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Key Messages

1. Intranasal vaccination using inactivated SARS coronavirus (SARS-CoV) vaccine with adjuvant can induce strong systemic (serum immunoglobulin [Ig] G) and respiratory tract local (tracheal-lung wash fluid IgA) antibody responses with neutralising activity.
2. RBD-Fc (protein-based vaccine) is able to induce effective neutralising antibodies able to provide protection from SARS-CoV infection in animal models.
3. A single dose of RBD-rAAV vaccination can induce adequate neutralising antibody against SARS-CoV infection.
4. Additional doses of vaccine increased the production of neutralising antibody 5 fold compared with a single dose.
5. RBD-rAAV vaccination provoked a prolonged antibody response with continually increasing levels of neutralising activity.
6. Intranasal vaccination with RBD-rAAV induced local IgA and systemic IgG neutralising antibodies and specific T-cell responses, able to protect against SARS-CoV infection in animal models.
7. When compared with the RBD-rAAV prime/boost vaccination, RBD-rAAV prime/RBD-peptide boost induced similar levels of Th1 and neutralising antibody responses that protected vaccinated mice from subsequent SARS-CoV challenges, but stronger Th2 and CTL responses.
8. Overall, our findings suggest that the inactivated vaccine, RBD-Fc and RBD-rAAV, can be further developed into effective and safe vaccines against SARS and that intranasal vaccination may be the preferred route of administration.

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Studies of SARS virus vaccines

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Introduction

Although SARS has been under control since June 2003, epidemiologists and scientists have predicted that an outbreak may recur in coming years. Therefore, development of a vaccine able to prevent SARS remains a high priority. This study was designed to investigate the hypothesis that inactivated vaccine, protein-based vaccine and rAAV live vaccine may be crucial means of preventing SARS. Our objectives were: (1) evaluation of an inactivated vaccine candidate, (2) construction and evaluation of an RBD-Fc vaccine candidate, and (3) construction and evaluation of an RBD-rAAV vaccine candidate.

Methods

This study was conducted from January 2005 to December 2006.

Study design
Inactivated vaccine
SARS coronavirus (SARS-CoV) strain GZ50 (GenBank accession number AY 304495) was used for generation of the inactivated vaccine candidate, because full-length sequencing and phylogenetic analysis showed that GZ50 lay between the reported Hong Kong strains and the Canadian and US strains.[1]

Protein-based vaccine
The RBD region of SARS-CoV S protein was selected as the target for the protein-based vaccine candidate, because it is the major determinant in eliciting neutralising antibody against SARS-CoV.[2] The RBD was fused with the Fc domain of human IgG1 (RBD-Fc), so that the fusion protein could be easily purified using a protein A-Sepharose column. Furthermore, the Fc domain of the fusion protein was able to bind to the Fc receptors on the surfaces of antigen presenting cells (APC) and enhance the immune responses initiated by the APC.

AAV-based vaccine
AAV was used as a delivery system for the construction of live vaccine candidates based on the following rationale: (1) respiratory and intestinal tracts are natural infection sites for both SARS-CoV and AAV; (2) intranasal vaccination of rAAV may induce strong local (respiratory tract) and systemic immune responses, including neutralising antibody responses; and (3) epithelial cells infected by the rAAV will be replaced by new epithelial cells derived from basal cells in the respiratory tract within several months, resulting in clearance of rAAV integrated cells, hence minimising the long-term risk caused by rAAV DNA integration into host chromosome DNA. On the other hand, it has been demonstrated that RBD can induce strong neutralising antibodies. We thus constructed RBD-rAAV as a vaccine candidate.

Study instruments
Construction and/or preparation of inactivated vaccine, RBD-Fc and RBD-rAAV
The vaccine candidates were prepared and/or constructed as described.[3-5]

Evaluation of immune responses to the vaccine candidates in mouse models
Mice were intramuscularly or intranasally immunised with the vaccine candidates. Systemic and local immune responses, including antibodies, Th and CTL, were

detected by enzyme-linked immunosorbent assays (ELISA), neutralisation assays, ELISPOT and triple staining of the cell surface and intracellular cytokines were measured by flow cytometry as described.[3-5]

Evaluation of the protective effect of the vaccine candidates in SARS-CoV animal models

