

**Short  
Communication****Amino terminus of the SARS coronavirus protein 3a elicits strong, potentially protective humoral responses in infected patients**Xiaofen Zhong,<sup>1</sup> Zufeng Guo,<sup>1,2</sup> Huanghao Yang,<sup>1,2</sup> Lisheng Peng,<sup>1</sup> Yong Xie,<sup>3</sup> Tin-Yau Wong,<sup>4</sup> Sik-To Lai<sup>4</sup> and Zhihong Guo<sup>1,2</sup>Correspondence  
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The 3a protein of severe acute respiratory syndrome (SARS)-associated coronavirus is expressed and transported to the plasma membrane in tissue cells of infected patients. Its short N-terminal ectodomain was found to elicit strong humoral responses in half of the patients who had recovered from SARS. The ectodomain-specific antibodies from the convalescent-phase plasma readily recognized and induced destruction of 3a-expressing cells in the presence of the human complement system, demonstrating their potential ability to provide immune protection by recognizing and eliminating SARS coronavirus-infected cells that express the target protein. In addition, when coupled to a carrier protein, the ectodomain peptide elicited 3a-specific antibodies in mice and rabbit at high titres. These results showed that the N terminus of the 3a protein is highly immunogenic and elicits potentially protective humoral responses in infected patients. Therefore, the short extracellular domain may be a valuable immunogen in the development of a vaccine for infectious SARS.

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Severe acute respiratory syndrome (SARS) is a new infectious disease that is caused by a new strain of coronavirus (CoV) (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003; Poutanen *et al.*, 2003). Immunogenicity of the viral pathogen has been a focal point of interest because of its central importance in the design of an efficacious vaccine. Several experimental vaccines have been developed successfully to induce protective humoral responses specific for the spike protein, suggesting that this is a major antigen responsible for the protective humoral immunity generated in infected SARS patients (Gao *et al.*, 2003; Bisht *et al.*, 2004; Buchholz *et al.*, 2004; Johnston, 2004; Subbarao *et al.*, 2004; Yang *et al.*, 2004; Zhao *et al.*, 2004). This is consistent with recent surveys of convalescent-phase serological samples from patients who had recovered from SARS, in which spike-specific antibodies were implicated in conferring long-term immune protection (Guo *et al.*, 2004; He *et al.*, 2004; Zhong *et al.*, 2005).

Besides the spike protein, the 3a protein and other viral proteins have also been found to be a target of humoral antibodies from SARS patients (Wang *et al.*, 2003; Chang *et al.*, 2004; Chen *et al.*, 2004; Leung *et al.*, 2004; Liu *et al.*, 2004; Shi *et al.*, 2004; Tan *et al.*, 2004a; Zhong *et al.*, 2005). While most of these antibodies are only of diagnostic value, 3a protein-specific antibodies might offer additional immune protection to infected patients and attracted our attention.

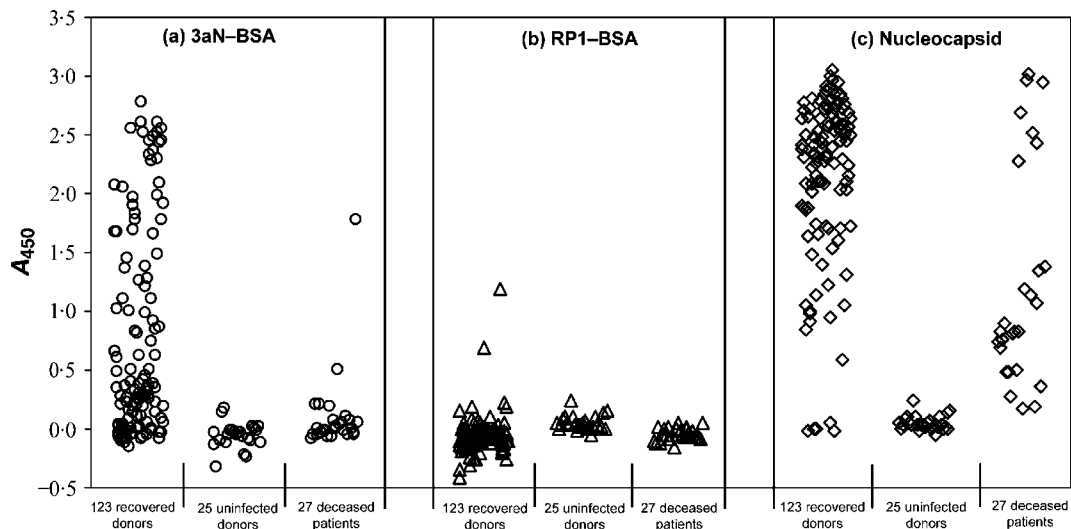
The 3a protein is a predicted 274 aa transmembrane protein. Recently, it has been shown to be expressed and transported to the plasma membrane in Vero E6 cells infected with SARS-CoV, with the N terminus (aa 1–35) exposed to the extracellular environment (Tan *et al.*, 2004b). Experimental evidence has also been provided for its *in vivo* expression in a lung section from a SARS-CoV-infected patient (Yu *et al.*, 2004). In addition, this protein has an intracellular perinuclear localization similar to all CoV surface proteins (spike, membrane and small envelope proteins) and interacts extensively with them (Tan *et al.*, 2004b; Zeng *et al.*, 2004), providing the rationale for its incorporation into the viral envelope in the replication process (Ito *et al.*, 2005). The role of the 3a protein as a newly discovered structural protein of SARS-CoV and the fact that it is a target of immune responses in infected patients suggest that its N terminus might be a valuable immunogen in vaccine development. In this study, therefore, we surveyed the prevalence of antibodies specific for the N terminus of the 3a protein (3aN) in serological samples from patients who had recovered from SARS, determined the capability of the antibodies to recognize and eliminate 3a-expressing cells, and tested the antigenicity of the N-terminal peptide in animals.

