Abstract

The availability of monoclonal antibodies (mAbs) specific for the SARS-coronavirus (SARS-CoV) is important for the development of both diagnostic tools and treatment of infection. A molecular characterization of nine monoclonal antibodies raised in immune mice, using highly purified, inactivated SARS-CoV as the inoculating antigen, is presented in this report. These antibodies are specific for numerous viral protein targets, and six of them are able to effectively neutralize SARS-CoV in vitro, including one with a neutralizing titre of 0.075 nM. A phylogenetic analysis of the heavy and light chain sequences reveals that the mAbs share considerable homology. The majority of the heavy chains belong to a single Ig germline V-gene family, while considerably more sequence variation is evident in the light chain sequences. These analyses demonstrate that neutralization ability can be correlated with specific murine VH-gene alleles. For instance, one evident trend is high sequence conservation in the VH chains of the neutralizing mAbs, particularly in CDR-1 and CDR-2. The results suggest that optimization of murine mAbs for neutralization of SARS-CoV infection will likely be possible, and will aid in the development of diagnostic tools and passive treatments for SARS-CoV infection.

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1. Introduction

SARS-coronavirus (SARS-CoV), the causative agent of severe acute respiratory syndrome (SARS) in humans, has

infected more than 8000 people in various countries worldwide and caused approximately 800 deaths (Drosten et al., 2003a,b; WHO, http://www.who.int/csr/sars/country/en/). The whole genomes of SARS-CoV isolates, implicated in the 2003 outbreak in Toronto, have been sequenced and characterized (Marra et al., 2003; Rota et al., 2003). Characterization of this virus continues at a phenomenal rate, and our understanding of the function of numerous viral proteins, the phylogeny of SARS-CoV, and the viral life cycle continues to grow (reviewed in Stadler et al., 2004; Eickmann et al., 2003; Thiel et al., 2003).

Currently, no effective vaccines or treatments for SARS-CoV infection are available. Until an effective vaccine is developed, the best hope for the treatment of infection and the
prevention and control of future outbreaks remains the development of passive immunotherapy with SARS-CoV-specific antibodies (Holmes, 2003). It has been suggested that protection might be afforded by passive immunotherapy with concentrated SARS-CoV-specific IgG antibodies (Li et al., 2003a), and reports have established that infected individuals can benefit from treatment with serum from recovered patients (Pearson et al., 2003). Recently, it was also reported that viral replication was inhibited in the lower respiratory tract of naive mice if they underwent passive immunization with neutralizing antibodies present in immune serum derived from infected mice (Subbarao et al., 2004). A monoclonal antibody developed from a human non-immune single chain variable region (scFv) library has also been shown to neutralize SARS-CoV in vitro, strengthening the argument that passive immunotherapy with highly specific mAbs might be very effective in controlling SARS-CoV infection (Sui et al., 2004).

The production of mAbs specific for SARS-CoV is vital for studies of viral pathogenesis, and the development of both diagnostic tools and vaccines. Since the development of serum antibodies after infection with SARS-CoV can take 1–3 weeks (Li et al., 2003a), assays that can accurately detect the presence of viral nucleic acids or proteins may be preferred for rapid diagnosis of SARS-CoV infection. The profiles of antibody responses to SARS-CoV have been well established (Li et al., 2003a). Analyses have also identified viral proteins that might serve as the best markers for immunological detection of infection by SARS-CoV (Tan et al., 2004; Lu et al., 2004). Similarly, the characterization of immunogenic peptides derived from SARS-CoV structural proteins has also allowed for the identification of epitopes that are recognized by antibodies present in patient serum (Wang et al., 2003). With this knowledge, the development of mAbs that can be used for both diagnostic assays and clinical treatments should be an attainable goal.

Herein, we further characterize the immunogenetics and neutralizing endpoints of purified murine hybridoma-derived mAbs raised in mice, using highly purified SARS-CoV as the inoculating antigen. Numerous reports exist, which characterize antibodies raised against surrogate SARS-CoV immunogens. Examples include polyclonal antibodies raised against peptides and a recombinant SARS-CoV nucleoprotein (Chang et al., 2004; Lin et al., 2003), and monoclonal antibodies raised against a SARS-CoV-derived recombinant protein fragment (Zhou et al., 2004). Similarly, a neutralizing mAb specific for the spike protein, derived from naive human plasmapheresis mononuclear cells, has been developed and characterized (Sui et al., 2004). Little has been published on mAbs developed to the native viral particle. The relevant immunogenetic characteristics of a panel of nine murine mAbs raised to whole SARS-CoV, six of which can effectively neutralize the virus in vitro, are presented. This development provides a distinct advantage in the search for an effective passive immune therapy, because these mAbs were raised against the intact virus, rather than any individual viral protein or immunogenic peptide.

