Antigenic and cellular localisation analysis of the severe acute respiratory syndrome coronavirus nucleocapsid protein using monoclonal antibodies

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Abstract

A member of the family of coronaviruses has previously been identified as the cause of the severe acute respiratory syndrome (SARS). In this study, several monoclonal antibodies against the nucleocapsid protein have been generated to examine distribution of the nucleocapsid in virus-infected cells and to study antigenic regions of the protein. Confocal microscopic analysis identified nucleocapsids packaged in vesicles in the perinuclear area indicating viral synthesis at the endoplasmic reticulum and Golgi apparatus. The monoclonal antibodies bound to the central and carboxyterminal half of the nucleocapsid protein indicating prominent exposure and immunogenicity of this part of the protein. Antibodies recognised both linear and conformational epitopes. Predictions of antigenicity using mathematical modelling based on hydrophobicity analysis of SARS nucleoprotein could not be confirmed fully. Antibody binding to discontinuous peptides provides evidence that amino acids 274–283 and 373–382 assemble to a structural unit particularly rich in basic amino acids. In addition, amino acids 286–295, 316–325 and 361–367 that represent the epitope recognised by monoclonal antibody 6D11C1 converge indicating a well-structured C-terminal region of the SARS virus nucleocapsid protein and functional relationship of the peptide regions involved. Alternatively, dimerisation of the nucleocapsid protein may result in juxtaposition of the amino acid sequences 316–325 and 361–367 on one nucleoprotein molecule to amino acid 286–295 on the second peptide. The monoclonal antibodies will be available to assess antigenicity and immunological variabilities between different SARS CoV strains.
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1. Introduction

At the end of 2002, the first respiratory infections attributed to the severe acute respiratory syndrome (SARS) occurred in the Guangdong province of the People's Republic of China. This was followed at the beginning of 2003 by worldwide spreading of the infection. The SARS-causing agent was identified as a coronavirus (CoV), probably of animal origin. The SARS outbreak was brought under control after 6 months except for a few sporadic cases diagnosed in the following months (Peiris et al., 2004). However, since until today the source of the virus is unknown and the virus may still exist in animals, there remains the risk of a new outbreak.

Coronaviruses are RNA viruses with a single positive-stranded genome. The genome of the SARS CoV comprises 29,751 nucleotides and contains 15 partly overlapping open reading frames (ORF). The ORFs 1a and 1b encode the viral transcriptase. Four other ORFs are assigned to viral structural proteins, the spike, envelope, membrane, and nucleocapsid (N) protein (Marra et al., 2003). In infected cells, the nucleocapsid protein is the most abundant of the viral proteins (He et al., 2004). In the virion, nucleoprotein molecules associate with viral RNA forming the helical nucleocapsid (Davies et al., 1981; Nelson et al., 2000). The protein contains both nuclear localisation and export signals (Rowland et al., 2005; Timani et al., 2005; You et al., 2005) suggesting shuttling of the protein between the nuclear and the cytoplasmic compartment in virus-infected

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cells. Electron microscopy, conventional immunofluorescence and confocal laser scanning microscopic analyses demonstrate that the N protein is located in the cytoplasm of infected cells. In addition, in cells transfected with expression plasmids that contain the nucleoprotein gene, the N protein was observed in the nucleus (Chang et al., 2004; Ksiazek et al., 2003; You et al., 2005). Moreover, nucleolar localisation of the SARS nucleoprotein in transfected cells was reported (Li et al., 2005). However, inconsistent data were obtained with respect to the presence of the N protein in the nucleus of virus-infected cells with some studies seeing no evidence of nuclear localisation (Qinfen et al., 2004; Rowland et al., 2005; Timani et al., 2005; You et al., 2005).

The SARS CoV nucleocapsid protein is highly immunogenic and detectable in serum and other clinical samples as early as 1 day after the onset of symptoms (Che et al., 2004). Computer modelling has been employed to predict antigenic regions of the protein (He et al., 2004; Wang et al., 2003) and antigenic sites have been mapped with sera from SARS CoV-infected individuals using overlapping peptides. With human sera, major antigenic sites have been assigned to the central and C-terminal part of the nucleocapsid protein (Wang et al., 2003). Further analysis indicated that the majority of human sera recognise conformational epitopes (Chen et al., 2004), but precise amino acid sequences of conformational epitopes have not yet been determined.

