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The expression of membrane protein augments the specific responses induced by SARS-CoV nucleocapsid DNA immunization

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Abstract

Nucleocapsid protein plays a critical role in SARS-CoV pathogenesis, and high-level anti-nucleocapsid antibodies are detected in the patients infected by severe acute respiratory syndrome-associated coronavirus (SARS-CoV). Several studies have shown that there exists an interaction between nucleocapsid (N) and membrane (M) protein. In this paper, we investigate whether the expression of membrane protein can affect the immune responses induced by nucleocapsid DNA immunization. Two recombinant plasmids containing M and N coding sequence were constructed. Moreover, in order to get the antigen for ELISA and in vitro stimulation assay, N protein were expressed and purified from *E. coli* bacteria. Injection of 20 μg of the mixture of pVAX1-M and pVAX1-N into the Balb/c mice could elicit the humoral and cellular responses. The ELISA analysis using the N antigen or inactivated SARS-CoV particles as capture antigen showed that co-injection of SARS-M could enhance N-induced antibody production, especially IgG2a subclass. After lymphocytes were stimulated with 10 μg/ml purified N antigen, The CD4+ and CD8+ T cells of N and M plus N group were increased compared with those of control groups, and the M protein could augment the activation of lymphocytes induced by N DNA vaccine. Cytokine ELISA analysis revealed that co-injection of M could enhance the levels of IFN-γ, IL-2 release induced by N antigen. Further experiments in field mouse also support the claim that membrane protein can augment the N-specific immune responses. Virus challenge test was conducted in BSL3 bio safety laboratory with Brandt's vole SARS-CoV model, and the results indicated that co-immunization of M and N antigens could reduce the mortality and pathological changes in lung from the virus infection.

Keywords: SARS-CoV; DNA immunization; Nucleocapsid; Membrane; Pathogenesis

1. Introduction

The syndrome, called severe acute respiratory syndrome (SARS), infected more than 8000 people early in 2003, and killed more than 770 (http://www.nature.com/nsu/031215/031215-9.html). The genome of SARS-CoV was sequenced by several independent groups. That the SARS genome from Singapore, Canada, Hongkong, Guangzhou and Beijing in China has remained unchanged, as the infection has spread (http://www.nature.com/nsu/030505/030505-10.html) suggests that genetic vaccine maybe an efficient approach to protect from SARS-CoV infection.

The SARS-CoV genome contains five major open reading frames (ORFs) that encode the replicase polyprotein; the spike (S), envelope (E), and M glycoproteins; and the N protein in the same order and of approximately the same size as those of other coronaviruses (Kathryn and Luis, 2003). The M gene encodes a protein of 221 amino acids. Analysis of the M protein revealed that a 121 amino acid hydrophilic domain on the inside of the virus particle is believed to interact with the N protein (Qin et al., 2003). It is further reported that there exists an interaction between SARS-CoV N and M protein and the stretch of

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amino acids (168–208) in the N protein may be critical for such protein–protein interactions (He et al., 2004). Whether the interaction plays role in immune responses induced by SARS-CoV infection is not clear.

The complete N protein contains 422 amino acids. It is possible that N protein plays a critical role in SARS-CoV pathogenesis. In addition, the basic nature of this peptide suggests it may assist in RNA binding (Marra et al., 2003). In previous study, N protein is a representative antigen for the T-cell response in vaccine setting (Gao et al., 2003), and the mice vaccinated with calreticulin (CRT)/N DNA were capable of significantly reducing the titer of challenging vaccinia virus expressing the N protein of SARS virus (Kim et al., 2004). It has been reported that N DNA vaccine could induce SARS-specific T-cell proliferation and cytotoxic T cells activity, further experiments demonstrate that SARS-N administration could induce virus-specific cellular responses in human cells using SCID-PBL/hu mouse model (Jin et al., 2005; Okada et al., 2005). Although more than 90% of sera obtained from convalescent SARS patients have antibodies against N (Tan et al., 2004), N antigen did not induce a detectable serum SARS-CoV-neutralizing antibody response in mice (Buchholz et al., 2004).

Taken these into account, we inserted the full M and N gene into pVAX1 and inoculated mice and Brandt's vole (*Microtus brandti* raddes) with M or N DNA alone or together to investigate the immune responses induced by these DNA immunizations. And in previous study, we have developed a SARS-CoV infection animal model with Brandt's vole (Gao et al., 2005), so we selected this animal for challenge test to assess the capacity of M plus N DNA immunization.

