the receptor binding site [30]. Upon binding to the target molecule, the S protein undergoes a conformational change leading to exposure of the fusogenic region in the S2 part resulting in membrane fusion between the viral and cellular membranes [31,32].

Mouse mAbs against recombinant protein fragments of S-II (aa485–625), S34 and S78, obtained by Zhou *et al.* [13], had potent neutralisation ability. The epitopes of S34 and S78 were localised to aa548–567 and aa607–627, respectively. These epitopes, however, are slightly downstream of the RBD shown by Wong *et al.* [9] and Xiao *et al.* [33]. On the other hand, among the mAbs against RBD fused with human IgG1-Fc fragment (RBD-Fc), two mAbs, 4D5 and 17H9 recognised linear epitopes such as aa435–451 and aa442–465, respectively [34], whereas the remaining mAbs recognized conformational epitopes, classified into six groups as Conf-I to Conf-VI. This study showed that the ability to inhibit ACE2 binding of SARS pseudovirus was retained in all conformational epitopes, especially in Conf-III, -IV and V, but not in the linear epitopes.

Likewise, human mAbs against S protein, such as 80R [35], CR3014 [36] and 201 recognise the RBD and have potent neutralising activity against SARS-CoV, as discussed below. The clone, CR3014, recognises the S1 fragment in the context with aa479, suggesting the importance of this amino acid in the high binding affinity of CR3014.

Spiga *et al.* tried to model the tertiary structure of the S1 and S2 S protein domains by the homology modelling and molecular dynamics methods [37]. Their model predicted two hydrophobic pockets, Phe850-Phe870 and Phe1077–1079, for the putative receptor binding site. This model would be useful in evaluating the antigen drift caused by the mutations in the S1 region, which has already been deposited in the NCBI database. Interestingly, when Yi *et al.* studied the immunogenicity of S protein in mice by DNA immunization, a single amino acid substitution, the R441A mutation, failed to induce neutralizing antibodies and abolished viral entry. The R453A mutation, however, retained the capacity to induce neutralizing antibodies, although it also abolished viral entry [38]. Thus, a single amino acid mutation here easily affects the virus-to-cell interaction, and therefore, this region is an ideal target structure for neutralization.

*Nucleocapsid (N) protein:* The nucleocapsid (N) protein of SARS-CoV is a highly basic structural protein of 422 amino acids. The N protein is thought to be involved in the packaging of the viral RNA-genome and, thus, crucial for viral replication and pathogenesis as in other CoVs [39,40] (Figure 1). Anti-N-protein antibodies are generally found in the sera of SARS patients, suggesting that the N-protein is one of the immunodominant structural proteins of SARS-CoV [41,42]. In addition, SARS patient's sera recognise the N-protein at a large number of linear epitopes [23,24] and, at least, two major conformational epitopes [43].

In patient's sera, Wang *et al.* identified the peptides N66 (aa66–87) and N371–401 (aa371–422) as the most immunogenic epitopes [24]. On the other hand, He *et al.* identified two major epitopes in the C-terminal region (aa362–412) and middle region (aa153–178), in addition to several minor immuno-dominant epitopes [44]. They also showed that the most potent antibody response was elicited against the C-terminal region, which contains a short lysine-rich sequence (aa362–381; KTFPPTEPKKDKKKKTDEAQ); however, the functional role of this stretch remains unknown. Van den Brink *et al.* established two human anti-N scFvs, CR3018 and CR3009, which recognised a linear epitope, RSAPRITFG (aa11–19) and a nonlinear epitope, respectively [18].

In mice immunised with inactivated SARS-CoV, we identified one major epitope in the middle part (aa110–210), which was recognised both by the SKOT-8 and SKOT-9 mAb [10], whereas He *et al.* identified one major epitope adjacent to the N-terminal region (aa76–101) in addition to epitopes in the C-terminal and middle regions [44].

Together, these mapping studies of the SARS CoV N protein suggest that there are three major epitopes localised to the N-terminal, middle and C-terminal regions, respectively, which are responsible for the potent immune response in both humans and mice.

*Envelop (E) protein:* The small E protein is a 76aa residue long protein involved in the envelop morphogenesis. As far as we know, there is only one mAb against this protein in humans [16], and this E protein antibody is detected in SARS convalescent patient sera [23].

*Matrix (M) protein:* The M membrane glycoprotein is a 221aa residue long integral membrane protein which is functionally involved in the budding of virions from cells. Pang *et al.* reported that rabbit antiserum raised against recombinant M protein has a potent neutralising ability *in vitro*, suggesting that the M protein could be one of the target proteins chosen for the vaccine development [45]. Wang *et al.* reported that the synthetic peptide M137 (aa137–158) is one of the most immunogenic regions of the SARS-CoV structural proteins [24]. Recently, Zhong *et al.* reported a systematic search for the viral epitopes by a biopanning of the M13 phage display dodecapeptide library using antibodies found in plasma samples of convalescent SARS patients [46]. They determined the continuous viral epitopes including an 'epitope-rich region' on the S2, M and E proteins. This contiguous epitope map of SARS-CoV would greatly help to develop an effective vaccine for SARS-CoV.

## Antibody cross-reactivity to OC43 and 229E

We confirmed the absence of cross-reactivity in the anti-N mAbs, SKOT-8 and SKOT-9, to human coronaviruses, HCoV-OC43 and HCoV-229E, by ELISA [10] and IFA (Figure 2). However, at the polyclonal level, there are reports of cross-reactivity between SARS and other human coronaviruses. Wo *et al.* reported false-positive results in their recombinant SARS-CoV N-protein-based ELISA assay, mainly due to the presence of cross-reactive

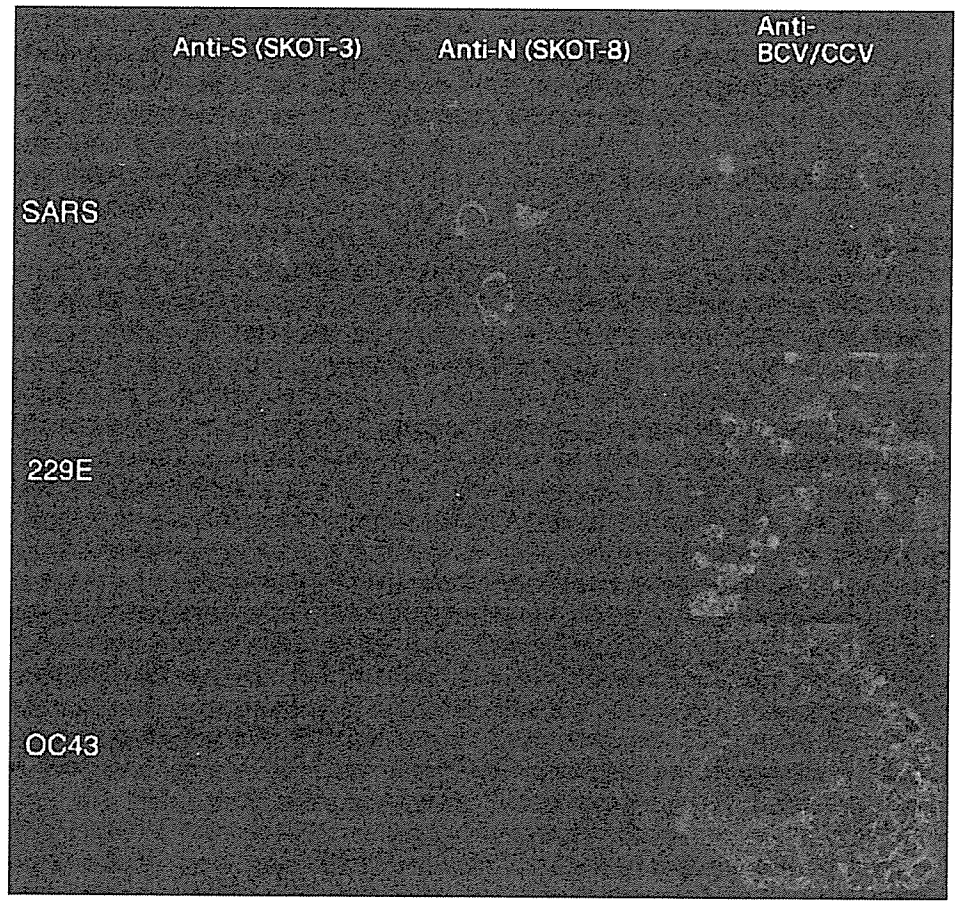


**Figure 2.** Immuno-fluorescent assay showing the absence of cross-reactivity with anti-SARS-CoV S-protein mAb, SKOT-3 and anti-N-protein mAb SKOT-8, with Hu-CoV 229E and OC43. SARS-CoV-infected VeroE6 cells (upper lane), Hu-CoV 229E-infected L132 cells (middle lane) and Hu-CoV OC43-infected HRT-18 cells are stained with anti-SARS-CoV S- and N-protein mAbs. SKOT-3 (left columns) and SKOT-8 (middle column) and polyclonal anti-BCV/CCV (right columns)

antibodies to SARS-CoV N-protein in the sera [47]. They found that 3 out of 21 and 1 out of 7 sera from convalescent patients from HCoV-OC43 and HCoV-229E, respectively, contained antibodies cross-reactive with the SARS-CoV N-protein, suggesting the presence of a common epitope(s) in these human CoV N-proteins. The sera from convalescent patients, however, did not react with SARS-CoV S-proteins in the Western blot analysis. Thus, highly specific SARS-CoV mAbs are required for sensitive and accurate laboratory diagnosis.

