Table 3. Identification of the essential amino acid motif within the epitope for SN5-25*)

Amino acid sequence	Reaction with SN5－25
ATVTKK	－
QAVTKK	－
QTATKK	－
QTVAKK	＋
QTVTAK	－
QTVTKA	－
QTVTKK	＋

*) The epitope Q^{245}TVTKK^{250} for SN5-25 was synthesized with substitutions on a membrane using the Fmoc method. The binding ability of SN5-25 to the various peptides was visualized with the immunostaining protocol described in Table 2. Bold "A" represents the substituted alanine residue.

Discussion

The 2002-2003 global epidemic of SARS had social impacts around the world. Although the epidemic was confined, the re-emergence of SARS is possible. In fact, several laboratory infections occurred after the epidemic. Recent epidemiological studies have suggested that bat species in southern China are the reservoirs for SARS-CoV-related viruses[11,13], and eradicating SARS-CoV in nature may prove impossible. Moreover, antiviral agents and vaccines have yet to be developed. Thus, reliable and sensitive diagnostic methods are critical for minimizing possible SARS epidemics. Detecting the infection in acute-phase patients is vital to guard against further infections, as the virus is shed in the acute phase in respiratory droplets. Although a variety of diagnostic methods have been developed, detecting SARS infection is still problematic. For example, antibodies are not detectable for ≥10 days after onset. The detection rates of RT-PCR and loop-mediated amplification (LAMP) 2-3 days after onset are only about 35-65%. Although an antigen-detection test by ELISA targeting NP has been developed, the assay takes several hours, which is not optimal for bedside diagnosis of SARS patients.

We established nine clones of mouse monoclonal antibodies to NP by immunization of mice with recombinant NP and a synthetic peptide. Kogaki *et al.*[8] used two different monoclonal antibodies to develop a sensitive antigen-detection method targeting NP by immunochromatography, which can detect $\geq 1.99 \times 10^2$ $TCID_{50}$/ml virus within 15 min. This method may be suitable for the rapid diagnosis of SARS, but the rationale for the high sensitivity was not determined and specificity was not evaluated. Therefore, in the present study, we analyzed epitopes and recognition sites of the monoclonal antibodies, as well as their specificity. Although all of the antibodies reacted with native SARS-CoV NP (Figs. 2 and 3), none reacted with the NP of the HCoV 229E strain. SARS-CoV and 229E were cross-reactive, but the monoclonal antibodies were not cross-reactive with 229E. In immunochromatography, rSN122 and rSN21-2 showed the highest specificity[8]. The binding sites of rSN122 and rSN21-2 are in the regions from amino acid residues 111-230 (trNP2 region) and 221-340 (trNP3 region), respectively. Therefore, two monoclonal antibodies recognizing the trNP2 and trNP3 regions may not inhibit their binding. On the other hand, when two monoclonal antibodies recognizing the same region were used, the immunochromatographic test showed less sensitivity. The 3D structures of NP in the N-terminal and C-terminal regions have been determined[7,27], but the actual distance between the trNP2 and trNP3 regions is unclear, as the 3D structure of the whole NP has not yet been ascertained. However, the distance between trNP2 and trNP3 may be large enough for rSN122 and rSN21-2 to bind NP at the same time, without interference. Using two different antibodies that bind to the different regions of NP may be essential to detect the protein with high sensitivity. Two monoclonal antibodies and the remaining seven antibodies bound to trNP2 and trNP3, respectively. This information may be also useful for functional analysis of SARS-CoV NP.

We determined the minimum epitope of the monoclonal antibody SN5-25 on NP. As the amino acid sequence of the epitope (Q^{245}TVTKK^{250}) is

unique to SARS-CoV, and no such sequence is found among the causative agents of human respiratory illnesses, SN5-25 is quite useful for diagnosing SARS.

In conclusion, our analysis of monoclonal antibodies to NP revealed that antibodies are very useful in establishing sensitive diagnostic methods for SARS as well as for analyzing the function of SARS-CoV NP.

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Search for potential target site of nucleocapsid gene for the design of an epitope-based SARS DNA vaccine

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ABSTRACT

It is believed today that nucleocapsid protein (N) of severe acute respiratory syndrome (SARS)-CoV is one of the most promising antigen candidates for vaccine design. In this study, three fragments [N1 (residues: 1–422); N2 (residues: 1–109); N3 (residues: 110–422)] of N protein of SARS-CoV were expressed in *Escherichia coli* and analyzed by pooled sera of convalescence phase of SARS patients. Three gene fragments [N1 (1–1269 nt), N2 (1–327 nt) and N3 (328–1269 nt)—expressing the same proteins of N1, N2 and N3, respectively] of SARS-N were cloned into pVAX-1 and used to immunize BALB/c mice by electroporation. Humoral (by enzyme-linked immunosorbent assay, ELISA) and cellular (by cell proliferation and $CD4^+$:$CD8^+$ assay) immunity was detected by using recombinant N1 and N3 specific antigen. Results showed that N1 and N3 fragments of N protein expressed by *E. coli* were able to react with sera of SARS patients but N2 could not. Specific humoral and cellular immunity in mice could be induced significantly by inoculating SARS-CoV N1 and N3 DNA vaccine. In addition, the immune response levels in N3 were significantly higher for antibody responses (IgG and IgG1 but not IgG2a) and cell proliferation but not in $CD4^+$:$CD8^+$ assay compared to N1 vaccine. The identification of antigenic N protein fragments has implications to provide basic information for the design of DNA vaccine against SARS-CoV. The present results not only suggest that DNA immunization with pVax-N3 could be used as potential DNA vaccination approaches to induce antibody in BALB/c mice, but also illustrates that gene immunization with these SARS DNA vaccines can generate different immune responses.

1. Introduction

A life-threatening and highly emerging disease called severe acute respiratory syndrome (SARS) originated in China in late 2002 and spread rapidly to many countries. Upon this outbreak, a worldwide collaboration network was coordinated by WHO. As a result of this extraordinary endeavor, in March 2003, SARS-CoV, a novel type of coronavirus, was identified as the etiologic agent of SARS [1]. The genomic sequence of SARS-CoV was completed and it was found that SARS-CoV has all the features and characteristics of other coronaviruses (groups I–III), but it is quite unique from them, representing a new group (group IV) [2,3]. SARS-CoV is believed to be a mutant coronavirus transmitted from a wild animal that developed the ability to productively infect humans [2,4].

Till date, there is no operational treatment for SARS. Quarantine or transmission-blocking measures have been the only means existing to curb its ruinous impact. Individuals convalescing from SARS have been seen to develop high titres of neutralizing antibodies [5]. Moreover, the appearance of antibodies coincides with the onset of SARS pneumonia [6,7]. These reports point to the possibility of vaccination as an effective therapy against SARS-CoV.

Prevention through vaccination would be a promising option that is less reliant on individual case detection to be effective. Even though there are no vaccines currently licensed for

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the human CoVs, vaccines have been successfully produced for some animal CoVs, such as certain strains of infectious bronchitis virus (poultry), bovine coronavirus and canine coronavirus [8–12].

The genome of SARS-CoV is a single-stranded plus-sense RNA ~30 kb in length and contains five major open reading frames (ORFs) that encode non-structural replicase polyproteins and structural proteins: the spike (S), envelope (E), membrane (M) and nucleocapsid protein (N), in the same order and of approximately the same sizes as those of other coronaviruses [2,4].

The SARS-CoV nucleocapsid (N) gene encodes a 50-kDa protein harbouring a putative nuclear localization signal [2]. However, the N protein is distributed predominantly in the cytoplasm of SARS-CoV-infected and N gene-transfected cells [13]. The SARS-CoV N protein is a highly charged, basic protein that can self-associate to form dimmers [14]. The three-dimensional structure of the N-terminal portion of the protein shares similarity with that of other RNA-binding proteins. The coronavirus N protein is thought to participate in the replication and transcription of viral RNA and to interfere with cell-cycle processes of host cells. As a result, it plays a critical role in SARS CoV pathogenesis [14–17]. In addition, the N proteins of many coronaviruses are highly immunogenic and expressed abundantly (90%) during infection [18]. High levels of IgG antibodies against N have been detected in sera from SARS patients [19]. The N protein can induce protective immunity or at least set up the protective response in some coronaviruses [20]. It is reported that N protein is a representative antigen for the T-cell response in vaccine setting [21], induces SARS-specific T-cell proliferation and cytotoxic T-cell activity and induces virus-specific cellular responses in human cells using mouse model [21,22].

From such observations, we hypothesize that N protein expressed in viral infected cells may be an effective mediator of the potential target for SARS-CoV vaccine. To address this issue, we therefore focused our studies on characterization of the N protein [N1 (residues: 1–422 aa, full-length sequence protein); N2 (residues: 1–109 aa, N-terminal region) and N3 (residues: 110–422 aa, middle plus C terminal region)] of SARS-CoV as a target antigen for vaccine development.