The nucleocapsid protein is a phosphorylated protein composed of 422 amino acids (aa). It is highly basic enabling it to interact with acidic genomic RNA (Rota et al., 2003; Wang et al., 2003). The structure of an N-terminal portion of the nucleoprotein (amino acid residues 45–181) of the virus comprising a putative RNA-binding domain has been analysed by NMR spectroscopy. The protein fragment consists of a five-stranded antiparallel β-sheet. An additional β-hairpin connects β2 and β3. The β3-strand contains the amino acid sequence FYYL-GTGP that is conserved among all coronaviruses (Huang et al., 2004). The structure of the central and C-terminal portions of the protein has been less well-characterized. Computer modelling by Tang et al. (2005) identified a region around amino acids 250–360 that exhibits a distinctively ordered structure. In addition, there is a lysine-rich region (amino acids 362–390) of unknown function possibly involved in RNA-binding (He et al., 2004) or nuclear localisation (Wang et al., 2003).

In this work, monoclonal antibodies against the SARS coronavirus N protein were produced to study the distribution of the nucleocapsid protein in virus-infected cells and to identify antigenic regions including linear and conformational epitopes of the protein that may reveal antigenic, structural and functional properties of the protein.

2. Methods

2.1. Virus culture

Cell culture with SARS CoV was performed under biosafety level 3 conditions. The SARS virus (strain Frankfurt 1) was propagated in Vero76 cells for 48 h at 37 °C and 5% CO_2. The supernatant was harvested, heat-inactivated for 15 min at 95 °C

and filtered through a 0.2 μm filter. The inactivated virus suspension was used for immunisation.

CoV 229E was cultured with MRC-5 cells, CoV OC43 with MDCK, and CoV NL63 with LLC-MCK2 cells (van der Hoek et al., 2004). The mouse hepatitis virus (MHV-A59) was cultured with 17Cl-1 cells. The porcine transmissible gastroenteritis virus (TGEV) was produced in swine testis (ST) cells. Virus cultures were incubated at 37 °C, 5% CO_2 or 34 °C (NL63) until a cytopathic effect was visible (229E, NL63, MHV, TGEV). For the non-cytopathic CoV OC43, virus infection was tested by hemagglutination assay (Schultze et al., 1991). Infected cells were harvested and sedimented by centrifugation. The cell pellets were resuspended in 500 μl PBS. For Western blot assays, the cell suspensions were mixed with lysis buffer (25% 1 M Tris–HCl pH 6.8, 40% glycerol, 0.08% bromophenol blue, 0.35% SDS and 10% β-mercaptoethanol) and heated for 15 min at 95 °C.

2.2. Preparation of recombinant SARS N antigen

The RNA of the nucleocapsid protein of inactivated SARS CoV was reverse transcribed and cloned into the bacterial expression vector pMALc2X (New England BioLabs, Inc.). To generate carboxyterminally truncated N proteins, the N gene was cut by endonuclease digestion to produce fusion proteins containing the N protein amino acids (aa) 1–69 (N69), 1–120 (N120), or 1–213 (N213). The recombinant nucleocapsid protein (molecular weight: ~46 kDa) was expressed in E. coli as a complete ~90 kDa fusion protein (N-MBP) of N together with the *E. coli* maltose-binding protein (MBP) and as carboxyterminally truncated N-MBP proteins after induction with 0.3 mM IPTG at 37 °C for 2 h. As control, the pMALc2X vector was used for expression of the ~51 kDa MBP. The bacterial pellets were solubilised and sonicated in column buffer (2% 1 M Tris–HCl, pH 7.4, 0.1 M NaCl, 0.2% 0.5 M EDTA, pH 8.0, 0.1% 1 M NaN_3, 0.07% β-mercaptoethanol). By exploiting the high affinity of MBP for maltose, the proteins were purified and concentrated with amylose resin solution (New England BioLabs, Inc.). Protein concentrations were determined by Bradford assay using bovine γ-globulin as standard. The purified N-MBP fusion protein was used for immunisation of mice and in immunological analyses.

2.3. Immunisation

Female BALB/c mice were immunised eight times over a period of 12 months beginning with 8-week-old mice. The animals received four times heat-inactivated cell culture supernatant of SARS CoV-infected Vero76 cells with or without complete and incomplete Freund's adjuvant (Sigma, Aldrich, Inc.) i.p. at intervals of 3–4 weeks to initiate an immune response against the natural viral proteins resulting in a SARS N protein-specific antibody response. Three months later a second immunisation schedule followed where mice were immunised subcutaneously three times with recombinant N-MBP fusion protein (25 μg, 3 μg, 3 μg) and Gerbu adjuvant (Gerbu Biochemicals, Gaiberg, Germany) at 4 and 5 week intervals. Mice received

a final antigen boost with recombinant protein (3 μg) shortly before preparation of B-lymphocytes.

