Amino acids 15–28 in the ectodomain of SARS coronavirus 3a protein induces neutralizing antibodies

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Abstract  A synthetic peptide corresponding to amino acids (aa) 15–28 of the severe acute respiratory syndrome coronavirus (SARS-CoV) 3a protein was used to raise polyclonal antibodies in rabbits. This anti-3a N-terminal antibody could detect 3a protein in infected cells, as did an anti-3a C-terminal antibody previously described. The latter targeted the C-terminal cytoplasmic domain of 3a (aa 134–274). The anti-3a N-terminal antibody could detect intracellular 3a as well as 3a expressed on the cell surface. Interestingly, only the anti-3a N-terminal antibody can inhibit SARS-CoV propagation in Vero E6 culture although the binding affinity of the anti-3a N-terminal antibody was lower than the anti-3a C-terminal antibody.

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1. Introduction

The genome of the severe acute respiratory syndrome coronavirus (SARS-CoV) encodes for eight group-specific proteins with no significance sequence homology to viral proteins of other coronaviruses [1–3]. The largest of these group-specific proteins is termed 3a and the 3a protein has also been shown to be expressed in SARS-CoV infected cells [4,5] and could be detected in tissues obtained from SARS patients [5–7]. Antibodies against 3a have also been detected in different cohorts of SARS patients [8–11].

The 3a protein consists of 274 amino acids (aa) and contains three putative transmembrane domains and it is expressed on the cell surface [4,12]. The topology of 3a on the cell surface was determined experimentally: its first 34 aa, i.e. before the first transmembrane domain, is facing the extracellular matrix and its C-terminal after the third transmembrane domain (i.e. aa 134–274) is facing the cytoplasm [4]. As 3a is a novel coronavirus structural protein [12,13], its N-terminal ectodomain would be expected to protrude out of the virion. Interestingly, in two separate cohorts of SARS patients, one from Taiwan [14] and one from Hong Kong [15], B cells recognizing the N-terminal region of 3a were isolated from patients. In addition, it was recently reported that the N terminal of 3a elicits strong and potentially protective humoral responses in infected patients [11]. In this study, rabbit polyclonal antibodies targeted against the N-terminal ectodomain and the C-terminal cytoplasmic domain of the 3a protein were tested for their abilities to inhibit SARS-CoV propagation in Vero E6 culture.

2. Materials and methods

2.1. Cell-line and virus

The Vero E6 cells and SARS-CoV isolate used in this study have been previously described [16]. Culturing of 293T cells have been previously described [4].

2.2. Synthesis of peptide and production of rabbit polyclonal antibodies

A peptide ((C)AQPVKIDNASPAST), which corresponds to amino acids 15–28 of SARS-CoV 3a protein, was synthesized by BioGenes GmbH (Berlin, Germany). The peptide was conjugated to a carrier, Limulus Polyphemus Hemocyanine (LPH) from horseshoe crab, and used to immunize two rabbits using standard protocols. All procedures were performed by BioGenes GmbH. The immunization schedule is showed in Table 1. All the sera were tested by Western blot analysis.

A rabbit polyclonal antibody raised against bacterially-expressed GST-3a (134-274aa) has been previously described [4]. This antibody targets the C-terminal cytoplasmic domain of 3a and the 6th bleed was used in this study. A neutralizing antibody (rabbit anti-SA10) that targeted the SARS-CoV spike (S) protein was also used in the neutralizing assays [17]. The last bleed obtained after 16 immunizations was used.

2.3. Western blot analysis and immunofluorescence experiments

In order to express recombinant 3a protein in mammalian cells, Vero E6 cells were transfected with a cDNA construct (pXJ-3a) for expressing full-length 3a protein, as previously described [4]. Transfected cells were then subjected to Western blot analysis and immunofluorescence experiments as previously described [4]. Briefly, cells were grown to 80% confluence in a 6 cm dish and transfected with 1 μg of the plasmid. The cells were harvested after ~16 h and washed with PBS and then lysed in 1 ml of lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.5% NP40, 0.05% deoxycholic acid, 0.005% SDS, 1 mM PMSF). After 6 rounds of alternate freezing and thawing, the cells suspension was centrifuged at 13000 rpm for 20 min at 4 °C. The lysates were used for Western blot analysis. Western blot analysis was also performed on Vero E6 cells infected with SARS-CoV at a multiplicity of infection (MOI) of 1. At 24 h post-infection, the cells were washed with PBS and lysed in 350 μl of lysis buffer and the lysate was subjected to Western blot analysis in a similar manner.
3. Results and discussion