We constructed eukaryotic expression plasmid encoding N [(N1 (nucleotide: 1–1269), N2 (nucleotide: 1–327), and N3 (nucleotide: 328–1296)) gene fragments of the SARS-CoV and compared their individual potential immune responses in BALB/c mice for use in the development of SARS vaccine candidates. Therefore, a safe and effective SARS vaccine would be highly beneficial to the human health system.

2. Materials and methods

2.1. Molecular cloning, expression and purification of bacterium-derived SARS-CoV N protein

DNA fragments containing the full length and truncated SARS-CoV N gene were generated by PCR using SARS-CoV (strain Hanoi 01–03) cDNA as the template. Table 1 shows the forward and reverse primers with PCR cycling conditions that were used in order to sub-clone the PCR product as a Bam H1-Sal1 fragment into the pET21a vector (Novagen, Germany). Plasmids containing the N gene were then transformed into *Escherichia coli* strain Origami™ B (DE3). SARS-CoV N protein expression was induced in transformed competent cells by adding 1 mM IPTG (Invitrogen, USA) for 4 h. The expressed N protein, containing a small hexahistidine tag at the C terminus, was subsequently purified by using the His Bind® purification kits (Novagen) through Ni-IDA resin column and confirmed by SDS-PAGE using 12% polyacrylamide gels followed by western blotting using mouse anti-SARS N protein monoclonal antibody (1:1,000 dilution, Zymed, USA) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:500 dilution, Zymed, USA) as a primary and secondary antibody, respectively. Purified proteins were then analyzed with pooled sera of convalescence phase of SARS patients as a positive control and healthy volunteers as a negative control.

2.2. Plasmid DNA constructs and DNA preparation

In the present study, we used pVAX-1 (Invitrogen, USA), containing a CMV promoter and BGH polyadenylation sequence, for high-level mammalian expression of our DNA vaccine. For the generation of pVAX-N1/N2/N3, DNA fragments encoding SARS-CoV nucleocapsid were amplified by PCR using a set of primers as a template (Table 1). The amplified products were cloned into pGEM®-T Easy vector (Promega, UK). pGEMT-N genes were digested with Bam HI and PSTI (Roche, Switzerland) and then inserted into vector pVAX-1. The accuracy of these constructs was confirmed by DNA sequencing (Genotech Company, Seoul, Korea). The sequences were identical to the reported sequence [Gene Bank accession number AY307165 (gi: 31540948)]. The DNA was amplified in *E. coli* DH5α strain and purified by plasmid-purification kit (Qiagen, USA) and dissolved in endotoxin-free PBS to a final concentration of 1 μg/μl, and stored at −20 °C. The 260/280 ratios ranged from 1.8 to 2.0. The endotoxin content from purified plasmid DNA was below 10 EU/ml in which the level had no effect on the immune response.

Table 1
Primer sequence, target site, restriction enzyme, product size and PCR conditions

Expression	Target site	Sequence (5′–3′)	Restriction enzyme	Product size (bp)	PCR conditions
Prokaryotic	N1 [1–1269]	F: CGG GGA TCC ATG TCT GAT AAT GGA CCC CAA	BamH1	1269	94 °C for 10 min, then 30 cycles of 94 °Cfor 30 s, 48 °C for 30 s, and 72 °C For 1 min, Followed By one cycle of 72 °C for 5 min
		R: ACGC GTC GAC TGC CTG AGT TGA ATC AGC	Sal1		
	N2 [1–327]	F: CGG GGA TCC ATG TCT GAT AAT GGA CCC CAA	BamH1	327	
		R: ACGC GTC GAC CCA TCT GGG GCT GAG CTC TTT	Sal1		
	N3 [328–1296]	F: CGG GGA TCC ATG TAC TTC TAT TAC CTA GGA ACT GGC	BamH1	942	
		R: ACGC GTC GAC TGC CTG AGT TGA ATC AGC	Sal1		
Eukaryotic	N1 [1–1269]	F: CGC GGA TCC ATG TCT GAT AAT GGA GCC CAA	BamH1	1269	
		R: CGG CTG CAG TTA TGC CTG AGT TGA ATC GC	Pst1		
	N2 [1–327]	F: CGC GGA TCC ATG TCT GAT AAT GGA GCC CAA	BamH1	327	
		R: CTG CAG TCA CCA TCT GGG GCT GAG CTC TTT	Pst1		
	N3 [328–1296]	F: CGG GGA TCC ATG TAC TTC TAT TAC CTA GGA ACT GGC	BamH1	942	
		R: CGG CTG CAG TTA TGC CTG AGT TGA ATC GC	Pst1		

The underline sequences are restriction sites.

2.3. Transfection of 293T cells with pVAX-N and Western blot analysis

The human embryonic kidney cell line ATCC 293T (CRL 11268) was maintained in Dulbecco's modified eagle's medium (DMEM, Gibco BRL) supplemented with 0.1% penicillin–streptomycin–neomycin and 10% fetal bovine serum (FBS). Cells were incubated at 37 °C in 5% CO_2. When the cell density was 80–90% confluent, transfection was performed using a transfection kit (Trans IT-LT1 reagent, Myrus). Experimental groups were treated with the pVAX-N1/N2/N3 plasmid and a negative control was treated with pVAX1 plasmid. Forty-eight hours after transfection, the cells were harvested and lysed using triple-detergent lysis buffer. The expressed proteins in the cells were separated on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (0.45 μm, Schleicher and Schuell GmbH, Dassel, Germany). The nitrocellulose membrane was incubated for 1 h with TNT [15 mmol/l Tris-base, 140 mmol/l NaCl, 0.05% (v/v) Tween-20] containing 5% (w/v) powdered skimmed milk. Membranes were probed with mouse anti-SARS nucleoprotein (Zymed Laboratories) at a 1:1000 dilution in T-PBS for 2 h, washed four times with T-PBS, and then incubated with peroxidase rabbit anti-mouse (CAPPEL) at a 1:500 dilution in T-PBS containing 5% non-fat milk. Membranes were washed four times with T-PBS and developed using Hyperfilm-enhanced chemiluminescence (Amersham, Piscataway, N.J.) [23].

2.4. Animals and immunizations

Eight-week-old female BALB/c mice, each weighing 18–20 g, free of adventitious rodent pathogens, ectoparasites and endoparasites, were used in this study. Mice were maintained in a certified animal house under supervision and standard conditions of 22 ± 2 °C temperature and 55 ± 10% relative humidity with a photoperiod of 12:12 h of light–darkness. Water and a dry pellet diet were given *ad libitum*. The mice were acclimatized for 4 days prior to the start of the experiments.

All procedures were performed in accordance with the guidelines [24] for the care and use of laboratory animals approved by Seoul National University [Approval number SNU070827-2].

Four groups ($n = 5$/group) of mice were used in this study: group 1 (negative control), group 2 (Sham control), groups 3 and 4 (experimental). Group 1 was immunized with 30 μl PBS, group 2 with 30 μg of pVAX-1, group 3 with 30 μg pVAX N1 and group 4 with 30 μg pVAX N3. All immunizations were carried out three times at 2-week intervals intramuscularly (i.m.) by electroporation in the right quadriceps muscle. Injections and electroporation treatments were made under light anesthesia induced by a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) into the tibialis anterior muscle. A pair of electrode needles with 5 mm between probes was inserted into the muscle to cover the DNA injection sites and electric pulses were delivered using an electric pulse generator (Electro Square Porator ECM 830; BTX, San Diego, USA). Four pulses of 100 V each were delivered to each injection site at the rate of one pulse per second, with each pulse lasting for 40 ms. This was followed by four pulses of the opposite polarity, as described previously [25].

2.5. Analysis of the humoral immune response

Serum antibodies against N1/N3 proteins were assessed by enzyme-linked immunosorbent assay (ELISA). Mouse sera (1:40) were collected the day before (day 0) and after 10 days of final immunization, for specific antibodies detection. N-specific immunoglobulin IgG and its subclasses (IgG1 and IgG2a) were assessed by ELISA. In this case, recombinant N (250 ng) protein expressed in *E. coli* was purified and used as the detection antigen. HRP-conjugated goat anti-mouse IgG, IgG1 and IgG2a (1:5000, Sigma) were used as secondary antibodies. Optical density (OD) was read at 490 nm (A_{490}) using a plate reader (Bio-Rad, USA). The values obtained for mouse sera from the experimental groups were considered positive when they were ≥2.1 times that of the control group. Values >0.05 were not included.