The protective effects of the vaccine candidates were evaluated in SARS-CoV–challenged mice. Viral load and pathological changes were tested using virus titration, real-time reverse transcription polymerase chain reaction, haematoxylin and eosin (H&E) staining etc, as described.[3-5]

Results

Antibody responses in inactivated vaccine–immunised mice

After two subcutaneous injections of 80 μg of inactivated virus, only low titres of ELISA antibody (1:8) were detected. Sera from the intranasal vaccination group were all negative, but showed neutralising antibody activity. Although a lower dosage of virus was used for intranasal immunisation of PEG-precipitated inactivated SARS-CoV, the serum neutralising antibody titre was 1:160 in all the mice in this group. When tracheal-lung-wash fluid was tested for anti-SARS immunoglobulin (Ig) A by immunofluorescence, no positive staining was detected in the group immunised with inactivated virus only. Nonetheless, strong immunofluorescence staining at 1:5 dilution was shown in all groups of mice immunised with the virus plus the adjuvants, and in mice immunised with PEG-inactivated virus.[3]

Vaccination with RBD-Fc induced long-term and potent SARS-CoV S-specific antibodies with strong neutralising activity

RBD-Fc vaccination induced a prolonged and potent humoral immune response with IgG specific to SARS-CoV S protein as tested by ELISA. Highest titres were reached during the third and fourth post-vaccination months. Although titres decreased slightly afterwards, specific IgG antibody levels increased rapidly after the third booster dose (12 months after the first vaccination). It was further demonstrated that the antibodies induced by vaccination with RBD-Fc exhibited a strong neutralising activity showing similar patterns to the ELISA antibodies to RBD. Furthermore, viral RNA copies for both the RBD-Fc group and the control group showed a reverse correlation with the titre levels of the neutralising antibodies (Table). These results suggest that the neutralisation test induced by RBD-Fc vaccination plays an important role in the prevention of SARS-CoV infection in virus-challenged mice.[4]

Mice vaccinated with RBD-Fc did not develop histopathological changes in their lung tissues

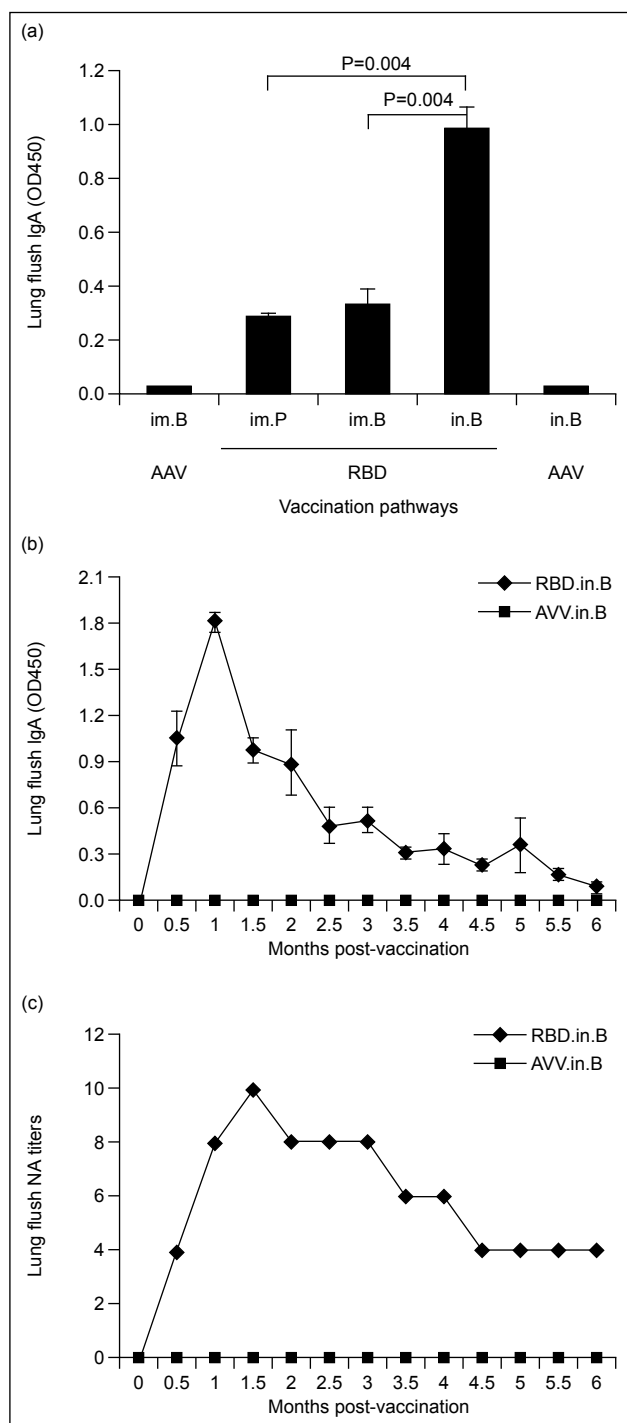Histopathological changes in lungs from the RBD-Fc vaccinated and control mice were observed using H&E-stained lung tissue sections. Lung tissues from the control mice revealed significant histopathological changes while lung tissues from the four RBD-Fc vaccinated mice exhibited no significant histopathological changes and were the same as normal mouse lung tissues.[4] These results indicate that RBD-Fc vaccine is able to prevent SARS-CoV infection in mice.