2. Materials and methods

2.1. Mice

The female Balb/c mice were purchased from the Institute of Genetics, Chinese Academy of Sciences. The *M. brandti* raddes were obtained from Dr. Wang De-Hua (Institute of Zoology, Chinese Academy of Sciences). Mice were kept under controlled conditions of light and temperature, with free access to a standard mouse chow and water. All experiments were conducted according to the guidelines of the Beijing Animal Care for Laboratory Animals, and the protocols were approved by the Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences.

2.2. Plasmid construction

The pcDNA3.1-M, N plasmid containing full-length SARS-CoV M and N cDNA were kindly provided by Professor Yang Huan-Ming (Institute of Genome, Chinese Academy of Sciences). The full-length M and N gene were cut with *Bam*HI and *Xba* I, and ligated into the corresponding restriction sites of pVAX1 vector (Invitrogen, Carlsbad, CA) to create pVAX1-M and pVAX1-N. The pair of primers: 1#, 5′-GGACATATGTCTGATAATGGACC-3′ and 2#, 5′-GATGGATCCGCCTGAGTTGAA-3′ containing NdeI and *Bam*HI enzyme sites at the 5′ and 3′ were used to amplify the N complete gene. The fragment digested by NdeI and *Bam*HI was inserted into pET21b (Novagen, Madison, WI, USA) that had been cut with the same enzymes. The three recombinants were confirmed by restriction digestion and sequencing.

2.3. Immunization and in vivo expression

Balb/c mice ($n = 36$) were divided into two control and four experimental groups. The voles ($n = 16$) were divided into one control and three experimental groups. The mice of one experimental group were injected with 20 μg pVAX1-N plus 20 μg pVAX1 in the leg muscle by three-spot injection once a week for three times. The mice of other experimental groups received 20 μg M or N alone or them together. Each experimental vole received 100 μg M or N recombinant alone or together. All animals were injected with 100 μl 0.25% bupivacaine-HCl (Sigma, Saint Louis, MO) 24 h before DNA inoculation. Control group was injected in the same way with equal volume of pVAX1 mock vector or saline using the same injection technique and schedule (Shi et al., 2005). To examine in vivo expression of the vaccine, total RNA was isolated from muscle of the injection sites and treated with the DNase I (Boehhringer Mannheim, Indianapolis, IN) to delete the interference of DNA. The pair of primers: 3#, 5′-ATGTCTGATAATGGACCCCAATCA-3′ and 4#, 5′-TTATGCCTGAGTTGAATCAG-3′, was used to detect the N expression at mRNA level. The pair of primes: 5#, 5′-ATGGCAGACAACGGTACTATTACC-3′ and 6#, 5′-TTACTGTACTAGCAAAGCAA-3′, was used for M mRNA amplification. The RT-PCR products were detected by agarose gel and sequencing. The PCR reaction without RT was also conducted in order to rule out the possibility of DNA contamination.

2.4. Expression, purification and detection of N proteins in bacteria

For ELISA and in vitro stimulation assay, N protein was expressed in bacteria and purified with affinity columns. *E. coli* BL21 was used as the cloning host for the pET21b-N and empty pET21b, and the cells were grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml). Two and four h after induction with 1 mM isopropyl-(-D-thiogalactopyranoside, the cells were harvested and washed twice with cold buffer (50 mM Tris–HC1, 2 mM EDTA, pH 8.0) and resuspended in the same buffer, the lysozyme and Triton X-100 were added to final concentrations of 100 μg/ml and 0.01%, respectively. Mixtures were incubated at 30 °C for 15 min, and centrifuged at 12,000 × *g*. Supernatants and cell lysates were resuspended in SDS-PAGE loading buffer, placed in a boiling water bath for 3 min and electrophoresed (Crack et al., 2002), then the protein samples in SDS PAGE gel were stained by coomassie blue.

The Hitrap affinity columns (Amersham Biosciences) were used for purification of the recombinant N-$(His)_6$ tagged proteins followed as the product instruction. The proteins after purification were detected by Western blot.