2.6. Measurement of lymphocyte proliferation index (LPI)

Antigen-specific T-cell proliferation assay (LPA) was performed as described [26]. In brief, 10 days following the final injection, mice were sacrificed and single-cell suspensions were prepared from the spleens for each group. Splenocytes (2×10^5/well) in RPMI-1640 medium (Sigma) supplemented with 10% FBS were seeded in 96-well plates, in triplicates. Cultures were stimulated under the following various conditions for 60 h at 37 °C and 5% CO_2: 5 μg/ml concanavalin A (positive control), 5 μg/ml purified N antigen (specific antigen), 5 μg/ml bovine serum albumin (irrelevant antigen), or medium alone (negative control). CellTiter 96 Aqueous One Solution Reagent (20 μl, Promega, USA) was added into each well according to the manufacturer's protocols. Following 4 h incubation at 37 °C, absorbance was read at 490 nm. Proliferative activity was estimated using the stimulation index (SI) calculated from the mean OD490 of antigen-containing wells divided by the mean OD490 of wells without the antigen.

2.7. Analysis of $CD4^+$ and $CD8^+$ T lymphocytes

Spleens of mice were excised and ground on a 200-mesh copper net. One hundred μl of a suspension containing 1×10^6 spleen cells were added into 5 ml PBS and centrifuged at $1500 \times g$ for 5 min. After washing twice with PBS, the cells were re-suspended to make a 0.5-ml cell suspension. Twenty microliters of an anti-mouse $CD8^+$ monoclonal antibody (mAb) labeled with FITC and a $CD4^+$ mAb labeled with PE were added into the suspension. The cells were washed twice and incubated at 4 °C for 30 min. Finally, 0.5 ml of fluorescence conservation solution was added, and the cells were then detected by a FACS Caliber flow cytometer and data were analyzed using the WinMDI software (Beckton Dickinson).

2.8. Statistical analysis

Statistical analysis was performed using Duncan's multiple range test (SAS v. 8.2, SAS Institute). p-Values <0.05 were considered statistically significant.

3. Results

3.1. Production and identification of SARS-CoV N protein in E. coli

The full-length (N1) and truncated (N2 and N3) fragments of the N gene were cloned and expressed in *E. coli* Origami TM B (DE3) pLysS using expression plasmids, pET21a as described in Fig. 1c and Table 1. The inserts in plasmid were sequenced and proved to be correct. After induction with IPTG, the pET-N (pET-N1, pET-N2 and pET-N3) from *E. coli* with a hexahistidine tag were over-expressed, with expected sizes of 46.95, 12.09 and 34.85 kDa, respectively. pET-N1, N2 and N3 were successfully purified through resin based affinity chromatography (Fig. 1d). The truncated recombinant N1 and N3 (but not N2) proteins were

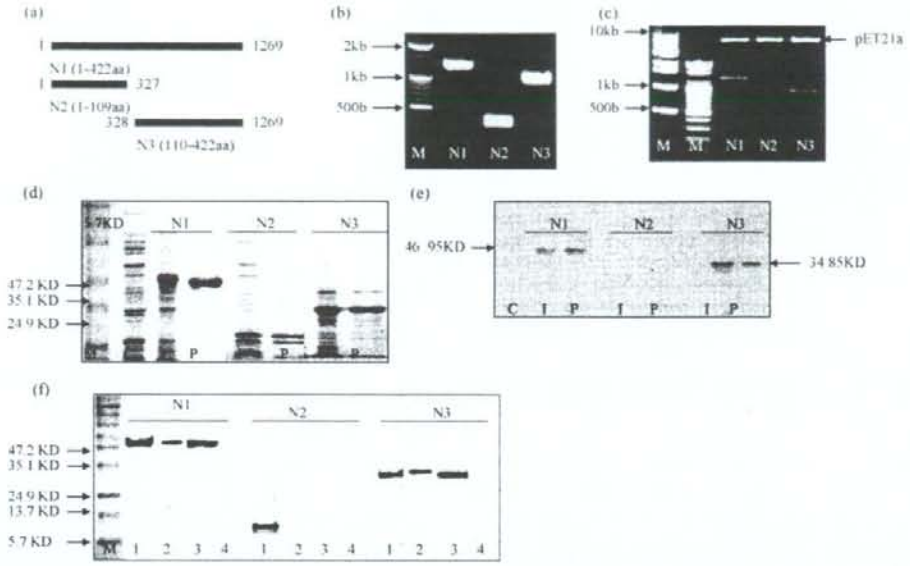

Fig. 1. Insert preparation, expression, purification and detection of N (N1/N2/N3) protein in *E. coli* Origami™ B (DE3). (a) Schematic representation of plasmid constructs expressing SARS-CoV N protein in prokaryotic expression vector and mammalian expression vector. Full-length N gene labeled as N1 (nucleotides: 1–1269) and truncated as N2 (nucleotides: 1–327) and N3 (nucleotides: 328–1296). The number is the amino acid position of N protein sequence. (b) PCR products of N1 (1269 bp), N2 (327 bp) and N3 (942 bp), M: DNA marker. (c) Prokaryotic expression vector (pET21a) cloning (BamH1 and Sal1 digestion). (d) Expression of N (N1/N2/N3) protein in *E. coli* by 12% SDS-PAGE. M: protein marker, C: control [protein from normal *E. coli* Origami ™ B (DE3)], I: 4 h after induction with 1 mM IPTG, P: N protein purification with His Band affinity Ni-IDA resin column. (e) SDS-PAGE gels were transferred onto cellulose nitrate membranes for western blotting for antigen confirmation. (f) Analysis of antigen. Coomassie blue stained from SDS-PAGE [M, protein marker and lane 1: positive control (purified protein)], western blotting with purified protein (lane 2), serum from convalescent SARS patient (lane 3) and serum from a healthy volunteer (lane 4).

identified by Western blotting with mouse SARS N protein antibody (Fig. 1e). We confirmed the same by using convalescent SARS patient sera as positive and healthy human sera as negative antibodies (Fig. 1f).

3.2. Structure and sequence analysis of DNA vaccine

To increase the potency of the specific immune response, the full-length N (N1) and truncated (N2 and N3) genes were ligated into pVAX-1 containing a CMV promoter and BGH polyadenylation sequence. The inserts in plasmid were indicated as pVAX-N1/N2/N3 and sequenced and proved to be correct (Fig. 2b).

3.3. Characterization of N protein in cells transfected with the various DNA vaccines

In order to characterize the expression of the SARS-CoV N protein in 293T cells transfected with the various DNA constructs, we performed a Western blot analysis using cell lysates derived from DNA-transfected cells. Mouse anti-SARS nucleoprotein was used for Western blot analysis. As shown in Fig. 2c, lysate from 293T cells transfected with N1 and N3 DNA revealed a protein band with a size of approximately 46.95 and 34.85 kDa, respectively. In contrast, N2 protein was not detected in lysates from 293T cells transfected with pVAX-N2 DNA Our data indicated that N DNA-transfected cells exhibited levels of N protein expression comparable to that in plasmid DNA with no insert-transfected cells (control).

3.4. Detection of antibody titer in mice immunized with the candidate vaccine

To evaluate the humoral immune response to DNA vaccines encoding SARS-CoV N (N1/N3) protein, we performed ELISA analysis using bacterium-derived N (N1/N3) protein and sera from mice ten days after the final vaccination with the two DNA vaccines (pVAX-N1 and pVAX-N3). As shown in Fig. 3, two groups (pVAX-N1

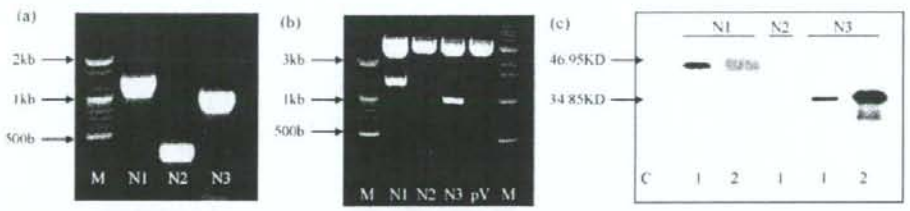

Fig. 2. Insert preparation, expression and confirmation of N (N1/N2/N3) protein in mammalian cells (ATCC 293T). (a) PCR products of N1 (1269 bp), N2 (327 bp) and N3 (942 bp), M: DNA marker. (b) Eukaryotic expression vector (pVAX-1) cloning (BamH1 and Pst1 digestion). (c) Expression of the constructs, determined by western blot analysis with antisera reactive with SARS CoV N was evaluated after transfection of the indicated plasmid expression vectors in ATCC 293T cells. C: control (whole cell lysate transfected with vector), lane 1: vector inserted gene (pVAX-N1/pVAX-N2/pVAX-N3), lane 2: Recombinant SARS N (N1/N3) protein as positive control.