A single dose of RBD-rAAV vaccination induced adequate SARS-CoV specific antibody responses with neutralising activity in mice

After a single dose of RBD-rAAV intramuscular vaccination, SARS-CoV specific antibodies in the sera of immunised mice reached 1:880 at 4 months post-vaccination. Inactivated virus vaccination stimulated antibodies that peaked earlier and at a slightly lower level or at a significantly lower level (P<0.05) at 2 months post-immunisation. The neutralising antibody levels rose continuously from 1:48 at 1 month to 1:108 at 4 months post-vaccination with RBD- which was similar to the peak level seen in those vaccinated with the inactivated virus suspended in Alum at 2 months post-vaccination, but three-fold higher than those given the inactivated virus suspended in PBS (P<0.05).[5]

Repeated vaccinations of RBD-rAAV effectively induced high levels of SARS-CoV–specific antibodies with neutralising activity

In a further experiment, mice were given two further doses of RBD-rAAV or inactivated virus intramuscularly. In the animals given the RBD-rAAV vaccine, the level of the SARS-CoV–specific antibody determined by ELISA increased continuously throughout the experiment, reaching a titre of 1:5120 at 5.5 months post-vaccination. In animals given the inactivated virus, the antibody levels peaked at 4 months post-vaccination. The neutralising antibody level in the RBD-rAAV group of animals also rose continuously throughout the 5.5 months of the study. The neutralising antibody levels seen in the RBD-rAAV group were similar to the peak neutralising antibody levels of those given the inactivated virus vaccine but five times higher than those induced by a single dose of this vaccine.[5]

Table. SARS coronavirus RNA copies in lung tissue, measured by Q-reverse transcription polymerase chain reaction

Group	Mouse	Neutralising antibody titres	RNA copies/g of lung tissues
RBD-Fc vaccination	M1	57	3.13×10^3
	M2	189	Undetectable
	M3	505	Undetectable
	M4	452	Undetectable
	M5	404	
Control	C1	<4	1.39×10^7
	C2	<4	3.77×10^7
	C3	<4	1.39×10^5
	C4	<4	1.98×10^6
	C5	<4	1.45×10^6

Fig 1. Local humoral immune response to RBD-rAAV vaccinations
(a) Comparison of mucosal IgA in intramuscularly and intranasally vaccinated mice. (b) Monitoring of mucosal IgA antibody levels in mice intranasally vaccinated with RBD-rAAV. (c) Detection of neutralising antibody titres in lung flush of intranasally vaccinated mice[6]

Intranasal vaccination induced a shorter-duration systemic humoral immune response but a stronger and prolonged mucosal immunoglobulin A response than intramuscular vaccination
A single-prime dose of intranasal vaccination of RBD-

rAAV did not induce a significant antibody response. After a booster dose, the vaccine rapidly stimulated a strong IgG antibody response, reaching its highest titre 1 month post-vaccination but the IgG antibody level dropped to a low level one month later. The neutralising antibody levels followed a similar pattern. These results indicate that intranasal vaccination induces similar neutralising antibody levels but shorter-duration systemic humoral immune responses than intramuscular immunisation.[6]