2.5. Detection of N-specific antibody in vaccinated animals

Ninety-six-well microtiter plates (Costar) were coated with 100 μl of 5 μg/ml of N protein per well diluted in 0.05 M bicarbonate buffer (pH 9.6) overnight at 4 °C, washed three times in PBST (0.1% Tween-20 and in PBS), blocked with 3% BSA-PBS at room temperature for 0.5 h. After washed three times, 100 μl sera with serial dilution starting at 1:10 with 10% normal goat sera supplemented with 0.1% Tween-20 was added to each well. After extensive washings, antibodies were detected with goat anti-mouse IgG, IgG1, and IgG2a conjugated with horseradish peroxidase (1:5000, Sigma) diluted with PBST contain 3% normal goat sera. After adding substract, the reaction was stopped with 2 M H_2SO_4. The absorbance was measured at wavelengths of 450 or 490 nm (Bio-Rad, 3550). Titer values of mouse antibody were assigned as the highest dilution at which the optical density was over or equal to 2.1 times than the absorbance produced by the serum of pre-immune mice at equivalent dilution.

2.6. FACS and cytokine production

Lymphocytes from the mice were isolated and cultured in six-well flat-bottomed plate at 2.5×10^6 cells/ml in RPMI-1640 (Gibco-BRL, Rockville, MD) containing 10% fetal calf serum (Hyclone) and 50 μM β-mercaptoethanol (2-ME) (Sigma) 4 weeks after immunization. The purified N protein was passed through filter (10 kDa cutoff, Millipore) to remove low molecular weight products in elution buffer, and 10 μg/ml N protein was added into the medium. On day 2, the culture medium was supplemented with recombinant IL-2 (PeproTech EC, London, UK) at 25 U/ml. Three days after stimulation; the lymphocytes were harvested and resuspended in flow cytometric analysis buffer and Fc block. Then the cells were stained with phycoerythrin (PE)-labeled anti-CD8a/Lyt-2 (clone 53-6.7, Souther Biotech, Birmingham, AL) and fluorescein isothiocyanate (FITC)-labeled anti-CD4/L_3T_4 (clone GK1.5, Souther Biotech), or isotype control antibodies for 1 h. After washing twice, the cells were analyzed on FACSCalibur flow cytometer (Cho et al., 2002) (Beckton Dickinson).

The supernatants of lymphocytes culture were harvested and subjected to measurement for IFN-γ, IL-2 and IL-4 with ELISA detection kits from R&D Systems. The values of cytokine were expressed as concentrations (pg/ml) according to the standard samples curves. The value of the well incubated with PBS was as blank.

2.7. Lymphocyte proliferation assay

About 50 μl per well of Dulbecco's modified eagle's medium (DMEM, supplemented with 10% fetal bovine serum (FBS) and 50 μM 2-ME) containing 2 or 10 μg/ml N antigen were added into 96-well plate (Costar). Bovine serum albumin (BSA, Promega, Madison, WI) at 10 μg/ml was set as a non-specific antigen control. After adding 50 μl (approximately 10^5 cells/well) lymphocytes suspension that from immunized voles per well, the plate was incubated in a humidified CO_2 incubator at 37 °C. On day 2, human recombinant IL-2 (Sigma) was added into the culture medium with 5 U/ml. After 3 days, 20 μl of CellTiter 96 Aqueous One Solution Reagent (Promega) was added into the wells, and the plate was incubated for 4 h at 37 °C. Then the absorbance of samples at 490 nm was read (Bio-Rad, 3550). The results were defined as stimulation index (SI) (ratio of A490 nm with N antigen and negative control).

2.8. Detection of the antibody reacted to inactivated SARS-CoV

The 96-well microtiter plate (Costar) was coated with inactivated SARS-CoV particles. The following experimental process was the same as above. The value at 490 nm over or equal to 0.22 was considered as positive. In previous study, the absorbance value of 0.22 from experimental mice is equal to 2.1-time of mean value of the mouse from the negative group (Liang GD, unpublished data).

2.9. Anti-SARS-CoV animal experiment

Fourteen adult's *M. brandti* raddes were divided into two groups. Two weeks after the animals immunized with mock vector ($n = 6$) or M + N vaccine ($n = 8$) for three times, the animals were challenged with live SARS-CoV (PUMC01) as previous report (Gao et al., 2005; Qin et al., 2005) Two voles died during anesthesia manipulation. Histology was performed on representative 5 μm sections of lung, liver, kidney, spleen, brain, heart, pancreas, intestine, stomach, thymus, lymph node, bladder, bone marrow, testicle, prostate, ovary, uterus tissue from field mice after challenge, which were paraffin embedded and then stained with Haematoxylin & Eosin (HE) staining. These experiments were conducted in BSL3 bio safety laboratory.

2.10. Data analysis

The values except special explanation are reported as mean ± S.E.M. Statistical analysis was performed using One Way-ANOVA. Differences were considered statistically significant with $p < 0.05$.