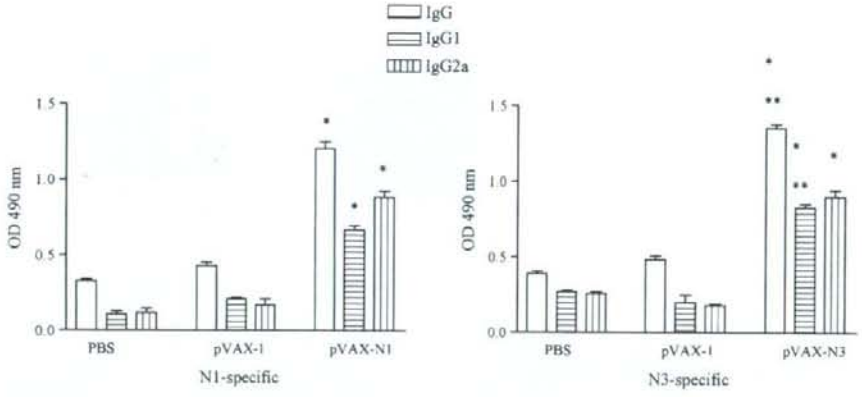

Fig. 3. Detection of humoral immune response in the immunized BALB/c mice. Mouse sera were collected 10 days after the final immunization. SARS-CoV N (N1/N3)-specific IgG, IgG1 and IgG2a were assessed. Data are presented as means ± S.D. *$p > 0.001$ and **$p > 0.01$.

and pVAX-N3) of vaccinated mice developed substantial antibody responses whereas the animals in the control groups (PBS and pVAX-1) did not show any detectable N-specific IgG and its subtype profile IgG1 and IgG2a antibody response. As shown in Fig. 3, the anti-SARS-CoV IgG, IgG1 and IgG2a were induced by all the immunization regimens. IgG, IgG1 antibody levels in the N3 groups showed higher degrees of increase compared to those in the N1 vaccinated groups, although the IgG2a antibody profiles were more or less similar in both groups.

3.5. *N-specific T cells proliferation*

Activation and proliferation of lymphocytes play a critical role in both humoral and cellular immune responses induced by vaccination. Therefore, we next evaluated whether pVAX-N1/N3 vaccination by electroporation and subsequent immunization could influence antigen-specific T-cell proliferation. As shown in Fig. 4, higher levels of lymphocytes stimulated by N protein were observed in mice immunized with pVAX-N1/N3 compared to controls (pVAX-1 and PBS) ($p < 0.01$). Cell proliferation in animals immunized with pVAX-N3 was appreciably higher than that in pVAX-N1 immunized animals ($p < 0.05$).

3.6. *$CD4^+$ and $CD8^+$ T lymphocytes responses*

Since activated $CD4^+$ and $CD8^+$ T lymphocytes are among the most crucial components of antiviral effectors, they were assessed in the vaccinated mice (Fig. 5). Flow cytometric analysis of unstimulated cells was used to standardize the background responses, and there was little variation in non-immunized mice. Compared with the control group ($p < 0.05$, the $CD8^+$ T cells increased and the ratio of $CD4^+/CD8^+$ decreased in the pVAX-N1 and pVAX-N3 (experimental groups), indicating that cytotoxic T lymphocyte (CTL) activity was induced by the recombinant plasmid. The $CD8^+/CD4^+$ ratio in the N3 groups was higher than that in the N1 groups but the difference was not statistically significant ($p > 0.05$).

4. Discussion

The 2002/2003 SARS epidemic is currently under control. However, the absence of an effective therapeutic agent against this relatively new lethal virus of animal origin, compounded by the threat of its potential for re-emergence, calls for the development of a SARS vaccine. Extensive efforts have been made for the

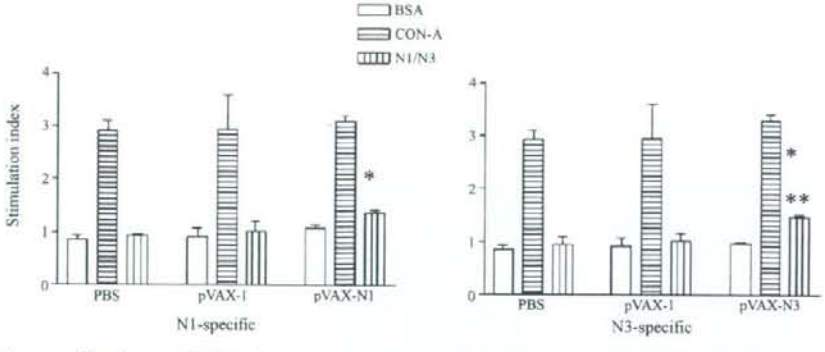

Fig. 4. N-specific lymphocyte proliferation assay. Pooled splenocytes were obtained from mice (five mice per group) immunized with the DNA vaccine on day 10 post-immunization. Splenocytes were stimulated *in vitro* with N protein (N1/N3-test groups), Con A (positive controls), and BSA (irrelevant antigen controls). Splenocytes from the control groups (pVAX-1 or PBS) were stimulated with N protein, and served as negative controls and sham controls. The stimulation index (SI) was calculated using the following formula: SI = (mean OD of Con A or antigen-stimulated proliferation)/(mean OD of non-stimulated proliferation). Each bar represents the mean SI ± S.D. of five mice. *$p > 0.01$ and **$p > 0.05$.

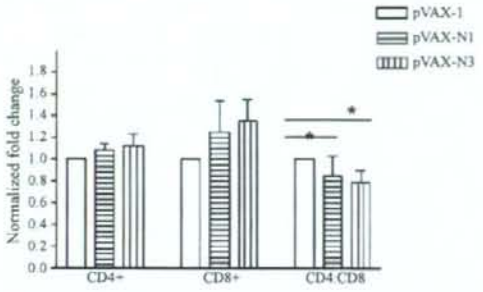

Fig. 5. Changes in CD4⁺CD8⁺ ratio of BALB/c mice in different groups. The CD4⁺ and CD8⁺ T subtypes were counted on day 10 post-immunization. Compared with the control group, the CD8⁺ T cells increased and the ratio of CD4⁺/CD8⁺ decreased in the experimental group, indicating that CTL activity was induced by the recombinant plasmid. *$p < 0.05$.

development of a SARS vaccine along a variety of approaches like inactivated whole virus vaccine, subunit vaccines, DNA vaccines, as well as live recombinant vaccines and various animal models of experimental infection by the SARS-CoV have been evaluated.

Results in a study by Tan et al. [5] suggest that as early as 2 days after the onset of illness, there are IgM and IgA anti-N antibodies present in the blood, and by 9 days, IgA levels against N protein are very high. This finding that N protein induces a strong IgA response parallels the findings by Tripp et al. [27] that monoclonal antibodies against N protein are present in the mucosal epithelia, as detected by immunohistochemistry on autopsy samples. These locations include the alveolar epithelium, trachea/bronchus, esophagus, gastric parietal cells and the intestinal tract. Strong immunoreactivity towards N protein suggests that the protein may be released from the virus or from infected cells into the circulation during infection. Alternatively, it may be presented by antigen presenting cells (APCs) for cytolysis of target cells. N protein does not appear to undergo rapid mutation like S protein. Coupled with the fact that S protein is more difficult to express, N protein could be a better target for the development of serological assays.

Similar to studies with S protein, putative antigenic sequences have also been identified for N protein. Using cDNA from full-length N protein expressed in *E. coli*, fragments were probed with SARS patients' sera in a study by Chen et al. [28]. Four regions with possible epitopes were identified: amino acids 51–71 (EP1), 134–208 (EP2), 249–273 (EP3) and 349–422 (EP4). While EP2 and EP4 were described as linear epitopes, EP1/EP2 and EP3/EP4 formed conformational epitopes that reacted with sera. As such, structural requirements appear to be important for antigenicity in N protein.

Keeping in mind these protein microarray data, we expressed recombinant N [amino acids: 1–422 (N1); 1–109 (N2) and 110–422 (N3)] proteins in *E. coli* and prepared N protein-specific monoclonal and polyclonal antibodies for developing a tool for SARS diagnosis. Two kinds of antibodies (N1-Ab, anti-full-length N protein and N3-Ab, anti-partial N protein) were obtained. We have shown N1 and N3 has 100% sensitivity and specificity for SARS-CoV antibody when testing with 10 SARS-CoV positive human sera compared to 50-healthy human sera by ELISA system. We also checked that the polyclonal or monoclonal antibodies do not have the cross-reactivity with the group 1 corona virus (human corona virus-229E and porcine epidemic diarrhea) and group II (human corona virus-OC43 and mouse hepatitis virus) [authors' unpublished data]. The identification of antigenic N protein fragments offered a potential target site for the design of an epitope-based vaccine against SARS.

In this report, we detected SARS-Cov N1 and N3 protein-specific immune response induced by pVAX-N1 and N3 DNA vaccination, respectively, and found significantly high titres of specific antibody and specific cell mediated immunity compared to control. These results indicate that N protein, which naturally exists in virus particles after binding of viral RNA, was able to induce strong humoral and cellular immune responses when induced by DNA vaccine, and it might be a prospective candidate gene for development of SARS-CoV vaccine. We have shown that DNA vaccination can successfully elicit SARS-CoV N-specific humoral and CD8⁺ T-cell responses in vaccinated mice. It is also evident that vaccination with pVAX-N3 is more effective than N1. We got the same result in ELISA system that the specificity and sensitivity of N3-antibody is higher than N1-antibody [authors' unpublished data].