## NEUTRALISING ABILITY OF ANTI-SARS-CoV ANTIBODIES

Neutralising antibodies are crucial for establishing protection from SARS-CoV infection. Notably, the analysis of the memory B cell repertoire at a clonal level suggested that the memory B cell repertoire in recovered SARS patients is biased towards neutralising antibodies recognising the S protein [48]. As described above, the main neutralising S protein epitope was at the aa318–510 position, which binds to the cellular receptor ACE2 (RBD; see Figure 1). Therefore, under the expectation that a passive immunotherapy that utilises neutralising antibodies against SARS-CoV could be a promising therapeutical method, many efforts have been focused on the development of mAbs as discussed above.

Analysis of rabbit anti-sera against recombinant S, N and M proteins suggested significant neutralising abilities in anti-S and anti-M antibodies, but not in anti-N antibodies [45]. The main neutralising epitope on S protein was in the RBD, whereas some parts irrelevant to the ACE2 binding site were also shown to be involved in neutralisation, presumably via steric hindrance. Furthermore, a competitive neutralisation assay suggested the presence of an additional target site other than ACE2 binding site [45]. In this regard, by continuous epitope mapping using the M13 phage display dodecapeptide library, Zhong *et al.* reported that the 'epitope-rich region' on the S2 protein (aa787–809) represents one of the major neutralising targets for SARS patients and that is blocked by the peptide of the corresponding region [46].

Human mAbs against SARS-CoV with neutralising activity are attractive reagents for the treatment of SARS patients. For example, all EBV-transformed memory B cells recovered from SARS patients have specificity for the S protein with neutralising activity [48]. Curiously, one of them, S3.1, conferred efficient protection in a mouse model with SARS-CoV infection [16]. In addition, one of mAbs established from the human scFv library by Sui *et al.*, designated 80R, was shown in a mouse model to display effective protection from infection with wild-type or mutant viruses isolated from patients [49]. Furthermore, when 80R IgG1 was administered into the mice prophylactically, SARS-CoV replication was reduced by more than four orders of magnitude as compared with untreated mice. Human mAbs, 201, established from transgenic mice that harboured human immunoglobulin genes [20] also had neutralising activity and significant protective effect in the mouse infection model upon administration with a 1.6–40 mg/kg dose [21]. Together, all these human mAbs could be candidates for chemoprophylaxis and therapy for SARS, and, in fact, clinical trials are being planned for some of these human mAbs [16,18,21,49].

## APPLICATION OF ANTI-SARS-CoV mAbs FOR LABORATORY DIAGNOSIS

Laboratory diagnosis of SARS-CoV infection was based on a combination of serologic tests, reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation [50–53]. Hence, the mAbs against SARS-CoV structural proteins have been established by several groups and their utility in antigen-capture ELISA, immuno-fluorescence assay, Western blot analysis has been tested [10,51,54](see Table 1).

### Immuno-fluorescence assay (IFA) and immuno-histochemistry (IHC)

IFA is the simplest serological test for detecting the pathogen. The IFA for SARS diagnosis is carried out by using several polyclonal and monoclonal antibodies against N-protein or those against S-protein, irrespective of the specificity for conformational or linear epitopes [10,15, 18,53]. We showed that SKOT-8 and SKOT-9 mAbs against N protein were quite useful for diagnosis, in terms of both specificity and sensitivity [10].

He *et al.* developed a system that uses insect sf9 cells expressing the N195-Sc fusion protein as a target cell, by which 23 serum samples from SARS patients were scored at a rate comparable

to that of conventional IFA and a commercial SARS-CoV IFA kit [55].

*Western blot:* The majority of anti-N mAbs are directed against linear epitope(s) and thus used in Western blot to detect the 47 kDa band of the N-protein [10,15]. In contrast, the epitopes on native S-protein are mostly conformational and the anti-S mAbs we obtained were not useful for Western blot [10]. However, by using SDS-denatured S protein or recombinant S protein fragments as an immunogen, several mAbs for Western blot were obtained [11,13]. Although the sera from patients and from immunised mice detected both the E and M protein [23,24,56], no useful mAb for detecting either of these proteins by Western blot have been established.

*ELISA:* Because the immunological test for the presence of SARS-CoV virions in a patient's specimen is an important complement to the RT-PCR test, three groups have so far reported the development of such an antigen capture ELISA system for SARS-CoV. We intended to establish an antigen-capture sandwich ELISA system by using a total of 29 mouse mAbs with specificity for S- and N-proteins, and found that a virus protein load as low as 40 pg/mL was successfully detected by use of the anti-N mAb, SKOT-8, as a capturing antibody and the biotinylated mAb, SKOT-9, as a probing antibody [10]. The sensitivity of the system did not differ between at least four strains of SARS-CoV tested. In contrast, any combination of anti-S mAbs failed to yield high titres.

Che *et al.* [15] utilised three mouse anti-N mAbs as a capturing antibody and a rabbit polyclonal anti-N antibody as a detecting antibody, and were able to detect the recombinant N protein at 50 pg/mL. This assay system scored positive for approximately 84% of SARS patients serologically confirmed and 1.5% in 1272 healthy individuals [15].

Di *et al.* tested 829 serum samples from 643 patients and compared them to 197 control sera from healthy donors [57]. After the onset of symptoms, the N protein was detected by day 10 with a sensitivity of 100% in the case of 27 patients who had been positive with the neutralisation test. After 10 days, the N-protein level in the sera decreased and was undetectable beyond days 19. The specificity of the assay calculated from the results of 66 serological test-negative patients and 197 healthy donors was reported to be 100%. The positive rate, 96–100%, was obtained from the sera at day 3–5 after the onset of symptoms for 27 neutralisation-test positive SARS patients and 298 serologically confirmed patients.

Lau *et al.* reported the development of polyclonal antibody-based ELISA for the N protein by utilising guinea pig and rabbit antibodies against recombinant $His_6$-tagged SARS-CoV N-protein [58]. They tested nasopharyngeal aspirates, urine and faecal samples, and detected N protein in the nasopharyngeal aspirate samples (day 6–24 after the onset of the disease), in urine samples (day 11–31) and in faecal samples (day 8–32), though the sensitivity was not very high except for the day 11–15 nasopharyngeal aspirates (83% sensitivity).

Apart from an antigen-capture detection system, the high-throughput assay for anti-SARS-CoV IgG antibody detection system, DETECT-SARS™, was reported to be highly sensitive (95.9%) for convalescent serum samples [59]. These immunological detection systems would be necessary compliments of RT-PCR-based diagnosis, and any future improvement in these detection systems will certainly contribute to control the SARS pandemic.

## IMMUNITY TO SARS-CoV AND ANIMAL MODELS (Table 3)

Immune responses have been studied using animal models including macaques, ferrets, cats, Golden Syrian hamsters and mice (review in [3]). However, these animal models showed only transient viral replication in the respiratory tracts without manifestation of the disease, except in ferrets and hamsters. Ferrets and hamsters develop pathological disorders in the lung and infected ferrets and cats transmit SARS-CoV to naïve counterparts housed together [60]. Thus, it seems that some aspects of the SARS-CoV infection in humans were reproduced in these animal models, but the disease entities themselves are quite different. For example, in humans, a rapid progression of pulmonary and intestinal complications is observed and almost 20% of SARS patients develop watery diarrhoea [61], which is not seen in animal models. Despite the different outcomes, animal models are still useful for vaccine development and evaluation.

While antibody responses in SARS patients have been studied extensively, we know little about T-cell responses in patients. Two A-2-restricted epitopes of the S2 domain were recognised by memory $CD8^+$ T cells of SARS-CoV-infected patients [62]. However, a study using HLA-A0201 transgenic mice or *in vitro* primed PBMCs of healthy donors identified a slightly different dominant epitope in the S2 domain [63]. In mice, epitopes of $CD8^+$ T cell were within the S1 domain [64]. Current strategies to elicit an effective immunity against SARS-CoV are listed in Table 3.

## VACCINE STUDIES

### Attenuated or inactivated SARS-CoV

Because we know little about the pathogenesis of severe acute respiratory syndrome associated with SARS-CoV infection, a live attenuated SARS-CoV vaccine will not be feasible as a candidate for a SARS vaccine. Instead, a whole inactivated virion will be the first choice. Mice subcutaneously or nasally injected with a whole virion, that had been inactivated either by UV-irradiation [56] or formalin [65–67], with or without a variety of adjuvant, induced a high level of antibodies against SARS-CoV. Surprisingly, we observed that the UV-inactivated virion *per se* elicited a considerably high level of serum IgG-type neutralising antibody without an alum adjuvant [56]. Furthermore, the level of serum IgG antibody was retained at the peak for more than 6 months after a single injection, probably reflecting the generation and maintenance of long-term AFCs (antibody-forming cells, i.e. effector memory cells) [68]. No IgA response was elicited in mucosal tissues by subcutaneous injection, whereas a response was elicited in mice immunised nasally with inactivated virions by the aid of potent mucosal adjuvants, such as cholera toxin (CT) or CpG [66]. The Chinese government recently announced that they completed a phase I study of an inactivated virion vaccine, and that no serious complications were observed so far (http://my.tdctrade.com/airnewse/index.asp?id=8856).

### Component vaccines

*Virus vector-based vaccines:* As described in the previous section, S and N proteins are two major viral proteins which induce a high level of antibody response. Current strategies to develop an effective vaccine generally rely on the induction of potent neutralising antibodies on the mucosal surfaces. The study of attenuated parainfluenza virus suggest that immunisation with recombinant S proteins, but not E, M or N proteins, results in protection by eliciting neutralising antibodies, indicating the advantage of S antigen-expressing vaccines currently in development.

