be induced by combining the inactivated virus and adjuvant. On the other hand, in mice immunized subcutaneously, with inactivated virus, aside from high titer of serum neutralizing antibodies, specific anti-SARS-IgG also could be detected in tracheal-lung wash fluid. However, no anti-SARS-IgA was detected, which indicated that the antibodies detected in the tracheal-lung fluid was not produced locally, but derived from serum antibodies.

Given that CTB was reported more or less toxic in hosts, only modified CTB shown to be nontoxic to humans could be expected to be approved for human use in the future [19]. CpG-ODN is non-toxic and induces effective humoral and cellular immune responses in hosts [20,21]. It is thus a promising adjuvant to be used with SARS-CoV for intranasal immunization. In our previous report of using CpG-ODN with HBsAg-ani-HBs complex by intranasal immunization in mice, it was suggested that a selective stimulation of humoral response or cellular response depended on different constructs of antigens [22]. Future studies of using other constructs of inactivated SARS-CoV with CpG ODN by intranasal delivery might induce both cell mediated immune immune responses and antibody responses. Due to the lack of available peptides and antigen for studying SARS-CoV CTL or cell proliferation responses, the cellular immune responses in the SARSV-CoV intranasally immunized mice were not studied. As both cell-mediated immune response and humoral immune responses are important for SARS protection, these studies will be done whenever production of more inactivated virus will be approved. PEG has been used for the purification of inactivated HAV vaccines [23], and PEG-interferon has already been used in clinical trials for treatment of viral hepatitis B and C [24]. So far no serious

Table 2
Anti-SARS-CoV indirect immunofluorescence assay of tracheal-lung wash fluid

Immunized groups	The way of inoculation	Mouse number	Fluorescence intensity of IF-IgA*				
SARSV- alum	s.c.	2	–	–			
SARSV	i.n.	5	–	–	–	–	±
SARSV -CpG	i.n.	5	++	+	+	±	±
SARSV - CTB	i.n.	5	+++	+++	++	+	±
SARSV -PEG	i.n.	5	++	++	++	++	++

s.c.: Subcutaneous immunization, i.n.: intranasal immunization, ±: non-significant fluorescent staining, only very slight staining, +: positive fluorescent staining mainly on the membrane of infected cells, ++: positive fluorescent staining on membrane and cytoplasm, +++: straong positive fluorescent staining on membrane and cytoplasm on all cells.

ill effects have been described. We therefore used PEG precipitated SARS-CoV inactivated virus for intranasal immunization of mice. Because the number of animals used in each intranasal immunized group was limited, no statistical differences could be drawn between groups. However, compared to the dosage being used with CPG. As the adjuvant, only half the dosage of inactivated virus was necessary to induce both local and serum specific antibodies by using PEG precipitate as the adjuvant. This precipitated inactivated virus is therefore also a good candidate for development of intranasal SARS-CoV inactivated vaccine.

Prior to the use of formaldehyde inactivated GZ50 for immunization; we tested whether the formaldehyde-inactivated virus retained its property of binding to cell receptors in vitro. The results on the blocking assay confirmed that the inactivated virus could block the replication of live virus in cells. Because no other strain of live virus was available, we could not study whether inactivated GZ50 could block other SARS-CoV strains. Recently, angiotensin-converting enzyme 2 has been reported as a functional receptor for the SARS-CoV [25]. Whether the successful blocking of inactivated GZ50 versus live GZ50 was associated with this receptor remains to be studied. Besdies, the present blocking effect was only shown in cell culture, it would be interesting to do blocking experiments in animal models. If similar results were obtained, inactivated virus could be used intranasally as an urgent preventive measure when an outbreak occurred. Though due to technical problems the neutralizing function of the IgA in tracheal-lung wash fluid has not been confirmed, the successful induction of IgA antibodies against SARS-CoV by intranasal immunization suggested that SARS-CoV could be blocked at he site of entry, and would protect the vaccinated recipients from SARS-CoV infection. Even if the virus broke through this front-line, serum-neutralizing antibodies could further interact and neutralize the virus. This presumption should however be validated in an animal model mimicking SARS [26].

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References

[1] Peiris JSM, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 2003;361(9366):1319–25.

[2] Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 2003;318(20):1967–76.

[3] Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003;348(20):1953–66.

[4] Marra MA, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YSN, et al. The Genome sequence of the SARS- associated coronavirus. Science 2003;300(5624):1399–404.

[5] Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 2003;300(5624):1394–9.

