Short Communication

Severe acute respiratory syndrome coronavirus nucleocapsid protein expressed by an adenovirus vector is phosphorylated and immunogenic in mice

Alexander N. Zakhartchouk,[1] Sathiyanarayanan Viswanathan,[1] James B. Mahony,[2,3] Jack Gauldie[2] and Lorne A. Babiuk[1]

Correspondence
Alexander N. Zakhartchouk
zakhartchouk@sask.usask.ca

[1]Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E3

[2]Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada L8N 3Z5

[3]St Joseph's Hospital, Hamilton, ON, Canada L8N 4A6

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Severe acute respiratory syndrome coronavirus (SARS-CoV) has been identified as the aetiological agent of SARS. Thus, vaccination against SARS-CoV may represent an effective approach towards controlling SARS. The nucleocapsid (N) protein is thought to play a role in induction of cell-mediated immunity to SARS-CoV and thus it is important to characterize this protein. In the present study, an E1/partially E3-deleted, replication-defective human adenovirus 5 (Ad5) vector (Ad5-N-V) expressing the SARS-CoV N protein was constructed. The N protein, expressed *in vitro* by Ad5-N-V, was of the expected molecular mass of 50 kDa and was phosphorylated. Vaccination of C57BL/6 mice with Ad5-N-V generated potent SARS-CoV-specific humoral and T cell-mediated immune responses. These results show that Ad5-N-V may potentially be used as a SARS-CoV vaccine.

Severe acute respiratory syndrome (SARS) is the latest in a series of emerging infectious diseases. This acute and often severe respiratory illness emerged in southern China in late 2002 and subsequently spread to other countries early in the following year. The SARS epidemic was contained at 8098 cases with 774 deaths. In addition to the human misery caused, there were direct economic consequences for those countries most affected (Christian *et al.*, 2004).

The causative agent of SARS was identified as a new type of coronavirus, the SARS coronavirus (SARS-CoV). SARS-CoV is an enveloped virus with a positive-sense, single-stranded RNA genome of 29 727 nt. The genome is composed of a region encoding an RNA-dependent RNA polymerase, a region representing four coding sequences for viral structural proteins (S, E, M and N) and several putative uncharacterized proteins (Marra *et al.*, 2003; Rota *et al.*, 2003).

The SARS-CoV nucleocapsid (N) gene encodes a 50 kDa protein harbouring a putative nuclear localization signal (Marra *et al.*, 2003). However, the N protein is distributed predominantly in the cytoplasm of SARS-CoV-infected and N gene-transfected cells (Chang *et al.*, 2004). The SARS-CoV N protein is a highly charged, basic protein that can self-associate to form dimers (He *et al.*, 2004; Surjit *et al.*, 2004). The three-dimensional structure of the N-terminal portion of the protein is similar to those of other RNA-binding proteins (Huang *et al.*, 2004). 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 (Parker & Masters, 1990; Kuo & Masters, 2002; He *et al.*, 2003). In addition, the N proteins of many coronaviruses are highly immunogenic and expressed abundantly during infection (Liu *et al.*, 2001; Narayanan *et al.*, 2003). Indeed, high levels of IgG antibodies against N have been found in sera from SARS patients (Leung *et al.*, 2004).

For some coronaviruses, there is evidence that the N protein can prime the protective response or, in some cases, induce protective immunity on its own (Seo *et al.*, 1997; Liu *et al.*, 2001). We therefore focused our studies on characterization of the N protein of SARS-CoV as a target antigen for vaccine development. We chose an adenoviral vector for SARS-CoV N protein expression, because adenovirus-vectored vaccines have been used to express antigens *in vivo* for the generation of both humoral and cellular immune responses and they can elicit potent and protective immune responses in a variety of animal models (Sullivan *et al.*, 2000, 2003; Shiver *et al.*, 2002).

To generate an E1/partially E3-deleted, replication-defective human adenovirus 5 (Ad5) vector expressing the N protein, cDNA of the SARS-CoV N gene (strain Tor-2) was cloned into the expression cassette of plasmid pH5L. The pH5L plasmid contains the left portion of the Ad5 genome

(nt 1–6100) with deletion of E1 and insertion of transcriptional control elements [the human cytomegalovirus immediate-early promoter and intron A, and the bovine growth hormone poly(A) signal]. The resulting plasmid, pH5L-N, was linearized and transfected into 293 cells together with a plasmid containing the right portion of the Ad5 genome with the 1878 bp deletion in the E3 region, using the ProFection mammalian transfection system (Promega). Homologous recombination led to generation of the recombinant virus, named Ad5-N-V. Adenovirus in crude lysate was plaque-purified by limiting dilution and agar overlay, and Ad5 vector clones were analysed by digestion of the viral genome with restriction enzymes and detection of N protein expression.

