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Molecular characterization of a panel of murine monoclonal antibodies specific for the SARS-coronavirus

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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...
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 naïve 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 fragment (Zhou et al., 2004). Similarly, a neutralizing mAb specific for the spike protein, derived from naive human phage display libraries, 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; Kehn 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 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 isotyped with a commercial murine antibody developed from infected mice (Subbarao 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.
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 manufacturer's instructions. Eluted antibody was dialyzed to remove salt using Centriprep YM-30 Centrifugal Filter Units (Millipore) with a 30 kDa cutoff. The mAbs so formed were steril filtered 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).

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 μg of RNA template, a 15 base oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen). Reactions were incubated at 94 °C for 15 min. RNA was removed from the reactions by incubating them with RNaseH (Invitrogen) at 37 °C for 15 min. cDNA was then used as a template for PCR amplification using Taq polymerase (Invitrogen) and various 5’ and 3’ primers specific for Vα and Vβ genes and. The primers used are as follows (in 5’-3’ orientation), and are described in detail elsewhere (Berry, 1999; Barbas et al., 2001; Dattamajumdar et al., 1996). 2.5. Identification of Ig germline sequences and assignment of relevant regions

Consensus nucleotide sequences were compared against the Mus musculus immunoglobulin (Ig) set database using IMGT/V-Quest (Lefranc, 2003; http://www.ncbi.nlm.nih.gov/igblast/home.html). The sequences were concurrently compared against the M. musculus Ig germline 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 Vβ and Vα 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 germline 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: Blossum). 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; V H 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 V H and V L 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 V H and V L sequences of F26G18 were chosen as follows: V H: GenBank accession no. BAC01727, level of identity: 83/115 amino acids; V L: 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 V H chains, only two Ig-V-gene

| mAb | Class | GenBank accession numbers | Targeta | Neutralizing titre (nM) | J-geneb | D-geneb | Most closely related Ig germline V-geneb | Percent identity to most closely related Ig germline V-geneb |
|------|------|--------------------------|---------|------------------------|--------|--------|-------------------------------------|----------------------------------|
| V H  | F26G1 | G2a/k AY605271 Spike – Jα4 | SP2-6/7/8 V05-1 | 92 | 85 |
|      | F26G6 | G2b/k AY605272 Spike – Jα2 | None | 85 | 89 |
|      | F26G8 | G2a/k AY605273 Spike – Jα2 | None | 95 | 89 |
|      | F26G18| G2b/k AY605269 Spike 0.075 Jα4 | FL16.2 J558.50 | 97 | 91 |
|      | F26G19| G2a/k AY605279 Spike 1 Jα4 | Q52.01 J558.50 | 97 | 91 |
|      | F26G3 | G2b/k AY605265 U 26 Jβ1 | FL16.1 J558.5 | 96 | 94 |
|      | F26G7 | G2b/k AY605266 U 6 Jβ4 | Q52.01 J558.50 | 97 | 91 |
|      | F26G9 | G2a/k AY605267 U 1 Jβ1 | SP2-3/4/5 J558.50 | 97 | 94 |
|      | F26G18| G2b/k AY605268 U 1 Jβ1 | SP2-3/4/5 J558.50 | 96 | 91 |

a Neutralizing mAbs are indicated by bold text.

b U: unknown.

c Most closely related genes, as determined by V-quest and NCBI IgBlast analysis of nucleotide sequences. N.A.: not applicable.

d As determined by NCBI IgBlast analysis of nucleotide sequences.

Expressed as percentage of identical residues (pairwise), as determined by NCBI IgBlast 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 J558 V-gene family members, 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 genes are represented in the VH chains, at a relatively even distribution. There appears to be a slight bias towards JH 4 (four mAbs) with JH 1 (three mAbs) and JH 2 (two mAbs) also represented (Table 1). 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 J- 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

| mAb    | Level of identity vs. J558.50 |
|--------|-----------------------------|
|        | Nucleotide | Amino acid |
| F26G18 | 26/72 (97%) | 86/92 (93%) |
| F26G19 | 26/72 (97%) | 84/92 (94%) |
| F26G7  | 26/72 (97%) | 87/92 (95%) |
| F26G8  | 26/72 (97%) | 89/92 (97%) |
| F26G10 | 26/72 (97%) | 86/92 (95%) |

Level of identity (pairwise) is expressed as number of identical residues/total number of residues and percent identical residues.