2. Materials and methods

2.1. Production of highly purified SARS-CoV for inoculation of mice

All of the procedures employed for the production of SARS-CoV are discussed in detail in Berry et al. (2004). Briefly, SARS-CoV (Tor-3 strain; Krokhin et al., 2003) was expanded after plaque purification in Vero-6 cell monolayers and partially purified through a sucrose cushion, and then further purified using iodixanol gradient centrifugation. Fractions were tested by Western immunoblot with convalescent patient serum, and the fractions that reacted with SARS-CoV were pooled, dialyzed against phosphate buffered saline (PBS), and further concentrated by ultracentrifugation for 1.5 h at 150,000 × g, resulting in highly purified whole virus particles.

2.2. Inoculation of mice and production of hybridomas and antibodies

All of the procedures employed for the production of hybridomas and mAbs are discussed in detail in Berry et al. (2004). Briefly, 5–6-week-old female BALB/c mice (Charles River, Wilmington, MA) were injected subcutaneous (S.C.) with 50 µl of SARS-CoV S.C. with 50 µg of beta-propiolactone-inactivated SARS-CoV (Tor-3 strain) with an equal part of Freund’s complete adjuvant (F.C.A.) (H37-Ra) from Difco (BD, Oakville, ON). Thirty days later, the mice received 50 µg of SARS-CoV S.C. in Freund’s incomplete adjuvant (F.I.A.) in a total volume of 100 µl. The mice received 5 µg of the same antigen in a total volume of 100 µl S.C. with F.I.A. on days 48 and 63. The mice received a final booster inoculation intraperitoneal (I.P.) with 5 µg of highly purified SARS-CoV in 200 µl PBS 3 days before euthanization by anesthesia overdose, spleen removal, and subsequent hybridoma fusion. After hybridoma fusion and establishment of stable clones, the hybridoma supernatants were screened via ELISA using purified SARS-CoV as the target antigen, and isotype with a commercial murine isotyping dipstick test (Roche), facilitating the selection of appropriate primers for subsequent RT-PCR. Monoclonal antibodies were produced in medium scale in 450 and 800 cm² culture flasks (Corning). Roller bottles containing 200 ml of complete hybridoma media [BD-Quantum yield (VWR, Canada), 10% fetal bovine serum (Wisent, Montreal), 10% Biogro-Hybridoma serum-free supplement (Biogro Technology, Winnipeg)] were inoculated with 50 ml of hybridoma cells such that a final concentration of more than 1 million viable cells/ml was achieved. The cultures were allowed to grow to extinction and were harvested at 7–10 days post-inoculation. Supernatants were clarified
by removing cell mass by centrifugation, and concentrated on Amicon 8400 stirred cell Ultrafiltration-nitrogen concentrators (VWR, Canada) with a YM-30 (Millipore, USA) membrane. Individual membranes were used for the concentration of each mAb. The concentrated supernatants were mixed 1:1 with protein A-binding buffer (Pierce) and purified on a equilibrated 1 or 5 ml protein G-sepharose column (Amersham Biotech) as per the manufacturers instructions. Eluted antibody was dialyzed to remove salt using Centriprep YM-30 Centrifugal Filter Units (Millipore) with a 30 kDa molecular weight cutoff. The mAbs were then sterilized through low-protein-binding 0.22 µm syringe filters (VWR). Protein concentration was determined using the micro-bca assay (Pierce) and the IgG concentration was standardized to 1 mg/ml in sterile PBS (Gibco).

2.3. Virus neutralization assay

The SARS-CoV in vitro neutralization assay was performed essentially as described (Berry et al., 2004), with the following modifications. The panel of purified mAbs was sterile filtered and each sample standardized to a concentration of 1 mg/ml of IgG before being subjected to serial dilutions and incubation with live SARS-CoV.