Likewise, the modified vaccinia virus Ankara (MVA) expressing the S protein (MVA-S) has been used successfully in mice [69], rabbits and monkeys [70]. MVA-S vaccination induced a high level of neutralising antibodies, which in turn results in protecting the monkeys from virus infection. In contrast, inoculation of attenuated vaccinia expressing the S protein into ferrets had no effect on the viral load and caused hepatitis upon challenge with SARS-CoV in MVA-S-immunised ferrets [71]. However, it remains unknown whether this has been caused by the mechanism of antibody-dependent enhancement of virus infectivity or some other immunopathological effect on the liver. In feline coronavirus infection, an antibody-dependent enhancement of the disease was observed in vaccinated animals with vaccinia vector [72]. Thus, such an unfavourable side effect of MVA-S vaccination has to be carefully considered for application.

Adenovirus type 5 vector expressing codon-optimised S, M or N was also developed as one of the candidate vaccines [73]. When six monkeys were immunised with these three recombinant adenoviruses, all developed antibodies against S1 fragments with neutralising activity. They also developed various levels of IFN-$\gamma$-producing T cells reactive to pooled 15-mer N peptides. However, no challenge experiment was carried out. Whether or not pre-existing immunity against adenovirus reduces the efficacy of recombinant adenovirus vaccine in humans needs to be further investigated.

Rhabdovirus-based vaccines were also developed, using a recombinant technology for rabies virus (RV) [74] and vesicular stomatitis virus (VSV) [75]. Both viruses, which encode the S protein in their genome, induced a high level of neutralising antibodies after a single injection into mice. In the case of VSV-based vector, pre-existing immunity is not a concern, and mice were protected from SARS-CoV infection by intranasal immunisation. RV can replicate in mucosal membrane cells and this is one of the advantages of

an RV-based vaccine. Intramuscular injection of RV-based vector expressing N protein did not elicit a neutralising antibody. Of note, the size of the S protein expressed by recombinant RV is smaller (~140 kDa) than that observed in other reports (~200 kDa) [10,69,75–77], suggesting that the S protein may not be modified by glycosylation during the replication of RV. In any case, the protective ability of the RV-based vaccine needs to be tested further.

*DNA vaccine:* DNA immunisation has been developed as a safe and stable vaccine technology for protection against a variety of infectious diseases. Since then, many efforts have been made to improve the efficiency of DNA vaccine, for example, by adding cytokines and costimulatory DNA adjuvants (review in [78]). Using modified codons to optimise expression, Yang *et al.* prepared plasmids encoding S protein and analysed their ability to elicit antiviral immunity in mice [79]. Both $CD4^+$ and $CD8^+$ T cells reactive to overlapping S peptides were detected and these mice generated neutralising antibodies, which accounts for their protection from respiratory infection. Depletion of both $CD4^+$ and $CD8^+$ T cells or adoptive T-cell transfer of immunised T cells did not affect protective immunity, whereas passive transfer of IgG from immunised mice provided immune protection. This study, along with that of attenuated parainfluenza virus [76], indicated that humoral immunity against S protein alone can confer protection. However, since SARS-CoV replicates in mice only transiently, it remains to be elucidated whether T-cell immunity is also important especially when neutralising antibodies fail to clear the virus.

The other studies used the plasmid-expressing N protein, expecting that a high level of intracellular N protein expression may induce strong CTL responses [80–82]. In order to increase the efficiency, Kim *et al.* [80] linked the *calreticulin (CRT)* gene to the N gene, which is a $Ca^{2+}$-binding, heat shock family protein abundantly present in the endoplasmic reticulum. When mice were injected three times with CRT/N DNA by a gene gun, humoral and T-cell immune response were enhanced and these immunised mice were protected from infection by recombinant vaccinia expressing N protein. This is the only approach so far which demonstrated the protective role of N-reactive T cells by vaccination. However, it remains unknown whether or not N-reactive T cells can contribute to suppressing the progression of SARS during natural infection.

Lastly, a combination of DNA vaccine with recombinant S protein [83] or whole-killed virus [84] were designed, with the expectation that this regimen may enhance not only the titre of neutralising antibody but also T-cell response, though the importance of the latter for protection is not yet clear.

*Recombinant proteins for vaccination:* Because the S protein in SARS-CoV is as heavily glycosylated as is the gp120 of HIV, the large scale production of recombinant protein is economically difficult. Nevertheless, a component vaccine has a great advantage with respect to safety, if it is effective enough. A baculovirus system was developed by Bisht *et al.* and they produced soluble recombinant polypeptide containing an N-terminal segment of the spike glycoprotein (nS) [85]. When mice were injected subcutaneously three times with nS with a Ribi (MPL + TDM) adjuvant, they were protected from SARS-CoV infection 4 weeks later. It would be interesting to study whether the neutralising antibody elicited by this protein subunit vaccine is sustained and is able to protect susceptible animals from disease.

## CONCLUSION

The mAbs against the structural proteins of SARS-CoV have been rapidly established and are being applied not only for basic research but also in clinical practice, including SARS patient therapy. Studies utilising these antibodies provide us enormous information with respect to the immune responses elicited by the SARS-CoV and for the development of vaccines against the SARS-CoV. What we learned from the emergence of SARS will be potently useful for preparing against any future outbreaks of new pathogens likely coming from wild animals.

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# Induction of protective immunity against severe acute respiratory syndrome coronavirus (SARS-CoV) infection using highly attenuated recombinant vaccinia virus DIs

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**Abstract**

SARS-coronavirus (SARS-CoV) has recently been identified as the causative agent of SARS. We constructed a series of recombinant DIs (rDIs), a highly attenuated vaccinia strain, expressing a gene encoding four structural proteins (E, M, N and S) of SARS-CoV individually or simultaneously. These rDIs elicited SARS-CoV-specific serum IgG antibody and T-cell responses in vaccinated mice following intranasal or subcutaneous administration. Mice that were subcutaneously vaccinated with rDIs expressing S protein with or without other structural proteins induced a high level of serum neutralizing IgG antibodies and demonstrated marked protective immunity against SARS-CoV challenge in the absence of a mucosal IgA response. These results indicate that the potent immune response elicited by subcutaneous injection of rDIs containing S is able to control mucosal infection by SARS-CoV. Thus, replication-deficient DIs constructs hold promise for the development of a safe and potent SARS vaccine.


*Keywords:* Vaccinia virus; Vaccine; SARS

## Introduction

Severe acute respiratory syndrome (SARS) has become a priority for healthcare agencies around the world given its communicability, associated mortality, and the potential for pandemic spread. As of 31 July 2003, 8,098 SARS cases had been identified worldwide, resulting in 774 deaths and a mortality rate of about 9.6% (World Health Organization statistics). SARS is now known to result from infection with a novel coronavirus (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). Evidence that SARS-CoV is the etiologic agent of SARS follows an experimental infection of macaques (*Macaca fascicularis*), fulfilling Koch's postulates (Fouchier et al., 2003). The clinical manifestations of SARS are hardly distinct from other common respiratory viral infections, including influenza. Because influenza epidemics might occur simultaneously with the eventual re-emergence of SARS, an effective SARS vaccine is urgently required, as well as more sensitive diagnostic tests specific for SARS.

Structural characterization of SARS-CoV and characterization of its complete RNA genome (Marra et al., 2003; Rota et al., 2003; Ruan et al., 2003) have provided us with the opportunity to develop a SARS vaccine. Like other coronaviruses, SARS-CoV is a plus-stranded RNA virus with a 30-kb genome encoding replicase gene products and the 4 structural proteins; i.e., spike (S), envelope (E), membrane (M), and nucleocapsid

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(N) (Marra et al., 2003; Rota et al., 2003). The S protein is thought to be involved in receptor binding, while the E protein has a role in viral assembly, the M protein is important for virus budding, and the N protein has a role in viral RNA packaging (for review, see reference (Holmes, 2003)). Recently, angiotensin-converting enzyme 2 (ACE2) has been identified as a cellular receptor for SARS-CoV (Li et al., 2003). Thus, the first step of infection likely involves binding of S protein to the ACE2 receptor. In a model of MHV infection, S protein is known to contain important virus-neutralizing epitopes that elicit neutralizing antibody responses in mice (Collins et al., 1982). Therefore, the S protein of coronavirus might be manipulated to induce immunity. However, S, M, and N proteins are also known to contribute to the host immune response (Anton et al., 1996; Jackwood and Hilt, 1995). A DNA vaccine encoding the S glycoprotein of the SARS-CoV induces T cell and neutralizing antibody responses, as well as protective immunity, in a mouse model (Yang et al., 2004). Vaccination with a plasmid expressing N protein is capable of generating strong N-specific humoral and T-cell-mediated immune responses in vaccinated C57BL/6 mice (Kim et al., 2004; Zhao et al., 2005; Zhu et al., 2004). In addition, N-specific $CD8^+$ T cells provide protective immunity against some coronaviruses (Collisson et al., 2000; Seo et al., 1997).

The DIs strain is a highly restricted host range mutant of the vaccinia virus isolated by successive 1-day egg passage of the DIE vaccinia strain, an authorized strain for smallpox vaccine and actually used in Japan until 1981. DIs does not replicate and is not pathogenic in mice, guinea pigs or rabbits. Furthermore, the DIs does not replicate in various mammalian cell lines (Tagaya et al., 1961). Recently, we established a system for foreign gene expression by inserting target genes into this strain, after which expression of (i) bacteriophage T7 polymerase, and (ii) the full-length HIV-$1_{NL432}$ *gag* gene, was observed (Ishii et al., 2002), thus demonstrating the usefulness of this system.

In the present study, we constructed a recombinant vaccinia virus DIs expressing one or more SARS-CoV structural proteins (E, M, N, and S, or a combination of E, M, and S (E/M/S), or E, M, N and S (E/M/N/S)). These rDIs vaccines were administered to mice either subcutaneously or intranasally, and the humoral and cellular immunity against SARS-CoV in vaccinated mice were analyzed. We demonstrated here that replication-deficient DIs constructs expressing S protein alone or in combination with other components, but not N alone, elicited strong protective immune responses against SARS-CoV infection.