Mucosal IgA SARS-CoV–specific antibody was further detected in the lung flush of vaccinated mice. RBD-rAAV intranasal prime-boost induced a strong IgA antibody response, which was significantly higher than that elicited intramuscularly by RBD-rAAV with a single-prime dose and prime-boost doses, respectively (Fig 1a). The IgA antibody titres and neutralising antibodies induced by the RBD-rAAV intranasal prime-boost in the mouse lung flush were further analysed at 0.5-month intervals. It was shown that the mucosal IgA antibody level reached its peak at 1 month post-vaccination, and gradually decreased to a low level in the following 5 months (Fig 1b). The lung flush from RBD-rAAV intranasal prime-boost vaccinated mice contained high-level and long-lasting neutralising antibody against SARS-CoV, which was highly detectable during the 6-month monitoring period (Fig 1c). The data indicated that intranasal vaccination with RBD-rAAV induced a long-term mucosal immune response with neutralising activity, implying that mucosal vaccination with RBD-rAAV should provide an effective protective immune response against SARS-CoV.[6]

Intranasal vaccination induced strong CTL responses in spleen and lungs
The CTL responses induced by RBD-rAAV vaccination were examined by measuring splenocytes and lung lymphocytes using ELISPOT and FACS. The intranasal vaccination with RBD-rAAV induced a markedly higher level of antigen specific IL-2+ T cells but a slightly lower level of IFN-γ+ T cells in the spleen. Specific CTL responses induced by RBD-rAAV vaccinations were further evaluated in the mouse splenocytes and lung lymphocytes using cell surface markers and intracellular cytokine staining followed by FACS. RBD-rAAV intranasal vaccination induced a markedly higher frequency of IL-2+ cells in the CD3+/CD8+ T cell population in both splenocytes and lung cells. In addition, IFN-γ-producing CD3+/CD8+ T cells were significantly higher in the splenocytes of RBD-rAAV intranasally versus intramuscularly vaccinated mice, but were similar or slightly lower in the lung lymphocytes from intranasally versus intramuscularly immunised mice.[6]