Our data show that middle or C-terminal region of SARS-CoV N protein has antigenicity, but N-terminal region does not, reflecting published reports [29]. The result suggests that the truncated recombinant protein except N-terminal of SARS N protein, containing a highly conserved motif, is more useful for designing a DNA vaccine [3,30].

The nucleocapsid of corona virus group contains T-cell dependent and independent epitopes. Nude (athymic) mice immunized with HBV-nucleocapsid alone develop high titers of IgM, IgG2a and IgG2b antibodies, which are the predominant antibodies in Th1 responses. There is evidence that the specific structural folding of viral nucleocapsid is responsible for its high immunogenicity [31]. Two more studies analyzed humoral and cell-mediated immune responses in mice immunized with DNA vaccines expressing nucleocapsid.

Kim et al. showed that linkage of nucleocapsid protein to calreticulin increased humoral and cellular immune responses in vaccinated mice compared to mice receiving DNA-nucleocapsid alone. Shi et al. showed that expression of membrane protein augments the specific responses induced by SARS-CoV nucleocapsid DNA immunization. Zhu et al. showed a high level of antibody titer in mice after three injections of DNA-nucleocapsid [7,32–34]. The success of immunization depends on several factors, such as type of antigen, route of administration and usage of adjuvants. Widera et al. increased DNA vaccine delivery and immunogenicity by electroporation *in vivo* [25]. In this study, mice were immunized by electroporation.

DNA immunization has been well modeled in mice for the evaluation of best possible parameters for immunization as also the types of immune responses produced, as is evident from data till date. DNA vaccines also seem promising in human application. However, effects in mice may often be more vivid than those in humans; hence, certain safety issues need to be taken care of regarding immunogenicity of DNA vaccines in humans. DNA vaccines in larger animals are still not totally effective due to significant restrictions with the recent technologies. Many characteristics still remain to be considered prior to the development of a DNA vaccine against SARS-CoV in humans. In our present report, we have illustrated that the presented SARS N DNA vaccine expressing plasmid induces specific immune responses in mouse. However, we did not run tests to check the protective effect with challenge SARS-CoV. Our next target would be to evaluate and establish the efficacy of this SARS DNA (pVAX-N3) vaccine in non-human primate model.

Acknowledgements

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BRIEF REPORT

Development of a serotyping ELISA system for Thailand virus infection

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Abstract To distinguish Thailand virus infection from infections with other hantaviruses, we established an ELISA serotyping system using a truncated nucleocapsid protein of Thailand virus lacking 49 amino acids at the N-terminus. In evaluations using patient and rodent sera, Thailand virus infection was readily distinguished from Hantaan and Seoul virus infections. Therefore, this ELISA system is an effective alternative to neutralization tests.

Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne viral zoonosis caused by viruses belonging to the genus *Hantavirus*, family *Bunyaviridae* [1]. Four hantaviruses are currently known to be causative agents of HFRS: Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava–Belgrade virus (DOBV), and Puumala virus (PUUV). Hantaviruses are considered to be closely associated with their rodent hosts due to a coevolution process [2, 3].

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Thailand virus (THAIV) was isolated from a greater bandicoot rat (*Bandicota indica*) captured in Thailand [4]. THAIV has a distinct focus reduction neutralization test (FRNT) pattern [5] and was shown to belong to a distinct lineage by phylogenetic analysis [6]. Recently, we reported the first HFRS case related to THAIV [7]. A seroepidemiological study revealed the existence of hantavirus infection among patients in Thailand with leptospirosis-like symptoms [7, 8]. Only a few cases of THAIV infection have been reported to date, but we believe that THAIV causes HFRS-like disease in Southeast Asia. Due to its immunological cross-reactivity with HTNV, SEOV, and DOBV, the FRNT is required to differentiate between hantavirus serotypes [9, 10]. However, the FRNT requires specific technical skill and a biosafety laboratory for handling viruses. To overcome these limitations, we developed a serotyping ELISA system using the truncated recombinant nucleocapsid proteins (NPs) of HTNV, SEOV, and DOBV [9]. The hantavirus NPs possess immunodominant, linear, and cross-reactive epitopes within their N-terminal 100 amino acids (aa) [9, 11, 12]. By removing 49 aa from the N-terminus, serotyping antigens that retained serotype-specific, multimerization-dependent epitopes in the C-terminal half of the NPs were prepared [12, 13]. In this study, the application of the serotyping ELISA system was expanded to the diagnosis of THAIV infection.

THAIV strain thai749 was kindly supplied by Dr. P. W. Lee (WHO Collaborating Center for Virus Research, Korea). The virus was propagated in the E6 clone of Vero cells (ATCC C1008, CRL 1586). Recombinant baculoviruses (Autographa californica nuclear polyhedrosis virus) containing regions encoding the whole and truncated NPs of hantaviruses (HTNV, SEOV, and THAIV) were propagated in High Five cells, as described previously [9]. The cDNA of THAIV strain thai749 was prepared as described previously

[14]. A portion of the gene encoding the entire THAIV NP was amplified using the polymerase chain reaction (PCR) with the primers Bam_THLS46F (5′-*GGATCC*ATGGCAA CTATGGAAGAG-3′) and Bam_THLS1344R (5′-TGTG*G GATCC*TAGAGTTTTAA-3′; the *Bam*HI sites are shown in italics) and that of aa 50–429 of THAIV NP was amplified by PCR with primers Bam_THLS193F (5′-AC*GGATCC*ATG GTGGCTGCATCAAT-3′) and Bam_THLS1344R. The amplified DNA was subcloned into the *Bam*HI site of the donor plasmid pFAST-Bac1 and then expressed using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA). The indirect immunofluorescent antibody (IFA) assay was carried out as described previously [15]. As shown in Table 1, the whole rNP of THAIV exhibited the same reactivity pattern to monoclonal antibodies (MAbs) as the corresponding authentic viral antigens, which showed that the rNP was well conserved. The MAb E5/G6, which recognizes a common epitope of hantavirus NPs [16], could detect full-length and truncated rNP of THAIV, demonstrating that MAb E5/G6 is useful as a capture antibody for THAIV antigens [9]. We can provide E5/G6 MAb upon request. HTNV-specific (C24B4 and BDO1) and SEOV-specific (DCO3) MAbs could not detect the THAIV rNPs. As an epitope of MAb ECO2 is localized between aa residues 1 and 33 of the N-terminus [12], the truncated NP of THAIV possess reactivity to MAb ECO1, but the reactivity was lower than that obtained with other truncated rNPs of HTNV and SEOV. The results for THAIV antigens shown in Table 1 were consistent with those reported previously for HTNV, SEOV, and DOBV [9].

A total of 96-well plates were coated for 1 h with MAb E5/G6 in PBS as a capture antibody, and then ELISA was performed as described previously [9]. Sera from HFRS patients previously diagnosed as being infected with HTNV, SEOV, and THAIV were used, and two human sera confirmed to contain no antibodies to any hantavirus by ELISA, IFA and Western blot were used as negative controls [7, 9, 17]. Figure 1a shows reaction patterns of patient sera showing OD values greater than 0.7 against whole NP from the homologous virus. All three whole rNPs reacted well with sera from patients infected with, HTNV, SEOV, or THAIV (Fig. 1a, left). In contrast, each truncated rNP reacted strongly with homologous sera, but reacted either not at all or with much lower intensity with heterologous sera (Fig. 1a, right). Figure 1b shows the effectiveness of the test for serotyping patient sera infected with HTNV, SEOV, or THAIV. The sera shown in Fig. 1a were re-examined and included in Fig. 1b (marked with asterisk). In Fig. 1b, upper panel, ELISA OD values of HTNV, SEOV, and THAIV patient sera to homologous or heterologous whole rNPs were plotted with different markers. As shown, the regression lines of markers for HTNV and SEOV patients crossed with each combination of antigens. Further markers for paired sera of THAIV patient were plotted close to the regression lines. Therefore, whole rNPs were not applicable for serotyping of THAIV infection. On the other hand, using the truncated rNPs, the serotypes of the infecting viruses could be distinguished clearly (Fig. 1b, lower panel). However, the ELISA OD value using truncated NP antigen was lower in some cases than that obtained with whole NPs. Therefore, the sensitivity of the ELISA using truncated rNPs might be lower than with whole rNPs. These results indicate that whole rNP antigens are effective for detecting anti-hantavirus antibodies and that the series of truncated rNPs (aa 50–429) is effective for serotyping.

