

Development, Characterization, and Application of Monoclonal Antibodies against Severe Acute Respiratory Syndrome Coronavirus Nucleocapsid Protein[∇]

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Five monoclonal antibodies (MAbs) against recombinant nucleocapsid protein (NP) of severe acute respiratory syndrome (SARS)-causing coronavirus (CoV) were developed by hybridoma technology. Epitope mapping by Western blotting showed that these anti-SARS-CoV NP MAbs bind to distinct domains of NP. These anti-SARS-CoV NP MAbs, with their high specificity, are potentially ideal candidates for developing early and sensitive diagnostic assays for SARS-CoV.

Severe acute respiratory syndrome (SARS) is a severe form of pneumonia due to infection with a coronavirus (CoV) that has adapted to human-to-human transmission (12). Several cell types in the human body are susceptible to SARS-CoV infection and replication, which in turn trigger a series of pathological changes (8). The development of specific diagnostic tests for SARS-CoV is of paramount importance for the effective treatment of infected patients and the prevention of future SARS outbreaks. Nucleocapsid protein (NP) is the most predominant virus-derived structural protein and is shed in large amounts in serum, nasopharyngeal aspirate (NPA), throat wash samples, fecal matter, and urine during the early days of infection (1–5, 15). Earlier studies have shown detection of NP in various body fluids using specific monoclonal antibodies (MAbs) raised against NP and its immunodominant epitopes using enzyme-linked immunosorbent assay (ELISA), indirect fluorescence assay, enhanced chemiluminescence immunoassay, and Western blotting (14). The lack of an effective, sensitive, and easy-to-use assay was considered to be one of the major drawbacks in the prevention of the 2003 SARS outbreak. Hence, the development of an easy-to-use, sensitive, and specific assay for NP could be a way forward to prevent a future SARS pandemic. Studies have shown that SARS-CoV NP can be detected in the acute phase of SARS infection by specific MAbs, compared to other structural proteins of SARS-CoV (15). The detection of NP in various samples from patients suspected of having SARS, which included serum, urine, fecal matter, NPA, throat wash samples, and saliva, during the early days of infection was also carried out, indicating that NP is rapidly shed in large amounts (2, 4, 7, 10, 11). Here we describe the development of SARS-CoV MAbs and characterize them by analyzing binding sites, epitope mapping, and cross-reactivity with related NPs of animal and human CoVs.

Five BALB/c mice were immunized with *Escherichia coli* expressing SARS-CoV NP (6) following a set immunization protocol (13). Based on high antibody titers, splenocytes were isolated from the immunized mice and fused with freshly grown SP2/0 myeloma cells using polyethylene glycol. After a third recloning step, five stable anti-SARS-CoV NP clones were generated against SARS-CoV NP and designated P140.20B7, P140.19B6, P140.19C7, P140.1D3, and P140.14D6. Each anti-SARS-CoV NP hybridoma clone was cultured, and supernatants were purified by protein G agarose chromatography. The range of immunoglobulin G (IgG) yields was 6 to 24 mg/liter of cell culture supernatant.

Isotyping of the five MAbs was done by using a commercially available isotyping kit (Sigma-Aldrich). The results demonstrated that the heavy chain of three of the anti-SARS-CoV NP MAbs (P140.20B7, P140.19C7, and P140.1D3) was of the IgG1 class while that of P140.19B6 and P140.1D3 was of the IgG2 class. The light chain was found to be kappa for all of the MAbs by Western blotting.

The purity of the anti-SARS-CoV NP MAbs was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot analysis showed the specificity of purified MAbs to SARS-CoV NP antigen. Epitope mapping studies were done for NP and its subfragments to determine if all of the anti-SARS-CoV NP MAbs bind to a specific region of NP or to separate or overlapping epitopes. To determine the binding regions of the MAbs on NP, three different truncated fragments, NP1.1, NP1.2, and NP1.3, were cloned and expressed in *E. coli* (6). The codon-optimized recombinant NP (422 amino acids [aa]) gene and its fragments (NP1.1, aa 1 to 140; NP1.2, aa 141 to 280; NP1.3, aa 281 to 422) were used to determine the specificity of the MAbs by Western blotting (6) (Fig. 1). When the protein G-purified antibodies were used (P140.20B7, P140.19B6, P140.19C7, P140.14D6, and P140.19D3), three of the MAbs (P140.20B7, P140.19B6, and P140.19C7) were found to react specifically to full-length SARS-CoV NP, as well as specific subfragments of the SARS-CoV NP antigen. P140.20B7 bound to full-length NP and to NP1.3, while P140.19B6 and P140.19C7 bound to full-length NP and to NP1.2, respectively, with different sensitivities. This