2.4. Generation of anti-N-specific monoclonal hybridomas

Four days after the final boost, the spleen was removed under aseptic conditions and splenocytes were isolated. Spleen cells were harvested in serum-free RPMI-1640 medium by grinding the organ cautiously through a cell strainer (BD Biosciences). Splenocytes were fused with murine myeloma SP2/0 cells at a cell-to-cell ratio of 1:5 in the presence of polyethylene glycol 1500 (Roche Applied Science) following a standard protocol (Kohler and Milstein, 1975; Pontecorvo, 1975). Cells were cultured in 96-well flat-bottomed plates in medium plus 10% FCS (Invitrogen Corporation and Biochrom AG), non-essential amino acids, L-glutamine, sodium pyruvate (Invitrogen) and 0.0005 μg/ml IL-6 (Strathmann Biotec AG) and growing cultures screened by ELISA for the production of anti-N-MBP antibodies. Positive cultures were regularly checked by light microscopy for single cell growth, cloned at least twice by limiting dilution, and expanded in 24-well plates and culture flasks to obtain suitable hybridoma cell lines.

2.5. SARS antibody ELISA, Western blot, and immunoglobulin isotyping

High binding 96-well ELISA plates (Greiner bio-one) were coated overnight at 4 °C with recombinant N-MBP or MBP (50 ng/well) in coating buffer (0.2 M NaHCO$_3$, pH 9.0). The plates were washed five times with PBS containing 0.05% Tween-20 (PBST) and blocked for 1 h at 37 °C in 3% BSA in PBST. Hybridoma culture supernatants (50 μl/well) were added and incubated for 2 h at 37 °C. Human serum from a person previously infected with SARS CoV and serum of the immunised mouse were used as positive controls, while serum from an uninfected individual and murine pre-immune serum were employed as negative controls. The ELISA plates were washed with PBST and 100 μl/well horseradish peroxidase (HRP)-conjugated anti-human (P0214, Dako, 1:6000) or anti-mouse (clone P0260, Dako, diluted 1:2000 in 3% BSA/PBST) secondary antibody was added to the plates for 1.5 h at 37 °C. Plates were washed twice and incubated for 30 min at room temperature (RT) with 100 μl/well substrate solution (0.42 mM TMB, 0.1 M sodium acetate/citric acid buffer pH 6.0, 0.006% H$_2$O$_2$). The reaction was stopped with 50 μl/well H$_2$SO$_4$ (0.5 M) and the optical density (OD) was read at 450 nm.

For Western blot analysis, recombinant N-MBP and MBP, a lysate of *E. coli*, or lysates of coronavirus-infected and uninfected cells were separated by SDS-PAGE using a 10% polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting. Non-specific binding to the membrane was blocked by treatment with 5% non-fat dry milk (Heirler, Radolfzell, Germany) in PBST for 1 h at RT. The blot was incubated overnight at 4 °C with hybridoma supernatants (1:500 diluted in PBST with 5% non-fat dry milk), heat-inactivated human serum (1:100), murine serum (1:10,000), or a rabbit anti-MBP antiserum (1:10,000, New England BioLabs$_{INC}$). After

washing with PBST, the membranes were incubated for 2 h at RT with HRP-conjugated rabbit-anti-human (1:1000), rabbit-anti-mouse (1:1000) or swine-anti-rabbit (P0217 1:1000 Dako) immunoglobulins, respectively. The blots were washed and incubated with substrate solution (PBST, 0.05% DAB and 0.006% H$_2$O$_2$).

Isotyping of the monoclonal antibodies was performed using the Mouse Immunoglobulin Isotyping ELISA kit (BD Biosciences) according to the manufacturer's instructions.

2.6. Affinity testing

Antibody-containing hybridoma culture supernatant was purified and concentrated using a protein-G column (GE Healthcare). The relative affinity of the antibodies was determined by ELISA using SARS N-MBP-coated microplates (100 ng/well) and a series of antibody dilutions from 10^{-8} to 5×10^{-13} mol/l. The relative affinity was defined as the antibody concentration that resulted in an optical density approximately three times above the background.