Table 1 Antigenic profiling of recombinant antigens using MAbs directed to hantavirus NPs in IFA

MAbs	IFA antigens								
	Vero E6 cells infected with			High Five cells infected with recombinant baculovirus expressing the whole N protein from:			High Five cells infected with recombinant baculovirus expressing a truncated N protein (aa 50–429) from:		
	HTNV	SEOV	THAIV	HTNV	SEOV	THAIV	HTNV	SEOV	THAIV
Cross-reactive clones									
ECO2	+	+	+	+	+	+	−	−	−
ECO1	+	+	+	+	+	+	+	+	±
E5/G6	+	+	+	+	+	+	+	+	+
HTNV-specific clones									
C24B4	+	−	−	+	−	−	+	−	−
BDO1	+	−	−	+	−	−	+	−	−
SEOV-specific clone									
DCO3	−	+	−	−	+	−	−	+	−

IFA profiles against Vero E6 cells infected with HTNV, SEOV, and THAIV were reported previously [7], and IFA profiles against High Five cells infected with recombinant baculovirus expressing the whole N protein and a truncated N protein (aa 50–429) from HTNV and SEOV were reported previously [9]

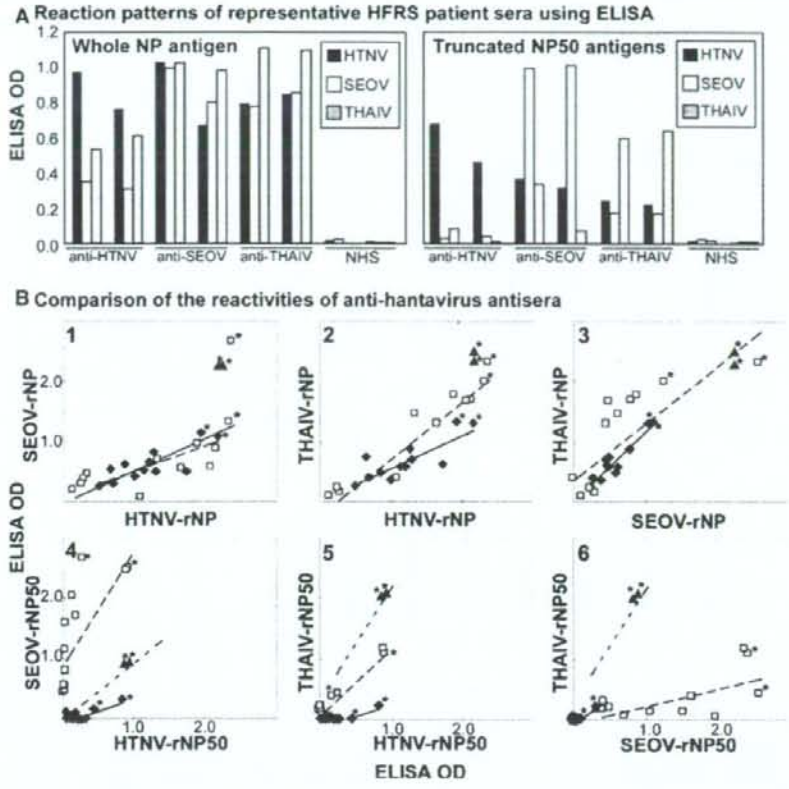
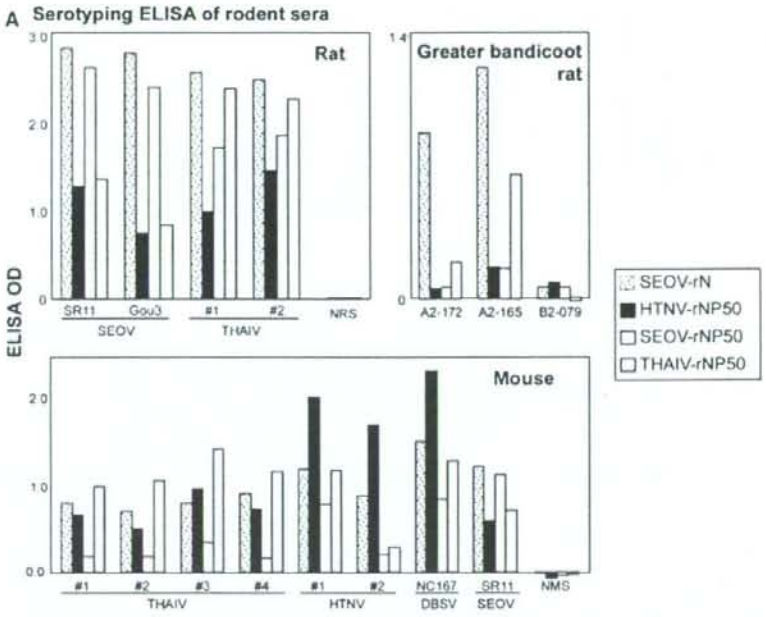


Fig. 1 Reactivities of representative and groups of patient sera against recombinant and truncated NP antigens in ELISA. **a** Reaction patterns of representative HFRS patient sera. Anti-HTNV patient sera were obtained from China. Anti-SEOV patient sera were obtained from Japan to Korea, associated with rat-borne disease outbreaks in laboratories. Two anti-THAIV serum samples were obtained from the same patient: one from the acute phase and another obtained 1 year after the first sample. NHS is a human serum obtained from a single individual confirmed to be negative for hantavirus-specific antibodies, obtained from Japan. The serotypes of infecting viruses were determined by FRNT. The ELISA OD values of sera against whole rNP antigens (*left panel*) and truncated NP antigens for aa 50–429 (*right panel*) of HTNV (*black bars*), SEOV (*white bars*), and THAIV (*gray bars*) are shown. **b** Comparison of the reactivities of anti-hantavirus antisera with the recombinant antigens used in this study. The horizontal and vertical axes show the ELISA ODs for sera from HTNV-infected patients (*diamonds*), SEOV-infected patients (*square*), and a THAIV-infected patient (*triangle*) for each antigen. A total of 23 serum samples were used to assess our serotyping ELISA system: 11 anti-HTNV samples obtained from China, 10 anti-SEOV samples obtained from Japan to Korea, associated with rat-borne outbreaks in laboratories, and two anti-THAIV samples were the same sera shown in panel A. The serotypes were determined by FRNT [7, 9, 17]. ELISA ODs were compared as follows: 1, HTNV-rNP versus SEOV-rNP; 2, HTNV-rNP versus THAIV-rNP; 3, SEOV-rNP versus THAIV-rNP; 4, HTNV-rNP50 versus SEOV-rNP50; 5, HTNV-rNP50 versus THAIV-rNP50; 6, SEOV-rNP50 versus THAIV-rNP50. The *lines* show linear regressions for each group of sera: *solid lines*, sera from HTNV-infected patients; *dashed lines*, sera from SEOV-infected patients; *broken lines*, sera from a THAIV-infected patient

To demonstrate the applicability of the serotyping ELISA, antisera derived from several rodents were prepared. Two WKAH/hkm rats and four Slc:ICR mice (SLC, Hamamatsu, Japan) were inoculated intraperitoneally with 10^4 FFU of THAIV strain thai749 per animal, and serum was collected after 5 weeks. All of the animals were treated in accordance with the laboratory animal control guidelines of our institute, which conform to those of the U. S. National Institutes of Health. All animal experiments were carried out in a BSL3 facility. Three serum samples from greater bandicoot rats from Thailand were obtained. Two of them were confirmed to be positive for anti-hantavirus antibody by IFA, ELISA, and FRNT in a previous study [7]. The third serum sample was used as a negative control. For the rat and greater bandicoot rat serum samples, bound antibodies were detected with peroxidase-conjugated



B Comparison of amino acid sequence of variable region in NPs

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HTNV   KDWSDRIEQWLIEPCKLLPDTAAVSLLGGPATNRDYLRQRQVALGNMETKESKAIRQHAEAAGCSMIEDIESP
AMRV   KDWGDRIEQWLSEPCKLLPDTAAVSLHGGXATNRDYLRQRQAALGNMETKESKAIRQHAETAGCSMIEDIESP
DBSV   KDWTDRIESWLGEPCKLLPDTSAVSLLGGPATNRDYIRQRQVALGNMETKEAKAVRQHAESAGCSMIDNIDSP

DOBV   KNWTERVEEWLDLPCKLLSEPSPTSLTKGPSTNRDYLNQRQGALAKMETKEAQAVRKHAIDAGCNLIDHIDSP
SAAV   KNWTERVEEWLDLPCKLLSEPSPTSLTKGPSTNRDYLSQRQGALAKMETKEAQVVRKHAIDAGCNIIDHIDSP

SEOV   KDWTSRIEEWLGAPCKFMAESLIAGSLSGNPVNRDYIRQRQGALAGMEPKEFQALRQHSKDAGCTLVEHIESP
Gou3   KDWTSRIEDWLGAPCKFMAESPIAGSLSGNPVNRDYIRQRQGALAGMEPKEFQALRQHSKDAGCTMVEQIESP

