Adenoviral expression of a truncated S1 subunit of SARS-CoV spike protein results in specific humoral immune responses against SARS-CoV in rats

Ran-Yi Liu [a], Li-Zhi Wu [a], Bi-Jun Huang [a], Jia-Ling Huang [a], Yan-Ling Zhang [a],
Miao-La Ke [a], Jun-Mei Wang [b], Wei-Ping Tan [c], Ru-Hua Zhang [a],
Han-Kui Chen [a], Yi-Xin Zeng [a], Wenlin Huang [a,*]

[a] *State Key Laboratory for Oncology in South China, Cancer Center, Sun Yat-sen University,*
651 Dong-feng Road East, Guangzhou 510060, China
[b] *Department of Histology and Embryology, Sun Yat-sen University, Guangzhou 510089, China*
[c] *The Second Affiliated Hospital, Sun Yat-sen University, Guangzhou 510020, China*

Abstract

The causative agent of severe acute respiratory syndrome (SARS) has been identified as SARS-associated coronavirus (SARS-CoV), but the prophylactic treatment of SARS-CoV is still under investigation. We constructed a recombinant adenovirus containing a truncated N-terminal fragment of the SARS-CoV *Spike* (S) gene (from −45 to 1469, designated Ad-S$_N$), which encoded a truncated S protein (490 amino-acid residues, a part of 672 amino-acid S1 subunit), and investigated whether this construct could induce effective immunity against SARS-CoV in Wistar rats. Rats were immunized either subcutaneously or intranasally with Ad-S$_N$ once a week for three consecutive weeks. Our results showed that all of the immunized animals generated humoral immunity against the SARS-CoV spike protein, and the sera of immunized rats showed strong capable of protecting from SARS-CoV infection in vitro. Histopathological examination did not find evident side effects in the immunized animals. These results indicate that an adenoviral-based vaccine carrying an N-terminal fragment of the *Spike* gene is able to elicit strong SARS-CoV-specific humoral immune responses in rats, and may be useful for the development of a protective vaccine against SARS-CoV infection.
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1. Introduction

Severe acute respiratory syndrome (SARS) is a life-threatening contagious disease caused by the SARS-associated coronavirus (SARS-CoV) (Ksiazek et al., 2003; Rota et al., 2003). Since the first SARS case emerged in China's Guangdong province in November 2002, the disease has affected over 8000 victims in 31 countries and devitalized near 1000 lives (Schlagenhauf and Ashraf, 2003). To date, no specific, effective medicine or vaccine has been developed against SARS (Berger et al., 2004), which is currently treated with heteropathy and/or conservative therapeutics. Although

the first SARS outbreak was controlled by the mains of quarantine, WHO's reports in 2004 (http://www.wpro.who.int/sars/docs/update/update_07022004_revisedfinal.asp and http://www.wpro.who.int/sars/docs/pressreleases/pr_31012004.asp) indicated that SARS remains a constant threat to public. Efforts to develop safe, efficient SARS vaccines are under way worldwide (Marshall and Enserink, 2004). It is, thus, important to develop a safe and effective vaccine against SARS-CoV. An inactivated SARS-CoV vaccine seems to be the most facile and convenient option, but inactivated vaccines have been associated with risks of infection. For example, monkey models and children immunized with inactivated measles virus vaccine may develop severe cases of atypical measles (Polack et al., 1999; Fulginiti et al., 1967). Therefore, developing a gene-based vaccine may be a more promising choice.