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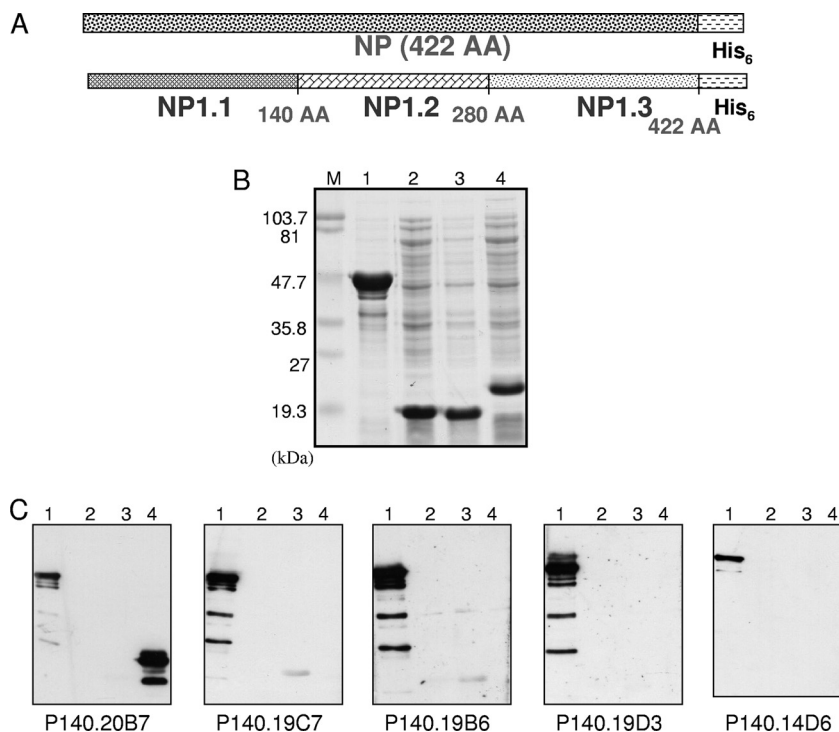


FIG. 1. SARS-CoV NP and its subfragments for epitope mapping. (A) Schematic representation of full-length NP and its subfragments used for epitope mapping analysis. (B) SDS-PAGE analysis of NP and NP fragment expression in *E. coli*. Lane M, standard molecular mass markers; lane 1, NP; lane 2, NP1.1; lane 3, NP 1.2; lane 4, NP 1.3. (C) Western blot analysis of NP and NP fragments probed with individual MAbs. Lane 1, NP; lane 2, NP1.1; lane 3, NP 1.2; lane 4, NP 1.3.

suggested a partial overlap of the epitopes or completely non-overlapping epitopes for P140.19B6 and P140.19C7. The remaining antibodies, P140.19D3 and P140.14D6, showed no signal with any of the fragments but exhibited binding to full-length NP.

MAb P140.20B7, which clearly showed specific binding to NP1.3, and MAbs P140.19B6 and P140.19C7, which bound to NP1.2, were useful in developing a sensitive heterosandwich ELISA to detect the SARS-CoV NP antigen. MAbs P140.19D3 and P140.14D6 showed no specific reactivity to NP subfragments and only weakly bound to the whole NP antigen, indicating their low specific reactivity to NP antigen. Though epitope mapping analysis may not determine the specific amino acids the MAbs bind to, it will indicate the specific regions of the antigenic protein, which will be helpful in developing pairs of MAbs that can be utilized in the development of heterosandwich immunoassays.

In the sandwich ELISA, an optimal concentration of 10 $\mu\text{g/ml}$ was used for the capture antibody and 1 $\mu\text{g/ml}$ was used for the detector antibody (Fig. 2). These concentrations were determined in initial experiments using checkerboard titrations (5, 13). A sandwich ELISA using checkerboard titrations showed that P140.19B6 as the capture antibody (C) had the highest optical density (OD) value when biotinylated P140.19C7 was used as the detecting antibody (D). Detection with biotinylated P140.19B6 as the detecting antibody and P140.19C7 as the capture antibody yielded comparatively lower OD values. The partial competition in the sandwich ELISA could be due to differences in the affinity of the MAbs for the NP antigen, steric hindrance, or antibody-induced con-

formational changes in the antigen upon antibody binding. Based on these data, we have further developed monospecific and bispecific antibody-based immunoswab assays for point-of-care application (9).

