Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 110–117
BBRC
www.elsevier.com/locate/ybbrc

Characterization and application of monoclonal antibodies against N protein of SARS-coronavirus

Bo Shang [a,1], Xiao-Yi Wang [b,1], Jian-Wei Yuan [a], Astrid Vabret [c], Xiao-Dong Wu [a], Rui-Fu Yang [b], Lin Tian [a], Yong-Yong Ji [a], Vincent Deubel [d], Bing Sun [a,d,e,*]

[a] *Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China*
[b] *State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Institute of Basic Medical Sciences, Academy of Military Medical Sciences, Beijing 100071, China*
[c] *Laboratory of Human and Molecular Virology, UPRES EA2128, C.H.U. de Caen, Avenue Georges Clemenceau, 14000 Caen, France*
[d] *Institute Pasteur of Shanghai, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China*
[e] *E-institutes of Shanghai Universities Immunology Division, China*

Received 15 July 2005
Available online 15 August 2005

Abstract

Severe acute respiratory syndrome-coronavirus (SARS-CoV) causes an infectious disease through respiratory route. Diagnosing the disease effectively and accurately at early stage is essential for preventing the disease transmission and performing antiviral treatment. In this study, we raised monoclonal antibodies (mAbs) against the nucleocapsid (N) protein of SARS-CoV and mapped epitopes by using different truncated N protein fragments. The mapping of those epitopes was valuable for constructing pair-Abs used in serological diagnosis. The results showed that all of the six raised mAbs were divided into two groups recognizing the region of amino acids 249–317 (A group) or 317–395 (B group). This region spanning amino acids 249–395 contains predominant B cell epitopes located at the C-terminus of N protein. One pair-Abs, consisting of N protein-specific rabbit polyclonal antibody and SARS-CoV N protein-specific mAb, was selected to construct a sandwich ELISA-kit. The kit was able to specifically detect SARS-CoV N proteins in serum samples.

Keywords: Severe acute respiratory syndrome; Nucleocapsid protein; Truncated N protein fragments; Monoclonal antibody; B cell epitope mapping; Human coronaviruses; Diagnosis

A new infectious disease, known as severe acute respiratory syndrome (SARS), broke out in Guangdong province of China in 2002. Based on a WHO report, there were 8437 persons being infected over the world and in which 813 patients died from the disease until July 11, 2003. The overall mortality rate of the disease was about 10.5%. A novel coronavirus was identified to be the etiologic agent of SARS [1]. The severe acute respiratory syndrome-coronavirus (SARS-CoV) is positive stranded RNA virus of the order of 29,727 nucleotides with 14 open reading frames. The structural proteins of SARS-CoV consist of four proteins: the surface spike protein (S), the nucleocapsid protein (N), the small membrane protein (M), and the envelope protein (E) [2].

Since the infectious disease of SARS spread by the respiratory route and until now there are no effective antiviral drugs and vaccines which have been developed,

* Corresponding author. Fax: +86 21 54921011.
E-mail address: bsun@sibs.ac.cn (B. Sun).
[1] These two authors contributed equally to this work.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.032

it is very important to have a rapid and accurate diagnostic tool to detect SARS-CoV at early stages of the disease.

At present, most of the SARS diagnosis methods mainly focus on detecting viral antigen-specific antibodies in the sera of suspected patients. Generally, the antibody response often develops on 10–14 days following SARS-CoV infection [3], so that the diagnosis based on a viral-specific Ab would miss a good time point for antiviral therapy effectively and quarantine of SARS patients. Therefore, the best option for the disease diagnosis is to detect viral antigens.

N protein reacted with most of SARS patient sera and serum samples from acute phase of SARS patients (5–10 days after SARS-CoV infection). In contrast, the sera samples from acute phase of SARS patients did not respond to S protein, suggesting that antibodies to N protein developed earlier than S protein-specific antibodies. This observation reflected that N protein was released into the blood of SARS-CoV patients during virus replication in vivo [4,5]. In addition, coronavirus N protein is highly immunogenic and abundantly expressed in vivo after the virus infects human being.

A recent study demonstrated that N protein-specific mAbs were capable of detecting a N protein from sera of SARS patients at early stage on day 5–10 following SARS-CoV infection, indicating that N protein was a good viral antigen for the disease diagnosis [3]. It was noted that N protein was easy to be degraded into small fragments in the lysates of SARS-CoV-infected Vero E6 cells, analyzed by proteomics [6]. Moreover, one study showed that one N protein fragment, N13 (amino acid residues 221–422), can react with 100% of sera from SARS-CoV patients (52/52) [7]. This indicates that the N13 fragment is highly immunogenic and contains predominant B cell epitopes within N protein.