3. Results

3.1. Expression of M and N in vivo

To identify the expression of pVAX1-M and pVAX1-N in vivo, the presence of M and N mRNA from mice muscle immunized with vaccine and mock DNA was analyzed. RT-PCR reaction with the designed primers was used to detect the M and N expression. The desired full-length of M cDNA is 666 nucleotide acids, while the N cDNA is 1269 nucleotide acids. Thus, pVAX1-M and pVAX1-N are proven to express in immunized mice muscle (Fig. 1). No bands were detected when the reaction was conducted without RT process (data not shown).

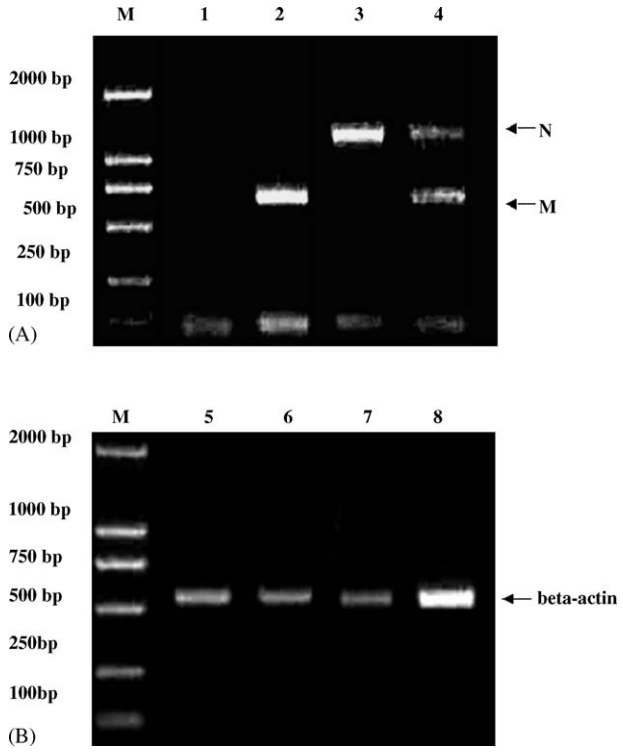

Fig. 1. Detection the SARS M and N mRNA expression in mice. After the total RNA was extracted and treated with DNase I, approximately 1 μg of total RNA was used for RT-PCR (A). Beta-actin was used as the internal control (B). M, 2 kb Marker. (1) pVAX1 mock, (2) pVAX1-M, (3) pVAX1-N, and (4) pVAX1-N plus pVAX1-M. The 5, 6, 7, 8 lanes represent the beta-actin expression of the 1, 2, 3 and 4, respectively.

3.2. Expression and purification of N protein

N protein with a C-terminal histidine tag was expressed in the bacteria BL21. After induction with IPTG, the volume of the desired protein increased (Fig. 2A). The soluble proteins in bacteria were treated with the Hitrap affinity columns. After treating, only two bands were remained in the SDS-PAGE gel (Fig. 2B), and the bold band represents the full-length of N protein with expected molecular weight (Fig. 2C).

3.3. Analysis of the N-specific antibodies induced by the vaccine

IgG titers specific to N antigen in mice sera at serial dilution were detected by standard ELISA. The log 10 titers were shown in Fig. 3, It showed that N-induced IgG increased when co-injection with M ($p < 0.01$). Whereas there is no significant difference between the group immunized with 20 μg N vaccine alone and the group with 20 μg N plus 20 μg mock DNA ($p = 0.276$). This indicates that the antibody enhancement by M is not contributed by plasmid DNA itself. The N antibody in sera immunized with M DNA vaccine at 1:10 was not over 2.1 times of that in pre-immune sera ($n = 6$), so the log value of antibody titer was expressed as one without bar.

From Table 1, we observed that IgG induced by N DNA immunization both in mouse and vole increased when co-injection with pVAX1-M. The mouse antibodies assay showed