## Results

### *Expression of SARS-CoV structural proteins by rDIs*

The structures of transfer vectors used in this study (pDIsSARS-E, pDIsSARS-M, pDIsSARS-N, pDIsSARS-S, pDIsSARS-E/M, pDIsSARS-E/M/S and pDIsSARS-E/M/N/S) were summarized in Fig. 1. Expression of SARS-CoV N and S proteins in chick embryo fibroblast (CEF) cells infected

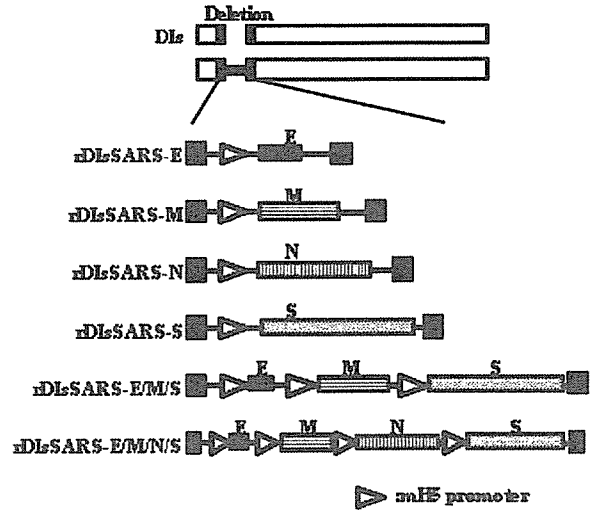


Fig. 1. Schematic diagram of rDIs constructs expressing SARS-CoV structural proteins. DNA fragments encoding E, M, N and S proteins were inserted into the location of the 15.4 kb deletion in DIs using the vaccinia virus transfer vector pDIsgptmH5. Six rDIs constructs are shown.

with rDIsSARS was detected by Western blotting using monoclonal antibodies (Fig. 2A) (Ohnishi et al., 2005). Purified SARS-CoV virion was used as a positive control (Fig. 2A, lane PC). A robust signal was detected at 50 kDa, corresponding to the N protein of SARS-CoV, as predicted by its genomic size (Marra et al., 2003; Rota et al., 2003). A band approaching 200 kDa likely corresponds to the S protein, which is known to be heavily glycosylated (Fig. 2A). Our results are consistent with data reported by Xiao et al. (2003) who expressed the full-length S glycoprotein of SARS-CoV Tor2 strain in 293 cells and demonstrated a protein approaching 180–200 kDa by SDS gel electrophoresis. Concerning the M protein, only a smear band in the stacking gel was detected using a polyclonal antibody against synthetic peptide of the M protein (Mizutani et al., 2004), presumably because it formed large oligomers with SDS-resistance in cells (Fig. 2A). Similar result was mentioned by the analysis of the M protein of SARS-CoV (Buchholz et al., 2004) and infectious bronchitis virus (Weisz et al., 1993).

The subcellular localization of S, M, and N proteins was analyzed by immunofluorescence staining. Cells infected with rDIsSARS-M demonstrated M proteins primarily co-localized with the Golgi marker GM-130 (Fig. 2B), which is consistent with the results of the recent study (Nal et al., 2005). Individually expressed SARS-CoV N protein could be detected partially with Golgi apparatus, but remained principally localized to the cytoplasm (Fig. 2B). Overexpressed recombinant SARS-S glycoprotein could be detected partially with Golgi apparatus, but also be detected throughout the cytoplasm (Fig. 2B). These results indicate that cells infected with rDIsSARS expressed significant levels of SARS-CoV proteins under the control of mH5 promoter with an expected post-translational processing (Nal et al., 2005; You et al., 2005).

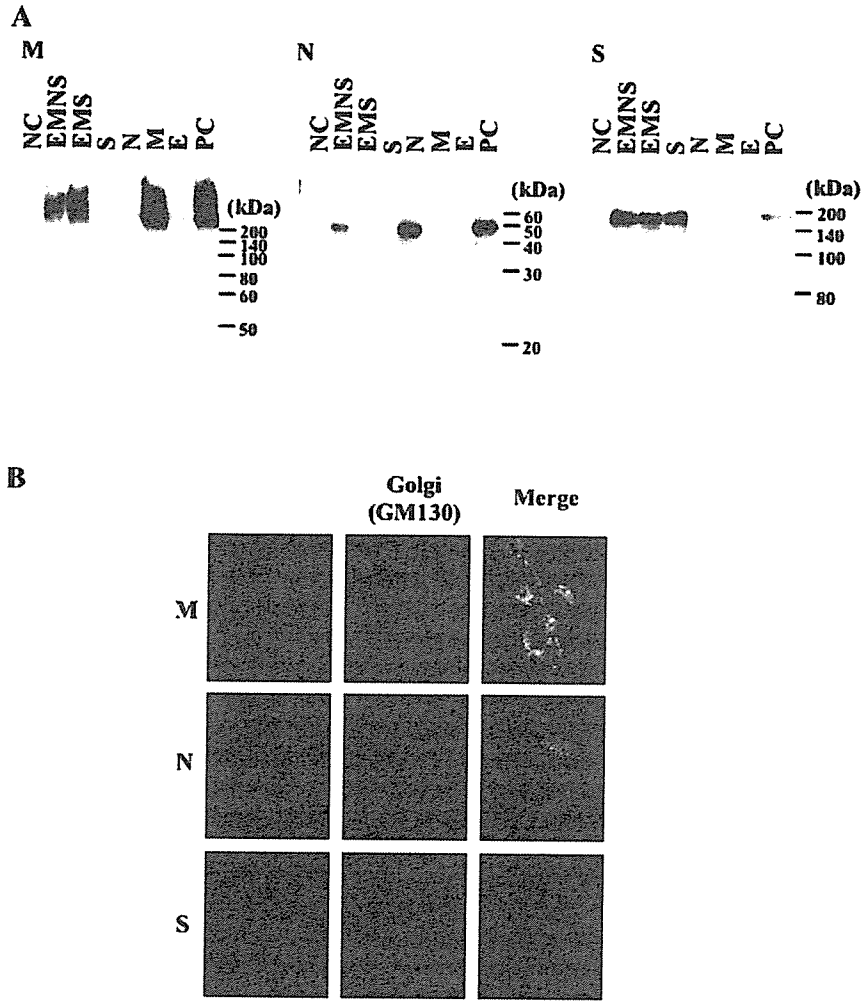


Fig. 2. Western blot analysis and indirect immunofluorescence analysis. (A) CEF cells were infected with rDIs constructs expressing SARS-CoV structural proteins (M, N and S, respectively). Purified SARS-CoV virion (0.5 μg) was used as a positive control. SARS-CoV proteins were detected using monoclonal antibodies (N and S) or polyclonal antibodies (M). Detection of bound antibodies was done with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibody, and visualized by chemiluminescence. (B) CEF cells were infected with rDIs constructs expressing SARS-CoV structural proteins (M, N and S, respectively). To detect SARS-CoV proteins, the cells were incubated with rabbit polyclonal antibodies against these proteins. The cells were further incubated with FITC-conjugated goat anti-rabbit IgG. To analyze subcellular localization of these proteins, monoclonal antibody against GM-130 (Golgi marker) and rhodamine-conjugated goat anti-mouse IgG were used. SARS proteins are shown in green, Golgi apparatus is shown in red and co-localization, where it occurs, is shown in yellow.

*rDIsSARS induces serum IgG antibody responses specific for SARS-CoV*

To examine the anti-SARS-CoV response in mice after inoculation with rDIsSARS, four mice in each group were subcutaneously or intranasally inoculated three times with 10 pfu of rDIsSARS-N, rDIsSARS-M, rDIsSARS-S, rDIs-SARS-E/M/S or rDIsSARS-E/M/N/S. Ten days after the final inoculation, vaccinated mice were observed to have high levels of anti-SARS-CoV IgG antibodies in their sera (Fig. 3).

In order to prove effective vaccination, we next examined whether neutralizing antibodies against SARS-CoV were elicited in these mice. Neutralizing antibodies against SARS-CoV were induced in mice following subcutaneous or intranasal injection of rDIsSARS-S, rDIsSARS-E/M/S, or rDIsSARS-E/M/N/S, but not in mice immunized with rDIsSARS-N or rDIsSARS-M. These results of ELISA data were incorporated into Fig. 3 by depicting the neutralization positive serum as closed circles. Thus, our results, consistent with others (Bisht et al., 2004; Buchholz et al., 2004; Yang et al., 2004), indicate that the S protein is a prerequisite for eliciting a sufficient IgG antibody response for neutralization. Similar neutralizing activity was obtained in mice receiving S alone or in combination with other components. Therefore, we expected that the rDIsSARS expressing E/M/N/S proteins in combination could be the best vaccine candidate among others.

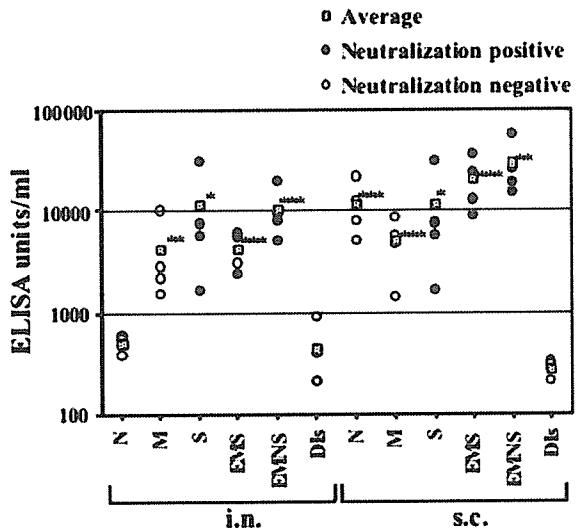


Fig. 3. Detection of anti-SARS-CoV IgG in vaccinated mice. IgG antibody levels against SARS-CoV were determined as described in Materials and methods. SARS-CoV-specific IgG titers were calculated as follows: SARS-specific IgG titer (ELISA units/ml) = (the unit value obtained for wells coated with virus-infected cell lysate) − (the unit value obtained for wells coated with non-infected cell lysate). $*P < 0.1$, $**P < 0.05$, $***P < 0.01$ vs. DIs-administered group. The data for neutralizing sera are represented by closed circles and the data for non-neutralizing sera are represented by open circles.