In this study, we compared the properties of sera obtained a rabbit that was immunized with bacterially expressed GST-3a (134–274 aa) (i.e. C-terminal, Rabbit #1, Ref. [4]), and two rabbits that were immunized with a synthetic peptide corresponding to 15–28 aa of 3a (i.e. N-terminal, Rabbit #2 and Rabbit #3). This peptide corresponds to aa 15–28 of the SARS-CoV 3a protein ectodomain which is predicted to face the extracellular matrix in infected cells [4]. Western blot analysis was performed to determine the specificity of the rabbit polyclonal antibodies. Total cell lysates obtained from Vero E6 cells transiently transfected with a DNA construct for expressing full-length 3a or SARS-CoV infected Vero E6 cells were used in Western blot analysis with sera collected from the immunized rabbits after different number of immunizations with the peptide. As shown in Fig. 1A, the sera from 1st to 5th bleeds of one of the immunized rabbits (Rabbit #2) could react specifically with the full-length 3a protein of ~35 kDa. Similar results were obtained with the other immunized rabbit (Rabbit #3, Fig. 1B), but the relative reactivity to 3a is lower than for Rabbit #2. Hence, subsequent experiments were performed with the sera from Rabbit #2.

Western blot analysis of SARS-CoV infected cells also demonstrated that the anti-3a N-terminal antibody (Rabbit #2, 5th bleed) could react specifically with the 3a protein expressed in infected cells (Fig. 1C, lane 2). For comparison, the anti-3a C-terminal antibody (Rabbit #1, 6th bleed) also detected 3a in infected cells (Fig. 1C, lane 4). Consistent with previous studies [10,11], the N-terminal of 3a is sufficient to stimulate specificity antibody response in rabbits. The anti-3a N-terminal antibody (Rabbit #2, 5th bleed) was used in indirect immunofluorescence experiments and it could detect the 3a protein expressed on the cell surface (Fig. 1D, left panel) and intracellularly (Fig. 1D, right panel). It was previously showed that properly folded 3a protein is efficiently transported to the cell surface with its N-terminal facing the extracellular matrix [4,21]; hence, the results showed that the anti-3a N-terminal antibody could recognize the native form of 3a expressed on the cell surface.

Next, the rabbit polyclonal antibodies were tested for their abilities to inhibit SARS-CoV propagation in Vero E6 culture. Microneutralization assays were performed by mixing a constant amount of virus with different dilutions of rabbit sera and then overlaying the mixture onto Vero E6 cells in 96-well plates. Calculations were made from the cytopathic effect induced in cell culture by the virus as described previously [20]. As shown in Table 2, the sera obtained from the rabbit immunized with the N-terminal of 3a (Rabbit #2) showed a neutralization titer of 1:67 for the 1st bleed and 1:80 for the 2nd to 5th bleeds, while the pre-immune serum did not have any neutralizing activity (titer <1:10). In contrast, the anti-3a C-terminal antibody did not show any neutralizing activity (titer <1:10). Neither the pre-immune serum from this rabbit. All experiments were performed in duplicates.

Table 1

| Day | Immunization no. | Bleed no. |
|-----|------------------|-----------|
| 0   | 1                | Pre-immune|
| 7   | 2                | –         |
| 14  | 3                | –         |
| 28  | 4                | –         |
| 35  | –                | 1         |
| 41  | –                | –         |
| 63  | –                | 2         |
| 77  | 6                | –         |
| 91  | –                | 3         |
| 120 | –                | 0         |
| 127 | –                | 7         |
| 141 | –                | 1         |
| 148 | –                | 5         |

For immunofluorescence experiments, the cells were grown on coverslips and transfected as described above. About 16 h later, the cells were fixed with 4% paraformaldehyde and/or permeabilized with Triton X-100, and incubated with the relevant antibodies as previously described [4].
tralizing assays. Indeed, the results showed that the sera from Rabbit #3 were also neutralizing, albeit with a lower titer of 1:40 (average value obtained for all the bleeds). For comparison, parallel experiments were also performed with a neutralizing antibody targeting 1055–1192 aa (SD10), which contains the heptad repeat 2 domain known to be important for mediating membrane fusion, of SARS-CoV S protein [17]. In this experiment, the rabbit anti-SA10 polyclonal antibody had a neutralizing titer of 1:160, which is consistent with our previous findings [17].