2.4. Cloning of the heavy (V₂H) and light (V₁L) chains of murine mAbs

Approximately 1–2 million mAb-secreting hybridoma cells were collected and homogenized via passage through a 20-gauge needle using an RNase-free 5 ml syringe. Total RNA was isolated using RNeasy® Mini Spin kit according to the manufacturer’s instructions (QIAGEN®). cDNA was produced via reverse transcription using 10 pg to 10 mg RNA template, a 15 base oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen). Reactions were incubated at 94 °C for 1 h, followed by inactivation of the reverse transcriptase at 10 °C. The SARS-CoV in vitro neutralization assay was performed essentially as described (Berry et al., 2004), with the following modifications. The panel of purified mAbs was sterile filtered and each sample standardized to a concentration of 1 mg/ml of IgG before being subjected to serial dilutions and incubation with live SARS-CoV.

2.5. Identification of Ig germline sequences and assignment of relevant regions

Consensus nucleotide sequences were compared against the Mus musculus immunoglobulin (lg) set database using IgM QUEST (Lefranc, 2003; http://imgt.cines.fr/home.html). The sequences were concurrently compared against the M. musculus Ig germine V-gene database using IgBLAST (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov/igblast/). This allowed the identification of the complementarity determining region (CDR) and framework (FR) regions of the V₂H and V₁L sequences, and provided numbering to the inferred amino acid sequences according to Kabat et al. (1991). Similarly, the IgBLAST results allowed for the identification of the most closely related murine Ig germine V-genes currently available in these databases. In all cases, the entire sequence, including those at the 5′ end of each sequence imposed by the specific primers used in the original PCR amplification, were examined.
2.6. Sequence alignments

The inferred amino acid sequences were trimmed to remove all residues encoded by the 5′ primer regions, to eliminate potential artefactual matches caused by the primers employed in PCR amplification of the heavy and light chains. The sequences were then aligned with ClustalW (Thompson et al., 1994; http://clustalw.genome.ad.jp/) using standard parameters (gap open penalty: 10; gap extension penalty: 0.05; no weight transition; hydrophilic gaps allowed; weight matrix: Blosum). The alignments were then analyzed using GeneDoc version 2.6.002 (Nicholas et al., 1997) to produce textual alignments and quantify the relatedness of the sequences.

2.7. Phylogenetic analysis

Analyses were performed using BioEdit version 5.0.9 (Hall, 1999) and MEGA version 2.1 software suite (Kumar et al., 2001). Only amino acid sequences were examined. The sequences, aligned via ClustalW as described above, were analyzed via neighbor joining analysis, bootstrapped using 1000 replicates. The naïve human mAb 80R (Sui et al., 2004; VH GenBank accession no. AAS19425; VL GenBank accession no. AAS19432) was initially used as an outlier sequence. This mAb was chosen because it is specific for the SARS-CoV spike protein and neutralizes SARS infection in vitro, but it was developed from a non-immune human single chain variable fragment (scFv) library. A second outlier, also human in origin, was found as follows. The VH and VL amino acid sequences of F26G18 were compared against the human Ig sequences in the NCBI databases using IgBlast, as described above. F26G18 was chosen because it was the most potent SARS neutralizing murine mAb isolated, and it is specific for spike. Human antibodies were chosen to ensure that similarity would be minimal, considering that mouse, and indeed human, antibodies inherently share considerable homology regardless of their specificity. The sequences that showed the lowest homology to the VH and VL sequences of F26G18 were chosen as follows: VH: GenBank accession no. BAC01727, level of identity: 83/115 amino acids; VH: GenBank accession no. CAD19025, level of identity: 83/132 amino acids. In all of the phylogenetic analyses, the human sequences clearly served as effective outliers, and only BAC01727 and CAD19025 are included in the relevant figures.