The SARS-CoV genome is a single strand RNA molecule encoding four structural proteins: spike (S) glycoprotein, membrane (M) protein, envelope (E) protein and nucleocapsid (N) protein (Rota et al., 2003). S protein contains two subunits, S1 and S2. S1 is responsible for recognizing and binding to receptors on host cells, while S2 directs fusion between the viral and cell membranes (Ingallinella et al., 2004; Yang et al., 2004a). Angiotensin-converting enzyme 2 (ACE2) has recently been identified as a cellular receptor of SARS-CoV (Li et al., 2003b), and the receptor-binding region of spike was defined as amino acid residues 318–510 of the S1 subunit (Wong et al., 2004). Both soluble ACE2 receptor and mAb to S1 subunit can interfere receptor association and block SARS-CoV infection (Hofmann et al., 2004; Sui et al., 2004). Presumably, a vaccine designed to interfere with the binding between the S protein and its receptor on host cells would also prevent the spread of SARS. Buchholz et al. (2004) reported that S glycoprotein, of the structural proteins, appears to act as the only significant SARS-CoV neutralization antigen in a hamster model. A human mAb to S1 subunit was found to neutralize SARS coronavirus by blocking receptor association (Sui et al., 2004). Moreover, previous studies concerning the development of vaccines against animal coronaviruses have suggested that the S1 subunit contains neutralizing epitopes that confer protection to animals (e.g., birds and pigs) upon viral challenge (Cavanagh, 2003; Correa et al., 1990; Delmas et al., 1990; Song et al., 1998).

It has recently been reported that viral expression of the full-length S protein or S1 subunit of SARS coronavirus elicits a high titer of neutralizing antibodies in monkey models (Bukreyev et al., 2004; Gao et al., 2003). However, they almost lack safety evaluation. Weingartl et al. (2004) reported recently that immunization with modified vaccinia virus expressing full-length spike (S) protein of SARS virus was associated with enhanced hepatitis, while developed a more rapid and vigorous neutralizing antibody response. S protein was presumed that induced strong inflammatory responses in ferret liver tissue. In this study, we constructed a recombinant adenovirus expressing a shorter, truncated S1 subunit of SARS-CoV (Ad-S_N), and investigated its ability to in-

duce humoral immunity against SARS-CoV and its safety in rats. Our study demonstrated that high titer antibodies against SARS-CoV were elicited in a rat model following both subcutaneous (s.c.) and intranasal (i.n.) administration of Ad-S_N, the latter of which is targeted at fostering the respiratory mucosal immunity for protection against airborne diseases such as SARS. This result indicates that our construct could be developed into a safe SARS-CoV vaccine.

2. Materials and methods

2.1. Cell lines and animals

The 293 cell line (CRL-1573) is a human embryonic kidney cell line containing nucleotides 1-4344 of adenovirus type 5 (Ad5) (Louis et al., 1997; Graham et al., 1977). The Vero-E6 cell line is an Africa green monkey cell line (CRL-1586). Both cell lines were purchased from the ATCC, and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C with 5% CO_2 and saturated humidity. RPMI 1640, supplements, and serum were obtained from Life Technologies (Gibco/BRL). Wistar rats (6–8 week old) were obtained from the Experimental Animal Center, Sun Yat-sen University (Guangzhou, China), and housed in SPF (specific pathogen free) animal facilities.

2.2. Construction of recombinant adenovirus

Adeno-X^{TM} expression system (Clontech Laboratories, Inc.), comprising adenovirus type 5 genome with a deletion in the E1 and E3 regions (ΔE1, 343–3465 bp; ΔE3, 28,756–30,561 bp), was utilized to construct a recombinant adenovirus carrying nucleotides −45 to 1469 of *Spike* gene of SARS-CoV (Ad-S_N) by in vitro ligation (Mizuguchi and Kay, 1998, 1999), which encoded a truncated S1 subunit of SARS-CoV S protein (490 N-terminal amino-acid residues) (Fig. 1). Ad-LacZ, a recombinant adenoviral vector containing β-galactosidase gene, was constructed according to the same procedure and used as vector control. Both of the two viruses were propagated in 293 cells, purified by CsCl-banding and titered with the Adeno-X^{TM} rapid titer kit (BD Biosciences, Inc.) (Eykholt et al., 2000). The prepared recombinant adenoviruses were monitored to prevent from contamination of bacterium, fungus, mycoplasma, wild type adenovirus and other viruses.