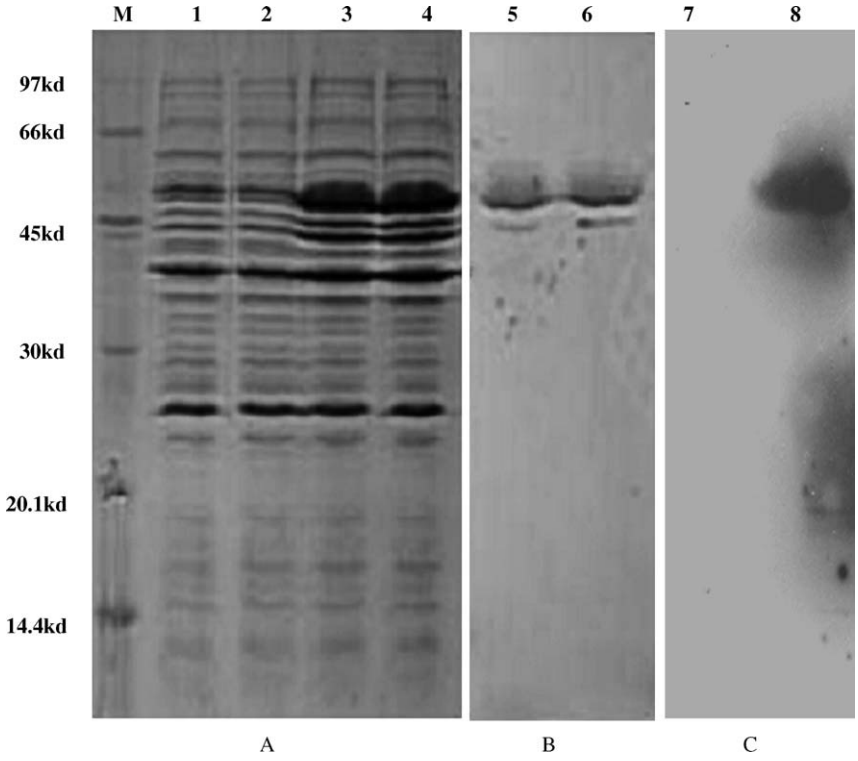

Fig. 2. Expression, purification and detection of the N protein in BL 21 *E. coli* bacteria. (A) Expression of N protein in BL21 *E. coli* bacteria. M: marker, lanes 1 and 2: 12 and 24 h after the pET21b-N entry into the host without induction. Lanes 3 and 4: 2 and 4 h after induction with 1 mM IPTG. (B) Purification the N protein with the Hitrap affinity columns. Lanes 5 and 6 were proteins purified from the lanes 3 and 4, respectively. (C) Detection the N protein by sera from the mice immunized with pVAX1-N using western blotting. Lane 7, protein from normal BL-21 bacteria. Lane 8, N protein after purification.

Table 1
Detection of IgG and IgG types against N antigen in sera 4 weeks after immunization

	Saline	pVAX1	M	N	M+N
IgG (Balb/c)[a]	0.07 ± 0.04	0.12 ± 0.01	0.15 ± 0.01	0.25 ± 0.02	0.65 ± 0.19
IgG1 (Balb/c)[a]	0.06 ± 0.01	0.09 ± 0.05	0.15 ± 0.03	0.12 ± 0.08	0.13 ± 0.08
IgG2a (Balb/c)[a]	0.05 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.50 ± 0.05
IgG (vole)[b]	ND[c]	0.09 ± 0.10	0.14 ± 0.06	0.89 ± 0.03	1.39 ± 0.05

[a] The sera samples from the Balb/c mice that had been immunized with mock or recombinant DNA three times were diluted to 1:20.
[b] The sera samples form vaccinated voles were diluted to 1:50.
[c] Not detected.

that the mostly increased subclass of N-specific IgG induced by M was IgG2a but not IgG1.

3.4. Analysis of CD8+ and CD4+ lymphocytes by flow cytometric analysis

The CD4+ and CD8+ stained cells were analyzed by the FACS scan. The activated CD4+ (64.8 ± 2.7% of gated cell) and CD8+ (16.6 ± 0.3) cells of the group immunized with N in combination with M were more than those (CD4+: 54.9 ± 3.0, CD8+: 6.3 ± 0.6) of the group immunized with N vaccine alone. In addition, there is no significant difference of activated CD4+ and CD8+ T cells between the group immunized with N DNA vaccine alone and mock plus N (CD4+: $p = 0.14$, CD8+: $p = 0.69$). This demonstrates that the N DNA vaccine could induce the T-cell mediated response in mice and that M expression could enhance the T lymphocytes activity induced by N vaccine.

3.5. Cytokines release

When stimulated with N antigen, the lymphocytes from the groups immunized with N or N in combination with mock secreted significant levels of IFN-γ, IL-2 and IL-4 compared with control groups. Moreover, the lymphocytes from the group of M plus N released more cytokines than the group only immunized with N vaccine. IFN-γ and IL-2 levels were increased about two- and 4.75-fold, respectively (Table 2).