2.7. Immunofluorescence analysis using confocal microscopy

Vero76 cells were cultivated on round glass coverslips, infected with SARS CoV and incubated for 48 h at 37 °C and 5% CO$_2$. Cells were fixed with methanol and the coverslips were stored at −80 °C. Uninfected Vero76 cells were used as a negative control. Non-specific binding was blocked using 100 μl 5% BSA in PBST for 30 min at 37 °C, followed by incubation of the coverslips with 50 μl of undiluted hybridoma supernatant for 2 h at 37 °C. After washing with 1% BSA in PBS, 50 μl of a FITC-labelled rabbit-anti-mouse antibody (F0479, Dako) diluted in 1% BSA/PBST was added and the slips were incubated for 1 h at 37 °C. Following additional washing, 50 μl propidium iodide (0.075 mM in PBS, Sigma) was added for 30 min at 37 °C in the dark. The coverslips were washed again, air dried and fixed on a microscope slide with 25 μl Entellan (Merck KGaA). The slides were examined with a confocal laser-scanning microscope (Leica Microsystems GmbH). The specimens were scanned in layers of 0.2 μm.

2.8. Epitope mapping

One hundred and thirty-nine peptides of 10 amino acids (aa) overlapping by 7 aa that span the complete sequence of the nucleocapsid protein (422 aa) of the SARS CoV strain Frankfurt 1 (Genbank accession number AP33707) were synthesised and spotted onto a cellulose membrane using an Intavis MultiPep peptide synthesiser (INTAVIS Bioanalytical Instruments AG, Bergisch Gladbach, Germany) at the Protein Core Unit, Interdisciplinary Centre of Clinical Research, University of Leipzig. Non-specific antibody binding was blocked with 5% non-fat dry milk in PBST for 1 h at RT. The membrane was incubated with the hybridoma culture supernatants for 2 h at RT at 1:5 to 1:25 dilution. The membrane was washed with PBST and incubated with HRP-conjugated rabbit-anti-mouse antiserum
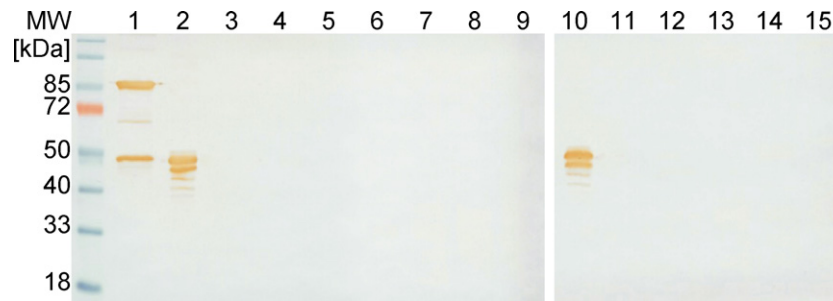
Fig. 1. Western blot and cross-reactivity analysis. Lysates of coronavirus-infected cells were separated by SDS-PAGE and reactivity of proteins with antibody 8G5 A1A was determined by Western blotting. Lane 1: N-MBP fusion protein (89 kDa) and protein degradation products; lane 2: SARS CoV-infected Vero76 cells; lane 3: uninfected Vero76 cells; lane 4: CoV-229E-infected MRC-5 cells; lane 5: uninfected MRC-5 cells; lane 6: CoV-NL63-infected LLC-MCK2 cells; lane 7: uninfected LLC-MCK2 cells; lane 8: CoV-OC43-infected MDCK cells; lane 9: uninfected MDCK cells; lane 10: SARS CoV-infected Vero76 cells; lane 11: uninfected Vero76 cells; lane 12: TGEV-infected ST cells; lane 13: uninfected ST cells; lane 14: MHV A59-infected 17-C11 cells; lane 15: uninfected 17-C11 cells. Similar results were obtained with the other monoclonal antibodies.

(P0260, Dako) for 2 h at RT and the membrane was developed using the ECL™ Western Blotting Analysis System (GE Healthcare) with a MultiImage™ Light Cabinet or on an X-ray film.