3. Results

3.1. Summary of the relevant genetic properties of the mAbs specific for SARS-CoV

Table 1 summarizes the relevant characteristics of the murine mAbs generated against highly purified, inactivated SARS-CoV. Examining the VH chains, only two Ig V-gene

<table>
<thead>
<tr>
<th>mAb</th>
<th>Class</th>
<th>GenBank accession numbers</th>
<th>Target</th>
<th>Neutralizing titre (nM)</th>
<th>J-gene</th>
<th>D-gene</th>
<th>Most closely related Ig germline V-gene</th>
<th>Percent identity to most closely related Ig germline V-gene</th>
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<td></td>
<td>SP2.6/7/8</td>
<td>Vκ1</td>
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<td>VJ58.33</td>
<td>95</td>
</tr>
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<td>J558.50</td>
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<tr>
<td>VL</td>
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<td>Jα4</td>
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<td>Spike</td>
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<td>N.A.</td>
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<td>α9</td>
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<td>AY055274</td>
<td>U</td>
<td>Jα2</td>
<td>N.A.</td>
<td></td>
<td>αβ20</td>
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<td>G2ark</td>
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<td>U</td>
<td>Jα1</td>
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<tr>
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<td>G2ark</td>
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<td>94</td>
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<td>F26G10</td>
<td>G2ark</td>
<td>AY055277</td>
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<td>Jα2</td>
<td>N.A.</td>
<td></td>
<td>αβ20</td>
<td>98</td>
</tr>
</tbody>
</table>

* Neutralizing mAbs are indicated by bold text.
* U: unknown.
* Jα2: closest related germline genes, as determined by V-quest and NCBI IgBlast analysis of nucleotide sequences. N.A.: not applicable.
* Jα4: closest related germline genes, as determined by NCBI IgBlast analysis of nucleotide sequences.
* Jα: closest related germline genes, as determined by NCBI IgBlast analysis of nucleotide or inferred amino acid sequences, as indicated.
families are represented. Eight out of the nine mAbs share high sequence identity with the J558 V-gene family, with the remaining mAb, F26G1, sharing high sequence identity with the VOx-1 gene. Of the eight mAbs belonging to the J558 V-gene family, five of the eight mAbs are most closely related to the J558.50 V-gene, and this set of five mAbs neutralizes SARS-CoV in vitro. mAbs F26G6, F26G8 and F26G3 are most closely related to the J558.33 and J558.5 V-genes, respectively, which are themselves closely related to J558.50.

A closer analysis of the VH chains of mAbs sharing significant homology with the J558.50 V-gene is presented in Fig. 1 and Table 2. An alignment of the nucleotide sequences of these VH sequences reveals a very high level of identity (Fig. 1A). Similarly, at the amino acid level, these sequences share considerable identity both amongst themselves and with the J558.50 V-gene (Fig. 1B), with some mutation away from the germline sequence, which is typical of immunoglobulin V-gene cDNAs from T-dependent B cell responses (Fish and Manser, 1987).

A quantification of the level of identity of these sequences is summarized in Table 2. An analysis of the J-genes shows that three JH gene families are represented, with a definite bias towards JH4 (four mAbs) with JH1 (three mAbs) and JH2 (two mAbs) also represented. Analysis of the CDR-3 sequences reveals that DH-regions are present in seven out of nine of the mAbs, with homology to numerous DH-gene sequences available in the databases. A detailed analysis of the DH-regions is presented in the following section.

Examination of the VL chains demonstrates that a wider variety of V-genes is represented. Six distinct V-genes are present, which suggests that a larger pool of V-genes can be selected, compared to the VH chains, to produce mAbs that are specific for SARS-CoV. As in the case for VH, three Jk-gene families are represented, with a definite bias towards Jk2 (five mAbs) and an even distribution of Jk1 and Jk4 (two mAbs each).

### Table 2

<table>
<thead>
<tr>
<th>mAb</th>
<th>Level of identity vs. J558.50</th>
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<tbody>
<tr>
<td></td>
<td>Nucleotide</td>
</tr>
<tr>
<td>F26G18</td>
<td>26/27/24 (97%)</td>
</tr>
<tr>
<td>F26G19</td>
<td>26/27/24 (97%)</td>
</tr>
<tr>
<td>F26G10</td>
<td>26/27/24 (97%)</td>
</tr>
<tr>
<td>F26G1</td>
<td>26/27/24 (97%)</td>
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Level of identity (pairwise) is expressed as number of identical residues/total number of residues and percent identical residues.
closely related (within each pair), with very few amino acid changes evident between these sequences.

An examination of the \( V_L \) sequences reveals that unlike the case of \( V_H \), CDR-1, -2, and -3 exhibit considerably higher levels of sequence variation. As is the case for the \( V_H \) chains, the \( V_L \) chains of the F26G6/F26G8, F26G7/F26G19, and F26G9/F26G10 pairs share a high level of sequence identity (within each pair) and are therefore likely clonally related. In the case of both the \( V_H \) and \( V_L \) sequences, mAb F26G1 stands out as being the least closely related to any of the other sequences in this panel of mAbs. A quantitative analysis of sequence relatedness is discussed in the subsequent sections.