To survey the prevalence of antibodies complementary to the identified 3aN antigenic site (Zhong *et al.*, 2005), a

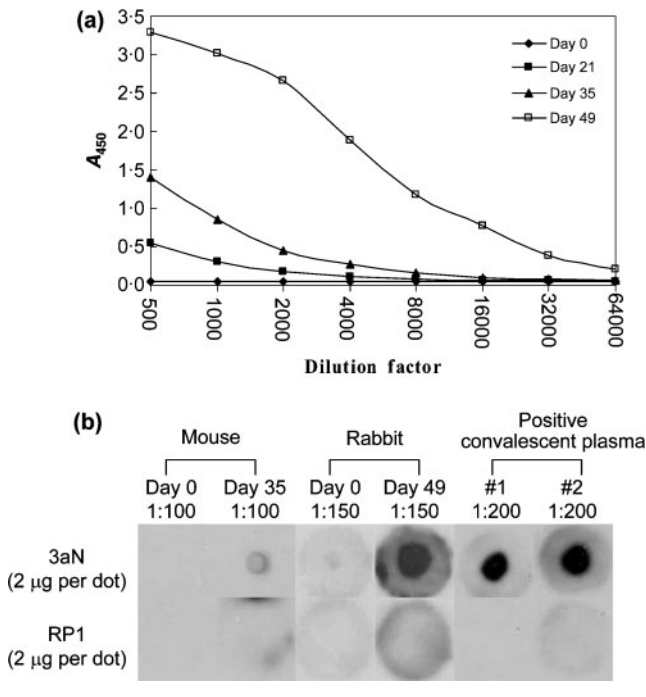
peptide with a sequence encompassing this epitope (aa 11–44, Ac-RSITAQPVKIDNASTVHATATIPLQASLP-FG-OH, where Ac = acetyl) was chemically synthesized and coupled to BSA for use as the antigen in ELISA screening of serological samples from SARS-CoV-infected patients. A total of 123 plasma samples collected from patients who had recovered from SARS (28 days after discharge) and 27 sera collected from patients who eventually died of SARS (28 days after hospitalization) were analysed. These serological samples were prepared between March and October, 2003, inactivated at 56 °C for 45 min and stored at –20 °C until used at the Princess Margaret Hospital, Hong Kong SAR, China. Under the given conditions, plasma samples from 25 uninfected donors collected from the Hong Kong Red Cross Blood Transfusion Service tested negative for antibodies against the peptide conjugate (Fig. 1). All patient blood samples tested negative for the BSA carrier protein, while only two tested positive for a BSA conjugate with an irrelevant peptide, RP1 (Ac-GPNLRNPVEQPLSVQA-OH). As a positive control, the nucleocapsid protein was found to be targeted by specific IgG antibodies in a high percentage of the serological samples from both recovered (95.1%) and deceased (92.6%) patients, consistent with the clinical diagnosis of infection by SARS-CoV for the patients and the high antigenicity of the nucleocapsid protein revealed in other investigations (Wang *et al.*, 2003; Chang *et al.*, 2004; Chen *et al.*, 2004; Leung *et al.*, 2004; Shi *et al.*, 2004; Tan *et al.*, 2004a). These control experiments established the validity of the ELISA screening method.