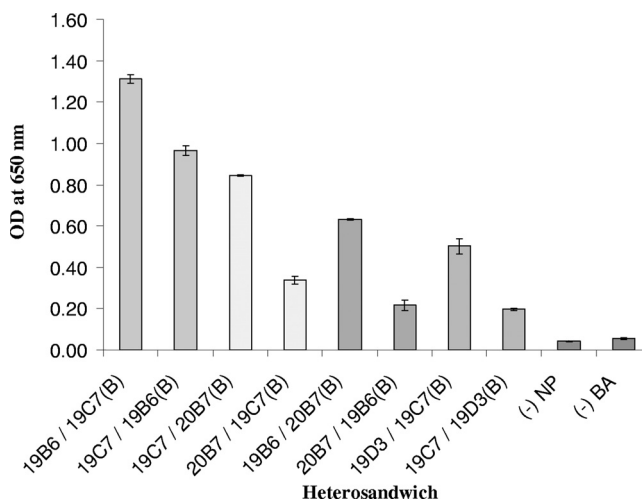


FIG. 2. Evaluation of heterosandwich pairs of anti-SARS-CoV NP MAbs at a low antigen concentration. P140.19B6 and P140.19C7 form an excellent heterosandwich pair compared to the other MAbs. Detection was carried out by the addition of streptavidin-horseradish peroxidase to the biotinylated (B) detecting antibodies after the NP was captured by capture antibodies. Controls used in the assay: (-) NP, without NP antigen; (-) BA, without biotinylated antibody. P140.19B6 was the capture antibody, and P140.19C7 was the biotinylated detecting antibody.

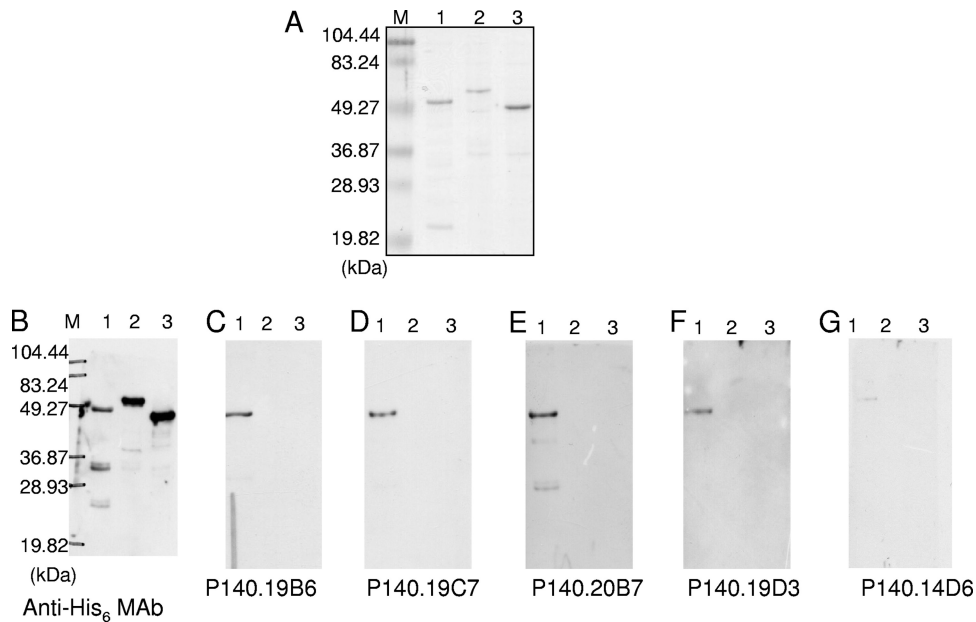


FIG. 3. Cross-reactivities of different anti-SARS-CoV NP antibodies. (A) SDS-PAGE analysis of SARS-CoV and bovine and human CoV (NL63) NP expression in *E. coli*. Lane M, standard molecular mass markers; lane 1, SARS-CoV; lane 2, bovine CoV; lane 3, NL63. B to G, Western blot analysis of SARS-CoV and bovine and human CoV (NL63) NPs probed with different MAbs. B, anti-His₆ MAb; C, P140.19B6; D, P140.19C7; E, P140.20B7; F, P140.19D3; G, P140.14D6. Lane M, standard molecular mass markers; lane 1, SARS-CoV; lane 2, bovine CoV; lane 3, NL63.

The SARS-CoV NP sequence shows only 20 to 30% amino acid homology with the NP sequences of other CoVs. There was no cross-reactivity between SARS-CoV and human CoV, as reported previously (3). The NP of SARS-CoV was cloned and expressed in *E. coli* (6). In this study, bovine CoV and human CoV (NL63) were cloned and expressed in *E. coli*. All of the clones expressed high levels of NP of the desired size (Fig. 3A), and expression was confirmed by Western blotting and probing with anti-His₆ MAb (Fig. 3B). The specificity of the newly developed MAbs for different NPs was evaluated by Western blotting (Fig. 3C to G). All five MAbs exhibited strong binding only with SARS-CoV NP, and no cross-reactivity with other NPs of bovine CoV and NL63 was observed.

In another study, we used these antibodies for the development of bispecific MAbs and established a novel bispecific-antibody purification method (1). Using different combinations of MAb, bispecific MAb, and IgY, we have developed a point-of-care immunoswab assay with high sensitivity (9). Thus, our new MAbs could be useful reagents for diagnostics, as well as functional analysis of NP during infection.

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