To corroborate the insertion of the N gene into the E1 region of Ad5, the viral genome was extracted from 293 cells at 24 h post-infection (p.i.) and digested with restriction enzymes. Due to the insertion of the N gene into the expression cassette, the genome of the recombinant virus Ad5-N-V contained a *Cla*I site and two *Bam*HI sites (Fig. 1a). Digestion with *Bam*HI resulted in three fragments of 18 208, 12 498 and 3553 bp, whilst digestion with *Cla*I produced two fragments of 30 737 and 3522 bp (Fig. 1b). This suggested that recombinant Ad5-N-V contained the SARS-CoV gene N in the E1 region and that the direction of gene transcription was the same as that of E1.

In order to detect N protein expression by recombinant adenovirus, we produced an antiserum against the SARS-CoV N protein. This was done by immunizing rabbits with a synthetic peptide corresponding to part of the N protein (YKTFPPTEPKKD), coupled to a carrier. The antiserum (named 03-52) recognized a major band of 50 kDa in SARS-CoV-infected cell lysates in a Western blot (data not shown).

To examine the product expressed by Ad5-N-V, 70–80 % confluent 293 cell monolayers in a six-well plate were infected with 10 TCID50 recombinant or wild-type Ad5 per cell. After virus adsorption for 1 h, cells were incubated in modified Eagle's medium (MEM) containing 10 % fetal bovine serum (FBS). At 24 h p.i., the medium was changed to methionine/cysteine-free MEM (for labelling with ^{35}S) (Sigma) or phosphate-free MEM (for labelling with ^{32}P) (Sigma), supplemented with 5 % FBS. After 1 h incubation, [^{35}S]methionine/cysteine [100 μCi (3·7 MBq) per well; Perkin Elmer] or trisodium [^{32}P]orthophosphate [300 μCi (11·1 MBq) per well; Amersham Biosciences] was added. After 4 h labelling, cells were harvested. Proteins were immunoprecipitated from cell lysates in radioimmunoprecipitation buffer by using rabbit serum 03-52 and samples were analysed by 12 % SDS-PAGE.

Immunoprecipitation of recombinant Ad5-N-V-infected cells labelled with [^{35}S]methionine/cysteine revealed a band of 50 kDa (Fig. 2a, lane 1). No similar band was observed in uninfected cells or in cells infected with Ad5 (Fig. 2a, lanes 2 and 3).

To test whether SARS-CoV N protein, expressed by Ad5-N-V, was phosphorylated, 293 cells infected with Ad5-N-V were labelled metabolically with [^{32}P]orthophosphate. The immunoprecipitation of recombinant Ad5-N-V-infected cells revealed a band of 50 kDa (Fig. 2b, lane 1) and no similar band was observed in uninfected cells or in cells infected with Ad5 (Fig. 2b, lanes 2 and 3).

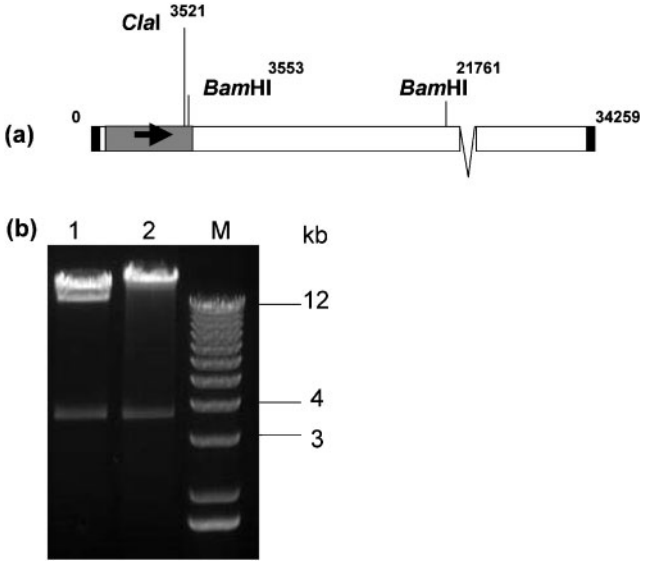

Fig. 1. Restriction-enzyme analysis of the recombinant Ad5-N-V genome. (a) Schematic representation of the Ad5-N-V genome, depicting the *Bam*HI and *Cla*I restriction-enzyme recognition sites. (b) Viral DNA extracted from 293 cells infected with Ad5-N-V and digested with *Bam*HI (lane 1) or *Cla*I (lane 2). M, 1 kb Plus DNA Ladder size markers.