2.3. Reverse transcription-polymerase chain reaction (RT-PCT) analysis

For detecting S_N expression at the mRNA level in Vero-E6 cells infected with recombinant adenoviruses, total cellular RNAs were isolated with the TRIZOL® reagent (Invitrogen), and then digested with RQ1 RNase-free DNase I (Promega) to remove residual DNA. The resulting mRNA was transcribed into cDNAs by the
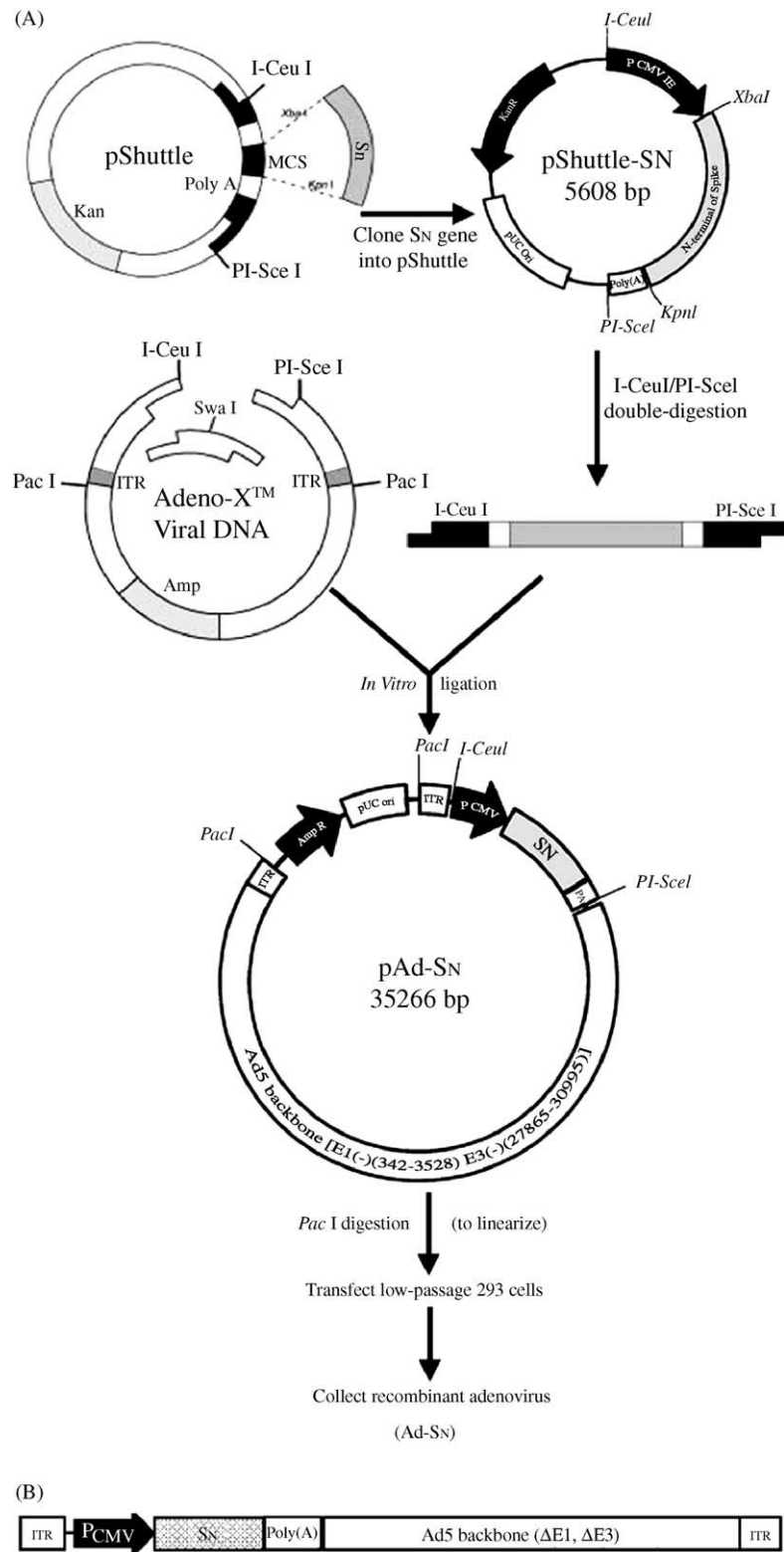
Fig. 1. (A) Construction of recombinant adenovirus Ad-S_N and (B) genomic structure of Ad-S_N.