THAIV  KDWTDRIQDWLGAPCKFLPDLSTQSLHASPPTNADYIAQRQGLLAGMETKESKGIRDHARDAGCTLVDHLDSP
Camb.  KDWTDRIQDWLGSPCKFLPDLSTQSLHASPPTNADYIAQRQGLLAGMETKESKGIRDHARDAGCTLVDHLDSP
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Fig. 2 Reactivities of rodent sera to truncated N antigens of HTNV, SEOV, and THAIV and comparison of the amino acid sequences of the variable region in NP. **a** Hantavirus-infected rat and mouse sera were subjected to serotyping ELISA. Sera from laboratory rats immunized with SEOV strain SR-11 or Gou3 were prepared previously [14], and serum from rats immunized with THAIV and non-immunized rat serum (NRS) were used for serotyping ELISA (*upper left*). Wild greater bandicoot rats A2-172 and A2-165 were both reported previously to have anti-THAIV antibody [7], and B2-079 serum was used as a negative control (*upper right*). The lower panel shows immune mouse sera to THAIV strain thai749, HTNV strain 76118 [14], DBSV strain NC167 [14], and SEOV strain SR-11 [14]. **b** Comparison of the amino acid sequences of the variable region of the NPs. The regions from aa 230–302 of representative Murinae-associated hantaviruses are shown for HTNV strain 76118 (M14626), AMRV strain AP61 (AB071183), DBSV strain NC167 (AB027523), DOBV strain Af19 derived from *Apodemus flavicollis* (AJ410615), Saaremaa virus SAAV strain 160 V derived from *Apodemus agrarius* (AJ009773), SEOV strains SR11 (M34881) and Gou3 (AB027522), THAIV strain thai749 (AB186420), and Cambodian hantavirus strain from *R. rattus* (AJ427511)

mouse anti-rat IgG (H + L) (Zymed, South San Francisco, CA, USA) and an *o*-phenylenediammonium dichloride (OPD) substrate tablet (Sigma, St. Louis, MO, USA). We also established an ELISA system for mouse sera. A 96-well plate was coated directly with recombinant and truncated NPs and negative control antigen, without capture antibody. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (Zymed) and OPD substrate tablets (Sigma). The amounts of the antigens were adjusted based on the ELISA OD value against pooled non-immunized mouse sera.

As shown in Fig. 2a, immune rodent sera against THAIV showed a typical THAIV infection reactivity pattern, while anti-SEOV immune sera against strains SR-11 (derived from *Rattus norvegicus*) and Gou3 [14] (derived from *R. rattus*) showed a typical SEOV infection reactivity

pattern. Similarly, immune sera to Da Bie Shan virus (DBSV) strain NC167 also showed a typical HTNV infection reactivity pattern. Figure 2b shows a comparison of the aa sequence in the type-specific region of the NP, which is thought to contain type-specific epitopes (aa 230–302). In fact, two PUUV-specific MAbs (3H9 and 5F4) were reported to bind to this region [18, 19]. From the sequence comparison, we identified four subgroups among Murinae-associated hantaviruses: HTNV, SEOV, DOBV, and THAIV. The HTNV subgroup includes DBSV and Amur virus (AMRV), which was recognized in far eastern Russia as a pathogenic hantavirus [20], and its animal reservoir is *Apodemus peninsulae* [21]. AMRV and other *A. peninsulae*-borne hantavirus from Korea (Soochong virus [22]) and China (lineage #2 [14]) were identified as distinct lineage from classical *A. agrarius*-borne HTNV. Previously, we identified AMRV patient sera using HTNV serotyping antigen [23]. Similarly, strains Gou3 and SR11 were found within the SEOV subgroup. In this study, both human and rodent antisera to THAIV showed THAIV-specific reactions in serotyping ELISA. In contrast, the Cambodian strain from black rats (*R. rattus*) [24] had an aa sequence similar to that of THAIV in this type-specific region. These observations indicated that THAIV and its relatives occur in both the greater bandicoot rat and black rat in South East Asia. Therefore, the serotyping antigen for THAIV developed in this study is useful for detecting THAIV and relative viruses from Cambodia and differentiating them from other hantaviruses.

Recently, there have been gradual increases in the number of case reports of hantavirus infection in Asian countries, including Thailand [7], Indonesia [25], Vietnam [26], and India [27, 28]. In terms of public health, it is important to develop rapid, convenient methods for epidemiological surveillance and studies. Our system will become a valuable tool for surveying human and rodent cases of THAIV infection. However, the reliability of the system is uncertain because we tested only two serum samples from one patient. More serum samples from THAIV patients are needed to assess the applicability of our serotyping ELISA system.

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Detection of Antibodies Against SARS-Coronavirus Using Recombinant Truncated Nucleocapsid Proteins by ELISA

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Severe acute respiratory syndrome (SARS) is a life-threatening emerging respiratory disease caused by the coronavirus, SARS-CoV. The nucleocapsid (N) protein of SARS-CoV is highly antigenic and may be a suitable candidate for diagnostic applications. We constructed truncated recombinant N proteins (N1 [1–422 aa], N2 [1–109 aa], and N3 [110–422 aa]) and determined their antigenicity by Western blotting using convalescent SARS serum. The recombinants containing N1 and N3 reacted with convalescent SARS serum in Western blotting. However, the recombinant with N2 did not. In ELISA using N1 or N3 as the antigens, positive results were observed in 10 of 10 (100%) SARS-CoV-positive human sera. None of 50 healthy sera gave positive results in either assay. These data indicate that the ELISA using N1 or N3 has high sensitivity and specificity. These results suggest that the middle or C-terminal region of the SARS N protein is important for eliciting antibodies against SARS-CoV during the immune response, and ELISA reactions using N1 or N3 may be a valuable tool for SARS diagnosis.

Keywords: ELISA, SARS, truncated recombinant nucleocapsid proteins

Severe acute respiratory syndrome (SARS) is a life-threatening, emerging respiratory disease. An outbreak of SARS first occurred in southern China (Guangdong province) in 2002. From there it has spread to several countries: Hong Kong (China), Vietnam, Singapore, Canada, Germany, Thailand, Taiwan, and beyond [6]. From November 2002 to July 2003, a total of more than 8,098 probable SARS cases with more than 774 deaths were reported in 26 countries [16].

The structural proteins of SARS-CoV are the spike (S) glycoprotein, the membrane (M) protein, the envelope (E) protein, the nucleocapsid (N) protein, and putative uncharacterized proteins [4, 6]. The N protein of SARS-CoV is a basic protein consisting of 422 amino acids. It has strong antigenicity and may play an important role in the induction of the host's immune response and even pathogenesis during SARS-CoV infection [15]. Moreover, this protein is expressed early during the infection period [11]. These data suggest that the SARS-CoV N protein is a suitable candidate for diagnostic applications.

An ELISA is a rapid, sensitive, and specific assay. It was proved in not only serological diagnosis of disease but also quantitative analysis of protein [5, 7, 10].

In this study, truncated recombinant N proteins (N1 [1–422 aa], N2 [1–109 aa], and N3 [110–422 aa]) were cloned, sequenced, expressed in an *E. coli* system, and purified. We developed an ELISA using the purified proteins and evaluated the sensitivity and specificity of the ELISA.

Materials and Methods

Serum Samples

Ten serum samples were collected from convalescent SARS-CoV patients in Vietnam (National Institute of Hygiene and Epidemiology). Fifty healthy serum samples were provided by volunteers. This work was approved by the institutional review board of Seoul National University (IRB No. 0705/001-001).

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Expression and Purification of SARS-CoV Nucleocapsid Protein

The cDNA of SARS-CoV strain Hanoi was used as a template for PCR using Accupower premix (Bioneer, U.S.A.). Different truncated fragments of the SARS-CoV N gene were acquired. The primers were synthesized according to the Urbani Accession No. AY278741; that is N1 forward (5'-CGG GGA TCC ATG TCT GAT AAT GGA CCC CAA-3') and N1 reverse (5'-ACGC GTC GAC TGC CTG AGT TGA ATC AGC-3'), N2 forward (5'-CGG GGA TCC ATG TCT GAT AAT GGA CCC CAA-3') and N2 reverse (5'-ACGC GTC GAC CCA TCT GGG GCT GAG CTC TTT-3'), and N3 forward (5'-CGG GGA TCC ATG TAC TTC TAT TAC CTA GGA ACT GGC-3') and N3 reverse (5'-ACGC GTC GAC TGC CTG AGT TGA ATC AGC-3'). The forward and reverse primers carried BamHI and SalI restriction sites (underlined). Products were amplified under the following conditions: 94°C for 10 min, then 30 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min, followed by one cycle of 72°C for 5 min, and then examined by 1% agarose gel electrophoresis.

The SARS-CoV N genes were cloned into the pGEM-T easy vector (Promega, U.K.) by standard techniques and then transformed into *E. coli* strain DH5α. The constructed pGEMT-N genes were digested with BamHI and SalI (Roche, Switzerland) and then inserted into the expression vector pET21a (Novagen, Germany), thereby introducing a small hexahistidine tag at the C-terminus of the protein to facilitate subsequent purification. The recombinant plasmids were transformed into *E. coli* strain DH5α. The constructed pET21a-N genes were sequenced.