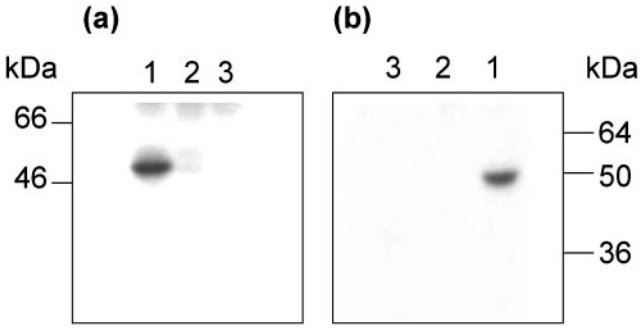

Fig. 2. Expression of SARS-CoV N protein in infected 293 cells. Proteins from lysates of [^{35}S]methionine/cysteine-labelled (a) or trisodium [^{32}P]orthophosphate-labelled (b) cells were immunoprecipitated with 03-52 antisera and analysed by SDS-PAGE under reducing conditions. Lane 1, Ad5-N-V-infected cell lysate; lane 2, Ad5-infected cell lysate; lane 3, uninfected cells. The positions of [^{14}C]-labelled protein molecular mass markers (Amersham Biosciences) (a) and SeeBlue Plus2 pre-stained standards (Invitrogen) (b) are indicated.

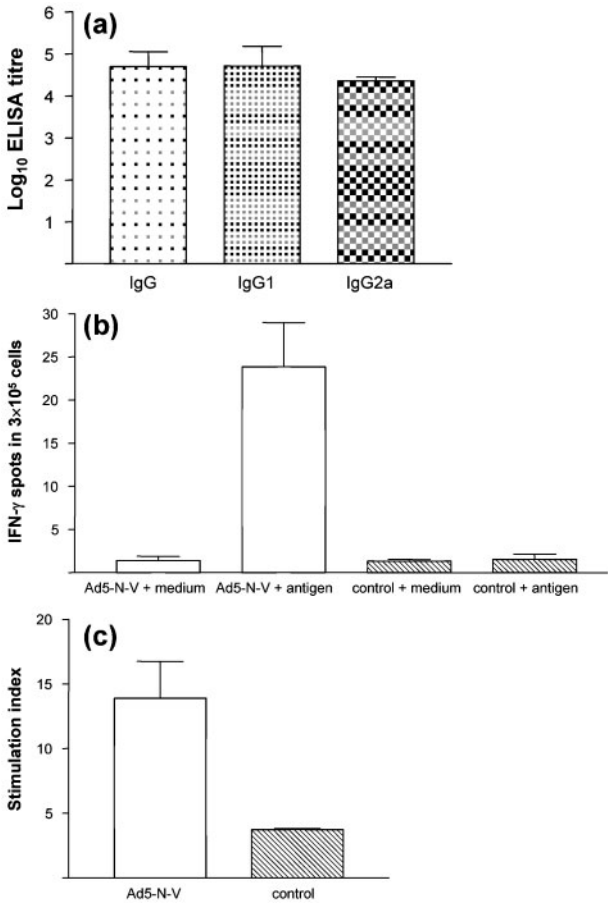

Fig. 3. SARS-CoV-specific immune responses in immunized C57BL/6 mice. (a) Antibody responses. Results are shown as means ± SD of results from five mice. (b) IFN-γ ELISPOT assay. (c) T-cell proliferation assay. Error bars in (b) and (c) represent SEM.

These data suggested that SARS-CoV N protein was expressed and phosphorylated in Ad5-N-V-infected 293 cells. Phosphorylation of the N protein is a well-documented phenomenon in many coronaviruses (Stohlman & Lai, 1979; Wilbur *et al.*, 1986; Lomniczi & Morser, 1981; King & Brian, 1982). Although the functional significance of N protein phosphorylation is still not clear, in the case of mouse hepatitis virus, phosphorylated N protein has been suggested to have a higher RNA-binding capacity than the unphosphorylated protein, and its dephosphorylation was found to be connected with initiation of infection (Wilson *et al.*, 1990; Mohandas & Dales, 1991). Interestingly, Ying *et al.* (2004) tried to confirm SARS-CoV N protein phosphorylation by several methods, but failed. Moreover, a review of SARS-CoV protein mass spectrometry data suggested that there was no phosphorylation of the N protein. Our data, showing metabolic labelling of Ad5-infected cells expressing SARS-CoV N, indicated clearly that the N protein is phosphorylated. A possible explanation for the ability of our laboratory to detect phosphorylation whereas others could not is that metabolic labelling using ^{32}P may be a more sensitive method. To our knowledge, this is the first experimental evidence of SARS-CoV N protein phosphorylation.