AMV Reverse Transcriptase System (Invitrogen) with oligo(dT)$_{12–18}$, and RT-PCR was performed with S_N-specific primers P1 (5′-CGAGTACATATCTGATGCC-3′) and P2 (5′-ACGCCATAGCACTTAAAGG-3′) to generate a 624 bp DNA fragment. β-actin was amplified with primers P3 (5′-CGTCTTCCCCTCCATCGTG-3′) and P4 (5′-CCCTCA TAGATGGGCACAG-3′) to produce a 417 bp fragment that was assayed as an internal control.

2.4. Western blot analysis

Cell lysates, culture supernatant or homogenized tissue samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane. Blots were visualized with a Phototope-HRP Western blot detection system (New England BioLabs). Briefly, blots were blocked with 5% non-fat dry milk powder in TTBS (20 mM Tris–HCl, pH 8.0, 0.8% NaCl, 0.1% Tween-20) overnight at 4 °C, followed by a 4 h incubation at room temperature with one of the following primary antibodies: rabbit anti-spike IgG (1:200) (Abgent Cat# AP6009b), serum from a convalescent SARS patient, or sera from Ad-S_N-immunized rats. Blots were rinsed with TTBS solution, and incubated with HRP-linked anti-rabbit/human/rat IgG (Santa Cruz, 1:1000) for 2 h. After rinsing, blots were incubated with LumiGLO^{TM} (New England BioLabs) for 1 min and exposed to X-ray film.

2.5. Immunization of rats and detection of SARS-CoV-specific antibodies

Rats ($n = 10$ per group) were intranasally or subcutaneously immunized with Ad-S_N (1×10^7 pfu/(dose·rat)), or with controls (Ad-LacZ or PBS). For intranasal immunization, viruses were dropped into the noses of rats after the animals were anaesthetized with amylobarbitone. Rats were immunized on day 0, 7 and 14. Sera were collected on day 0, 7, 14, 21 and 28. SARS-CoV-specific antibodies (IgGs) were titered by enzyme-linked immuno-sorbent assay (ELISA). Briefly, 96-well microtiter plates coated with SARS-CoV full antigens (provided by the Chinese Academy of Military Medicine Chinese Academy of Sciences, Huada Co. Ltd., Beijing) were blocked with PBS (pH 7.4) containing 1% BSA. Serial dilutions of rat sera were added to the wells and incubated at 37 °C for 30 min. Wells were rinsed five times with PBST (PBS containing 0.05% Tween 20), followed by a 20 min incubation with HRP-linked anti-rat IgG (1:5000) at 37 °C, five times of rinsing and another 10 min incubation with the substrate solution at 37 °C for in darkness. The reactions were terminated by stop solution, and optical densities (ODs) were determined using a microplate reader set at 450 nm with a wavelength correction at 630 nm. The end-point titer was defined as the reciprocal of the highest dilution resulting in an OD ≥ 0.16 above that of the negative control. Samples were analyzed in duplicate.

2.6. Neutralizing activity analysis

The neutralizing activities of sera from immunized rats were tested in an in vitro micro-neutralization assay. Briefly, heat-inactivated rat sera were diluted two-fold and incubated with 100 TCID50 of SARS-CoV (BJ01 stain) at 37 °C for 1 h. Samples were then added into 96-well plates seeded with Vero-E6 cells (1×10^4 cells/well). The plates were incubated at 37 °C for 3–4 days, and the cytopathic effects (CPE) were observed daily. The neutralization titer was measured as the reciprocal of the highest serum dilution that completely inhibited Vero-E6 cell lysis in at least 60% of tested wells. All procedures were done at biosafety level 3.

2.7. Histopathological examination

Immunizated rats were sacrificed at day 28, various tissues (including lung, liver, colon, heart, brain, spleen, kidney, etc.) were collected, fixed with 10% PBS-buffered formalin, embedded in paraffin. Histopathological examination was performed on tissue sections stained with hematoxylin and eosin.