Table 2
Cytokine production of the lymphocytes stimulated with N antigen

	pVAX1	M	N	pVAX1+N	M+N
IFN-γ	205 ± 21	217 ± 12	460 ± 43	432 ± 56	939 ± 75
IL-2	231 ± 10	195 ± 41	185 ± 37	289 ± 35	879 ± 91
IL-4	71 ± 21	69 ± 15	300 ± 67	190 ± 47	274 ± 22

Lymphocytes (2.5×10^6/ml) were cultured in presence of N protein (10 μg/ml). Cell-free supernatants were harvested after 72 h and cytokine levels were measured by ELISA kits. The values are expressed as mean ± S.E. of the cytokines concentration (pg/ml) according to the standard samples curves.

3.6. Lymphocyte proliferation assay

Activation and proliferation of lymphocyte play a critical role both in the humoral and cellular immune responses induced by vaccination. As shown in Fig. 4, the higher level of activity of lymphocytes stimulated by 1 or 5 μg/ml N antigen was observed both in the animals immunized with N alone and the mixture of M and N compared with control. Moreover, the lymphocytes proliferation specific to N antigen was stronger in voles immunized with M + N than that in animals immunized with only N.

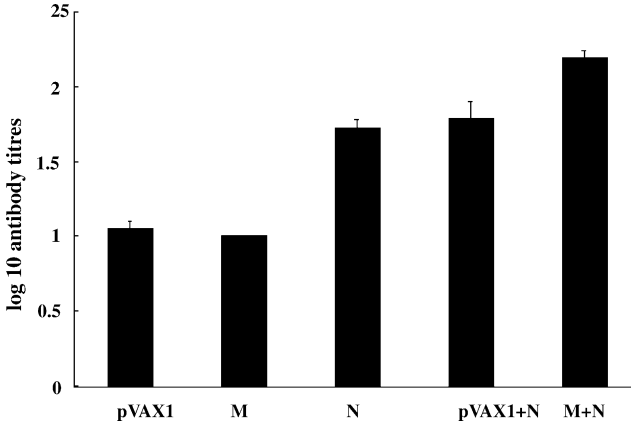

Fig. 3. Detection of the N-specific antibody in immunized mice. Antibody responses to N ($n = 6$) that were bled on day 28 after last immunization. The mean titers of antibodies were evaluated by ELISA. The values were expressed in log 10, and defined as positive by using the absolute ratio of Post/Naive serum at a cutoff of 2.1.

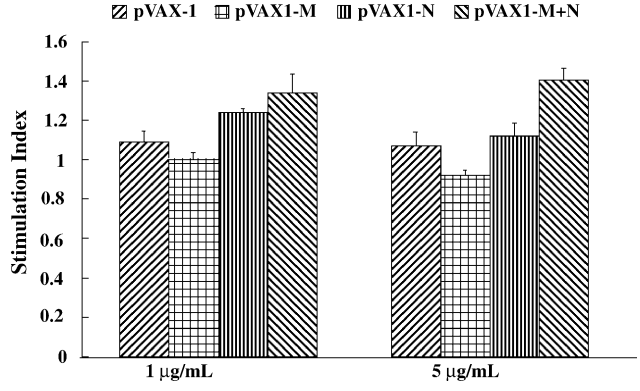

Fig. 4. Lymphocyte proliferation assay. Four weeks after the last immunization, the voles were sacrificed and the single lymphocyte suspension was cultured in medium. Approximately 10^5 cells were added into the 96 wells, 1 or 5 μg/ml purified N antigen was used to stimulate the lymphocyte proliferation activity. After 3 days, 20 μL MTS solution was added into the wells, and the plate was incubated for 4 h at 37 °C. Then the absorbance of samples at 490 nm was read. The results were expressed as stimulation index (SI, ratio of the absorbance at OD 490 nm with N antigen and negative control). The data are expressed as mean ± S.E.M.