RBD-rAAV vaccination suppressed SARS-CoV replication in mouse lungs
The vaccine protective efficacies were further investigated in mice challenged with 10^5 $TCID_{50}$ of SARS-CoV strain GZ50. Viral loads (RNA copies/μg of lung tissues) in all mice immunised with RBD-rAAV were significantly lower
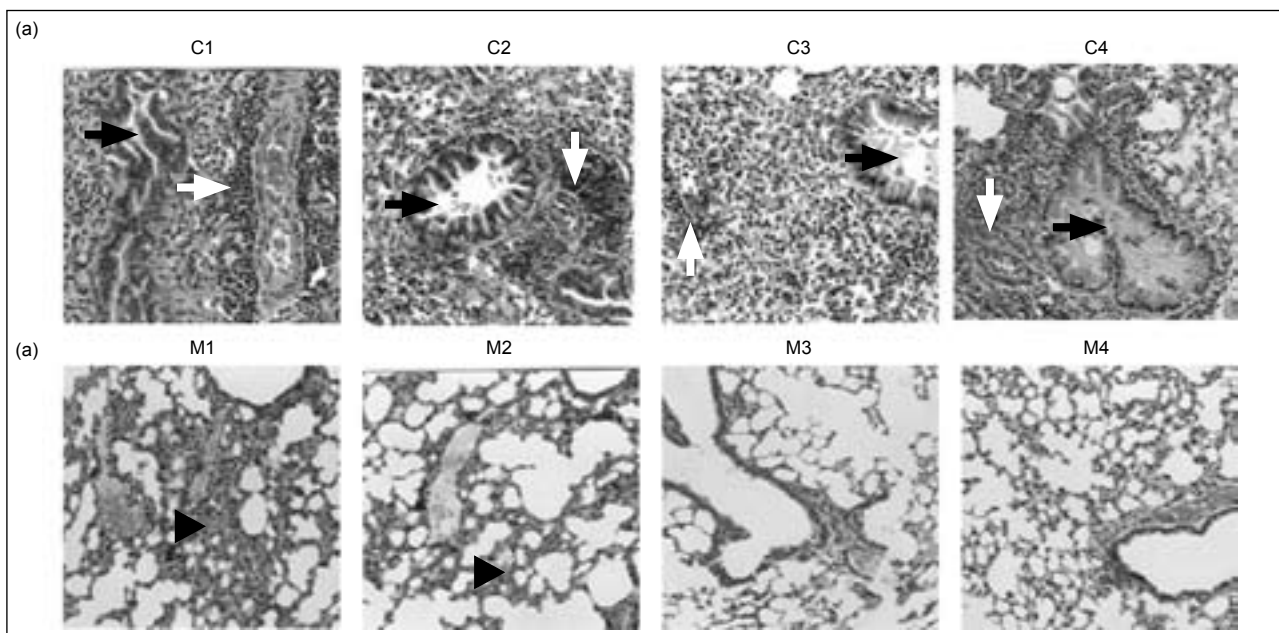
Fig 2. Histopathology of mouse lungs infected with severe acute respiratory syndrome coronavirus 8 days' post-infection[6]
All sections of mouse lung tissue were examined under the microscope (H&E, x100). (a) Representative images of histopathological damage to lung tissue from control mice given blank AAV in either intramuscular (C1-C2) or intranasal (C3-C4) booster vaccinations. These control mice developed interstitial pneumonia. Infiltrating lymphocytes and mononuclear cells were identified around small blood vessels (white arrows). Pulmonary vascular peripheral lymphocyte infiltration was also seen, with bronchial epithelial cell degeneration, necrosis, desquamation (black arrows), broadening interstitial spaces, and exudates. (b) Representative images of the histopathological changes in lung tissue from mice intramuscularly vaccinated with a single-prime dose (M1) or prime-booster doses (M2), and intranasally vaccinated with prime-booster doses (M3-M4) of RBD-rAAV. The lung tissue from single-prime dose intramuscularly vaccinated mice shows mild interstitial pneumonia changes with focal broadening of the interstitial spaces and lymphocytic infiltration (arrowhead) [M1]. Mice given intramuscular prime-booster vaccinations developed slight interstitial pneumonia with normal alveolae, slightly widened pulmonary intervals and small lymphocytic infiltrations (arrowhead) [M2]. Mice given intranasal prime-boost vaccinations showed almost normal vascular structures, bronchioles, alveolae, and alveolar lung spacing (M3-M4)

than those in the corresponding control group immunised with blank AAV via intramuscular and intranasal routes (P<0.05). This indicates that SARS-CoV replication was effectively suppressed in the vaccinated mice.[6]

RBD-rAAV vaccination provided long-term protection against SARS-CoV challenge

Mice were given RBD-rAAV booster doses 12 months after the first RBD-rAAV immunisation, and challenged with 10^5 TCID$_{50}$ of SARS-CoV. Challenged mice were sacrificed 8 days post-challenge and examined for histopathological changes. Serious pulmonary interstitial pneumonias were observed in the lung tissues of all control mice vaccinated with blank AAV after the SARS-CoV challenge (Fig 2a). In contrast, the mice vaccinated with RBD-rAAV showed no significant pulmonary effects after the viral challenge (Fig 2b). These results demonstrate that RBD-rAAV vaccinations provide long-term protective immunity against SARS-CoV infection in mice.[6]

Priming with RBD-rAAV and boosting with RBD-specific peptides elevated humoral and cellular immune responses against SARS-CoV infection

The immune responses to and protective effects of the immunisation with RBD-rAAV prime/RBD-specific T cell peptide boost were further evaluated. Compared with the RBD-rAAV prime/boost vaccination, the RBD-rAAV prime/RBD-peptide boost induced similar levels of Th1 and neutralising antibody responses that protected the vaccinated mice from subsequent SARS-CoV challenges, but stronger Th2 and CTL responses.[7] Since T cell epitopes are highly conserved and boosting with peptides may induce the production of effector memory T cells, which may be effective against viruses with mutations in the neutralising epitopes, these results suggest that the vaccination protocol used may be ideal for providing effective, broad and long-term protection against SARS-CoV infection.[7]

Conclusion

This study has shown that inactivated vaccine candidates, RBD-Fc, RBD-rAAV and T cell specific peptides, have the potential to be further developed into safe and effective vaccines able to prevent SARS-CoV infection. It has also shown that intranasal vaccination may be the preferred route of administration.

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