3. Results

3.1. Immunological and biochemical characteristics of monoclonal SARS N-specific antibodies

Ten stable monoclonal hybridoma cultures were established that secreted antibodies reactive with SARS N protein in ELISA, Western blot, and the immunofluorescence test. The antibodies recognised proteins of 46 and 48 kDa in lysates of virus-infected cells that most likely represent the phosphorylated and non-phosphorylated forms of the nucleocapsid protein, and nucleoprotein degradation products. None of the antibodies showed cross-reactivity against the human coronaviruses 229E, NL63 and OC43 or the mouse hepatitis or porcine transmissible gastroenteritis coronaviruses in the Western blot (Fig. 1). Isotyping

Table 1
Biochemical and immunological properties of anti-SARS N monoclonal antibodies

Antibody	Immunoglobulin type	Light chain	Epitope localisation (amino acid residues)
1G3B3F	IgG1	λ	Between 213 and 422
1G7D1	IgG1	κ	274–283 373–382
2G8D4	IgG1	κ	274–283 373–382
4C11 A3	IgG1	λ	Between 213 and 422
4E10A3A1	IgG1	κ	400–409
4F3C4	IgG1	λ	373–382
5G6C4	IgG1	λ	Between 213 and 422
6D11C1	IgG2a	κ	286–295 316–325 361–367
8G5A1A	IgG1	κ	Between 120 and 213
8G7B2C	IgG1	κ	193–202

of the antibodies (Table 1) demonstrated that nine of ten antibodies were IgG1. Four of these had a light λ-chain, five a κ-chain. One of the hybridoma cultures produced IgG2a antibodies with a light κ-chain. The relative affinity of the antibodies towards the recombinant protein defined as the minimum antibody concentration at which a significant signal was obtained in the ELISA was in the range of 1.25×10^{-10} to 7.5×10^{-12} mol/l.

3.2. Localisation of the SARS nucleoprotein in infected cells

Using confocal laser scanning microscopic analysis of SARS CoV-infected cells, the site and distribution of the nucleoprotein were examined. The protein was dispersed in granules primarily in the perinuclear region. The stained area corresponded to the region occupied by the rough endoplasmic reticulum, which extends into the Golgi apparatus through the intermediate compartment. The granules were identified as vesicles containing virus particles or nucleocapsids with some granules found to move towards the periphery of the cells demonstrating transport of the virus to the cytoplasmic membrane (Fig. 2). Z-sectioning was performed with 0.2 μm slices. In superficial sections and on the edges, green fluorescence of the nucleoprotein occasionally superimposed the nuclei representing fluorescence of labelled nucleoprotein in the cytoplasm above or below the nucleus. All mAbs recognised the nucleoprotein in immunofluorescence analysis.

3.3. Identification of linear and conformational epitopes

To determine the epitope specificity of the antibodies, peptide spot analyses with 139 overlapping peptides bound to a cellulose membrane were performed. Six of the 10 antibodies reacted strongly with at least one of the peptides in the peptide spot analysis. Three antibodies (4F3C4, 1G7D1, 2G8D4) recognised peptide 373–382 suggesting prominent exposure and high immunogenicity of this protein region. Antibodies 4E10A3A1, 4F3C4 and 8G7B2C that reacted with a single peptide, bound to linear epitopes (Fig. 3C, D and F). Three of the antibodies (1G7D1, 2G8D4, 6D11C1) reacted with at least two peptides that were from spatially separated sites of the nucleocapsid protein. Recognition of discontinuous peptides indicates that in the
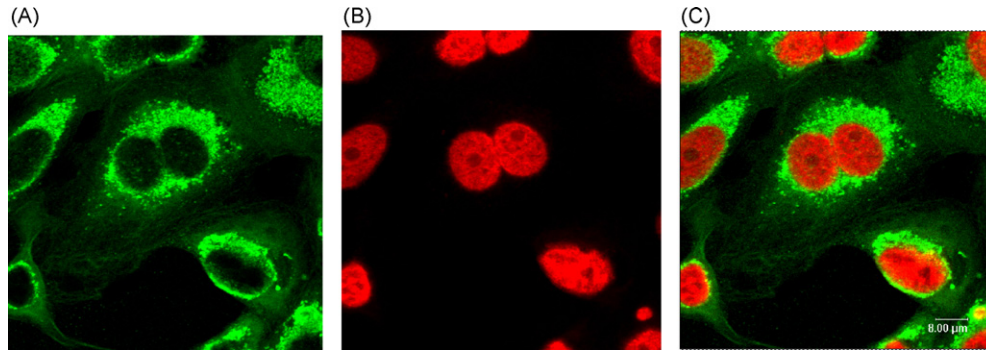
Fig. 2. Intracellular distribution of the SARS CoV nucleocapsid protein. Confocal laserscan microscopy of SARS CoV-infected Vero76 cells. Superimposition of 6 consecutive sections of 0.2 μm. (A) Staining with the mAb 4C11A3 and a secondary FITC-conjugated rabbit anti-mouse antiserum; (B) propidium iodide staining of the nuclei; (C) overlay of A and B shows granular distribution of the SARS N protein concentrated around the nucleus.