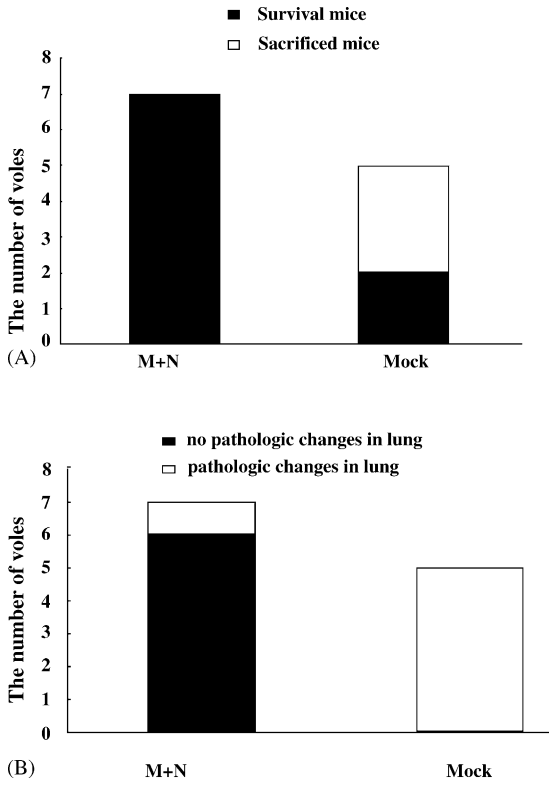

Fig. 5. Anti-SARS-CoV animal experiment. The adult's *Microtus brandti* raddes that had been inoculated M + N ($n = 7$) and mock ($n = 5$) were challenged with SARS-CoV. Fourteen days after challenge, the number of mice that did not die and had no pathological changes in lung were showed.

3.7. Reaction of the antibody to inactivated SARS-CoV

The reaction of IgG antibody to complete SARS-CoV particle in sera (1:10) was detected by ELISA. The result indicated that only the mice immunized with N together with M vaccine could produce the positive reaction to inactivated SARS-CoV ($n = 4$), and the value is 0.69 ± 0.35. However, the sera from the mice immunized with mock, M or N plasmid alone ($n \geq 4$) could not.

3.8. Anti-SARS-CoV experiment in voles

Fig. 5 shows that no *M. brandti* raddes immunized with M plus N died 2 weeks after challenged with SARS-CoV (0/7), whereas most animals that had been immunized with mock vector died (3/5), and from the results of carefully observation and microscopic examination, the syndrome of the voles challenged with virus is like the infection syndrome in human being. The data of microscopic inspection showed that all voles (5/5) challenged with virus had the obvious pathological changes in lung, whereas only one of seven animals that had been inoculated with the mixture of M and N had the pathologic changes in lung.

3.9. Histological analysis of the pulmonary sections

After the voles challenged with live SARS-CoV, pulmonary alveoli of the control animals were destroyed extensively with haemorrhage. The inflammation cells invasion and the pulmonary space compression were also observed in the lung section (Fig. 6). Moreover, the variable features include kidney, spleen and liver haemorrhage, variable amounts of multinucleated giant cells and monocytes were detected. However, the lung pathological changes were found in only one of seven voles immunized with M plus N with the features of alveolar cell hyperplasia and calcify of kidney tubule, and no obvious pathological changes were observed in the lung, liver, kidney and the other organs inspected in other six vaccinated animals.

4. Discussion

In this study, we successfully constructed pVAX1-M and pVAX1-N expression plasmid containing the full-length M and N cDNA isolated from the SARS patient who was killed by the disease. In vivo and in vitro assay (data not shown) demonstrated that the vaccines could express in eukaryotic cells. In order to get the N protein for detection of specific antibodies and the T-cell mediated responses, we constructed the prokaryotic expression plasmid pET21b-N. The recombinant could correctly express N protein in BL21 bacteria and the expression volume increased after induced by IPTG.

Further ELISA analysis demonstrates that co-expression of M with N protein could enhance the production of the N-specific antibody in Balb/c, and the major increased antibody subclass in Balb/c is IgG2a, which is consistent with Th1 immuno-response. This result is helpful for us to explain the observation that high-level nucleocapsid antibodies were found in the patients infected by SARS-CoV.

Since plasmid DNA itself is a potential adjuvant, in order to rule out the possibility that the immunity enhancement by M is contributed by different amounts of plasmid DNA, the N-specific antibody from the mice had been immunized with mock plus SARS-N was measured and the result revealed that there was no significance between the group of mock plus N and only N. Using the complete SARS-CoV as the capture antigen, ELISA assay shows that the absorbance value of samples from mice immunized with M and N together are positive, whereas all the sera from only M or N vaccine immunized mice is negative.

CD4+ T-cell mediated cellular response is very important when assess the efficacy of anti-viral vaccine, our study show that when stimulating lymphocytes with 10 μg/ml N antigen, the CD4+ lymphocytes of the mice immunized with N or M + N vaccine is more than the mice injected with mock or M alone, and co-expression of M with N can enhance CD4+ T cells activity against N antigen.