3. Results

3.1. Construction and characterization of the recombinant adenovirus, Ad-S_N

To construct the recombinant adenoviral vector, an N-terminal fragment of the SARS-CoV *Spike* gene (from −45 to 1468, S_N), derived from a Guangdong patient suffering from SARS, was firstly cloned into the *Xba*I and *Kpn*I sites of pShuttle. And then S_N expression cassette was cloned into pAdeno-X^{TM} by in vitro ligation at the unique restriction sites, I-*Ceu*I and PI-*Sce*I, to produce plasmid pAd-S_N, which contained the Ad5 genome lacking the E1/E3 region, with *Pac*I sites at each end of the Ad5 genome, and the S_N expression cassette inserted into the E1 deletion site (Fig. 1). PCR analysis showed that pAd-S_N contained the S_N expression cassette, and endonuclease restriction digestion generated the expected fragments (data not shown). Sequencing analysis showed that the cloned S_N gene has three variant bases (723G-T, 1024G-A, 1294C-T) compared with the SARS-CoV (BJ01 isolate) *Spike* gene, which encodes two deduced amino acid changes (342Gly-Arg, 432Pro-Ser) (GenBank accession no. AY862402).

Recombinant adenovirus Ad-S_N was obtained by transfecting *Pac*I-linearized pAd-S_N DNA into 293 cells. PCR analysis showed that recombinant adenovirus Ad-S_N contained the inserted S_N gene, and restriction analysis showed that Ad-S_N had the same digestion patterns as pAd-S_N/*Pac*I (data not shown). In addition, no contaminations of bacterium, fungus, mycoplasma, wild type adenovirus or other viruses were detected in prepared Ad-S_N (data not shown).

3.2. Expression of the truncated S1 subunit of spike protein (S_N)

For detecting S_N in vitro expression at the transcriptional level, Vero-E6 cells were infected with Ad-S_N or Ad-LacZ at the indicated multiplicities of infection (MOIs) for 48 h, and the expression of S_N mRNA was tested by RT-PCR. Our results showed that S_N mRNA was detected in Vero-E6 cells
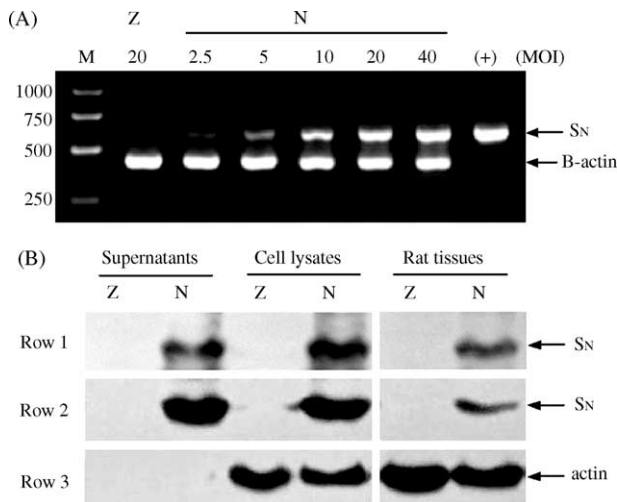
Fig. 2. Expression of the S_N fragment in vitro and in vivo (Z: Ad-LacZ; N: Ad-S_N). (A) Vero-E6 cells (2×10^5 cells/well) were seeded into a 6-well plate and cultured for 16 h until cells reached 80% confluence. Cells were then infected with Ad-S_N or Ad-LacZ at the indicated MOIs for 48 h. Total cellular RNAs were isolated, and S_N mRNA was detected by RT-PCR amplification, with β-actin used as the internal control (S_N 624 bp, β-actin 417 bp). (B) Vero-E6 cells were infected with Ad-S_N or Ad-LacZ at 20 MOI for 48 h, then cells and the culture supernatants were collected. Alternatively, rats were injected subcutaneously with Ad-S_N or Ad-LacZ (1×10^7 pfu/rat), after 48 h, tissues near the injection site were sampled and homogenized. Western blotting was used to detect S_N protein in the infected cells, culture supernatants and rat tissues using the Phototope-HRP Western blot detection system (New England BioLabs). Serum from convalescent SARS patients (Row 1) or rabbit anti-spike IgG, SARS spike N-term D204 antibody (Row 2) were used as the primary antibodies, with an anti-actin mAb (C-2) (Santa Cruz, sc-8432) (Row 3) as the control.

infected with Ad-S_N, but not in cells infected with Ad-LacZ. Moreover, the expression of S_N mRNA in cells infected with Ad-S_N was dose-dependent (Fig. 2A).