Among the 123 recovered patients, 60 (48.8%) tested positive for 3aN-specific antibodies (Fig. 1), whereas only two (7.4%) of the 27 deceased patients developed humoral responses to the antigenic peptide. This high immunoreactivity of the 3aN peptide was consistent with the high positive rate (71%) of convalescent-phase SARS sera found for the whole recombinant 3a protein (Tan *et al.*, 2004a) and the positive immunoreactivity of SARS patient sera for a different N-terminal peptide (Zeng *et al.*, 2004; Zhong *et al.*, 2005). Noticeably, both the prevalence and the levels of 3aN-specific antibodies were significantly lower for the deceased patients compared with the recovered patients, despite both groups of samples having a similar percentage of nucleocapsid-specific antibodies. Thus, these results showed that a substantial proportion of the recovered patients developed antibodies specific for 3aN.

To test the antigenicity of the N-terminal peptide of the 3a protein in animals, the peptide was coupled to a carrier protein (BSA or KLH) and the resulting conjugates were used to immunize three mice and a rabbit. A 12-week-old New Zealand white rabbit was immunized with 1 ml of peptide–KLH conjugate (0.84 mg) emulsified in an equal volume of Freund's complete adjuvant (Sigma) at more than 20 sites by intradermal injection. Booster injections were made with the same amount of the peptide conjugate emulsified in Freund's incomplete adjuvant (Sigma) at an interval of 14 days. The mice were immunized by intraperitoneal injection using a lower dose (0.2 mg peptide–BSA conjugate). As shown in Fig. 2(a), antibodies specific



**Fig. 1.** ELISA analysis of plasma samples from infected patients and uninfected donors using 3aN–BSA conjugate (a), an irrelevant peptide RP1–BSA conjugate (b) and recombinant SARS-CoV nucleocapsid protein (c) as the antigen (1 µg per well). The value of  $A_{450}$  in the plot was obtained by subtracting the reading of a parallel control experiment using BSA as the blank antigen from the sample value. Among 123 patients who had recovered from SARS, 60 (48.8%) were positive for 3aN-specific antibodies and 117 (95.1%) were positive for nucleocapsid-specific antibodies. Among 27 patients who had died from SARS, two (7.4%) were positive for 3aN-specific antibodies and 25 (92.6%) were positive for nucleocapsid-specific antibodies. All 25 uninfected donors were negative for 3aN-specific antibodies, but one was found to be positive for antibodies targeting the nucleocapsid protein.