*Intranasal inoculation of rDIsSARS expressing E/M/N/S induces SARS-CoV-specific IgA in nasal mucosa and a high level of mucosal IgG in parallel with that of serum IgG*

Mucosal IgA response is believed to be crucial for the protective immunity against various pathogens (Meeusen et al., 2004). We, next, examined mucosal immunity in the respiratory tracts of mice inoculated with rDIsSARS either subcutaneously or intranasally. The level of anti-SARS-CoV IgA within nasal wash fluid of vaccinated mice was determined by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4A, substantial levels of anti-SARS-CoV IgA were detected only in mice received intranasal inoculation of rDIsSARS-E/M/N/S, compared to those inoculated with parental DIs ($P = 0.0010$). The level of IgA detected in intranasally rDIsSARS-E/M/N/S-inoculated mice was similar to that observed following intranasal immunization with UV-inactivated, purified SARS-CoV virion (positive control). On the other hand, subcutaneous injection of all forms of rDIsSARS produced only slightly higher levels of IgA than those observed in DIs -injected control mice. Therefore, the results indicated that the subcutaneous route of injection is inefficient, especially when mucosal IgA response is required.

Since neutralizing activity was, nevertheless, detected in the nasal washes of mice following subcutaneous immunization (data not shown), we also measured anti-SARS-CoV IgG levels in the nasal washes of these mice (Fig. 4B). High levels of IgG were detected in the nasal washes of mice following nasal immunization, which were observed to correspond well with IgG levels in the serum (Fig. 4C). A similar trend was observed in mice following subcutaneous immunization, despite at a lower level than in mice immunized intranasally. These results suggest that neutralizing IgG antibodies are capable of reaching the mucosal surface if plasma levels are high enough.

*Protection of rDIsSARS-immunized mice from nasal SARS-CoV challenge is achieved without mucosal IgA response*

The level of protection against SARS-CoV challenge in mice following inoculation with rDIsSARS is a critical issue for the vaccine development. We inoculated three times with 10 pfu of rDIsSARS-N, rDIsSARS-E/M/S or rDIsSARS-E/M/N/S into four mice in each group either subcutaneously or intranasally. One week after final inoculation, the mice were challenged intranasally with $10^4$ tissue culture 50% infectious dose ($TCID_{50}$) of SARS-CoV. The results were shown in Fig. 4D. In mice inoculated with saline, $10^3$ $TCID_{50}$/ml of SARS-CoV were recovered from lung wash fluid on day 3. In contrast, titers of SARS-CoV from the lungs of mice subcutaneously immunized with rDIsSARS-E/M/S or rDIsSARS-E/M/N/S were below the limits of detection. The same was true for mice intranasally immunized with rDIsSARS-E/M/N/S, whereas the virus was recovered in mice similarly immunized with rDIsSARS-E/M/S. Taken into consideration of a relatively low or marginal level of mucosal IgA antibody in mice intranasally immunized with rDIsSARS-E/M/N/S or rDIsSARS-E/M/S, or even no IgA response by subcutaneous route as described above, it was suggested that mucosal IgG antibody, but not IgA antibody, likely contributed to the protective immunity, especially in mice simultaneously immunized with recombinant rDIsSARS-E/M/N/S.

On the other hand, titers of SARS-CoV from the lung wash fluid of mice intranasally or subcutaneously immunized with rDIsSARS-N, were similar or slightly lower than the titers of negative controls, suggesting that intranasal or subcutaneous administration of rDIsSARS-N does not protect mice from SARS-CoV challenge, which is highly reflected by the non-neutralizing nature of anti-SARS-CoV N antibodies.

*Cellular immunity induced by rDIsSARS*

Although now we know that the systemic neutralizing IgG antibody against SARS-CoV S protein is a major component of protective immunity, T cell responses are also important to protect hosts from various viral infection. In a previous study of coronaviruses, S protein was shown to play an important role in viral pathogenesis, as well as induction of protective immunity (Holmes, 2003). In order to assess the ability of rDIsSARS to induce SARS-CoV S-specific T cells, T cells from axillary lymph nodes (ALN), superficial cervical lymph nodes (CLN) and spleens of mice subcutaneously immunized with rDIs-SARS-S or DIs were isolated and stimulated in vitro with UV-inactivated, purified SARS-CoV virion. Culture supernatant was collected 4 days later, and the levels of interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, IL-5 and tumor necrosis factor-α (TNF-α) were measured. T cells in ALN produced the greatest cytokine levels (Fig. 5, and data not shown). This is not surprising in light of the subcutaneous route of immunization.

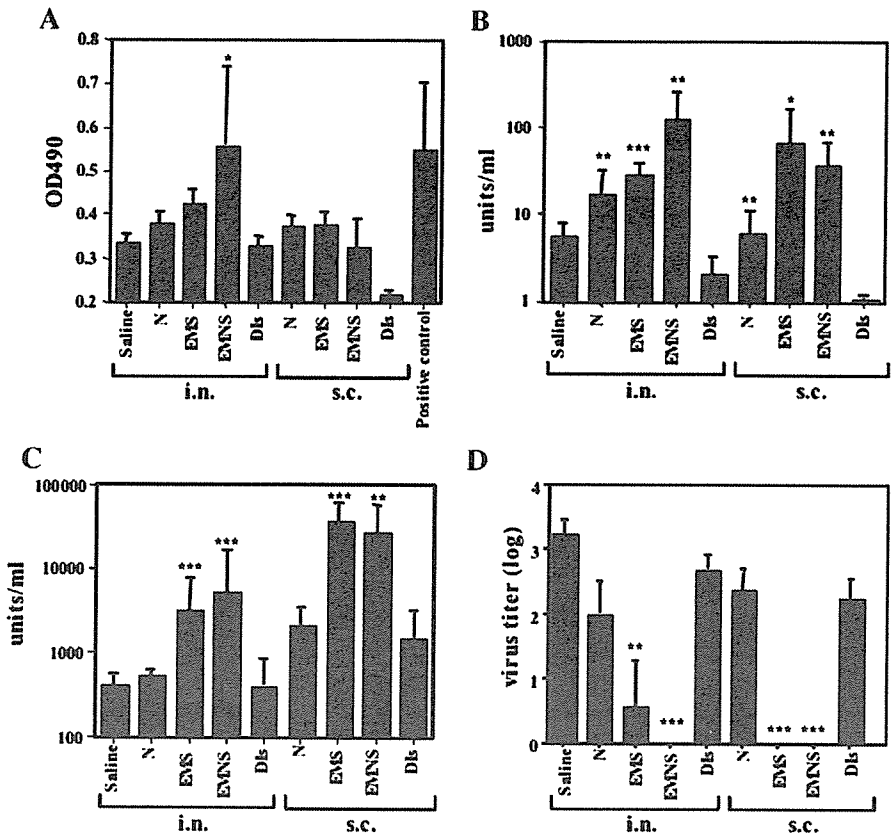


Fig. 4. Mucosally secreted anti-SARS-CoV IgG, but not IgA, antibodies are protective from nasal SARS-CoV challenge in vaccinated mice. The levels of Ig A and IgG antibodies against SARS-CoV were determined as described in Materials and methods. (A) Titers of anti-SARS-CoV IgA in the nasal washings of vaccinated mice. Error bars represent the mean ± SD. (B) Titers of anti-SARS-CoV IgG in the nasal washings of vaccinated mice. Error bars represent the mean ± SD. (C) Titers of anti-SARS-CoV IgG in the sera of vaccinated mice. Error bars represent the mean ± SD. (D) The titers of SARS-CoV in the lungs of vaccinated mice challenged 1 week later with $10^4$ $TCID_{50}$ of SARS-CoV. Virus titers are expressed as $\log_{10}TCID_{50}$. Error bars represent the mean ± SD. $^*P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.01$ vs. DIs-administered group.

Notably, mice immunized with rDIsSARS-S produced a high level of IFN-γ upon in vitro stimulation with UV-inactivated, purified SARS-CoV virion. The production of TNF-α, an inflammatory cytokine, was significantly elevated in T cells in ALN of rDIsSARS-S immunized mice after in vitro stimulation with virion antigens. However, TNF-α production was observed also in mice immunized with parental DIs without in vitro stimulation with virion antigens. Since T cells from the

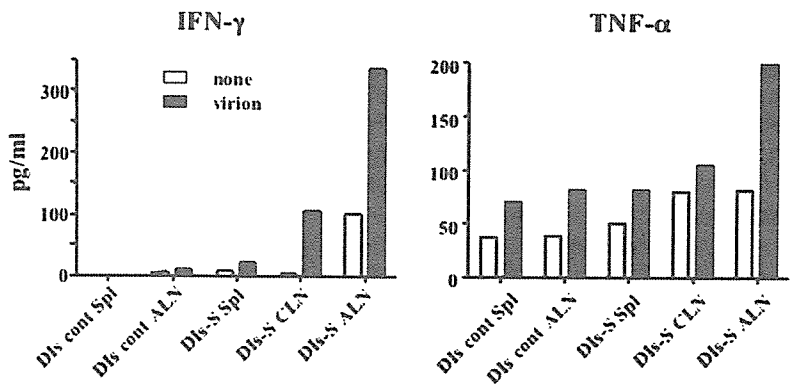


Fig. 5. In vitro response of SARS-CoV-specific T cells in mice subcutaneously immunized with rDIsSARS-S. CLN, ALN and spleens were obtained from mice 1 week after the third vaccination of either DIs control or rDIsSARS-S. After preparation of single cell suspensions, T cells were purified and cultivated with irradiated and T-cell depleted normal BALB/c mouse splenocytes as APCs in the presence or absence of 10 μg/ml of purified UV-irradiated SARS-CoV virion. Four days later, IFN-γ and TNF-α concentrations in the culture supernatant were measured.

lymph nodes of naïve mice did not produce cytokines even after in vitro stimulation with virion antigens (data not shown), it is possible that injection of DIs induces mild local inflammation, even when viral proliferation does not occur at the injection site. The pattern of IL-2, IL-5 and IL-4 production were similar to that of IFN-γ, and the maximum level of these cytokines in ALN T cells from rDIsSARS-S-immunized mice were 254, 227 and 88 ng/ml, respectively.