For detecting expression of S_N protein in vitro, Vero-E6 cells were infected with Ad-S_N or Ad-LacZ at 20 MOI for 48 h, and the infected cells and their culture supernatants were collected. For detecting S_N protein expression in vivo, rats were injected subcutaneously with Ad-S_N or Ad-LacZ (1×10^7 pfu/rat). Fourty-eight hours later, injection locus tissues were sampled and homogenized. Western blotting was performed to detect S_N protein in the various samples. A specific ∼60 kDa protein band, consistent with the predicted size of the S_N protein, was clearly observed in Ad-S_N-infected Vero-E6 cells, their culture supernatants and Ad-S_N-treated tissues, as visualized by both commercial rabbit anti-spike IgG (Abgent) and sera from convalescent SARS patients, whereas no S_N band was found in Ad-LacZ-infected cells or tissues (Fig. 2B).

3.3. Immunization and detection of SARS-CoV-specific antibodies

ELISA analysis showed SARS-CoV-specific antibody responses were detected in rats subjected to three i.n. or s.c. immunizations with Ad-S_N, whereas no specific immune re-

sponses were found in animals treated with Ad-LacZ or PBS (Fig. 3A). SARS-CoV-specific IgGs were detected in Ad-S_N-treated rats 2 weeks after the first immunization, with levels gradually increasing to maximum IgG titers of $1:2^9$ and $1:2^{9.1}$ in the i.n. and s.c. groups, respectively, by 2 weeks after the last treatment.

The antibody response specific for S_N protein in Ad-S_N-vaccinated rats was tested by Western blot analysis. Our results showed that all of the sera from Ad-S_N-immunized rats (i.n. and s.c.) showed visible Ag-Ab reactions with S_N protein expressed in Ad-S_N-infected Vero-E6 cells (Fig. 3B), while sera from Ad-LacZ-treated or PBS-treated rats did not (data not shown).

3.4. Neutralizing activity assay

For detecting the neutralizing activity of sera from immunized rats against SARS-CoV, Vero-E6 cells were challenged with SARS-CoV (BJ01 isolate). As expected, the sera of rats immunized with Ad-S_N protected Vero-E6 cells against SARS-CoV (BJ01 stain) challenge in vitro, with neutralization titers up to $1:2^7$ and $1:2^{7.3}$ in the i.n. and s.c. groups, respectively, while the sera from control animals did not display any neutralization activity (Fig. 3C).

3.5. Histopathology

To evaluate the safety of Ad-S_N vaccine candidate, histopathological examination was performed on tissue sections from immunizated rats. There was not any evident pathological change in either Ad-S_N-immunized or Ad-LacZ-treated rat tissues (data not shown).

4. Discussion

Various strategies have been explored in the search for an effective vaccine against SARS-CoV, including the use of inactivated whole virus particles, attenuated live virus, purified viral structural proteins and gene vaccines (Tang et al., 2004; Xiong et al., 2004; Choy et al., 2004; Zhang et al., 2004; Zhou et al., 2004; Yang et al., 2004b; Bukreyev et al., 2004; Bisht et al., 2004; Gao et al., 2003). Recently, the gene vaccines, which express SARS-CoV structural proteins and induce host immunity against SARS-CoV, have become increasingly popular.