The discernible \( D_H \)-regions present in the CDR-3 of the \( V_H \) domains show considerable variation, and alignments of these regions with available related \( D_H \)-region sequences identified by Vquest analysis are shown in Fig. 2 B. mAb F26G1 contains the most well delineated but shortest \( D_H \)-region, with high homology to SP2.6/7/8 \( D_H \)-genes over six nucleotides. mAb F26G3 shows high homology to FL16.1 and considerable homology with SP2.7/8 \( D_H \)-genes, which also overlaps with FL16.1. mAbs F26G7 and F26G19 contain identical \( D_H \)-regions, which share homology with both SP2.7/8 and Q52.01/02, although delineation of the regions of homology is less clear. Similarly, mAbs F26G9 and F26G10 contain identical \( D_H \)-regions, with homology to SP2.3/4/5, DP2.1, and FL16.2. Manual alignment of the sequences suggests that the central portion of the \( D_H \)-region of F26G18 is most likely encoded by an SP2 or DP2 element, with considerable junctional diversity (\( D \)) on either end (Fig. 2 B). mAb F26G18 contains a \( D_H \)-region with homology to SP2.5/7, DP2.1, and FL16.2. Discernable \( D_H \)-regions are lacking in mAbs F26G6 and F26G8, and these mAbs are therefore not included in this figure. It is clear from the analysis that the \( D_H \)-regions of all of these mAbs are highly variable, and likely resulted from complex somatic gene rearrangements and junctional diversification during assembly.

3.3. Quantitative analysis of the relatedness of the regions encoded within the \( V_H \) and \( V_L \) chains

Examining the full length of the \( V_H \) sequences, as outlined in Fig. 2A, a clear pattern emerges regarding amino acid sequence identity. The most closely related \( V_H \) sequences are F26G9/F26G10 (97% identical), F26G7/F26G19 (95% identical), F26G6/F26G8 (92% identical) and F26G18/F26G19 (82% identical). Both \( V_H \) CDR-1 and CDR-2 exhibit high levels of identity, with all of the neutralizing mAbs showing remarkable sequence conservation, while \( V_H \) CDR-3 shows a high level of sequence diversity. One neutralizing mAb F26G3 shares little identity with any of the other neutralizing mAbs because it is derived from a different germline \( V_H \) allele (see the following section for a more detailed analysis...
of the full length of V_H, it is evident that the average level of subdivided into pertinent regions, is shown in Fig. 3. Across a small margin (Fig. 3A). An examination of the V_H CDRs identity is highest amongst the neutralizing mAbs, albeit by bars: CDR-3. (B) Summary of the percentage of identity at the amino acid level of all, neutralizing, non-neutralizing) as indicated on the X-axis. White bars: full length of sequences; hatched bars: CDR-1; grey bars: CDR-2; black bars CDR-3. (B) Summary of the percentage of identity at the amino acid level of the V_L sequences. This figure is labeled the same as (A).

The average level of identity of the heavy and light chains, subdivided into pertinent regions, is shown in Fig. 3. Across the full length of V_H, it is evident that the average level of identity is highest amongst the neutralizing mAbs, albeit by a small margin (Fig. 3A). An examination of the V_H CDRs reveals that CDR-1 and CDR-2 exhibit the highest level of sequence identity, whether subdivided into groups containing all mAbs, neutralizing mAbs, or non-neutralizing mAbs. However, this finding is most apparent when examining the neutralizing mAbs, whose CDR-1 and CDR-2 regions share 73% and 82% average sequence identity, respectively. On the other hand, CDR-3 exhibits the highest sequence variability, whether grouped into all mAbs, neutralizing mAbs, or non-neutralizing mAbs, with an average level of identity ranging from 24% to 47%. An extended CDR-3 domain is characteristic of the V_H chains of the SARS-CoV neutralizing mAbs. This domain of the neutralizing mAbs contains, on an average, 12 amino acid residues, while the average amongst all of the mAbs is 10 residues (not including D-}