Next, we analyzed the antigenic epitopes of SARS-CoV-specific T cells in the spleen. We carried out IFN-γ enzyme-linked immunospot (ELISPOT) analysis using four 20-mer peptides corresponding to the ACE2 binding region of the S protein selected using the SYFPEITHI score (S44-47), as well as overlapping 20-mer peptides pool covering a whole N protein. When the splenic T cells of mice were analyzed following intranasal or subcutaneous immunization with the most potent vaccine, rDIsSARS-E/M/N/S, a high level of reactivity against S46 was observed especially in the T cells of subcutaneously immunized mice (Fig. 6A). Zhi et al. recently identified a $CD4^+$ T cell epitope, known as NYNYKYRYL, in BALB/c mice (Zhi et al., 2005). S46 contains this sequence, thus, these IFN-γ-producing T cells are likely $CD4^+$ T cells. To detect N-specific T cells, mice subcutaneously or intranasally immunized with rDIsSARS-N were analyzed by ELISPOT. In this case, ten peptides were pooled from amino-terminus of N protein, resulting in 5 pools of peptides. We thus detected N-reactive T cells capable of recognizing the first 10 peptides pool (Fig. 6B), and observed a greater proportion of N-specific T cells following nasal immunization than subcutaneous immunization. These results indicate that S- and N-specific T cells are generated systemically by rDIs.

In order to elucidate whether or not SARS-CoV-specific $CD8^+$ T cells were induced by immunization with the rDIs, the splenic T cells of mice subcutaneously or intranasally immunized with rDIsSARS-E/M/N/S were further analyzed by ELISPOT using a stably S-expressing A20.2J B cell S6.2 clone, as an antigen presenting cells (APC). Expression of S protein on the S6.2 clone was confirmed by FACS analysis using anti-SARS S monoclonal antibody (Fig. 7A). An empty vector transfectant, BOS-5, was used as a negative control APC. Subcutaneous and intranasal immunization with the most potent rDIsSARS-E/M/N/S generated a significant level of S-specific T cells (Fig. 7B), and a dramatic decrease in S-specific T cells was observed following partial depletion of $CD8^+$ T cells (Fig. 7C). Therefore, rDIsSARS-E/M/N/S was able to induce both SARS-CoV-reactive $CD4^+$ and $CD8^+$ T cells.

*Histopathological findings*

The immunogenicity of rDIs expressing SARS-CoV structural proteins was further evaluated by histopathological and immunohistochemical analysis of lung tissue in mice, the primary infection site of SARS-CoV (Fig. 8). Slight migration of inflammatory cells and mild disruption of the bronchial epithelium were detected in lung tissue of mock-vaccinated mice. SARS-CoV antigens were diffusely observed within the bronchial and alveolar epithelium. In contrast, significant lymphocytic infiltration into peribronchial sites, with little to no detection of SARS-CoV antigens, was observed in mice intranasally immunized with rDIsSARS-E/M/S or rDIsSARS-E/M/N/S (Fig. 8). The infiltrating lymphocytes were found to be CD3-positive T-cells, as determined by immunohistochemistry with anti-CD3 antibody (Fig. 8). On the other hand, intranasal or subcutaneous immunization by only N-expressing DIs induced neither T-cell infiltration nor protective immunity against SARS-CoV, despite of the induction of N-specific antibodies and T cells. These results suggest that marked induction of T-cell response in mice immunized with rDIsSARS-E/M/S and rDIsSARS-E/M/N/S help to eliminate SARS-CoV from the lung tissue. On the other hand, intranasal or subcutaneous immunization by only N-expressing DIs did not induce protective immunity against SARS-CoV, despite of the

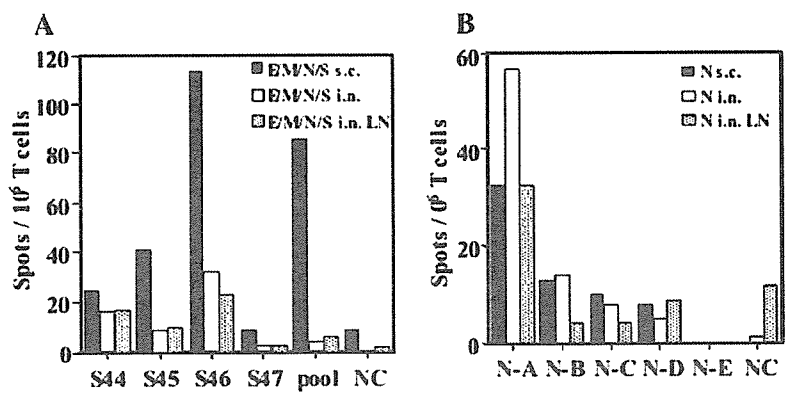


Fig. 6. Detection of SARS-CoV-specific T cells elicited by rDIsSARS-E/M/N/S or rDIsSARS-N vaccination. Splenic and lymph node (LN) T cells of mice s.c. or i.n. immunized with recombinant rDIsSARS-E/M/N/S (A) or rDIsSARS-N (B) were separated using a MACS system (Miltenyi Biotec), and IFN-γ ELISPOT analysis was performed. (A) T cells ($5 \times 10^5$ cells) from mice immunized with rDIsSARS-E/M/N/S were cultured with irradiated A20.2J B cells ($1 \times 10^4$), in triplicate, in a 96-well membrane plates coated with IFN-γ capture antibody in the absence or presence of 5 μM of S peptides (S44–S47). The numbers of IFN-γ spot-forming cells were then counted and are depicted. (B) T cells from mice immunized with rDIsSARS-N and A20.2J B cells were cultured as described in A. N protein (422 amino acids) was divided into 5 parts by 100 amino acids (A, B, C, D and E) and 10 peptides each of pooled 20-mer overlapping peptides specific to the each stretch of N sequence were used as an antigen.

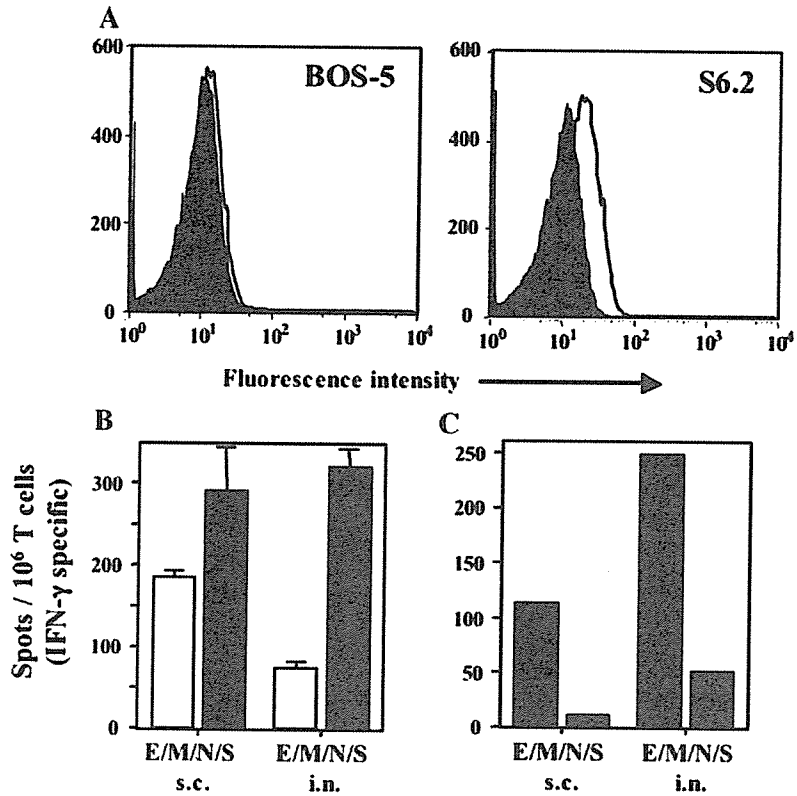


Fig. 7. Detection of SARS-CoV S-specific $CD8^+$ T cells in mice immunized with rDIsSARS-E/M/N/S. (A) A20.2J B cell clone expressing S (S6.2) or empty vector (BOS-5) was stained with biotinylated anti-SARS-CoV S monoclonal antibody (solid line) or control IgG (shadowed line), followed by the incubation with streptavidin-APC, and then analyzed by FACScalibur. A histogram of APC fluorescence of gated live cells (PI negative) is depicted. (B) Splenic T cells from mice immunized s.c. or i.n. with recombinant rDIsSARS-E/M/N/S were purified and IFN-γ ELISPOT analysis was carried out using γ-irradiated S6.2 and BOS-5 as APCs. The number of T cells reactive for BOS-5 control (white column) and S6.2 (black column) cells are shown. Error bars represent the mean ± SD. (C) Using the same splenic T cells as in panel B, $CD8^+$ T cells were partially removed using anti-CD8 mAb-coated magnetic beads (Miltenyi Biotec), and the number of T cells reactive for BOS-5 and S6.2 cells were counted by ELISPOT. The number of BOS-5-reactive T cells was subtracted and the number of S-specific T cells is depicted. Black columns: total T cells; grey columns: partially CD8-depleted T cells.

induction of N-specific antibodies and T cells. Thus, rDIs expressing the S protein, along with other membrane components (E/M and E/M/N), are capable of inducing strong immunity of both humoral and cellular arms and are fully competent to clear SARS-CoV infection.