N protein these peptides are tightly connected. Thus, the areas comprising amino acids 274–283 and 373–382, as well as amino acids 286–295, 316–325 and 361–367 are in close proximity in the natural N protein and constitute specific structural units (Fig. 3A, B, E and G). Comparative protein sequence analyses

indicated that none of the amino acid sequences recognised were conserved in other coronaviruses.

Four antibodies did not react with any of the peptides. To further define the binding region of these antibodies, Western blot analysis was performed using truncated N proteins. This
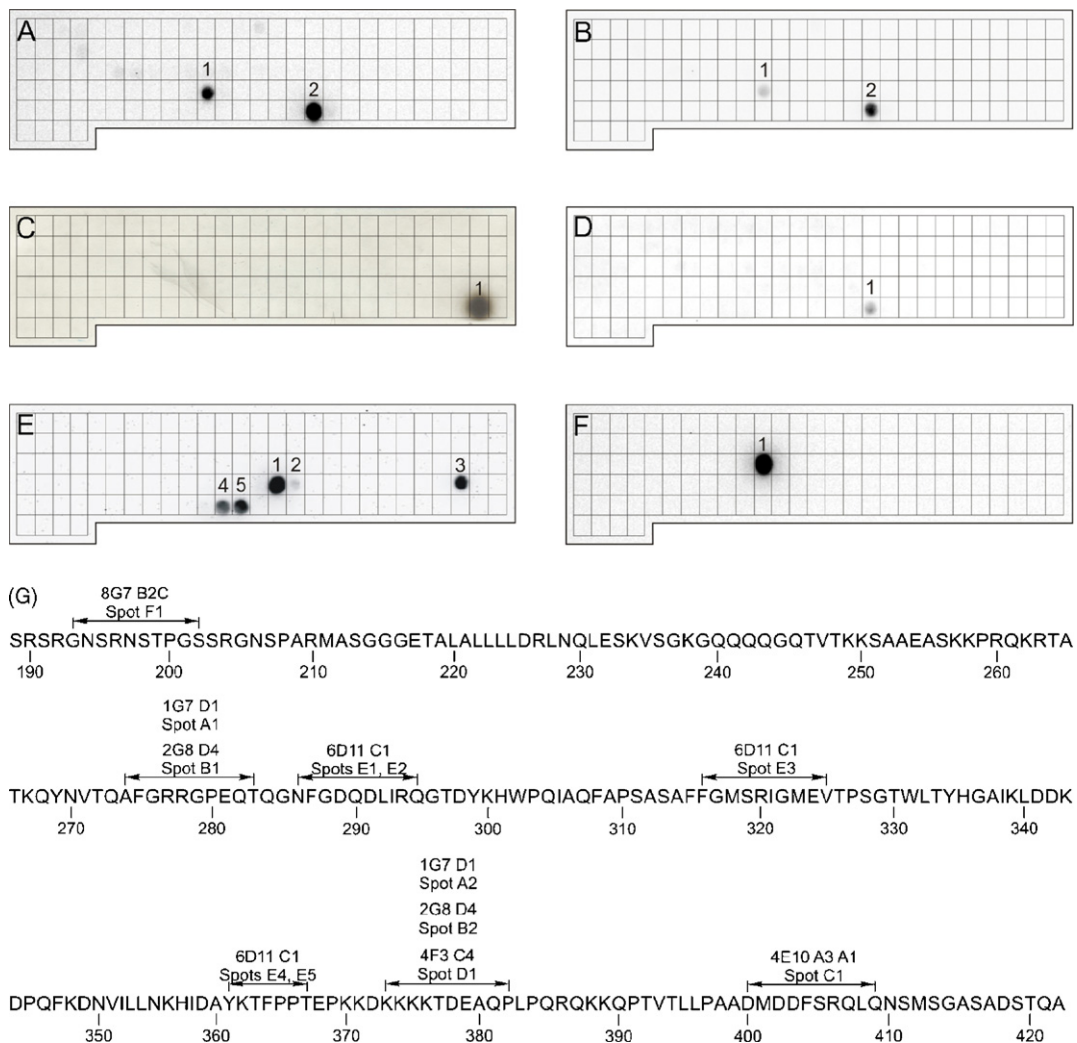

Fig. 3. Overlapping SARS nucleoprotein peptides on a cellulose membrane recognised by anti-SARS N monoclonal antibodies: (A) clone 1G7D1; (B) clone 2G8D4; (C) clone 4E10A3A1; (D) clone 4F3C4; (E) clone 6D11C1; (F) clone 8G7B2C; (G) antibody epitopes are indicated on the amino acid sequence of the C-terminal portion of the nucleoprotein.

demonstrated that 3 of the epitopes were in the 209 carboxyterminal amino acids of the nucleocapsid protein. The position of the epitope of the fourth antibody could be narrowed down to amino acids 120–213 (Table 1).