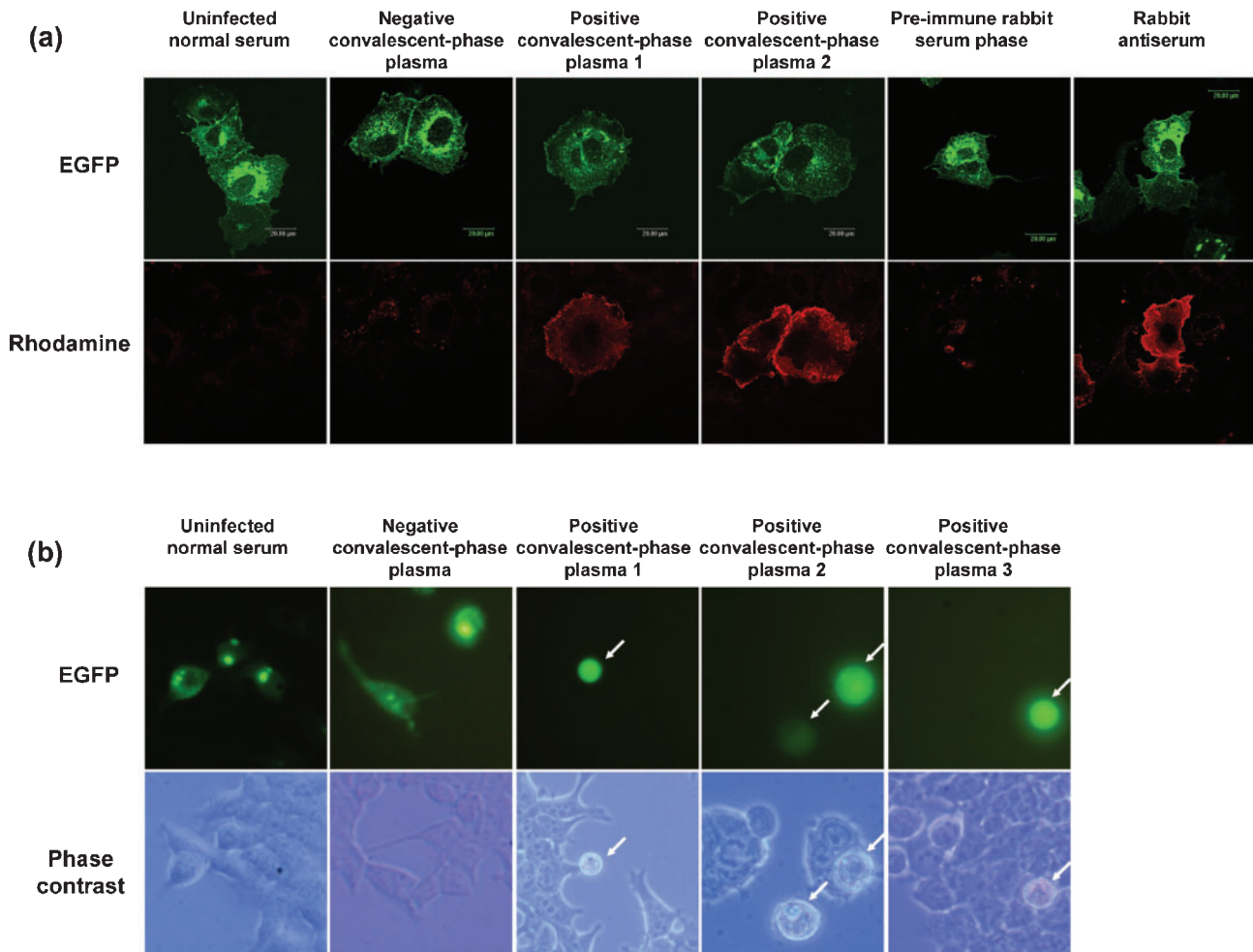
**Fig. 2.** Antibody responses to the 3aN conjugates in mice and rabbit. (a) Titration curves for 3aN-specific antibodies in rabbit antiserum. The rabbit was immunized with a 3aN-KLH conjugate on days 1, 14, 28 and 42 and test bleeding was conducted on days 0, 21, 35 and 49 for ELISA titration using a 3aN-BSA conjugate. Day 0 serum was collected before immunization as a negative control. In a similar experiment, a titre of 6400 for 3aN-specific antibodies was determined for a combined serum collected on day 35 from three mice immunized with a 3aN-BSA conjugate on days 1, 14 and 28. (b) Western dot-blot analysis of 3aN-specific antibodies in the antisera collected from the immunized mice and rabbit on days 35 and 49 after immunization, respectively.

for the 3aN peptide were readily induced and reached a titre of 6400 and 64 000 for the mice and rabbit, respectively. The titration experiments showed that the induced antibodies could recognize the 3aN peptide. This was further supported by a Western dot-blot analysis of the antiserum antibodies with the pure and unconjugated 3aN peptide absorbed onto a PVDF membrane (Fig. 2b). Due to the short length of the peptide, which is unlikely to form a stable conformation, the antiserum antibodies most likely target a consecutive amino acid sequence in the 3aN peptide in the range from aa 12 to 37 as determined in phage-panning experiments (Zhong *et al.*, 2005). These results showed that the short 3aN is indeed highly antigenic and is able to elicit humoral responses in animals, in accordance with its being a target of the humoral responses in humans.

The 3a protein is expressed as a plasma transmembrane protein in SARS-CoV-infected cells with its short N terminus exposed to the extracellular environment and its C-terminal end in the cytoplasm (Ito *et al.*, 2005; Tan *et al.*,

2004b). The 3aN-specific antibodies in the plasma of recovered patients should be able to offer immune protection by recognizing SARS-CoV-infected cells for elimination by the complement system. To test this, the 3a protein was fused to enhanced green fluorescent protein (EGFP) at its C terminus and expressed in Vero E6 cells. Fluorescent microscopic analysis found that the 3a-EGFP fusion protein was located on the perinuclear region and the plasma membrane (Fig. 3a), a subcellular distribution indistinguishable from that found for the viral protein without a fusion (Yu *et al.*, 2004; Tan *et al.*, 2004b). After staining with positive human plasma samples or the rabbit antiserum as the primary antibody and an appropriate anti-IgG-rhodamine conjugate as the secondary antibody, the green fluorescent cells were labelled with the red fluorescent rhodamine under a microscope, indicating that the 3a-specific antibodies in patient plasma and animal antiserum could indeed recognize the ectodomain of the 3a fusion protein on the cell surface. Under identical conditions, such cell labelling was not found with pre-immune rabbit serum, uninfected plasma samples or convalescent-phase plasma samples that tested negative for 3aN-specific antibodies. To assess the ability of 3aN-specific antibodies to induce elimination of 3a-expressing cells, HEK293T cells transiently transfected with the 3a-EGFP plasmid were sub-cultured into 96-well microplates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. After 24 h, cells were incubated for 1 h with 200 µl of the convalescent plasma (1:10 dilution) tested for 3aN-specific antibodies in the immunofluorescent experiments. Cells were then washed with PBS and incubated in DMEM supplemented with 10% normal human serum. After another 24 h, all 3a-expressing fluorescent cells showed signs of death with obvious morphology changes and detachment from the poly-D-lysine matrix, whereas non-fluorescent cells were not affected (Fig. 3b). When the human serum was heat-inactivated before incubation with the cells treated with 3aN-specific antibodies, the 3a-expressing fluorescent cells were not affected. These results indicate that the 3aN-specific antibodies were able to activate the human complement cascade through the classical pathway, leading to elimination of the 3a-expressing cells. Taken together, these experiments demonstrated that antibodies elicited by 3aN in humans can specifically recognize and, in the presence of the human complement system, eliminate cells in which the 3a protein is expressed.