## Discussion

The DIs strain, which replicates well in CEF cells but not in most mammalian cells, was isolated from the DIE strain of the vaccinia virus during serial 1-day egg passage, and it is characterized by the induction of tiny pocks on chicken chorioallantoic membrane (Tagaya et al., 1961; Kitamura et al., 1967; Ishii et al., 2002). The DIs-derived recombinant viruses express high levels of viral and inserted genes, even in non-permissive cell lines without any cytopathic effects (Ishii et al., 2002). In earlier studies, MVA strain of vaccinia virus, which is also replication-incompetent in most mammal cells, was used to express a variety of foreign genes and some of these recombinant viruses were studied as candidate vaccine vectors and appeared to be more effective than many replication-competent vaccinia virus vaccines (Sutter and Moss, 1992; Sutter et al., 1994; Belyakov et al., 1998; Nam et al., 1999; Stittelaar et al., 2000). rDIs does not replicate nor produce infectious virions in most mammalian cells, therefore the DIs strain has a safety advantage when used as a recombinant vaccine vector as for MVA. Recently, a recombinant DIs, rDIsSIVGag, expressing a full-length *gag* gene of SIV, was developed, and demonstrated to have a potential for use as an HIV/AIDS vaccine (Someya et al., 2004).

Attempts at vaccine development against SARS-CoV are ongoing by a number of organizations using various techniques (see review, (Groneberg et al., 2005)). DNA vaccines (Kim et al., 2004; Yang et al., 2004; Zhu et al., 2004; Zhao et al., 2005) and viral vectors such as vaccinia virus (Bisht et al., 2004; Weingartl et al., 2004), parainfluenza virus (Bukreyev et al., 2004), adenovirus (Zakhartchouk et al., 2005) and rhabdoviruses (Faber et al., 2005; Kapadia et al., 2005) are used as recombinant vaccines. Yang et al. (2004) showed that viral replication was reduced by more than six orders of magnitude in the lungs of mice vaccinated with these S plasmid DNA expression vectors, and protection was mediated by a humoral but not a T-cell-dependent immune mechanism. Bisht et al. (2004) showed that inoculation of BALB/c mice with a

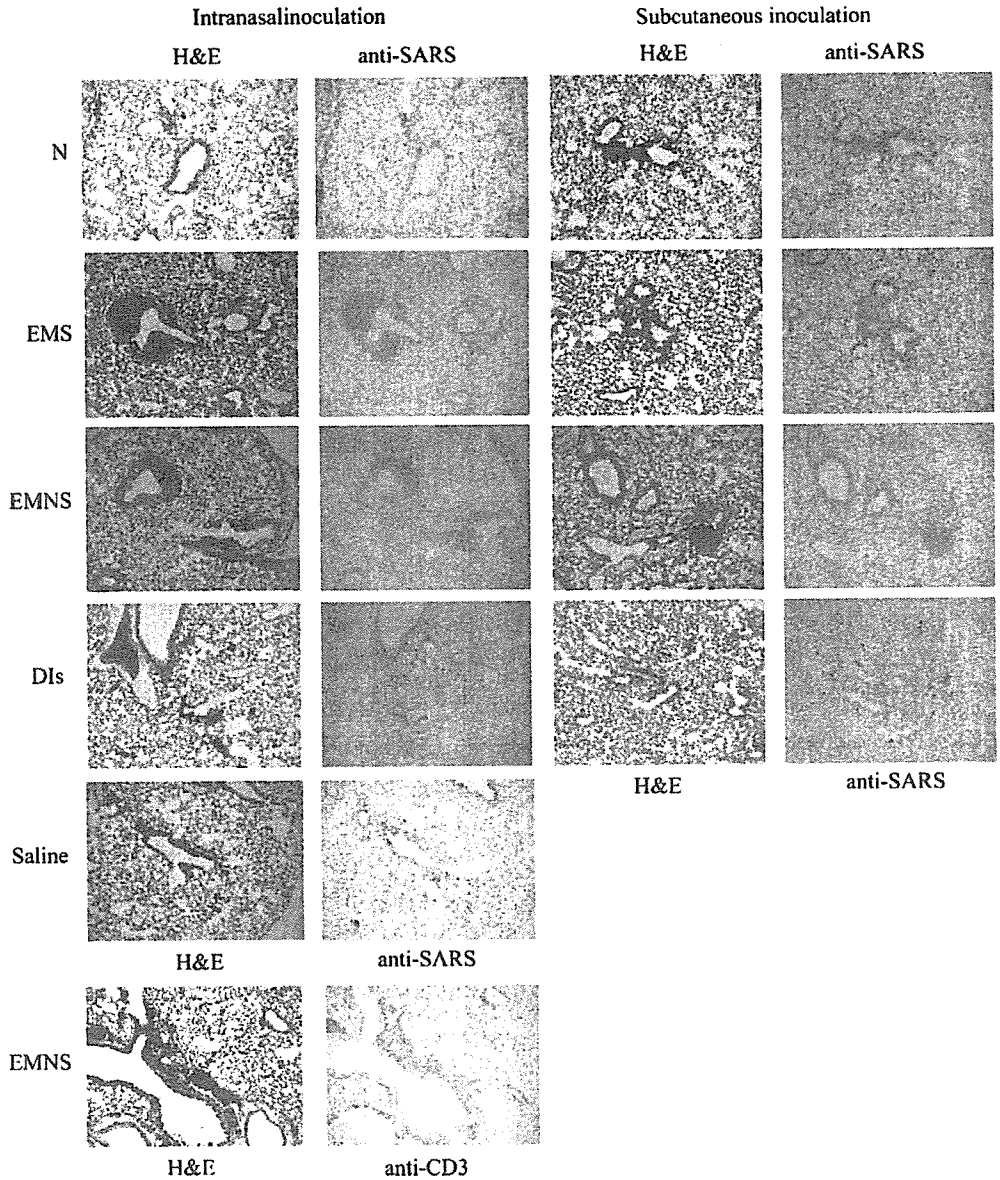


Fig. 8. Histopathology and immunohistochemistry. Lung specimen of mice immunized with rDIs expressing structural proteins of SARS-CoV and challenged with SARS-CoV. Mice were immunized i.n. or s.c. with DIs, rDIsSARS-N, rDIsSARS-E/M/S, rDIsSARS-E/M/N/S or saline. Mice were challenged with SARS-CoV 2 weeks after the final vaccination. Lungs were harvested 3 days after the challenge. The section was stained with hematoxylin and eosin (H&E) or immunohistochmically stained with anti-SARS-CoV antibody or anti-CD3 antibody.

recombinant MVA expressing SARS-CoV S protein elicited serum antibodies to SARS-CoV S protein and protective immunity against SARS-CoV infection. Previous studies demonstrated passive transfer of serum from immunized mice conferred protection against SARS-CoV in the respiratory tract following inoculation with either SARS CoV or recombinant MVA expressing S protein (Bisht et al., 2004; Subbarao et al., 2004). These results suggest that S protein is a crucial antigen in generating protective immunity. We observed that intranasal or subcutaneous inoculation of BALB/c mice with rDIs expressing S protein (rDIsSARS-S, rDIsSARS-E/M/S or rDIsSARS-E/M/N/S) produced serum antibodies capable of recognizing the SARS-CoV virion by ELISA, also capable of neutralizing SARS-CoV in vitro. The subcutaneous route appears to elicit stronger immunity than intranasal immunization with respect to the level of anti-SARS-CoV IgG antibody produced. Important finding here is that although the mucosal IgA antibody response was induced only in mice intranasally immunized with rDIsSARS-E/M/N/S, the mice administered with rDIsSARS-E/M/N/S by either route elicited strong protective immunity. Therefore, the protection was achieved in the absence of a mucosal IgA response in mice subcutaneously immunized with rDIsSARS-E/M/N/S. Thus, our results clearly show that mucosal infection might be prevented in the presence of a

high level of neutralizing serum IgG antibody. Control mice vaccinated with DIs not expressing envelope proteins were not protected, indicating that the effect was specific for the expressed envelope proteins of SARS-CoV and was not due to enhanced nonspecific immunity.

A recent report suggests that a combination of three adenovirus vector expressing SARS-S, -M and –N protein is capable of eliciting neutralizing antibodies in serum and N-specific T cell responses in rhesus macaques (Gao et al., 2003). However, in this report, the relationship between these immune responses and actual protection was not presented. In another study, the importance of SARS-CoV structural proteins in generating protective immunity was investigated by expressing them individually and in combination using a recombinant parainfluenza virus (PIV) type 3 vector. The expression of S with the two other putative virion envelope proteins, M and E protein, did not augment the neutralizing antibody response. In the absence of S, expression of M and E, or the nucleocapsid protein N, did not induce a detectable serum SARS-CoV-neutralizing antibody response (Buchholz et al., 2004). Our results were consistent with this in that expression of M or N proteins by administration of DIs harboring SARS-CoV M or N gene singly did not induce a neutralizing antibody response, although anti-SARS-CoV antibodies were detected by ELISA.