3.4. Characterisation of the minimum epitope size

Overlapping peptides have 7 amino acids in common. Antibodies 4E10A3A1, 4F3C4 and 8G7B2C bound to only one peptide indicating that the minimum size required for anchoring the antibody is 8–10 amino acids. One antibody (6D11C1) reacted with adjacent overlapping peptides. Comparable signals with the adjacent peptides 358–367 and 361–370 indicate that binding is mediated by the overlapping 5–7 amino acids. Significantly different staining intensity of adjacent peptides, such as that observed with 6D11C1 and peptides 286–295 and 289–298, indicate that without the three amino acids unique to the former peptide, robust antibody binding is not possible. Thus, the putative minimum anchoring sequence for antibody 6D11C1 in this area comprises aa 286–295 (Fig. 3E). On these premises, the minimum size of the epitope recognised by antibodies 1G7D1 and 2G8D4 that bind to 2 separate peptides is larger than 14 amino acids. Similarly, the epitope of antibody 6D11C1 that binds to 5 peptide spots, 2 of them adjacent to each other, contains 3 anchoring regions with more than 23 amino acids (Fig. 3G).

4. Discussion

Confocal microscopic analysis shows that in virus-infected cells the SARS nucleocapsid protein is primarily located in the perinuclear region. The large area stained and the strong fluorescence signal indicates synthesis of large amounts of this protein in the infected cell. The granular structure of the stained area indicates that the nucleoprotein is associated with virus particles or nucleocapsids packaged in cytoplasmic vesicles and is not diffusing freely in the cytoplasm. This corresponds well with the demonstration of SARS virus nucleocapsid particles in the rough endoplasmic reticulum and in virus morphogenesis matrix vesicae (VMMV) as well as virus particles fully assembled in the smooth vesicles from the Golgi apparatus (Ksiazek et al., 2003; Qinfen et al., 2004). The nucleoprotein of other coronaviruses has been located in both cytoplasm and nucleus with a predilection for the nucleolus (Hiscox et al., 2001; Wurm et al., 2001). Moreover, the SARS CoV N protein contains putative nuclear localisation and export regions (Chang et al., 2004; Marra et al., 2003; Rowland et al., 2005; Timani et al., 2005; Wang et al., 2003; You et al., 2005) suggesting nuclear shuttling. Nuclear localisation of the SARS N protein has been observed with transfected cells (Chang et al., 2004; Li et al., 2005; You et al., 2005). However, in virus-infected cells, the findings by different research groups regarding the nuclear localisation of the N protein were consistent with the retention of the N protein in the cytoplasm (Rowland et al., 2005; Timani et al., 2005; You et al., 2005). Moreover, the study of Rowland and colleagues demonstrated that the lysine-rich domain covered by aa 369–389 lacked nuclear transport activity (Rowland et al., 2005).

In the study presented, green fluorescence of antibody-labelled N protein projecting onto the nuclei was frequently observed in the confocal laser scanning analysis upon Z-sectioning, but fluorescence was not observed in the core of the nuclei. This finding provides evidence that nucleoprotein is found in the perinuclear cytoplasm above or below the nucleus rather than localised in the nucleus.

To characterise nucleoprotein antigens and binding of the monoclonal antibodies, epitope mapping using overlapping peptides was employed. The 7 epitope regions identified were located in the central and C-terminal portion of the protein (Table 1). In addition to these regions, two other epitopes (aa 84–93 and aa 388–404) have been described by He and colleagues (He et al., 2004). The latter epitope, aa 388–404, overlaps with the epitope recognised by antibody 4E10A3A1 in this study. Conformational B-cell epitopes in the nucleoprotein have not been described in the past.

Epitope mapping allowed comparison of the antigenicity of the N protein with previous studies. For instance, Chen and colleagues described superior immunogenicity of the C-terminal compared to the aminoterminal part of the nucleocapsid protein (Chen et al., 2004). In this study, more than 75% of the sera of infected individuals reacted with peptides from the C-terminal (aa 362–412) and central (aa 153–178) region, but few sera reacted with the aminoterminal part of the protein (He et al., 2004). The evidence provided in this study, in which region aa 362–412 was recognised by at least 5 of 10 monoclonal antibodies, supports these findings. Thus, the carboxyterminal part of the protein is highly immunogenic in both humans and mouse.