Fig. 1. Alignments of the nucleotide and amino acid sequences of VH regions of the neutralizing mAbs belonging to the J558 V-gene family. (A) Nucleotide alignment of the VH regions of the mAbs most closely related to the J558.50 V-gene. The consensus sequence used is that of J558.50 (nucleotide sequence GenBank accession no. AF303881). A dot in the individual sequences denotes nucleotides that are the same as the consensus. A dash in the individual sequences denotes a deletion. Neutralizing clones are shown in bold text. (B) Amino acid alignment of the VH regions of the mAbs most closely related to the J558.50 Ig V-gene. The consensus sequence used is that of J558.50 (amino acid sequence GenBank accession no. AAG39162). A dot in the individual sequences denotes amino acids that are the same as the consensus. A dash in the individual sequences denotes a deletion. The framework and complementarity determining regions (CDR) are indicated above the appropriate sequence segments in the figure.
closely related (within each pair), with very few amino acid changes evident between these sequences.

An examination of the \( \text{V}_L \) sequences reveals that unlike the case of \( \text{V}_H \), \( \text{CDR}-1, -2, \) and \( -3 \) exhibit considerably higher levels of sequence variation. As is the case for the \( \text{V}_H \) chains, the \( \text{V}_L \) 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 \( \text{V}_H \) and \( \text{V}_L \) 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 DH-regions present in the \( \text{CDR}-3 \) of the \( \text{V}_H \) domains show considerable variation, and alignments of these regions with available related DH-regions identified by Vquest analysis are shown in Fig. 2B. mAb \( F26G18 \) contains a DH-region with homology to SP2.5/7, DP2.1, and FL16.2. Discernable DH-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 DH-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 \( \text{V}_H \) and \( \text{V}_L \) chains

Examining the full length of the \( \text{V}_H \) sequences, as outlined in Fig. 2A, a clear pattern emerges regarding amino acid sequence identity. The most closely related \( \text{V}_H \) sequences are \( F26G9/F26G10 \) (97% identical), \( F26G7/F26G19 \) (95% identical), \( F26G6/F26G8 \) (92% identical), and \( F26G18/F26G19 \) (82% identical). Both \( \text{V}_H \) CDR-1 and CDR-2 exhibit high levels of identity, with all of the neutralizing mAbs showing remarkable sequence conservation, while \( \text{V}_L \) 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 \( \text{V}_H \) allele (see the following section for a more detailed analysis...
of this mAb. The most closely related V \textsubscript{L} sequences are F26G7/F26G19 (98% identical), F26G9/F26G10 (98% identical), and F26G6/F26G8 (90% identical). The V \textsubscript{L} CDRs exhibit more sequence variability than those found in the V \textsubscript{H} chains, and as was the case for F26G3 V \textsubscript{H}, the V \textsubscript{L} chain of this mAb shows very little sequence identity with the V \textsubscript{L} chains of the other neutralizing mAbs (Fig. 2A). For both the V \textsubscript{L} and V \textsubscript{H} chains, mAb F26G1 demonstrates the lowest sequence identity compared to any of the other mAbs in the panel (Fig. 2A and B).

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 \textsubscript{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 \textsubscript{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 \textsubscript{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-}

### 3.4 Summary of the phylogenetic relationship of the mAbs

The overall relatedness of the V \textsubscript{H} and V \textsubscript{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 \textsubscript{H} and V \textsubscript{L} chains. Fig. 4A summarizes the V \textsubscript{H} chains. Clearly, the neutralizing mAbs F26G7, F26G9, F26G10, F26G18, and F26G19 cluster together and are closely related. Amongst these V \textsubscript{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 \textsubscript{L} chains sharing the highest identity with the J558.50 V-gene, 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 \textsubscript{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.
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 naïve 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 (Sui 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 VH and VL chains (Table 3). Examining the VH 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
Comparison of the VH and VL regions of the most efficiently neutralizing mAb (F26G18) with the corresponding regions of the remainder of the neutralizing mAbs.

| mAb   | Neutralizing titre (nM) | Region examined and level of identity vs. F26G18\(^a\) |
|-------|-------------------------|--------------------------------------------------------|
|       |                         | Full length    | CDR-1    | CDR-2    | CDR-3    |
| VH    |                         |              |          |          |          |
| F26G19| 1                       | 95(116) (82%) | 3/5 (60%)| 15/17 (88%)| 3/13 (23%)|
| F26G9 | 1                       | 96(116) (83%) | 5/5 (100%)| 16/17 (94%)| 2/13 (15%)|
| F26G10| 1                       | 95(116) (80%) | 5/5 (100%)| 15/17 (88%)| 2/13 (15%)|
| F26G7 | 6                       | 99(116) (93%) | 4/5 (80%)| 16/17 (94%)| 4/13 (31%)|
| F26G3 | 26                      | 75(116) (66%) | 3/5 (60%)| 11/17 (64%)| 4/13 (31%)|
|       | Average identity\(^b\) (%) | 81 ± 10 | 80 ± 20 | 86 ± 12 | 21 ± 8   |
| VL    |                         |              |          |          |          |
| F26G19| 1                       | 69/99 (70%)  | 8/11 (72%)| 2/7 (28%)| 3/9 (33%)|
| F26G9 | 1                       | 63/99 (66%)  | 5/11 (45%)| 2/7 (28%)| 7/9 (77%)|
| F26G10| 1                       | 64/99 (65%)  | 5/11 (45%)| 2/7 (28%)| 6/9 (66%)|
| F26G7 | 6                       | 70/99 (71%)  | 8/11 (72%)| 2/7 (28%)| 3/9 (33%)|
| F26G3 | 26                      | 50/103 (57%) | 5/15 (33%)| 3/7 (42%)| 2/9 (22%)|
|       | Average identity\(^b\) (%) | 66 ± 6   | 57 ± 17 | 31 ± 6  | 46 ± 24  |

\(^a\) 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.