In this study, we constructed a replication-incompetent recombinant adenovirus containing nucleotides −45 to 1469 of *Spike* gene of SARS-CoV, named Ad-S_N, which encodes a truncated S1 subunit of SARS-CoV S protein. Sequencing analysis revealed that the cloned S_N fragment had three nucleotides difference compared with SARS-CoV BJ01 isolate, resulting in two amino-acid alterations in the predicted S_N protein (GenBank accession no. AY862402). Computational prediction of antigenic peptides (Kolaskar and Tongaonkar, 1990; Singh and Raghava, 2001)
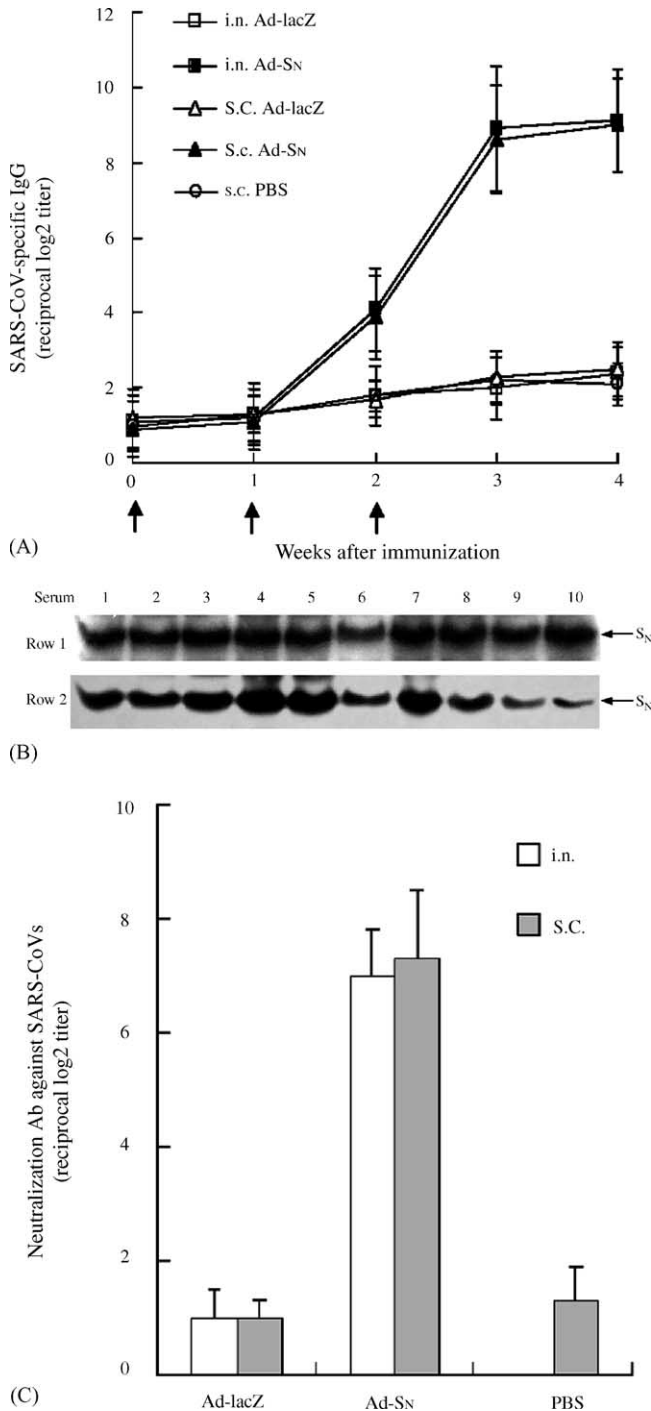
Fig. 3. Ad-S_N induced rats to produce high titers of antibodies (A, B) and neutralization activity (C). (A) ELISA assay for SARS-CoV-specific serum IgG in the rats immunized subcutaneously (s.c.) or intranasally (i.n.) with Ad-S_N. Arrows (↑) indicate time points of immunization. (B) Western blot analysis of S_N-specific antibodies in sera of immunized rats. Western blotting was performed with the Phototope-HRP Western Blot Detection System (New England BioLabs). The culture supernatants of Vero-E6 cells infected with Ad-S_N (separated by 10% SDS-PAGE) were used as antigen, and sera from rats immunized with Ad-S_N subcutaneously (Row 1) or intranasally (Row 2) were used as the primary antibodies. Bands indicate the existence of S_N-specific antibodies in the sera of immunized rats. (C) Analysis of neutralizing activities of sera from the rats immunized subcutaneously or intranasally with Ad-S_N.