Recent studies have shown that vaccination with a plasmid expressing N protein can elicit SARS-CoV nucleocapsid-specific humoral and cellular immune responses (Kim et al., 2004; Zhu et al., 2004; Zhao et al., 2005). They showed that linkage of N protein to calreticulin, $Ca^{2+}$-binding protein known to enhance immune response, in a DNA vaccine resulted in the significant enhancement of the humoral and cellular immune responses to N protein in vaccinated mice. They also showed that the N protein-specific DNA vaccine elicited partial protection against N protein expressing vaccinia virus challenge, however, the efficacy of N-specific cellular immune responses in protection of SARS-CoV infection is not clear. Although we here showed that N protein expression by rDIs was capable of eliciting N-specific humoral and cellular immunity, vaccination with rDIsSARS-N failed to elicit a neutralizing antibody response against SARS-CoV infection in vitro, and failed to confer full protection in vaccinated mice against SARS-CoV challenge, suggesting that SARS-CoV N protein-specific antibodies and CTLs were not sufficient to provide full protection against SARS-CoV infection.

Histopathological analysis of lung tissues in the present study revealed a marked lymphocytic infiltration in peribronchial sites in mice immunized intranasally or subcutaneously with recombinant vaccinia virus DIs expressing E/M/S or E/M/N/S. Almost no SARS-CoV antigens were detected in these areas upon immunohistochemical analysis. The infiltrating lymphocytes were shown to be CD3 positive T-cells. This is the first evidence of induction of protective immunity against SARS-CoV associated with marked infiltration of T cells at a SARS-CoV infection site. We were able to detect SARS-CoV S-specific $CD8^+$ T cells in the spleen of these immunized mice by INF-γ ELISPOT. Thus, these results suggest that T cell induction in mice immunized with rDIsSARS-E/M/S and rDIsSARS-E/M/N/S might provide additional help to completely eliminate SARS-CoV in the lung. Of note, Weingartl et al. (2004) reported a low level of neutralizing antibody response in ferrets (*Mustela putorius furo*) immunized with a recombinant MVA expressing SARS-CoV S protein. They also showed that there are more rapid and vigorous neutralizing antibody responses in immunized ferrets, compared to control animals after challenge with SARS-CoV. However, SARS-CoV infection and spreading in the immunized ferrets is not prevented. Moreover, upon infection with SARS-CoV, strong inflammatory responses were noted in ferrets immunized with either recombinant MVA expressing SARS-CoV S or N protein, suggesting that vaccination with the recombinant MVA expressing SARS-CoV S or N protein may, in some case, lead to enhanced pathology during SARS-CoV infection (Czub et al., 2005; Weingartl et al., 2004). It was also reported that antibodies that neutralized most human SARS-CoV S enhanced entry mediated by the civet virus S, suggesting the possibility that such kind of vaccines might enhance viral infection (Yang et al., 2005). Although we observed no such pathology in rDIs-immunized mice after virus challenge, the potential for widespread tissue damage following administration of SARS-CoV proteins should be carefully investigated. In addition, further studies are required to clarify whether the recruited T-cells indeed play an important role in clearance of SARS-CoV from sites of infection.

In this study, we constructed rDIs containing genes encoding four structural proteins of SARS-CoV that were individually or simultaneously expressed. Intranasal or subcutaneous inoculation of BALB/c 3T3 mice with rDIs expressing S protein with or without other structural proteins elicited a high level of neutralizing antibodies against SARS-CoV and protective immunity, in the lungs of mice after intranasal challenge. Furthermore, both cellular and mucosal immunity against SARS-CoV structural proteins were also induced following administration of the rDIs. Therefore, the replication-deficient DIs strain is a feasible, safe and effective SARS vaccine vector.

## Materials and methods

### *Cells*

CEF cells and Vero E6 cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FCS), 1% penicillin-streptomycin and L-glutamine (GIBCO BRL/Life Technologies, Gaithersburg, MD). A20.2J murine B cells were maintained in RPMI1640 supplemented with 10% FCS, 50 μM β-mercaptoethanol, L-glutamine and antibiotics.

### *Plasmid DNA constructs and DNA preparation*

cDNA encoding SARS-CoV structural proteins were generated by reverse transcription of SARS-CoV HKU39849

(Accession No. AY278491) using superscriptII (Invitrogen Corp., Carlsbad, CA), followed by amplification using expand high fidelity PCR system (Roche diagnostics), as described previously (Ohnishi et al., 2005). These DNA fragments encoding E, M, N and S proteins were cloned into the vaccinia virus transfer vector pDIsgptmH5, which also harbored E. coli xanthine-guanine phosphoribosyltransferase, under control of a vaccinia virus p7.5 promoter in the cloning site of pUc/DIs (Ishii et al., 2002), to generate pDIsSARS-E, pDIsSARS-M, pDIsSARS-N, and pDIsSARS-S, respectively. To construct transfer vectors to generate rDIs expressing E/M, E/M/S, or E/M/N/S, DNA fragments encoding M, N and S proteins controlled by the mH5 promoter of the vaccinia virus (Wyatt et al., 1996) were inserted into pDIsSARS-E *Sma*I, *Not*I and *Sac*I sites, respectively, thus generating pDIsSARS-E/M, pDIsSARS-E/M/S and pDIsSARS-E/M/N/S (Fig. 1). A plasmid expressing S driven by the EF-1α promoter was constructed using pEF-BOS-bst (Yoshizawa et al., 2001), and designated pEF-S-bst.

*Generation of recombinant vaccinia virus*

Recombinant forms of DIs were obtained by hypoxanthine-guanine phosphoribosyltransferase selection (Falkner and Moss, 1988). Monolayers of CEF cells in 6-well plates were pre-incubated with DMEM containing 10% FCS, 25 μg/ml of micophenoic acid (MPA), 250 μg/ml of xanthine and 15 μg/ml of hypoxanthine. Infection was performed onto CEF cells grown in 8 cm dishes with DIs at a multiplicity of infection (moi) of 1.0. Transfection was performed using 20 μg of each DIs transfer vector and Lipofectamine (GIBCO BRL/Life Technologies). rDIs expressing SARS-CoV structural proteins were selected following four consecutive rounds of plaque purification of CEF cells in 6-well plates pre-incubated with DMEM containing 10% FCS, 25 μg/ml of MPA, 250 μg/ml of xanthine, and 15 μg/ml of hypoxanthine. Resultant rDIs expressing SARS-CoV structural proteins were designated as rDIsSARS-E, rDIsSARS-M, rDIsSARS-N, rDIsSARS-S, rDIsSARS-E/M, rDIsSARS-E/M/S and rDIsSARS-E/M/N/S, respectively, and subsequently maintained in CEF cells for use in further studies.

*Western blot analysis*

CEF cells infected with rDIs constructs harboring ORFs of SARS-CoV structural proteins were fractionated by SDS-PAGE under reduced conditions. Purified SARS-CoV virion (0.5 μg) was used as a positive control. The proteins were then transferred to an Immobilon-P PVDF membrane (MILLIPORE, Bedford, MA) and incubated with monoclonal antibodies against N or S proteins (Ohnishi et al., 2005) or polyclonal antibody against M protein (Mizutani et al., 2004). After washing, the membrane was reacted with HRP-conjugated Fab fragment of anti-mouse or rabbit IgG (H + L) (1:20,000, Santa Cruz Biotechnology, Santa Cruz, CA), followed by visualization of the bands using chemiluminescent reagents (Pierce, Rockford, IL).

*Indirect immunofluorescence analysis*

CEF cells were infected with rDIs expressing SARS-CoV structural proteins. After 48 h of incubation, the cells were washed with PBS and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were then permeabilized with 0.2% Triton X-100 for 3 min at room temperature, and then blocked with a non-fat milk solution, Block Ace (Yukijirushi Co., Tokyo, Japan). The cells were incubated with polyclonal antibodies against M, N or S proteins (Mizutani et al., 2004) for 60 min at 37 °C. The cells were further incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (TAGO, Burlingame, CA) diluted to 1:500 in PBS to detect SARS-CoV M, N or S proteins. To analyze the subcellular localization of SARS structural proteins, anti-GM-130 monoclonal antibody (BD Biosciences, Mississauga, ON, Canada) and rhodamine-conjugated goat anti-mouse IgG (TAGO) were used to stain the Golgi apparatus.

*Vaccination*

Animal studies were carried out under a protocol approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases, Japan. Five- to 6-week-old female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and immunized with $10^6$ pfu of rDIs, either subcutaneously (s.c.) or intranasally (i.n.). After 2 and 6 weeks, identical titers of recombinant virus were re-administered. One week later, the mice were intranasally challenged with $10^4$ $TCID_{50}$ of SARS-CoV in 20 μl of saline as previously described (Subbarao et al., 2004). Three days later, serum, nasal wash fluid and bronchoalveolar wash fluid were collected to measure viral titers and antibodies against SARS-CoV from mice that were sacrificed under anesthesia with chloroform.

*Detection of SARS-CoV specific IgA and IgG antibodies*

IgA and IgG titers against SARS-CoV were determined by ELISA, as previously described (Takasuka et al., 2004). Briefly, microtiter plates (Dynatech, Chantily, VA) were coated overnight at 4 °C with SARS-CoV-infected or mock-infected Vero E6 cell lysate samples previously treated with 1% NP40, followed by UV-inactivation. The plates were blocked with 1% OVA in PBS–0.05% Tween 20, and then incubated with serially diluted sera (1:10–$1:25^5$) for 1 h at room temperature. The plates were then incubated with either peroxidase-conjugated anti-mouse IgG (1:2000, Zymed, South San Francisco, CA) or IgA (1:2000, Southern Biotechnology, Birmingham, AL) antibody. The plates were washed three times with PBS-Tween at each step. The substrate mixture (o-phenylenediamine (Zymed) and hydrogen peroxide) was added to each well, and the absorbance of each well was read at 490 nm using a model 680 microplate reader (Bio-Rad, Hercules, CA). To provide a standard for IgG detection, serum was obtained from a hyper-immunized mouse and the OD490nm value of 100 ELISA units/ml of standard serum was around three in every assay.