Cell-mediated cytotoxicity is another essential defense against viral pathogens, and most cytotoxic T cells are CD8+ and recognize specific antigen presented on MHC class I. That SARS-M enhances the CD8+ T cells activity specific to N antigen indicates that M could augment the cytotoxicity provoked by N DNA immunization.

T-cell-derived cytokines release represents an important defense mechanism against virus infection. The production of Th1 cytokine IFN-γ and IL-2 of lymphocytes immunized with M plus N by stimulation is significant increased compared with those of lymphocytes immunized with only N, which is in accor-

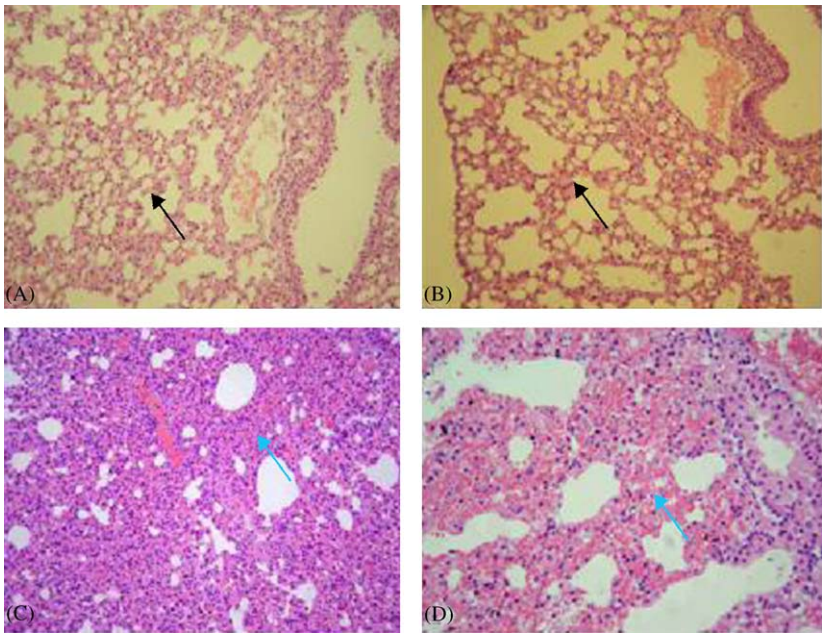

Fig. 6. Histological analysis of the pulmonary morphological structure. After the voles challenged with live SARS-CoV. The pulmonary tissue was harvested and the 5 μm sections were prepared. The sections were stained with standard hematoxylin and eosin. (A) The vole without challenge. (B) The vole immunized with M and N and challenged with virus. (C) and (D) The vole immunized with mock vector and challenged. Black arrow show normal pulmonary space, and blue arrow represent abnormal pulmonary space. Results of the representative experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

dance with the result that the major increased antibody subclass against N antigen induced by M is IgG2a.

The experiments of anti-SARS-CoV in *M. brandti* radde support that the immunity induced by N in combination with M DNA immunization could protect the animals from the virus challenge.

It is not clear why the M administration can enhance the N-induced antibody production and T lymphocyte activity. Since analysis of the M protein revealed that a 121 amino acid hydrophilic domain on the inside of the virus particle is believed to interact with the N protein, and additional mammalian two-hybrid assay had proved the possible interactions between SARS-CoV N and the M protein (He et al., 2004). We suppose that the N antigen maybe reveal more B-cell and T-cell epitopes when the M protein interacts with it. From previous study, linking a Marek's disease virus type 1 VP22 or new castle disease virus to an antigen could improve the DNA vaccine potency (Hung et al., 2002; Termeer et al., 2000). Because M antigen is also a virus antigen, we suppose that it can enhance the efficacy of the N DNA vaccine. According to this, M antigen is a potential new adjuvant.

It is predicted from the basic nature of N peptide that it may assist in RNA binding and has a novel nuclear function with playing an important role in pathogenesis for SARS-CoV. DNA construct encoding CRT linked to a SARS-CoV N antigen is capable of generating strong N-specific humoral and cellular immune responses and may potentially be useful for control of infection with SARS-CoV (Gao et al., 2003). Although antibody specific to N had no significant protective efficacy in mouse model, the data that M protein can enhance the specific immunity induced by N DNA immunization is helpful to investigate the SARS pathogenesis during the course of SARS-CoV infection and further to design potential vaccines based on T-cell-medicated responses.

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