To determine the ability of Ad5-N-V to induce SARS-CoV-specific immune responses, five 6–8-week-old female C57BL/6 mice were immunized intraperitoneally with 2×10^{10} Ad5-N-V particles in 0·1 ml sterile PBS on days 0 and 28. Mice in the control group ($n=5$) were each immunized with 0·1 ml sterile PBS, using the same route of injection.

On day 45 after the first immunization, sera were analysed for the presence of SARS-CoV-specific IgG by ELISA. For ELISA, each well of a 96-well plate was coated with 0·1 ml purified inactivated SARS-CoV (1 μg ml^{-1}). Ad5-N-V induced a SARS-CoV-specific IgG antibody response (Fig. 3a). Both IgG1 and IgG2a antibody subclasses were found in the sera of immunized mice. The presence of both IgG antibody types (IgG1 and IgG2a) in equal levels in sera indicated that the immune response was a balanced Th1/Th2 response.

To evaluate SARS-CoV-specific cellular immune responses, splenocytes were isolated from Ad5-N-V-immunized mice or PBS-vaccinated control mice on day 45. Antigen-specific responses were measured by gamma interferon (IFN-γ) enzyme-linked immunospot assay (ELISPOT) and cellular proliferation. For ELISPOT, unifilter 96-well plates coated overnight with 0·1 ml rat anti-mouse IFN-γ (1·25 μg ml^{-1}; BD PharMingen) per well were washed once with RPMI 1640 (Life Technologies) containing 10 % FBS and incubated in triplicate with 3×10^5 splenocytes per well in 0·1 ml RPMI 1640 medium with 10 % FBS containing 10 μg purified inactivated SARS-CoV ml^{-1}. After incubation for 48 h at 37 °C in a CO_2 incubator, plates were washed five times in PBS containing 0·05 % Tween 20 and incubated overnight at 4 °C with 0·1 ml biotinylated rat anti-mouse IFN-γ antibody (1·25 μg ml^{-1}; BD PharMingen) per well. After washing with PBS containing 0·05 % Tween 20, plates were incubated for 1·5 h with a 1 : 500 dilution of streptavidin–alkaline phosphatase (Jackson ImmunoResearch). After eight washes with water, the plates were developed with Sigma Fast BCIP/NBT. Development was stopped by washing with tap water and plates were air-dried and read under a microscope.

As shown in Fig. 3(b), mice immunized with Ad5-N-V developed SARS-CoV-specific IFN-γ ELISPOT responses. The presence of IFN-γ indicated antigen-specific Th1-type $CD4^+$- and $CD8^+$-cell activation. There was no response in control-immunized mice or in splenocytes stimulated by cell-culture medium alone.

SARS-CoV cellular proliferative responses were assessed by a [methyl-^{3}H]thymidine incorporation assay. Mice splenocytes were resuspended at a concentration of 3×10^6 cells ml^{-1} in RPMI 1640 containing 10 % FBS. A 100 μl aliquot

was added to each well of a 96-well plate together with 0·5 μg purified inactivated SARS-CoV per well or medium alone. After 3 days culture, 0·4 μCi [methyl-^{3}H]thymidine (Amersham Biosciences) was added to each well. After incubation for 16 h, cells were harvested and thymidine uptake was measured by scintillation counting. Proliferative responses were calculated as mean values of triplicate cultures and were expressed as a stimulation index (counts min^{-1} in the presence of antigen/counts min^{-1} in the absence of antigen). As shown in Fig. 3(c), mice immunized with Ad5-N-V developed a significant SARS-CoV-specific proliferative response, whereas control mice did not.

These results showed that Ad5-N-V is capable of generating strong SARS-CoV-specific humoral and cellular immunity and may potentially be used as a SARS-CoV vaccine. Kim *et al.* (2004) and Zhu *et al.* (2004) have also used SARS-CoV N protein for induction of immune responses; however, they used a DNA-based vaccine strategy.

Gao *et al.* (2003) used adenoviral vectors that expressed the SARS-CoV spike protein S1 fragment and the M and N proteins. Monkeys immunized with all three adenovirus vectors had an antibody response against the S1 protein and a T-cell response against the N protein, as measured by IFN-γ ELISPOT analysis of peripheral blood mononuclear cells. Our data are in agreement with those of Gao *et al.* (2003); however, we used only one recombinant adenovirus for mouse immunizations and the immune responses were assessed by using murine splenocytes.

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