SARS-CoV induces strong humoral responses in infected human patients or animals, targeting various structural and non-structural proteins. So far, the only identified antibodies that can neutralize the virus and provide immune protection exclusively target the major envelope glycoprotein, the spike protein. The other viral surface glycoproteins – the matrix protein and small membrane protein – have not been found to elicit antibody responses in infected SARS patients. In this study, we found that the N terminus of the 3a protein, a plasma transmembrane protein expressed in



**Fig. 3.** (a) Recognition of 3a-expressing cells by antibodies in rabbit antiserum and convalescent-phase plasma. Rhodamine staining of Vero E6 cells transfected with a plasmid containing the 3a-EGFP gene was negative for the rabbit pre-immune serum, two normal uninfected sera and two convalescent-phase plasma samples that tested negative for 3aN-specific antibodies, but was positive for the rabbit antiserum and five positive convalescent-phase plasma samples from the ELISA screening. (b) Cytotoxicity of 3aN-specific antibodies towards 3a-expressing cells in the presence of the human complement system. HEK293T cells were transfected with the 3a-EGFP-expressing plasmid, treated with control human serum (heat inactivated) and convalescent-phase plasma, incubated in 10% human serum (not heat inactivated) and imaged with a fluorescent microscope. Fluorescent cells (white arrows) treated with convalescent-phase plasma containing 3aN-specific antibodies deformed and started to detach from the matrix after incubation in human serum, whereas those treated with serological samples without 3aN-specific antibodies were not affected. The deformed cells were confirmed to be dead by trypan blue staining. All experiments were performed in triplicate.

infected cells, elicited strong humoral responses in a high percentage of patients who had recovered from SARS and was highly antigenic in animals. These antibodies were also found readily to bind cells expressing the 3a protein and induce elimination of these cells in the presence of the human complement system. Such antibodies can provide immune protection *in vivo* by recognizing and binding to the surface 3a protein of SARS-CoV-infected cells for destruction by the host complement system. Although protein 3a is a newly discovered structural protein of SARS-CoV (Ito *et al.*, 2005), the 3aN-specific antibodies are unlikely to offer protection by blocking cellular entry of the pathogenic virus.

This can be seen from the inability of antibodies specific to the matrix or small membrane protein to neutralize the infectivity of corresponding animal coronaviruses (Rottier, 1995; Siddell, 1995). Nevertheless, the high prevalence of 3aN-specific antibodies in the plasma of patients who have recovered from SARS and their ability to induce destruction of infected cells suggest that such antibodies can confer long-term immune protection.

Current efforts to develop a SARS vaccine rely on the spike protein to elicit protective humoral responses (Gao *et al.*, 2003; Bisht *et al.*, 2004; Buchholz *et al.*, 2004; Johnston,

2004; Subbarao *et al.*, 2004; Yang *et al.*, 2004; Zhao *et al.*, 2004). However, evasion of neutralization by SARS-CoV subtypes identified in the latest outbreak has been found for the spike-targeting antibodies, especially those specific for the receptor-recognition site (Yang *et al.*, 2005). This is probably a result of molecular evolution of the pathogen under immune pressure and raises concern about the efficacy of spike-based vaccines. In contrast to the high mutation rate of the spike protein, the 3aN antigenic site has a much higher stability; no mutations have been identified at this site in molecular epidemiological studies of known SARS-CoV genome sequences (Ruan *et al.*, 2003; Chinese SARS Molecular Epidemiology Consortium, 2004; Yeh *et al.*, 2004). Its high genetic stability and the potential ability to elicit long-term immunity make 3aN a highly valuable supplementary immunogen in the development of a vaccine, which is urgently needed for the infectious SARS disease.

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