The overall relatedness of the V_H and V_L sequences of the mAbs can be quickly summarized via phylogenetic neighbor joining analysis. Fig. 4 shows phylogenetic trees generated by an examination of the amino acid sequences of the V_H and V_L chains. Fig. 4A summarizes the V_H chains. Clearly, the neutralizing mAbs F26G7, F26G9, F26G10, F26G18, and F26G19 cluster together and are closely related. Amongst these V_H chains, F26G18 is the least closely related to the remaining members of this group. Interestingly, F26G18 and F26G19, which are both spike-specific neutralizing mAbs, cluster further apart, and are in fact on separate branches of the tree. Another neutralizing mAb, F26G3, clusters closer to mAbs F26G6 and F26G8, which are non-neutralizing, spike-specific mAbs. Unfortunately, the protein target of mAb F26G3 is currently unknown. However, one would not be surprised if this mAb does in fact target spike, but this interaction has been undetectable by the methods employed thus far (Berry et al., 2004).

Although these analyses cannot determine whether F26G6/F26G8, F26G7/F26G19, and F26G9/F26G10 are in fact mAbs derived from the same clones, it is clear that they cluster in related branches according to their overall properties (e.g. neutralizing, spike-specific). It is also clear that mAb F26G1 is least related to any of the other mAbs in this panel, occupying its own branch well separated from the rest of the mAbs. Examining the V_L chains sharing the highest identity with the J558.50 V_L, all of which are neutralizing, it is evident that a similar pattern emerges. Monoclonal antibodies F26G9/F26G10 and F26G7/F26G19 are encoded by V-genes that cluster closely together, with F26G18 occupying a divergent branch on the tree (Fig. 4B). Essentially the same pattern emerges when examining the V_L chains (Fig. 4C), with neutralizing and spike-specific mAbs clustering in related branches, and F26G1 occupying a separate branch on the tree, illustrating that this mAb is the least related to any of the other mAbs in the panel.

Fig. 3. Summary of the percentage identity of the SARS-CoV-specific mAbs. (A) Summary of the percentage of pairwise identity, at the amino acid level (Y-axis), between the V_H chains of various categories of mAbs (all, neutralizing, non-neutralizing) as indicated on the X-axis. White bars: full length of sequences; hatched bars: CDR-1; grey bars: CDR-2; black bars CDR-3.

3.4. Summary of the phylogenetic relationship of the mAbs

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Fig. 4. Phylogenetic analysis of the mAbs specific for SARS-CoV. Trees are based on neighbor joining analysis of the inferred amino acid sequences of the relevant V\text{H} and V\text{L} sequences, as outlined in Section 2. (A) V\text{H} regions of all mAbs. (B) V\text{H} regions of all mAbs sharing significant identity with the J558.50 Ig germline V\text{-}gene. (C) V\text{L} regions of all mAbs. In each case, the outliner sequences are of human origin, chosen as outlined in Section 2. Neutralizing mAbs are shown in bold text. The number at each node represents the level of bootstrap support (expressed as a percentage) for the node over the total number of replicates performed. Only those values above 50% are reported.

3.5. Detailed analysis of the neutralizing mAbs

Table 3 presents a summary of the level of identity evident in the group of mAbs that exhibit SARS-CoV neutralizing ability, relative to the most efficiently neutralizing mAb, F26G18. As shown in this table, the neutralizing titre varies from a low of 0.075 nM (F26G18) to a high almost 350-fold greater, at 26 nM (F26G3). As a comparison, it was recently reported that a human mAb, 80R, developed from a naive human immune scFv library and specific for the angiotensin-converting enzyme 2 (ACE2) binding domain of spike, exhibited a neutralizing concentration as low as 0.37 nM (Su et al., 2004). The relationship between F26G18 and the rest of the neutralizing mAbs was therefore examined as an attempt to identify any common theme or relationship that exists amongst them. Clearly, mAb F26G3 is least related to F26G18 or any of the other neutralizing mAbs, in both the V\text{H} and V\text{L} chains (Table 3). Examining the V\text{H} chains, the average level of identity (compared to F26G18) for all of the neutralizing mAbs is high across the entire sequence of the