\(^b\) The average level of identity (±S.D.) is calculated from the values of the specified regions for each given mAb.
sequence variability is exhibited by the VH CDR-3, while, not of four have not yet been identified, and work is currently in process. Of the panel of six neutralizing mAbs, the targets not involved in viral adhesion or other steps in the infection spike protein, some of which are in regions of spike that are panel of mAbs recognizes a diverse set of epitopes within the SARS-CoV infection in vitro. These results suggest that this panel of mAbs 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 less efficiently. The clustering of the sequences of the neutralizing mAbs based on their relatedness 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 conserved than the VL regions, and suggests that the sequence of the VH chains plays a smaller role in neutralizing ability than 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 in vitro. These results suggest that this 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/F10 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 transcription 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 $V_{\text{H}}$ 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 contain several hundred members in the genome of the BALB/c mouse (Livant et al., 1986) and this family is highly selected in murine B cells (Haines et al., 2001). It is clear that a restricted pool of $V_{\text{H}}$ 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 thymus has also been reported (Kasturi et al., 1994). $J_{\text{H}}$ gene usage is distributed relatively evenly in the mAbs generated during this work, which agrees well with the observation that $J_{\text{H}}$ gene usage in several murine innate antibodies belonging to the J558 V-gene family is approximately evenly distributed (Seidl et al., 1999). These results suggest that a typical variety of joining (J) genes is selected during the generation of $V_{\text{H}}$ chains in these clones. Conversely, a bias was evident in the selection of J genes in the generation of the $V_{\text{L}}$ chains in these clones, which agrees with published reports of preferential selection of $J_{\text{L}1}$ and $J_{\text{L}2}$ genes in adult mouse splenic cells (Wood and Coleclough, 1984; Nishi et al., 1985). This observation suggests that the $V_{\text{L}}$ chains of these mAbs appear to be derived from a more diverse pool of V genes, and may be less critical for contributing contact residues, relative to $V_{\text{H}}$, for binding to SARS-CoV proteins. A complete absence of $D_{\text{H}}$ regions, and as a result a small CDR-3 in the V-domain, in two of the non-neutralizing, spike-specific mAbs, F26G6 and F26G8, suggests that this region contributes significantly to neutralization. The diversity of the length, sequences, and apparent order of assembly of the $D_{\text{H}}$ regions present in these mAbs suggests that significant somatic alteration of sequences flanking the $D_{\text{H}}$ regions occurred during junctional assembly, or that somatic mutations occurred during affinity maturation in a germinal center. The presence of identical $D_{\text{H}}$ elements in the CDR-3 regions of F26G7/F26G19 and F26G9/F26G10 supports the notion that these pairs are clonally related, and derived from the same parental V-gene rearrangement.

In summary, multiple mAbs able to neutralize SARS-CoV infection in vitro were generated in this work, and they display neutralization titres in the nanomolar to picomolar range. The majority of the $V_{\text{H}}$ chains of the neutralizing mAbs share significant homology to the J558 Ig V-gene family, suggesting selection of a limited pool of $V_{\text{H}}$ genes occurred in mice exposed to highly purified SARS-CoV. High levels of sequence identity in CDR-1 and CDR-2 of the $V_{\text{H}}$ chains, compared to low levels of sequence identity in all of the $V_{\text{L}}$ chains, suggests that neutralization ability is imparted to these mAbs by contact residues within the $V_{\text{H}}$ chains. Chain shuffling experiments will be employed to determine the relative contributions of the $V_{\text{H}}$ and $V_{\text{L}}$ chains to SARS-CoV neutralization (Berry et al., 2003).

Passive immune therapy is a viable treatment for exposure to numerous viruses. The development of the mAbs reported here is an important first step in isolating mAbs generated against the intact SARS virus that could be used for passive immunotherapy. Humanization of murine mAbs is a viable method for the development of potential passive immunotherapeutics (Roguska et al., 1994; Rader et al., 1998), and these methods can be applied to the mAbs developed in this work. One caveat to this analysis is that it is not an exhaustive statistical analysis of the relationships between 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 encode virus-neutralizing antibodies is becoming more evident and detailed molecular analyses become available (Kalinke et al., 1996). Studies with animal models are currently under way to determine the in vivo neutralization properties of the neutralizing mAbs summarized here.

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