[6] Anton IM, Gonzalez S, Bullido MJ, Corsin M, Risco C, Langeveld JP, et al. Cooperation between transmissible gastroenteritis coronavirus (TGEV) structural proteins in the invitro induction of virus-specific antibodies. Virus Res 1996;46(1–2):111–24.

[7] Nicholls JM, Poon LLM, Lee KC, Ng WF, Lai ST, Leung CY, et al. Lung pathology of fatal severe acute respiratory syndrome. Lancet 2003;361(9371):1773–8.

[8] Weiss RC, Scott W. Antibody-mediated enhancement of disease in feline infectious peritonitis: comparison with dengue hemorrhagic fever. Comp Immunol Microbiol Inf Dis 1981;4(2):175–89.

[9] Ruan YJ, Wei CL, Ee AL, Vega VB, Thoreau H, Su ST, et al. Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection. Lancet 2003;361(9371):1779–85.

[10] Liu L, Zhou XH, Shi JY, Xie X, Yuan ZH. Toll-like receptor-9 induced by physical trauma mediates release of cytokines following exposure to CpG motif in mouse skin. Immunology 2003;110(3):341–7.

[11] Walgate R., SARS vaccine race. http://www.biomedcentral.com/news/20030502/03 The Scientist May 2, 2003.

[12] Gao WT, Tamin A, Soloff A, D'Aiuto L, Nwanegbo E, Robbins PD, et al. Effects of a SARS-associated coronavirus vaccine in monkeys. Lancet 2003;362(9399):1895–6.

[13] Lowell GH, Kaminski RW, VanCott TC, Slike B, Kersey K, Zawoznik E, et al. Proteosomes, emulsomes and cholera Toxin B improve nasal immunogenicity of human immunodeficiency virus gp160 in mice: induction of serum, intestinal, vaginal and lung IgA and IgG. J Inf Dis 1997;195(2):292–301.

[14] Balmelli CE, Roden R, Potts A, Schiller J, De Grandi P, Nardelli-Haefliger D. Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. J Virol 1998;72(10):8220–9.

[15] McNeal MM, Rae MN, Bean JA, Ward RL. Antibody-dependent and-independent protection following intranasal immunization of mice with rotavirus particles. J Virol 1999;73(9):7565–673.

[16] Fries LF, Montemarano AD, Mallett CP, Taylor DN, Hale TL, Lowell GH. Safety and immunogenicity of a proteosome-shigella flexneri 2a lipopolysaccharide vaccine administered intranasally to healthy adults. Infect Immun 2001;69(7):4545–53.

[17] Kang SM, Compans RW. Enhancement of mucosal immunization with virus-like particles of simian immunodeficiency virus. J Virol 2003;77(6):3615–23.

[18] Malley R, Lipsitch M, Stack A, Saladino R, Fleisher G, Pelton S, et al. Intranasal immunization with killed unencapsulated whole cell prevents colonization and invasive disease by capsulated pneumococci. Infect Immun 2001;69(8):4870–3.

[19] Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Yamamoto Miho, Fujihashi K, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci USA 1997;94(10):5267–72.

[20] McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. J Immunol 1998;161(9):4463–6.

[21] Grierynska M, Kumaraguru U, Eo S-K, Lee S, Frieg A, Rouse BT. Induction of CD8 T-cell-specific systemic and mucosal immunity against herpes simplex virus with CpG-peptide complexes. J Virol 2002;76(13):6568–76.

[22] McCluskie MJ, Wen YM, Qu D, Davis HL. Immunization against hepatitis B virus by mucosal administration of antigen-antibody complexes. Viral Immnol 1998;11(4):245–52.

[23] Hagen AJ, Oliver CN, Sitrin RD. Optimization of poly(ethylene glycol) precipitation of hepatitis A virus used to prepare VAQTA, a highly purified inactivated vaccine. Biotechnol Prog 1996;12(3):406–12.

[24] Pepinsky RB, LePage DJ, Gill A, Chakraborty A, Vaidyanathan S, Green M, et al. Improved pharmacokinetic properties of a polyethylene glycol-modified form of interferon-beta-1a with preserved in vitro bioactivity. J Pharmacol Exp Ther 2001;297(3):1059–66.

[25] Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiostensin- convering enzyme 2 is a functional receptor for the SARS coronavirus. Nature 2003;426(6965):450–4.

[26] Fouchier RAM, Kuiken T, Schutten M, van Amerongen G, van Doornum GJJ, van den Hoogen BG, et al. Aetiology: Koch's postulates fulfilled for SARS virus. Nature 2003;423(6937):240.