Table 3

<table>
<thead>
<tr>
<th>mAb</th>
<th>Neutralizing titre (nM)</th>
<th>Region examined and level of identity vs. F26G18*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Full length</td>
</tr>
<tr>
<td>V\text{H}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F26G19</td>
<td>1</td>
<td>95/116 (82%)</td>
</tr>
<tr>
<td>F26G9</td>
<td>1</td>
<td>96/116 (83%)</td>
</tr>
<tr>
<td>F26G10</td>
<td>1</td>
<td>93/116 (80%)</td>
</tr>
<tr>
<td>F26G7</td>
<td>6</td>
<td>99/116 (93%)</td>
</tr>
<tr>
<td>F26G3</td>
<td>26</td>
<td>75/116 (66%)</td>
</tr>
<tr>
<td>V\text{L}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F26G19</td>
<td>1</td>
<td>69/99 (70%)</td>
</tr>
<tr>
<td>F26G9</td>
<td>1</td>
<td>65/99 (66%)</td>
</tr>
<tr>
<td>F26G10</td>
<td>1</td>
<td>64/99 (65%)</td>
</tr>
<tr>
<td>F26G7</td>
<td>6</td>
<td>70/99 (71%)</td>
</tr>
<tr>
<td>F26G3</td>
<td>26</td>
<td>59/103 (57%)</td>
</tr>
</tbody>
</table>

* Level of identity (pairwise) is expressed as number of identical residues/total number of residues and percent identical residues. F26G18 is the most efficiently neutralizing mAb, with a neutralizing titre of 0.075 nM.

* The average level of identity (± S.D.) is calculated from the values of the specified regions for each given mAb.
VH chain, and within CDR-1 and CDR-2. Conversely, CDR-3 is the least closely related region in all of the neutralizing mAbs. The same pattern is evident when the average level of sequence identity of the VH CDR-3 of all mAb sequences is examined (Fig. 3A). Examining the VH chains, the average level of identity between the rest of the neutralizing mAbs and F26G18 is relatively low (Table 3), which is similar to the relatively low average level of identity exhibited by all of the mAbs in the panel (Fig. 3B). The average level of identity in CDR-1 and CDR-2 of VH is lower, compared to F26G18, than the corresponding regions in VH, while CDR-3 exhibits a higher average level of identity. In the VH chains, a slightly lower average level of identity is evident in CDR-2, compared to CDR-1 and CDR-3; however, this is far less noticeable than the difference in relatedness between CDR-3 compared to CDR-1 and CDR-2 in the VH chains.

4. Discussion and conclusions

This panel of mAbs derived from mice immunized with highly purified whole SARS-CoV exhibits diverse specificity for binding targets and variable in vitro neutralization ability. Neutralization titres amongst the mAbs vary from the lowest, at 0.075 nM for F26G18, to a high of 26 nM, for F26G3. Interestingly, two of the mAbs that exhibit the highest neutralizing efficiency, F26G18 and F26G19, are specific for the spike protein (Berry et al., 2004). As has been established conclusively, spike is required for binding of SARS-CoV to its receptor, ACE2, and mAbs that block this interaction can neutralize SARS-CoV (Li et al., 2003b; Sui et al., 2004). These results suggest that F26G18 and F26G19 likely neutralize SARS-CoV infection by directly targeting spike, although whether the interaction between spike and its receptor is targeted specifically remains to be determined, and ongoing work to answer this question is in progress. Although five of the mAbs are confirmed to be spike-specific, three of these (F26G1, F26G6, and F26G8) are unable to neutralize SARS-CoV infection in vitro. These results suggest that the panel of mAbs recognizes a diverse set of epitopes within the spike protein, some of which are in regions of spike that are not involved in viral adhesion or other steps in the infection process. Of the panel of six neutralizing mAbs, the targets of four have not yet been identified, and work is currently underway to determine them.

Examining the level of pairwise identity of the neutralizing mAbs at the amino acid level reveals that the highest sequence variability is exhibited by the VH CDR-3, while, not surprisingly, CDR-1 and CDR-2 of the VH chains demonstrate a greater level of sequence identity. Conversely, all three CDRs of the VL chains exhibit similar levels of sequence identity, which are, on average, lower than the levels of identity in the corresponding VH regions. The variability in CDR-3 of the VH chains is therefore likely a significant contributing factor to the target specificity of these mAbs, and the VH CDRs likely influence the neutralization ability of the mAbs to a lesser extent than the VH CDRs. The average length of CDR-3 also appears to influence neutralizing ability. The VH CDR-3 of the neutralizing mAbs contains more amino acids than the average number in the whole panel of mAbs, while the CDR-3 regions of the non-neutralizing mAbs contain fewer residues. Conversely, the average length of VH CDR-3 is constant. These results suggest that both the length and sequence of the VH CDR-3 influence the neutralization ability of the mAbs, most likely by determining the specificity of contact residues for key neutralizing epitopes on SARS-CoV proteins.