To be certain that the inability of the anti-3a C-terminal antibody to neutralize the SARS-CoV is not due to lower antibody concentrations, we determined the relative binding affinities of the anti-3a N-terminal and C-terminal rabbit polyclonal antibodies to 3a protein expressed in 293T cells by ELISA. Diluted rabbit sera were added to wells coated with total cell lysates from untransfected cells or cells transiently transfected with a DNA construct for expressing the full-length 3a protein. The OD difference (in arbitrary units) represented the specific binding of antibody to the 3a protein. As shown in Fig. 2, the serum from Rabbit #1 (i.e. C-terminal, 6th bleed) has relatively stronger binding affinity to the 3a protein than the sera from different bleeds of Rabbit #2 (i.e. N-terminal). The pre-immune sera from both rabbits showed little binding to 3a. Hence, it is clear that the anti-3a C-terminal antibody did not have any neutralizing activity, even though it contains a relatively higher level of 3a-specific antibodies than the anti-3a N-terminal antibody.

Interestingly, B cells recognizing the N-terminal ectodomain of the 3a protein were found in two separate cohorts

Table 2
Neutralizing titers for the pre-immune sera and the different bleeds obtained from rabbits that have been immunized with either the N- or C-terminal of the SARS-CoV 3a protein

| Bleed no. | N-terminal (Rabbit #2) | C-terminal (Rabbit #1) |
|-----------|------------------------|------------------------|
| Pre-immune sera | <1:10 | <1:10 |
| 1 | 1:67* | ND* |
| 2 | 1:80* | ND* |
| 3 | 1:80* | ND* |
| 4 | 1:80* | ND* |
| 5 | 1:80* | ND* |
| 6 | ND* | <1:10 |

*Neutralizing titers were determined by the Reed–Muench method and computed as the average of three independent experiments.

ND means not determined.
of SARS patients [14,15]. In another study, it was reported 48.8% of patients who recovered from SARS had antibodies against the N terminal of 3a while only 7.4% of the diseased patients has such antibodies [11]. It was further demonstrated that anti-3a antibodies in the patient serum could bind cells expressing 3a and induce the elimination of these cells in the presence of the human complement system [11]. Several other studies also found that anti-3a antibodies were presented only in a subset of SARS patients [4,9,10,22]. In this study, we showed that rabbit polyclonal antibody targeting a 14 aa epitope (aa 15–28) in the N-terminal ectodomain of 3a could neutralize the SARS-CoV replication in Vero E6 cells in the absence of the complement system [11]. Several experiments were performed in duplicates and the average values with standard deviations are plotted.

Fig. 2. Binding affinity of rabbit polyclonal antibodies to 3a protein expressed in 293T cells as determined by ELISA. Rabbit sera were diluted 1:2000 (A) or 1:8000 (B) and added to wells coated with total cell lysates from untransfected cells or cells transiently transfected with a DNA construct for expressing the full-length 3a protein. The OD difference (in arbitrary units) represented the specific binding of antibody to the 3a protein. Rabbit #1 was immunized with bacterially expressed GST-3a (134–274 aa) (i.e. C-terminal) and the pre-immune serum and the serum from the 6th bleed were tested. Rabbit #2 was immunized with a synthetic peptide corresponding to amino acids 15–28 of 3a (i.e. N-terminal). For Rabbit #2, the pre-immune serum and the sera from the 1st to 5th bleeds were tested. All experiments were performed in duplicates.

are immunogenic but only the ectodomain stimulates neutralizing antibodies.

To our knowledge, this is the first report of a SARS-CoV group-specific protein that can induce neutralizing antibody. Our results showed that antibody targeting the N-terminal of 3a can inhibit SARS-CoV replication, suggesting that 3a can stimulate protective humoral responses during SARS infection. This finding is consistent with a recent report that a higher percentage of patients who recovered from SARS infection have antibodies against the N-terminal of 3a, when compared to the patients who died from SARS infection [11]. Even though the 3a protein is not essential for SARS-CoV replication in cell culture and the murine model [23], it is very likely that 3a contributes to viral replication or pathogenesis in the natural host(s). In future studies, it will be crucial to determine the role of 3a during SARS-CoV infection in non-human primate models, where the animals could develop a disease comparable to that in SARS patients [24].
15-28 in the N-terminal ectodomain of the 3a protein during SARS-CoV infection also remains to be elucidated.

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