Semi-empirical methods are being used to predict protein regions of particular antigenicity. For instance, peptides 371–390 and 355–376 were predicted to be immunogenic on the basis of a hydropathy plot (Li et al., 2003). In fact, these peptides contained two epitopes recognised by four of the mAbs in this study. However, an additional five epitope peptides recognised by the antibodies were only partially or not represented by the predicted peptides. Similarly, computer modelling by the method of Kolaskar and Tongaonkar predicted 16 antigenic peptides in the central and C-terminal part of the N protein (He et al., 2004; Wang et al., 2003). A comparison of the peptide sequences with the epitopes identified in this study showed that none of the epitopes associated with a B-cell immune response contained any of the 16 peptides predicted in the semi-empirical model. This indicates that the specificity of the B-cell response to SARS nucleoprotein in mice is poorly amenable to predictions based on hydrophobicity analysis.

The structure of an N-terminal portion of the nucleoprotein (amino acid residues 45–181) of the virus has been determined by NMR spectroscopy and a putative RNA-binding domain has been identified (Huang et al., 2004). In contrast, the structure of the central and C-terminal part of the SARS CoV nucleocapsid protein has not yet been clarified. Identification of conformational epitopes in the C-terminal area permits postulations on the protein configuration. For instance, to be accessible to the antibodies, the epitope sequences must not be covered by other parts of the molecule. Furthermore, comparison with predictions of the tertiary structure (Wang et al., 2003) indicates that

the converging amino acids 286–295, 316–325, 360–370 recognised by antibody 6D11C1 are located in or close to different helical sequences. Moreover, a major conformational epitope in this area provides experimental evidence for the highly ordered structure of the region around aa 250–360 that has been previously predicted (Tang et al., 2005). Biochemical studies demonstrate that aa 270–370 comprise a dimerisation domain of the nucleoprotein (Yu et al., 2005) which is analogous to a dimerisation domain in the nucleoprotein of other coronaviruses (Fan et al., 2005). Recent crystal structure analysis of this region in both the SARS coronavirus (Yu et al., 2006) and of the structural homolog in infectious bronchitis virus (Jayaram et al., 2006) demonstrate that epitope residues aa 316–325 and 360–370 recognised by antibody 6D11C1 are in close proximity in the monomeric form of the N protein. In contrast, aa 286–295, which represent part of the antibody epitope, are distantly located. Dimerisation of the protein results in juxtaposition of aa 286–295 of one nucleoprotein molecule to aa 316–325 and 360–370 of the second peptide molecule. Thus, the epitope identified independently supports the structural analysis results reported by Jayaram et al. (2006) and Yu et al. (2006) and suggests that the monoclonal antibody 6D11C1 cross-links two nucleocapsid protein molecules. It would be interesting to examine whether cross-linkage of SARS nucleoprotein dimers by the monoclonal antibody affects infectivity of the virus.

The three peptides recognised by 6D11C1 are flanked by the amino acid sequences 274–283 and 373–382 that converge to an epitope recognised by antibodies 1G7D1 and 2G8D4. Peptide 373–382 comprises part of a lysine-rich region (aa 362–381, KTFPPTEPKKDKKKKTDEAQ). The highly basic nature of this sequence suggests a role in RNA-binding (He et al., 2004). The basic character of this domain is further enhanced in combination with peptide 274–283 (AFGRRGPEQT) that contains two additional arginine residues indicating that the converging regions jointly exercise a specific function in the nucleoprotein.

In conclusion, several monoclonal antibodies reactive with the SARS CoV nucleocapsid protein were produced. Confocal analysis demonstrated the nucleoprotein in cytosolic vesicles but not in the nucleus. Data of this and previous work with human sera indicates that the C-terminal part of the nucleoprotein is highly immunogenic in both humans and mice. Predictions of protein areas with particular antigenicity based on hydrophobicity analysis of the nucleoprotein could not be confirmed fully. A structural unit identified by antibody epitope mapping consisting of a lysine-rich unit (aa 373–382) in combination with two additional arginine residues of peptide aa 274–283 might have a specific function. The produced antibodies will be available for the analysis of the structure, function and variability in antigenicity of the nucleoprotein of different SARS CoV strains.

Acknowledgements

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