Within the group of neutralizing mAbs, a trend is evident that relates homology in the VH chains of the most potent neutralizing mAb, F26G18, with those that neutralize SARS-CoV less efficiently. The clustering of the sequences of the neutralizing mAbs based on their relatedness to F26G18 tends to correlate well with their ability to neutralize SARS-CoV in vitro. For both VH and VL, the mAb with the lowest neutralizing efficiency, F26G3, has the lowest overall sequence identity when compared to mAb F26G18, and when compared to the average sequence identity across the whole panel of neutralizing mAbs. This trend is more apparent for the VH sequences, since the percentage of identity of the VH chains relative to F26G18 is lower overall. This observation is consistent with the dominant role of VH compared to VL in previously identified neutralizing mAbs against a viral pathogen (Barbas et al., 1993). CDR-1 and CDR-2 of the VH chains of the neutralizing mAbs are most closely related to the same regions in F26G18, while CDR-3 is less conserved. From this analysis, one can infer that high homology in VH CDR-1 and CDR-2 (relative to F26G18) of the neutralizing mAbs appears to correlate with neutralizing ability. Interestingly, F26G7 exhibits a six-fold higher neutralizing titre than F26G19, even though their sequences are closely related both to each other and to F26G18, revealing that the trend for relatedness to F26G18 correlating with neutralizing ability does not follow for all of the mAbs in this panel. This observation also suggests that small changes in the amino acid composition of these mAbs can significantly alter neutralization efficiency. Overall, this analysis clearly demonstrates that the VH regions of the neutralizing mAbs are more highly related than the VL chains, and suggests that the sequence of the VH chains plays a smaller role in neutralizing ability than the VH chains.

Analysis of the sequences of the mAbs reveals that the VH and VL chains of several mAbs are closely related. The pairs F26G6/F26G8, F26G7/F26G19, and F26G9/G10 exhibit few differences at the amino acid level, within each pair. This observation could be explained in several ways. First, each pair might have originated from the same B cell progenitor and they are clonally related (Fish et al., 1989), with the few observed sequence variations resulting from somatic mutation. Second, identical CDR-3 rearrangements might have occurred in the B cell progenitors, and antigen selection may have produced several independently rearranged but closely related B cell clones. Third and alternatively,
sequence changes might have occurred during reverse tran-
scription or PCR amplification, and the mAbs originated
from the same, independently picked hybridoma clone. While
these analyses do not allow a differentiation between these
potential causes of this high sequence similarity, we would
speculate that the first explanation is most likely true.

The VH chains of eight of the mAbs belong to the J558
V-gene family, and six of these mAbs neutralize SARS-CoV
in vitro. This result suggests that a small pool of B cells,
expressing related Ig V-genes, was selected for in response
to exposure to SARS-CoV. This is not entirely surprising,
considering that the J558 V-gene family is estimated to con-
tain several hundred members in the genome of the BALB/c
mouse (Livan et al., 1986) and this family is highly selected
in murine B cells (Haines et al., 2001). It is clear that a re-
stricted pool of VH genes was selected to encode SARS-CoV
neutralizing mAbs. A bias towards the selection of genes in
the J558 V-gene family to generate autoantibodies in the tight-
skin mouse has also been reported (Kasturi et al., 1994). JH
gene usage is distributed relatively evenly in the mAbs gen-
erated during this work, which agrees well with the observa-
tion that JH gene usage is distributed relatively evenly in the mAbs
described here. Due to the small numbers of mAbs examined,
only trends and general relationships regarding the levels of sequence identity between any of the mAbs and
their immunological properties can be inferred. Regardless,
this analysis provides a useful summary of murine mAbs that
possess the potential for development into viable immune
therapies and diagnostic tools for SARS-CoV infection. The
recurrent usage of particular murine V-gene elements to en-
code virus-neutralizing antibodies is becoming more evident
and detailed molecular analyses become available (Kalinke
et al., 1996). Studies with animal models are currently un-
derway to determine the in vivo neutralization properties of
the neutralizing mAbs summarized here.

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