[http://immunax.dfci.harvard.edu/Tools/antigenic.html and http://www.imtech.res.in/raghava/propred/] demonstrated that these variations did not influence the antigenicity of the recombinant protein. The secretory ∼60 kDa truncated S_N protein, expressed in Vero-E6 cells and in rats infected by Ad-S_N, was recognized by rabbit anti-spike IgG, sera from convalescent SARS patients and Ad-S_N-immunized rats (Figs. 2B and 3B). This indicates that the truncated S_N fragment is a potential antigen capable of inducing specific antibody responses in rats, rabbits and humans.

Previous studies have reported that several antigenic epitopes on the spike protein are recognized by sera from convalescent SARS patients and are capable of inducing neutralizing antibodies in humans (Hua et al., 2004; Guo et al., 2004). Spike protein or its S1 subunit have been reported that can induce efficient immunity against SARS-CoV and protect from the attack of SARS-CoV in animal models (Yang et al., 2004b; Bukreyev et al., 2004; Gao et al., 2003). In this study, we demonstrated that a truncated S1 subunit of spike protein (490 N-terminal amino-acid residues) could induce a strong humoral immune response against SARS-CoV in rats (Fig. 3A and B), and the sera from Ad-S_N-immunized rats could effectively protect Vero-E6 cells against SARS-CoV (BJ01 isolate) infection in vitro (Fig. 3C). No evident pathological changes (e.g., inflammatory response) were observed in various tissues (including lung, liver, colon, heart, brain, spleen, kidney, etc.) of Ad-S_N-immunized animals (data not shown), contrast to the finding of Weingartl et al. that immunization with modified vaccinia virus expressing the SARS-CoV spike (S) protein exhibited strong inflammatory responses in liver tissue in ferrets (Weingartl et al., 2004). This difference may be due to the difference of experimental animals, protocols and *Spike* gene fragments that vectors carried. Our present data cannot conclusively demonstrate whether truncated S1 gene vaccine will be a safer vaccine candidate. However, it can be presumed that truncated S1 gene carries less risk for spontaneous recombination with wild type virus to generate new virus types. Considering these favorites, truncated S1 subunit gene vaccine could be a safer vaccine candidate than the full-length *Spike* gene and may act as a pharmacologically active vaccine.

Replication-incompetent adenovirus vectors are widely used as foreign gene carriers for vaccines (Xiang and Ertl, 1999; Casimiro et al., 2003; Wu et al., 2003). Previous work has shown that immunization via the respiratory tract (i.e. intranasal) could induce protective mucosal immunity (Sakaue et al., 2003; Bukreyev et al., 2004; Xiang and Ertl, 1999). As respiratory epithelial cells are the natural hosts for adenovirus, recombinant adenovirus Ad-S_N immunized through the respiratory tract should induce mucosal immunity as a first line of defense against viral attack. This is particularly important for SARS, which mainly spread through the respiratory tract. In this study, it was demonstrated that SARS-CoV-specific antibodies and neutralizing activities could be induced by intranasal immunization at the same

serum level as that elicited by subcutaneous immunization (Fig. 3A and C). This indicates that recombinant adenovirus encoding truncated S1 subunit is a good candidate for development as a mucosal SARS-CoV vaccine.

Clinical observations in SARS patients imply that SARS-CoV seemed to elicit effective humoral immunity but inhibit cellular immunity, especially CD8^{+} memory T lymphocytes over time (Huang et al., 2005), so developing a vaccine to induce both humoral and cellular immune responses may be required to prevent from SARS-CoV infection (Tang et al., 2003; Li et al., 2003a). It is necessary to make clear whether Ad-S_N can elicit T-cell immunity in rats. Comprehensive examinations of the associated immunological parameters will be further needed to evaluate the benefits of the various immunization strategies. In addition, the rat model may not faithfully replicate the immunological characteristics of humans. It will be necessary to test potential SARS vaccine candidates for their immunogenicity, safety and efficacy in other animal models, such as rabbit, primates, raccoons, dog and human.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2005.02.009.

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