The pET21a-N genes were transformed into *E. coli* strain Origami B (DE3) pLysS Competent Cells. Bacterial cells were treated with 1 mM IPTG (Invitrogen, U.S.A.) at 37°C for 4 h in LB broth containing selective antibiotics. Cells were harvested by centrifugation, and the pellets were extracted with BugBuster Protein Extraction Reagent (Novagen, Germany). The supernatants of the extracts were filtered with a 4.5-μm syringe filter, and the proteins were purified from the filtered supernatants using His-Bind kits. The filtered supernatants were loaded onto an Ni-IDA resin column, followed by washing with binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9), and then washing buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH 7.9). The proteins were eluted with elution buffer (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazole, pH 7.9).

Western Blotting

The recombinant N proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels and were then transferred onto cellulose nitrite membranes. After blocking with 5% skim milk for 2 h, the membranes were incubated with either a mouse anti-SARS-CoV N protein monoclonal IgG_{2a} (Zymed, U.S.A.) or a serum from a convalescent SARS patient or a healthy volunteer. After washing, the membranes were stained with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG_{2a} Ab (Zymed, U.S.A.) or goat anti-human IgG Ab (Sigma, U.S.A.) for 2 h. The blots were developed using ECL detection reagents (Amersham Pharmacia Biotech, Sweden).

ELISA

Purified N protein (250 ng) dissolved in coating buffer (0.016 M Na_2CO_3, 0.034 M $NaHCO_3$, pH 9.6) was added to a 96-well microplate and incubated at 4°C overnight. The wells were then blocked with 1% BSA (Sigma, U.S.A.) in phosphate buffer saline (PBS) for 1 h at 37°C. For detection of antibodies (Abs), sera were diluted in PBS (1:40) and then incubated at 37°C for 2 h. Each well was washed three times with PBS containing 0.05% Tween-20 and then incubated with a HRP-conjugated anti-human IgG Ab for 2 h at 37°C. After washing, the peroxidase reaction was visualized using OPD (*O*-phenylenediamine) as a substrate (Sigma, U.S.A.) for 10 min at room temperature. The reaction was stopped by adding 100 μl/well of 1 N H_2SO_4, and the absorbance was read at 492 nm with a 692 nm reference filter. The serum samples were run in triplicate. The cutoff value was defined as the mean optical density (OD) of the control samples plus three standard deviations (SD).

Results

Expression and Purification of SARS-CoV Nucleocapsid Protein

The truncated recombinant N proteins (N1 [1–422 aa], N2 [1–109 aa], and N3 [110–422 aa]) were designed for an ELISA to detect Abs against the SARS-CoV N protein. The truncated recombinant N proteins were efficiently expressed in the *E. coli* strain Origami B (DE3) pLysS Competent Cells with a hexahistidine tag, and the purified truncated recombinant N proteins were visually detected by SDS-PAGE analysis (Fig. 1).

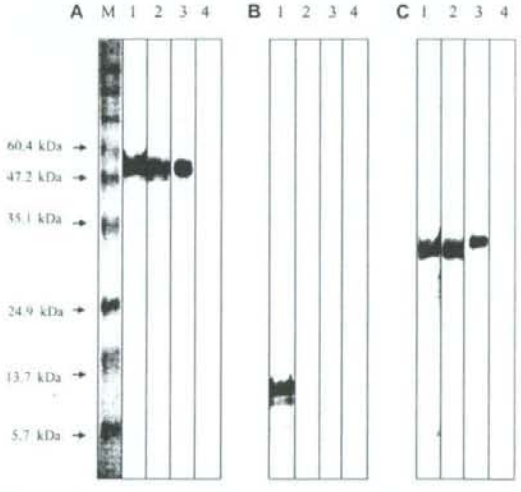

Fig. 1. SDS-PAGE and Western blotting analysis of the recombinant truncated N proteins (**A: N1, B: N2, C: N3**).
Purified recombinant truncated N proteins were run in SDS PAGE 12% gels with molecular mass markers in Lane **M**. One of the gels was stained with Coomassie blue (Lanes **M** and **1**). Protein bands in the other gel were transferred onto cellulose nitrite membranes for Western blotting with a monoclonal serum (Lane **2**), a serum from a convalescent SARS patient (Lane **3**), and a serum from a healthy volunteer (Lane **4**). This figure was edited from our published data [2].

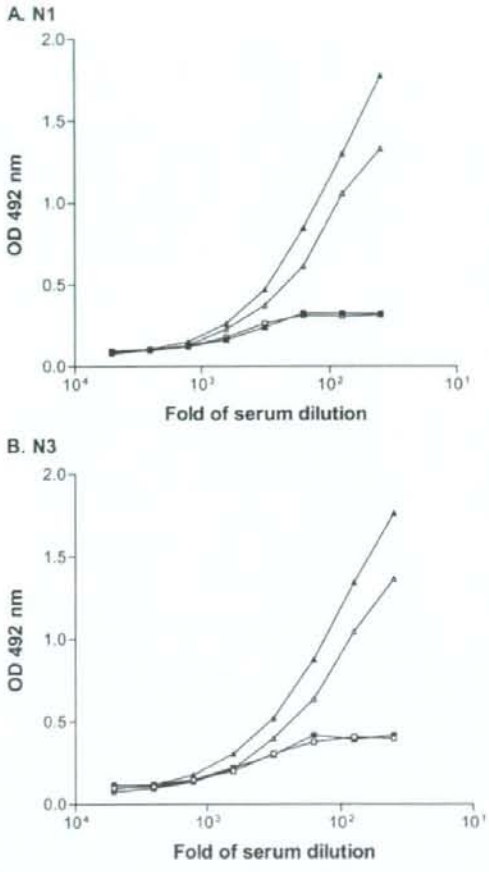

Fig. 2. Sensitivity of the ELISA using N1 and N3.
ELISA plates were coated with recombinant N1 and N3. Serum samples from two convalescent SARS patients (▲, △) and two healthy individuals (■, □) were serially diluted and dispensed into the wells. HRP-conjugated goat anti-human IgG Ab was used as the secondary antibody with OPD as the substrate. The results are expressed as an absorbance reading at a wavelength of 492 nm with a 692 nm reference filter.

Western Blotting

The truncated recombinant N proteins were identified by Western blotting with a mouse anti-SARS-CoV N protein monoclonal IgG_{2a} and a convalescent SARS serum. N1 and N3 reacted with the mouse anti-SARS-CoV N protein monoclonal IgG_{2a} and the convalescent SARS serum, but N2 did not (Fig. 1).

ELISA

Sera from two convalescent SARS patients and two healthy individuals were serially diluted and tested in an ELISA using the N1 or N3. When the antigens were coated at a concentration of 250 ng and the sera were diluted 1:40, the ELISA detected Abs against the SARS-CoV in a specific and sensitive manner (Fig. 2).

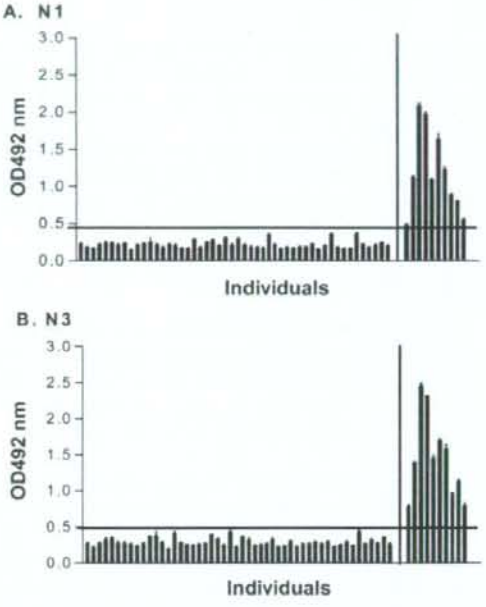

Fig. 3. Comparison of the ELISA using N1 and N3.
Ten convalescent SARS sera (right side of vertical line) and fifty healthy sera (left side of vertical line) were screened with the ELISA using N1 (**A**) and N3 (**B**). The serum samples were diluted 40-fold. The results are expressed as absorbance readings at a wavelength of 492 nm with a 692 nm reference filter. The horizontal line shows the cutoff value (N1: 0.373; N3: 0.456).

The cutoff value was calibrated from the ELISA results using 50 healthy sera. All serum samples from the 50 healthy volunteers were negative (Fig. 3). When serum samples (diluted 1:40) from 10 convalescent SARS patients were analyzed sequentially, all of them were positive, suggesting a high sensitivity for the ELISA using the N1 or N3 constructs (Fig. 3).

DISCUSSION

SARS-CoV N protein is known as a useful antigen for the diagnosis of SARS, and the antigenic site of the SARS-CoV N protein has been investigated by many researchers [3, 9, 15]. Studies of ELISA systems using the SARS-CoV N protein been have also reported [8, 14, 18].

In this study, we constructed N1 (1–422 aa: full protein sequence), N2 (1–109 aa: N-terminal region known to have weak antigenicity), and N3 (110–422 aa: middle and C-terminal regions known to have strong antigenicity) to