In this study, we generated six mAbs and one polyclonal Ab that were specific to N protein of SARS. Four truncated N protein fragments were used to locate the epitopes recognized by the mAbs within N protein. It was interesting to find that the epitopes recognized by six mAbs were divided into two groups and were located in the C terminus (221–422) of N protein. The selected pair-Abs were able to detect N protein in samples of SARS patients but not in sera of SARS unrelated patients.

Materials and methods

Animals. New Zealand rabbits and BALB/c mice were purchased from Shanghai Laboratory Animal center, Chinese Academy of Sciences. Animals were kept in conventional conditions and were handled in compliance of Chinese Academy of Sciences Guidelines for Animal Care and Use.

Expression and purification of SARS-CoV nucleocapsid protein. The full length and different truncated fragments of SARS nucleocapsid (N) gene were inserted into vector pETs [7]. After transformation of *Escherichia coli* strain, BL21 (DE3), bacterial cells were induced by 10 mM IPTG at 22 °C for 16 h in tryptone–phosphate medium. Proteins were extracted with buffer A containing 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mM imidazole, and 0.5 mg/ml lysozyme. The high-speed supernatant of the extract from 1 liter of culture was loaded onto 8 ml Ni–NTA agarose column, followed by washing with 100 ml of 20 mM imidazole in buffer A. Proteins were then eluted with 50 mM imidazole in buffer A.

Western blotting. Recombinant N proteins and inactivated cell lysates from SARS-CoV-infected Vero-E6 cells, HCoV-OC43-infected HRT18 cells, HCoV-229E-infected MRC5H cells, and CoV-NL63-infected LLC-MK2 cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% polyacrylamide gels and were then transferred onto a membrane of polyvinylene difluoride (PVDF) as described previously [8]. After blocking with 3% BSA for 1 h, the membrane was incubated with anti-SARS N protein mAbs and stained with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h. The blots were developed using ECL detection reagents (Amersham Pharmacia Biotech).

Immunization and raising polyclonal antibodies. Rabbits were immunized subcutaneously at multiple sites on the back of rabbits with 1 mg of *E. coli*-expressed N protein in emulsion 1:1 v/v with complete Freund's adjuvant. The rabbits were boosted three times at 2-week interval with N protein in emulsion with incomplete Freund's adjuvant. The blood was collected after 1 week of last immunization. The titers of the antisera from rabbits were determined by ELISA.

ELISA assay. Enzyme-linked immunosorbent assay (ELISA) was performed as described previously [8]. In brief, 96-well microtiter plates were coated with the SARS N protein in 0.1 M carbonate buffer (pH 9.6) (20 μg/ml, 50 μl/well) at 4 °C overnight. After being blocked with phosphate-buffered saline (PBS) containing 10% bovine serum and 0.1% Tween 20, the plates were incubated with diluted antisera at various concentrations at 37 °C for 2 h. Bound antibodies were detected with HRP-coupled goat anti-rabbit IgG antibody (Bio-Rad). Tetramethylbenzidine (TMB) was used as the substrate (Sigma, USA), and the absorbance was measured by microplate autoreader (Thermo) at 450 nm.

HRP enzyme labeling purified polyclonal antibody. Anti-SARS N protein antisera from rabbits (pAbs) were initially purified by 33% saturated ammonium sulfate precipitation and then the purified antibodies were labeled with HRP by 1.25% glutaraldehyde (GA). The reactivity was stopped by 0.2 M lysine for 2 h. The enzyme labeled complex was dialyzed with PBS at 4 °C overnight. At last, the HRP-coupled pAbs were precipitated by 33% saturated ammonium sulfate and then dialyzed.

Preparation of monoclonal antibodies against SARS N protein. Female BALB/c mice were immunized with 100 μg *E. coli*-expressed N protein in emulsion 1:1 v/v with complete Freund's adjuvant and were boosted three times at 2-week interval in incomplete Freund's adjuvant. The mouse with the highest Ab titer tested by ELISA was boosted intraperitoneally with 100 μg of the N protein in 0.5 ml PBS. On the third day after boosting, the mouse was sacrificed and the spleen cells were harvested. The spleen cells were fused with murine myeloma cells, SP2/0, by 50% PEG and distributed in 96-well culture plates. The cultured supernatants from each well were screened by detecting their reactivity to recombinant N proteins by ELISA. The positive hybridoma clones were selected by limiting dilution method [9]. After three cycles of cloning, the stable hybridoma clones were obtained. The antibody isotypes were identified by mouse sub-isotype panel (Bio-Rad).

In order to acquire abundant mAbs, the pristine-primed BALB/c mice were injected intraperitoneally with 1×10^6 hybridoma cells per mouse. The ascites were collected and mAbs were purified by protein G affinity column.